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Construction and mutagenesis of an anti-insulin single chain Fv (scFv) using protein engineering techniques: Identification of peptide mimotopes which inhibit scFv-insulin binding

Lake, Douglas Fletcher, Ph.D.

The University of Arizona, 1993
CONSTRUCTION AND MUTAGENESIS OF AN ANTI-INSULIN SINGLE CHAIN Fv (scFv) USING PROTEIN ENGINEERING TECHNIQUES: IDENTIFICATION OF PEPTIDE MIMOTOPES WHICH INHIBIT scFv-INSULIN BINDING

by

Douglas Fletcher Lake

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As members of the Final Examination Committee, we certify that we have read the document prepared by Douglas F. Lake, entitled "Construction and Mutagenesis of an Anti-insulin Single Chain Fv (scFv) using Protein Engineering Techniques: Identification of Peptide Mimotopes which Inhibit scFv-insulin Binding" and recommend that it be accepted as fulfilling the requirements for the Degree of Doctor of Philosophy.

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ABSTRACT

Peptide mimotopes were found using random peptide library technology (Selectide technology) which bind to the paratope of an anti-insulin monoclonal antibody. These peptides inhibit insulin from binding to the anti-insulin antibody, indicating that they share at least part of the paratope for insulin. The 50% inhibitory concentrations (IC50) of certain peptides were determined to be less than one order of magnitude from that of insulin. The immunoglobulin variable region genes of the anti-insulin antibody were rescued and cloned independently as VH and VK. A single chain Fv (scFv) was constructed using a polymerase chain reaction (PCR) assembly technique from the anti-insulin VH and VK. The scFv demonstrated similar, but not identical binding properties to the parental anti-insulin antibody. Binding of four of six peptide mimotopes was localized to the variable region of the anti-insulin heavy chain. The heavy chain alone was also able to bind insulin. None of the peptides bound to the variable region of the kappa light chain. Mutagenesis studies indicated that complementarity determining region (CDR) 1 and CDR3 of the heavy chain were important for binding to insulin and to the peptides. Position 99 in CDR3 of the heavy chain was found to be most critical for binding to insulin. Mutational analysis of position 99 revealed that glycine and alanine are acceptable residues for position 99, whereas valine and threonine are not. This finding suggested that residue size, not hydrophobicity determined whether the antibody could bind to insulin.
CHAPTER 1

INTRODUCTION

As early as 1798, Edward Jenner vaccinated humans with cowpox. The cowpox vaccinations induced cross-reactive, protective antibodies against smallpox. Nearly one hundred years later the natural role of antibodies was discovered as von Behring and Kitasato in 1890 and Ehrlich in 1897, recognized that antigen binding molecules from animals immunized with diphtheria or tetanus toxins could neutralize those agents. Since then, antibodies have become an increasingly useful tool, both diagnostically and clinically in the fight against infectious agents and cancers that cause disease.

The adaptive immune response can be divided into two parts: cell mediated and humoral responses. Antibodies are the product of the humoral immune response and are secreted by plasma cells derived from B lymphocytes. There is close collaboration between the two arms of the immune system. The immune response to an invading agent is very complex and even today is only partially understood. A brief description of the antibody response to an invading agent will be given below.

Upon introduction of a foreign agent into a host, the first cellular host response is phagocytosis of the immunogen by macrophages and various antigen presenting cells. Circulating B cells may also encounter and bind to antigen. Once a B cell has bound to the immunogen, the antigen is endocytosed, processed into peptides and presented in the context of major
histocompatibility complex (MHC) class II proteins to T-helper lymphocytes which are members of the cell mediated arm of the immune system. The MHC class II antigen, together with the antigenic peptide form a complex with the T cell receptor resulting in signal transduction and activation of the T-helper cell. T cell activation by an antigen causes a series of cytokines to be released, including interleukins (IL)-4 and IL-5. The interleukins may cause the specific B cell clone to proliferate and differentiate, respectively, in response to the antigen it encountered. Depending on the antigen and the antigen presenting cell, the class of the antibody also switches during proliferation from cell surface IgM and IgD to either μ, δ, α, ε, or γ. Different classes have various effector functions. B cells terminally differentiate into plasma cells which are short lived (three to four days) but secrete large quantities of immunoglobulin. In addition, memory B cells are generated which have cell surface antibody and the potential to rapidly proliferate if the antigen should be encountered again.

In the following sections I shall review several significant aspects of antibodies which include antibody structure, antigen-antibody interactions, immunoglobulin genes, applications of antibodies and some emerging technology which promises to revolutionize the development of diagnostic and therapeutic antibodies.

**Antibody Structure**

Biochemical and serological characterizations were performed on antibodies long before the crystal structures were solved. For example, under reducing conditions, heavy and light chains can be separated and
their individual molecular weights determined. The heavy chain of IgG has a molecular weight of approximately 50 kilo Daltons (kD) and the light chains have a molecular weight of 25kD. Digestion of an intact IgG with papain yields 2 products: Fab fragments (Fab = antigen binding fragment) and the disulfide bonded Fc (Fc = crystallizable fragment). The Fab contains antigen-binding properties and the Fc mediates the effector functions of the molecule since it contains the complement binding site and the binding site for Fc receptors found on various cells of the immune system such as phagocytic cells.

X-ray crystallographic analysis of immunoglobulin Fabs confirmed that antibodies (IgG) are composed of four polypeptide chains: two identical light chains and two identical heavy chains. The heavy and light chains pair with each other forming two symmetrical units connected by disulfide bonds. The light chains are composed of two domains, one variable domain (VL) and one constant domain (CL). The heavy chains for IgG contain four domains, one variable (VH) and 3 constant domains (CH1, CH2, and CH3). Each domain is approximately 110 amino acids in length. DNA sequence analysis also showed that immunoglobulins have individual domains that are similar. The domains are characterized by anti-parallel β-sheets and an interchain disulfide bond. The anti-parallel β-sheets of opposing domains pack together via lateral hydrogen bonding in a β-barrel structure. At the ends and between anti-parallel β-sheets, are loops. In the variable regions, these loops are hyper variable in length and amino acid composition. The loops contain the complementarity determining regions (CDRs) which have been implicated in binding to antigen (1).
The relationship between sequence and structure in CDRs has been used to develop canonical structure models for each of the heavy and light chain variable region loops except for heavy chain CDR3. Canonical structure modeling of antibody CDRs is based on positions within and adjacent to the complementarity determining regions involved in structure rather than specificity. Analysis of CDR sequences and known X-ray crystallographic structures have shown that there are only a few loop conformations for each CDR. The conformation of each loop is determined by conserved residues in and adjacent to the loops. For example, residues 48, 50, 51, 52 and 64 in CDR2 of light chains are conserved positions that determine light chain CDR2 canonical structure number 1. Ninety-five percent of both human and murine sequences fit this canonical structure. Crystal structure analysis has shown that different antibodies having hyper variable loops of the same size, but with different sequences have the same main chain conformation (2, 3). As the immunoglobulin structural database increases in size, canonical models may be used to model combining sites of antibodies of unknown structure. If accurate canonical models can be predicted for all types of antibodies, residues not involved in the structure of the hypervariable region, should be involved in binding to antigen. However, this remains to be proven.

**Antigen-Antibody Interactions**

The heavy and light chain variable regions have a total of six CDRs: three from the \( V_H \) and three from the \( V_L \). However, all six CDRs do not always participate equally in binding to antigen (4, 5). For example, heavy
chain CDR3 of an anti-lysozyme antibody, D1.3, contributes more contact residues than its other CDRs (6). On the other hand, light chain CDR3 of HyHEL-5 has more contact residues for lysozyme (7). The antigen binding domain(s) in the variable region are called the paratope of the antibody and the portion of the antigen which binds to the paratope is called the epitope. Just as the antibody paratope is discontinuous when it binds antigen, most epitopes on protein antigens are discontinuous (8, 9).

A few antibodies have been crystallized both bound to antigen and alone. One of the first antibodies to be co-crystallized with its antigen was McPC603 (5). In this study, Segal et al. showed that upon binding to phosphorylcholine, McPC603 did not change its conformation to accommodate antigen. Most other later studies, however, have shown that there is a conformational change in the antibody (and antigen) upon binding, even in hapten-antibody interactions (4, 10, 11). Such a case is the anti-neuraminidase antibody, NC-41 in which Colman et al. described the interaction between antigen and antibody similar to that of a handshake where both proteins change structure upon binding (4, 10). Similarly, Rini et al. demonstrated structural evidence for induced fit as a mechanism for peptide-antibody recognition (11). The antigen they used was a nonapeptide which bound primarily to light chain CDR3 and heavy chain CDR2 and CDR3. These and other structural studies have shown that the antigen combining site of an antibody is flexible and is composed of multiple pockets for antigen binding with a total area of 600 to 700Å² (12). The implications of these observations suggest that one antibody may be able to bind to at least two unrelated antigens by utilizing different portions of its paratope (9, 12).
**Immunoglobulin Genes**

Immunoglobulin genes are discontinuous. In humans, the heavy chain genes reside on chromosome 14, the kappa light chain genes reside on chromosome 2 and the lambda light chain genes reside on chromosome 22. In mice the heavy chain genes reside on chromosome 12, the kappa chain genes on chromosome 6 and the lambda chain genes on chromosome 16. On a single chromosome containing heavy chain genes, multiple variable region (V-region) genes, diversity (D) region genes and joining (J) region genes are separated by over 6 kilo bases from the constant region genes: m, d, a, e, and g. The different genes fuse together in a recombinatorial process, forming an antibody with a specificity defined by the joining of the V, D and J genes. A brief description follows below.

In the process of B cell development, heavy and light chain germline immunoglobulin (Ig) genes undergo rearrangement. First, one of the twelve germline D (diversity) segments is paired with one of four germline J (joining) segments. Then one of over 300 variable region gene segments pairs with the DJ, resulting in a heavy chain VDJ rearrangement. Only one allele from each V, D and J region rearranges. This is called allelic exclusion. Following heavy chain rearrangement, the light chain genes, kappa and lambda, undergo a similar rearrangement except that only VL and JL genes rearrange. Kappa chain genes rearrange first. If they do not rearrange successfully, then the lambda chain genes rearrange. Only one type, kappa or lambda is expressed and associates with the heavy chain. Allelic exclusion holds true for light chains as well as heavy chains.
Once both heavy and light chain genes successfully rearrange, the pre-B cell expresses cytoplasmic IgM. Then surface IgM (monomer) and IgD are expressed as the pre-B cell continues its ontogeny towards becoming a B cell, ready for antigen stimulation. If a B cell expressing surface Ig encounters a specific antigen during immune surveillance, it can be stimulated to proliferate. Proliferation of a specific B cell clone in response to a specific antigen occurs in a series of complex events. Once cell surface antibody has bound a specific antigen, the complex is internalized and the antigen is degraded into peptides and presented on the surface of the B cell in the context of MHC class II. Specific T cell receptors on T-helper cells recognize the processed antigen presented in context of MHC class II on the B cell surface which causes the T-helper cell to secrete various cytokines such as IL-4 and IL-5 which stimulate proliferation and differentiation of the B cell, expanding one specific clone of antibody-producing cells. One can imagine that the response to a bacterial glycoprotein could involve thousands of B cell clones, each proliferating in response to a different 600-700Å² epitope on the glycoprotein.

Immunoglobulin gene rearrangement from multiple germline genes constitutes the first mechanism of antibody diversity. Any D segment can pair with any J segment, while any V segment can pair with any DJ rearrangement. The same is true for light chains except that there is only VJ rearrangement and pairing, however, both kappa and lambda genes add to antibody diversity. In addition, imprecise V(D)J joining and N-nucleotide addition upon V(D)J joining adds to diversity. Also, any
rearranged light chain can pair with any heavy chain, resulting in the potential for even more antibody diversity.

Another mechanism of B cell diversity occurs when the B cell undergoes clonal proliferation. During an antigen-driven proliferation of B cells, mutations occur in the variable region genes of the antibody, causing antibodies produced by a few B cells to bind more strongly to antigen. This process is called affinity maturation. These B cells are preferentially stimulated over those that bind less strongly, possibly resulting in better neutralization of the infectious agent. When all the mechanisms for antibody diversity are taken into consideration, there are more than $10^7$ different variable region combinations.

**Molecular Cloning of Immunoglobulin Genes**

Genetic engineering technology has made it possible to clone, express and manipulate antibody genes. Rearranged immunoglobulin genes can be rescued from any given B cell population or from a hybridoma producing a potentially useful monoclonal antibody. Once the heavy and light immunoglobulin genes have been cloned and sequenced, they can be genetically engineered to possess properties not normally found in nature. Mouse / human recombinant, chimeric antibodies have been constructed (13) which retain the murine characteristic (variable regions) of binding to a tumor associated antigen, but contain human constant regions for increased serum half life (14). Chimeric antibodies have also been constructed where one arm of the antibody binds to a receptor on a tumor cell and the other arm consists of an anti-cancer agent such as tumor
necrosis factor (TNF) (15, 16) or a cytotoxic agent such as ricin A chain (17). These genetic constructs allow one to select the desired antigen-binding variable regions and clone those genes into a vector able to express human constant regions resulting in a humanized chimeric antibody with the specificity of the original variable regions. Some of these humanized antibodies and immunotoxins are in clinical trials, so it remains to be determined if these antibodies will be useful in the clinic.

**Single Chain Antigen Binding Proteins**

Single chain Fvs (scFv) are composed of immunoglobulin variable heavy (V\text{H}) and variable light (V\text{L}) chains joined together by a flexible peptide linker. The first reports of scFvs demonstrated that rearranged heavy and light chain immunoglobulin variable regions could be engineered for expression from bacteria as a single polypeptide which retained the ability to bind antigen (18, 19). Earlier, it was known from the work of Skerra and Pluckthun that Fvs could be expressed in *E. coli* and assembled into a V\text{H}-V\text{L} heterodimer which retained binding specificity (20). Different types of scFv peptide linkers have been designed to join the heavy and light variable chains. The most common linker used is (Gly4-Ser)3 because it is hydrophilic, flexible and relatively free of side chains that might interfere with scFv refolding or the capacity to bind to antigen (21). However, other more rigid linkers have been designed and used with success (18).

Various single chain Fvs have been generated for use in many diverse applications. One application of scFvs is in tumor imaging
diagnostics and therapy. Many murine monoclonal antibodies have been made against many different tumor types (22-25). When these murine monoclonal antibodies are infused into cancer patients, a human anti-mouse antibody (HAMA) response usually develops (26). Most of the HAMA response is directed toward the constant regions of the murine anti-tumor antibody, so after two or three infusions of the antibody, it is rapidly cleared from circulation. Once the antibody is no longer in circulation, it can no longer target its antigen. Since scFvs contain only the variable regions, they are less immunogenic (26). However, the small size of scFvs also causes them to be rapidly cleared from circulation.

In addition to decreased immunogenicity of the scFvs, Yokota et al. have reported that scFvs penetrate the micro-vasculature of solid tumors faster and more evenly than intact IgG, F(ab')2 and Fab fragments in an experimental colon carcinoma xenograft model (27). Furthermore, when directly compared to intact anti-tumor IgG, the scFv demonstrated deeper penetration distal to blood vessels. They also reported faster whole body clearance than intact IgG and Fab fragments. This suggests that the relatively small size of a scFv might make them more effective for solid tumor therapy.

Other studies have used protein engineering techniques to develop cytotoxic fusion proteins. Brinkmann et al. expressed a scFv-immunotoxin fusion protein from bacteria using gene cloning techniques (28). The scFv was generated from the variable region genes of the murine monoclonal antibody B3, which recognizes a carbohydrate antigen on the surface of some human carcinomas. The immunotoxin used was truncated
*Pseudomonas* exotoxin which lacked the cell-binding domain. This study showed selective cytotoxicity to human tumor cells bearing the B3 antigen in cell culture and caused complete regression of human tumors growing in immunodeficient mice.

Hoogenboom et al. constructed an anti-transferrin receptor-tumor necrosis factor (TNF) Fab-like conjugate from anti-transferrin receptor antibody genes and TNF DNA. This study demonstrated cytotoxicity towards TNF-sensitive L929 cells and human MCF-7 cells mediated by the anti-transferrin receptor-TNF chimera *in vitro*. Although TNF alone is toxic to these cells, excess soluble transferrin receptor blocked the cytotoxicity, suggesting that the anti-transferrin receptor-TNF chimera was mediating the specific cytotoxicity. Since the anti-transferrin receptor antibody variable regions were of murine origin and the constant regions were human the study also demonstrated that a humanized immunotoxin-like chimeric molecule may have potential use in cancer immunotherapy.

One area of scFv antibody technology which is rapidly growing is the generation of combinatorial immunoglobulin libraries displayed on the surface of filamentous bacteriophage (29-33). This approach for expressing the variable regions of antibodies fused to a phage gene product is based upon the initial phage display work of Parmley and Smith (34). This powerful technology is performed by harvesting mRNA from rearranged immunoglobulin variable region genes from immunized mice (35) or humans (36). Polymerase chain reaction (PCR) (37) is then employed to amplify the immunoglobulin variable regions. The variable regions can then be cloned, randomly, into a phagemid vector for Fab phage display (38).
or as scFvs (29), creating a combinatorial phage display antibody library. The phage display library can then be used for screening against a specific target antigen bound to a solid support.

Just as a B cell clone is selected by antigen to proliferate in vivo, phage expressing scFv from a combinatorial library can be selected by a target antigen to "proliferate." Once specific phage displaying antibody variable region gene products have been selected by antigen, they can be enriched by infecting susceptible bacteria and harvesting progeny phage. Then the antigen selection process can be repeated several times to obtain high affinity antibody fragments on the phage surface. Burton et al. used this strategy with bone marrow aspirates from HIV-1 positive donors to generate anti-HIV-1 Fabs (Phabs) displayed on the surface of phage which bound to gp120 (31).

The most recent and perhaps the most promising area employing phage display libraries is the selection of antigen-binding phage from non-immunized combinatorial immunoglobulin libraries. Marks et al. used the strategies described above to generate an immunoglobulin combinatorial library from non-immunized human peripheral blood lymphocytes which contained a theoretical $10^7$ different members for phage display (39). Screening the library with turkey egg-white lysozyme (TEL), bovine serum albumin (BSA), and 2-phenyloxazol-5-one (phOx) hapten resulted in selection of several phage displayed scFvs which bound to each antigen except for BSA which just selected one scFv. Binding affinities of the scFvs were $10^7$ M$^{-1}$ or TEL and $2 \times 10^6$ M$^{-1}$ for phOx. Higher affinity antibodies could be generated by randomizing the codons in the CDRs as was
demonstrated by Ahrweiler et al (40). Rescue of specific antigen binding antibody fragments from naive lymphocytes represents a major step toward antibody development without the need to immunize laboratory animals. In addition, human monoclonal antibodies could be developed against pathogens when a source of immunized lymphocytes is not available.

**Random Peptide Libraries**

Recent advances in drug discovery processes have included screening peptide libraries to discover peptide ligands which are biologically active. Generally, the number of peptides in a library range between $10^4$ to $10^8$, depending on the method used to generate the library. One method which has been developed involves the use of filamentous bacteriophage (41). Random peptide libraries on the surface of bacteriophage (phage) can be generated by inserting a randomly generated oligonucleotide into gene III of the phage. The resulting phage display random peptides at the amino terminus of the viral pIII coat protein. Phage with peptides which bind to the acceptor molecule of interest will be selected and can be amplified through multiple rounds of screening. Gene III of selected phage can be sequenced to elucidate the active peptide.

Another method of generating random peptide libraries consists of the chemical synthesis of free peptides in solution (42). For this method, sequential positions in the peptide are fixed while all other positions are randomized. In this manner, the sequence of the active peptide may be obtained by defining which amino acid at each sequential position shows the greatest amount of reactivity with the acceptor molecule. The
methodology used to generate random peptide libraries in this dissertation is based on Selectide technology, or the one-bead one-peptide approach (43). The Selectide process utilizes "split synthesis" in which the beads are divided and exposed to only one amino acid at a time so that each bead contains a single peptide species. The above peptide library technologies each have advantages and disadvantages, but allow identification of peptide ligands which bind with high affinity to acceptor molecules.

**Rationale for Study**

Since molecular biology techniques have become available for cloning and manipulating antibody genes, it became possible to investigate, at the molecular level, interactions between ligand and receptor. These molecular interactions were studied by mutating specific amino acids in the CDRs of the receptor (antibody).

Peptide ligands specific for the antibody variable regions were obtained by screening random peptide libraries with the antibody of interest using the Selectide process. Upon screening the library, the antibody, labeled with biotin, bound specifically to peptides coupled to beads. The peptide-antibody-bead complex was detected with streptavidin coupled to an enzyme after which the reactive beads were stained. Individually stained beads were then subjected to microsequencing and the peptide sequences which bound to antibody were elucidated. Characteristically, peptide families or sequence motifs were obtained after sequencing several reactive beads.
The following study is important because it elucidates, through protein engineering, the critical antibody variable region residues responsible for binding to a protein antigen. The ability to mutate an antibody variable region so that it loses and then regains binding activity is a powerful tool. By knowing which variable region residues are important for binding to a given ligand and which residues are not, antibody variable regions may be designed, constructed and tested for the ability to bind a complementary ligand.

This study also demonstrates that peptide ligands can be found which mimic a conformational determinant on a globular protein and that the affinity of specific peptides for an antibody can be increased through randomization of certain positions in the peptide. One might hypothesize that a peptide which bound to a clinically relevant receptor with a higher affinity than the natural ligand itself, would have the potential to be used in a therapeutic application such as specific peptides for IL-2 receptors or the 170kD protein on the surface of tumor cells responsible for multi-drug resistance. Understanding the complementary relationship between amino acids on the receptor and ligand at the molecular level will enable the rational design of potent biologically-based drugs.
CHAPTER 2

BACKGROUND

Cloning and Expression of OCILY8 Lymphoma Immunoglobulin

The initial intent of this receptor-ligand study was to rescue the rearranged immunoglobulin (Ig) genes from an IgM, surface positive B cell lymphoma and then clone and express the immunoglobulin heavy and light variable regions as well as a Fab from bacteria. Specific peptide families would then have been found with random peptide library technology (43) using pure recombinant, biotinylated lymphoma immunoglobulin variable regions as probes. It was hypothesized that the peptides selected from the library by the recombinant lymphoma Ig fragments might have therapeutic potential by inducing the lymphoma cells to terminally differentiate, undergo apoptosis or be able to directly kill the cell using a radioactive or toxin-labeled peptide. Antibody-induced apoptosis has been demonstrated with a B cell lymphoma (44).

Peptide ligands against the lymphoma would have the advantage of being quickly identified using the purified receptor as a probe in random peptide library screening as well as the advantage of being easily modified and elongated by the addition of amino acids at any position in the peptide. The cloned antibody fragments would have the advantages of being a pure source of a receptor unique to the surface of one particular tumor cell type. The cloned antibody fragments could also be quite easily mutated to model
somatic mutation that has been reported (45-47) in humans undergoing anti-idiotype therapy.

As described in the following Methods chapter, polymerase chain reaction (PCR) was used to amplify the rearranged heavy and light immunoglobulin genes. The immunoglobulin genes were then cloned and sequenced. Figure A1 and A2 in Appendix A show the nucleotide and amino acid sequences of both the heavy and light variable regions of a human B cell immunoblastic lymphoma, designated OCILY8.

Variable heavy and CH1 as well as variable lambda and constant lambda light chain were cloned into the pComb3 phagemid expression vector system (36, 48) and expressed from transformed bacteria as a Fab and on the pIII gene product (Phab) of filamentous bacteriophage. A diagram of the pComb3 phagemid expression vector system is shown in figure A3, Appendix A.

Expression of the antibody fragments from the pComb3 phagemid transformed E. coli was transient and unstable at best. As a result, expression levels of Fab were very low. Minute amounts of l light and m heavy chain were detected in the bacterial culture medium in western blotting analysis as shown in figure A4, Appendix A. However, most of the antibody fragments were cell lysate associated.

A murine anti-idiotype antibody had previously been generated against the OCILY8 lymphoma antibody from "rescue fusion" immunoglobulin. The rescue fusion was generated by fusing the OCILY8 human B lymphoma cells with a human-mouse hetero-myeloma fusion partner resulting in a hybridoma which had the ability to secrete OCILY8
immunoglobulin. Subsequently, it was found that the rescue fusion secreted mainly lambda chain and mu heavy chain only at very low levels. As a result the anti-idiotype antibody recognized the lambda light chain homo-dimer paratope, not the mu-lambda heterodimer paratope. This result is shown in figure A5 of Appendix A.

The cloned OCILY8 antibody fragments were produced in bacteria, purified, biotinylated and used as probes for screening a random peptide library. Only a few beads stained positively and they were subjected to amino acid sequencing. There was no consensus sequence obtained from the sequence analysis. In any case, one sequence was chosen, RFYIL, and the peptide was synthesized on beads for reconfirmation. Attempts to re-stain the beads with the OCILY8 lambda chain resulted in positive results, however, attempts at re-screening other libraries for peptides with the same or similar motifs did not prove fruitful.

Based upon the inability to select peptide families using the OCILY8 immunoglobulin fragments as probes and the limited reactivity of the anti-idiotype (reacted only with lambda chain, not mu-lambda heterodimer), it was decided to pursue another model system which consisted of the following characteristics:

1. Intact parent IgG readily available from hybridoma.
2. Parent IgG binds to a defined antigen.
3. Parent IgG has the capacity to select peptides from random peptide libraries.
A murine hybridoma, designated HB125, which produces an anti-insulin IgG1 with a kappa light chain was used as a model system to study peptide ligand-receptor interactions because it possessed the above attributes. Table B1 in Appendix B lists some of the characteristics of HB125 antibody. HB125 anti-insulin producing antibody was generated by Schroer et al. in 1983 for studies to map antigenic determinants on the insulin molecule (49). HB125 antibody was found to bind to a conformational determinant on insulin. When the A chain and B chain of insulin were dissociated, HB125 antibody failed to bind to either chain. This result suggests that the epitope recognized by HB125 antibody is conformational. Therefore, by definition, any peptide found to bind to the paratope of HB125 and inhibit insulin from binding to HB125 would be a mimotope (50). Geysen defined a mimotope as, "... a molecule able to bind to the antigen combining site of an antibody molecule, not necessarily identical with the epitope inducing the antibody, but an acceptable mimic of the essential features of the epitope." Indeed, several peptides were selected from random libraries by the parental HB125 antibody. The motifs of the selected peptides from primary screenings are shown in table B2 in Appendix B. Based upon the sequences of the selected peptides, a secondary library was generated by fixing specific positions in the peptides which showed a preference for a specific amino acid while the other positions were randomized in sequential screenings. Ligands with higher affinities were generated in this manner.
The remainder of this dissertation concerns the study of HB125 antibody variable regions binding to insulin and insulin mimotopes at the molecular level.
CHAPTER 3

METHODS

Messenger RNA Isolation and cDNA synthesis

Lymphoma or hybridoma cells were cultured in RPMI 1640 medium containing 10% fetal calf serum and 100μg/ml gentamycin sulfate in a 5% CO2 humidified chamber. Cells were grown to a density of 1 x 10^6 cells/ml after which 10m! of cells were pelleted by centrifugation at 1000g. The cells were washed once in phosphate buffered saline and re-suspended in lysis buffer in the presence of proteinase K at 50°C for 30 minutes. The chromosomal DNA was fragmented with a 20-gauge needle and syringe so that the mRNA was able to be recovered with oligo dT cellulose. The mRNA was eluted from the oligo dT cellulose in distilled water and reverse transcribed for 2 hours at 42°C using oligo deoxythymidine (dT) as the reverse transcriptase primer to synthesize cDNA from the mRNA template. Reverse transcriptase was subsequently inactivated for 2 minutes at 99°C.

Polymerase Chain Reaction (PCR) of OCILY8 Lymphoma Immunoglobulin Variable and Constant Regions

PCR amplification (37) was performed for both immunoglobulin heavy and light chains using cDNA as a template. PCR amplification of lambda light chain was initially carried out using reported degenerate oligonucleotide primers (51) for the NH2 terminal leader sequence of the
lambda light chain (sense) and the N-terminal portion of the lambda constant region (anti-sense) (Appendix C). PCR amplification of the heavy chain was performed using a mixture of 3 degenerate primers for the leader sequence of human Ig heavy chain and the N-terminal portion of the \( \mu \)-constant region (Appendix C). PCR cycling temperatures were as follows: Melt 94°C, 1 minute; primer anneal 50°C, 2 minutes; primer extension 74°C, 3 minutes. PCR was run in a thermocycler for 30 cycles in a reaction volume of 50 or 100μl.

Additional primers for the heavy and light chains were synthesized with specific restriction endonuclease sites for cloning into the pCOMB3 phagemid expression vector (48).

**Cloning of OCILY8 Immunoglobulin Fragments into Sequencing and Expression Vectors**

The heavy and light chain PCR products were electrophoresed on a 0.9% agarose gel, excised from the gel and purified on glass beads. The DNA fragments were then blunt ended using the Klenow fragment of DNA polymerase I. A 5' phosphate was added to the blunt ended PCR product by T4 polynucleotide kinase treatment. Klenow fragment and T4 polynucleotide kinase were removed from the DNA by purifying the DNA once again on glass beads. Then the sequencing vector, pTZ, was cut with the restriction endonuclease \( SmaI \), treated with calf intestinal phosphatase (CIP), purified on an agarose gel, excised from the gel and further purified on glass beads.
Both the immunoglobulin DNA insert and the vector were quantitated on a 0.9% agarose gel by ethidium bromide staining before ligating the insert into the vector. The ligation reaction consisted of either 5 or 10 fold excess insert over vector concentration. Ten units of T4 DNA ligase were added and the ligation reaction was incubated at 16°C for 12 hours.

The ligated DNA was used to transform competent *E. coli* (XL-1 blue). Competent XL-1 blue cells (50µl) were thawed from -70°C and mixed with one half of the ligation reaction for 30 minutes on ice. The cells were heat shocked at 42°C for 60 seconds, 100µl S.O.C. media was added to the cells after which the cells were incubated at 37°C for 1 hour. Finally, the transformed cells were plated on Luria Bertoni (LB) agar containing 50µg/ml carbenicillin and incubated at 37°C overnight. Individual colonies were picked from the plate and grown to stationary phase in LB broth containing 50µg/ml carbenicillin.

Plasmid DNA was isolated from the transformed XL-1 blue culture and to ensure successful ligation of the immunoglobulin insert the immunoglobulin fragment was digested out of the plasmid with the appropriate restriction enzymes. Plasmid DNA was obtained from the bacterial cells using the following methodology. Plasmid DNA was isolated by pelleting the transformed XL-1 blue cells from 1.5ml of stationary phase culture at 10,000g for 5 minutes. The cell pellet was re-suspended in 150µl 50mM Tris-HCl, pH 8.0 containing 10 units/ml RNase. The cells were then lysed with 150µl of 1% SDS in 0.2M NaOH after which the chromosomal DNA and cellular proteins were precipitated by adding 150µl of 2.55M
potassium acetate, pH 4.8. The precipitate was pelleted in a microfuge for 10 minutes at 10,000g after which the supernatant was pipetted into a separate tube. The plasmid DNA in the supernatant was then precipitated by the addition of 100% ethanol and pelleted for 15 minutes at 10,000g in a microfuge at 4°C. Then the DNA pellet was washed with 70% ethanol, pelleted again as above and lyophilized to dryness. The plasmid DNA was then reconstituted with water and digested with appropriate restriction endonucleases both 5' and 3' to the insert and electrophoresed on a 1% agarose gel to confirm that the immunoglobulin was inserted into the vector.

**PCR Screening of Transformed Bacterial Colonies**

As an alternative to picking transformed bacterial colonies, growing the colony in liquid culture, isolating plasmid DNA from the cells, and performing a restriction endonuclease digest, PCR screening of transformed colonies was performed. After the transformed colonies from a ligation had grown to an approximate diameter of 1mm on an LB plate containing carbenicillin, a micropipet tip was used to pick the colony from the plate. The colony was then resuspended in 40µl of water and boiled at 100°C for 3 minutes. The boiled bacteria was then pelleted by centrifugation at 12,000g. Ten microliters of the supernatant from the boiled bacteria colony was used as template in a PCR with primers specific for either heavy or light chain immunoglobulin 5' and 3' variable regions. After a 20 cycle PCR using the conditions described above, the PCR products were electrophoresed on a 0.9% agarose gel.
**DNA Sequencing of the Immunoglobulin Insert**

Double-stranded, Sanger dideoxy-nucleotide sequencing methodology was used (52). Prior to sequencing the plasmid template was purified on glass beads. The template was then denatured in 0.2M NaOH, 0.2mM EDTA for 30 minutes, neutralized in 3M sodium acetate, precipitated with 100% ethanol, washed in 70% ethanol, dried and then reconstituted with 7μl distilled water. The appropriate primer was added and the sequencing reaction was carried out using Sequenase (modified T7 DNA polymerase, United States Biochemical Corp.) and Sequetide (dithiothreitol, 35S-dideoxyATP (dATP), dideoxyATP (ddATP), ddCTP, ddGTP and ddTTP. Finally the sequencing reactions were loaded onto a 7.8M urea, 5% acrylamide gel and electrophoresed at 50 watts. The gel was dried, and exposed to Kodak XAR-5 film for 24-48 hours after which the film was developed. The sequence was compared to the Genbank database to ensure that the sequence was immunoglobulin.

**Expression of Soluble OCILY8 Immunoglobulin Fragments from the pCOMB3 Vector**

Prior to expression of the soluble immunoglobulin fragment, it was necessary to remove the phage surface protein, gene III, from the expression construct. This was done by digesting the phagemid with NheI and SpeI, gel purifying the digested plasmid away from gene III and self ligating the compatible cohesive ends. The resulting phagemid then produced soluble Ig fragments that were directed toward the periplasm of
the bacteria via the pel B signal peptide. The pel B signal peptide was cleaved in the periplasm of the bacteria resulting in secretion of a soluble Ig fragment.

In practice, bacteria harboring the phagemid were inoculated into a flask containing Superbroth (Tryptone, Yeast extract, NaCl/liter) and grown at 37°C until the cells were in mid log phase (0.8 O.D.). Then indolylpyranoside thio-galactose (IPTG) was added to the culture for a final concentration of 1mM and the cells were further incubated at room temperature for 4 to 16 hours.

**Preparation of Bacterial Cell lysates for HB125 or OCILY8 Immunoglobulin Fragment Isolation**

Three to five hour cultures containing *E. coli* producing either VH, VK or OCILY8 Ig fragments were subjected to centrifugation for 10 minutes in an SS-34 rotor at 8,000 rpm. In the case of pCOMB3 transformed cells, the supernatant was saved and sampled for SDS-PAGE analysis. The cell pellet was washed 3 times in refolding buffer (50mM Tris-HCl, 50mM KCl, 10mM CaCl2, pH 8.0) followed by sonication at 50 Watts for 45 seconds (15 seconds each) and centrifugation at 12,000 rpm after each wash. After the third centrifugation, the cell lysate was resuspended in 6M urea, pH 8.0 and sonicated once more as above. The lysate was then incubated at 4°C for 4 hours or overnight. Finally the urea solublized cell pellet was subjected to centrifugation at 12,000 rpm in the SS-34 rotor and the supernatant was removed for SDS-PAGE analysis and further purification.
At this point in the OCILY8 study, the decision was made to concentrate efforts on the HB125 study for the reasons enumerated in the Background chapter.

Construction on HB125 scFv

HB125 VH and Vk PCR products were subjected to a second round of PCR so that a (Gly4-Ser)3 could be constructed between the two variable regions. VH was amplified by PCR using a 5' sense primer containing an NcoI site and a 3' anti-sense primer with one unit of Gly4-Ser. Vk was amplified by PCR using a 5' sense primer with three units of Gly4-Ser and a 3' anti-sense primer containing an XhoI site. One microliter of each of the VH-Gly4-Ser and (Gly4-Ser)3-Vk PCR products was added to a third and final PCR along with the 5' sense NcoI and 3' ant-sense XhoI primers. The product from this reaction was the assembled scFv. All primers were designed so that the resulting PCR products, once cloned into the expression vector, were ready for "in frame" expression of the antibody fragments. Sequences of primers are listed in Appendix C.

Cloning and Expression of Immunoglobulin Fragments in pET Vectors

Once the variable regions were amplified by PCR using primers with the proper restriction endonuclease sites on the ends of the products, they were cloned into a sequencing vector designated, pT7blue (53) via the 3' adenosine that Taq polymerase has been reported to add to the end of PCR products (54). The immunoglobulin inserts were then digested out of pT7blue, gel purified and ligated into the pET vector. The ligation reaction
was used to transform Novablue cells. Transformed cells were plated and colonies were allowed to grow overnight after which they were screened by PCR. Colonies which showed a positive PCR, were grown in liquid culture and then the plasmid DNA was isolated from them by a mini-prep procedure described above. The plasmid DNA was then used to transform BL21 (DE3) E. coli. Transformed colonies were picked from the plate and grown to mid log in LB medium after which they were pelleted by centrifugation and resuspended in 50ml of Superbroth. The culture was grown for one hour and IPTG was added and further cultured for 3 hours to induce over production of the immunoglobulin fragment. The liquid culture was then subjected to centrifugation at 10,000 rpm in an SS-34 rotor. The cell pellet was washed once with refolding buffer, pelleted again and then resuspended in 2ml of 6M urea. The 6M urea-solublized bacterial lysates were then sonicated three times for fifteen seconds each time.

**Superose 12 column fractionation of HB125 Immunoglobulin Fragments**

Bacterial cell lysates solublized in 6M urea were pelleted at 10,000 rpm in an SS-34 rotor for 10 minutes and filtered through a 0.45μm membrane. The lysate was then loaded onto a 32 cm Superose 12 column in a maximum volume of one milliliter. The elution solvent was 2M urea in PBS.

**Biotinylation of Immunoglobulin Fragments**

Purified fractions containing immunoglobulin fragments (e.g. VH, Vk or scFv) were dialyzed against 2 liters of 50mM NaHCO3, pH 8.0 for at
least 6 hours. The protein content of the purified material was then quantified (Bradford). NHS-LC-Biotin was then solubilized in 50mM NaHCO₃, pH 8.5 and added to the dialyzed fractions such that there was between 9 and 17 biotin molecules per molecule of immunoglobulin fragment on a molar basis. Biotinylation was performed at room temperature for 2 hours after which Tris-HCl, pH 7.9 (15mM final) was added to stop the Biotinylation reaction. The immunoglobulin fragments were tested in a streptavidin-Ig fragment-streptavidin-HRP after each biotinylation against a control biotinylated molecule.

**Variable Heavy Chain Inhibition ELISA**

Fractions from the Superose 12 molecular weight sieving column were dialyzed and coated onto an ELISA plate for 1 hour and then the plate was blocked using PBSTw-0.1% gelatin. Unlabeled insulin (40μg/ml) was added to one set of fractions while 0.1% gelatin was added as a control to the other. After a one hour incubation, the plate was washed once in PBSTw-0.1% gelatin and 4μg/ml of biotinylated insulin was added in either 0.1% gelatin or in the presence of 40μg/ml of unlabeled insulin. One hour later the plate was washed 3 times with PBSTw-0.1% gelatin and streptavidin-coupled to alkaline phosphatase was added. The plate was incubated and washed as before after which 1mg/ml p-nitrophenyl phosphate (pnpp) substrate in carbonate buffer pH 9.6 was added and the optical densities at 405nm were quantified on a Titertek multiskan ELISA reader.
**Peptide Inhibition of HB125 Immunoglobulin ELISA**

Flat bottom ELISA plates coated with 1μg/ml insulin were used. Ten-fold serial dilutions of peptides starting at 5 x 10^{-5}M were incubated with either biotinylated HB125 antibody or biotinylated, recombinant HB125 antibody fragments in PBSTw-gel in the insulin-coated plates for a minimum of 2 hours. The plates were washed three times in PBSTw-gel after which streptavidin-horse radish peroxidase was added to the plates and further incubated for 1 hour. The plates were then washed as before and 0.225μg/ml TMBZ substrate was added. The plates were allowed to develop and the optical densities at 450nm were quantified.

**Control Experiment: Peptide Inhibition of MOPC315**

This control experiment was performed exactly as the insulin-based inhibition ELISA. 2,4-Dinitrobenzene (DNB) was coupled to bovine serum albumin (BSA) by mixing the two together in 50mM NaHCO3 at a 10:1 (DNB to BSA) ratio in the dark for 2 hours. The BSA turned a bright yellow upon coupling to DNB. Two micrograms per milliliter of DNB-BSA were coated onto an ELISA plate at 37°C for 1 hour, after which the plate was blocked with PBSTw-gel at 37°C for 1 hour. Then either 10-fold dilutions of peptides or DNB-BSA were pre-incubated with MOPC315 for 1 hour and then added to the DNB-BSA coated plate. The mixture was incubated in the DNB-BSA coated plate for 1 hour and then washed out. MOPC315 was detected with anti-IgA-HRP anti-sera. The remainder of the experiment was performed identically to the insulin-base HB125 peptide inhibition ELISA described above.
Purification of HB125 scFv on an Insulin-Agarose Column

Bacterial cell lysates solublized in 6M urea were dialyzed overnight against four liters of PBS alone, 2M urea in PBS, or in 2M urea, 0.1mM glutathione, 5mM dithiothreitol (DTT) in PBS. After dialysis, precipitated material was removed by centrifugation at 12,000 rpm for 10 minutes in an SS-34 rotor. Soluble material containing HB125 scFv was filtered through a 0.45μm membrane and loaded onto a PBS-equilibrated insulin-agarose column. The column was washed with 20 bed volumes of PBS. HB125 scFv was eluted from the column with 5ml of 100mM glycine, pH 2.3 in fractions of 1ml each. Fractions were neutralized to pH 7.0 with 1M Tris-base. Ten microliters from each fraction were analyzed by SDS-PAGE followed by Coomassie blue staining.

Purification of Immunoglobulin Fragments on a Nickel-Agarose Column

Immunoglobulin fragment-producing bacterial cultures were processed as in the section, "Preparation of Bacterial Cell lysates for Ig Fragment Isolation." A nickel-agarose column was prepared by charging the metal chelating linker on the agarose with 50mM NiSO₄·6H₂O (55, 56). Then clarified and 0.45μm filtered bacterial cell lysates in binding buffer (6M urea, 0.5M NaCl, 5mM imidazole, 20mM Tris-HCl, pH 7.9) were run through the pre-equilibrated column at a flow rate of 0.3ml/minute. The column was then washed with 15 bed volumes of binding buffer followed by 10 bed volumes of wash buffer (6M urea, 0.5M NaCl, 60mM imidazole, 20mM Tris-HCl, pH 7.9). The poly-histidine-containing Ig fragment was then eluted from the column in 1ml fractions with elute buffer (6M urea,
1M imidazole, 0.25M NaCl, 10mM Tris-HCl, pH 7.9) The purity of the fractions were then analyzed by SDS-PAGE followed by Coomassie blue staining. Fractions containing purified proteins were pooled and dialyzed against refolding buffer.

**PCR-based mutagenesis**

Multiple site-directed mutagenesis of the immunoglobulin CDRs was performed using PCR single overlap extension (PCR soeing)(57, 58). Overlapping sense and anti-sense primers were designed with one or more mutations which changed amino acids in the CDRs. An example is shown below.

**VH CDR1**

\[
\text{GCTTCT GGTTACCTTCACA GACTATTCATGCAC TGGGTGAAGCAGGCT CCAGGA} \\
\text{A S G Y T F T D Y S M H W V K Q A P G} \\
\text{5'--AAATATGGCATGAAC TGGGTGAAGCAGGCT--3'}-> \\
\text{GCTTCT GGTTACCTTCACA GACTATTCATGCAC TGGGTGAAGCAGGCT CCAGGA} \\
\text{<-3'--CCAATATGAAGTGT TTTATCCTGACTTG--5'}
\]

Figure 1. Diagram of PCR mutagenesis in CDR1 of HB125 variable heavy chain gene. Mutated amino acids are shown in bold and mutated codons incorporated into the primers are shown in italics. In the example shown above, aspartic acid (D), serine (S), and histidine (H) were changed to lysine (K), glycine (G) and asparagine (N), respectively. The mutations were made using the primer set above and the 5' and 3' scFv flanking primers containing NcoI and XhoI restriction endonuclease sites (Appendix B).
**Single chain Fv insulin binding ELISA**

Purified scFv mutants were serially diluted and coated onto 96-well microtiter ELISA plates in carbonate buffer for 1 hour at 37°C. The plates were blocked with PBSTw-gel at 37°C for hour. Biotinylated insulin was then added to the plates at a concentration of 0.5μg/ml and incubated at room temperature for 1 hour. The plates were then washed twice with PBSTw-gel followed by the addition of streptavidin-horse radish peroxidase (HRP). The plates were further incubated for 1 hour at room temperature and washed three times as before. Finally, TMBZ substrate was added to the plates and the optical density of each well was quantified on an ELISA reader.

**Random Peptide Library Synthesis**

Random peptide libraries were synthesized such that each resin solid-phase support (bead) possesses a single peptide species. This process is called the "one-bead one-peptide" concept (43). In the one-bead one-peptide concept, each bead is exposed to a single amino acid in each coupling cycle. Separate coupling cycles may be repeated after thorough mixing (randomization) of the beads until a peptides of desired length are obtained.

Polydimethylacrylamide resin beads (50-300μm in diameter, 0.3-0.5mmol/g substitution) were used for the random peptide library synthesis. The chemical peptide synthesis was performed according to the methods of Atherton and Sheppard (59). Approximately two million beads (three grams of resin) were mixed with ethylenediamine overnight and
then washed thoroughly. Two repeating units of e-aminocaproic acid, β-alanine (βεβε-bead) were coupled to the resin using Fmoc chemistry without a cleavable linker. The next five positions were randomized using split synthesis methodology (43, 60) with all nineteen eukaryotic amino acids except cysteine, each separately, at each coupling step. After the five randomization steps were completed, the beads were exposed to 20% piperidine in dimethylformamide (DMF) to cleave the N-terminal Fmoc from the peptide. The side chain protecting groups were removed from the peptides with a mixture of trifluoroacetic acid-phenol-anisole-ethanedithiol (94:2:2:2, v/w/v/v). The deprotected beads were then washed with DMF and neutralized with 10% diisopropylethylamine in DMF and stored until use in DMF.

**Screening and Selection of Peptide Ligands from the Peptide Library**

Prior to screening, the beads were washed free of DMF with increasing amounts of water and 0.1% gelatin to block any non-specific binding. Biotinylated HB125 antibody was then incubated with the library at a concentration of 10-100ng/ml for at least 4 hours. The beads were then washed free of unbound HB125 antibody. Streptavidin coupled to alkaline phosphatase was then added to the beads at a pre-determined dilution and incubated for 1 hour. The beads were washed as before and then BCIP substrate was added. The library was then transferred to petri plates where the reactive beads were allowed to develop for 2 hours. Only the beads with peptides which reacted with HB125 turned turquoise, while the majority of the beads remained colorless.
Peptide Sequencing

Positive beads were physically removed from the remainder of the library and treated with 8M guanidium hydrochloride, pH 1.0. The beads were then washed with distilled water, individually placed on glass filters and inserted into an Applied Biosystems microsequencer where the amino acid sequences of the peptides were obtained.
CHAPTER 4

RESULTS

CLONING AND SEQUENCE ANALYSIS OF IMMUNOGLOBULIN HEAVY AND LIGHT CHAIN VARIABLE REGIONS

Rescue of Anti-Insulin Immunoglobulin Heavy and Light Variable Regions

Messenger RNA (mRNA) was isolated from an anti-insulin immunoglobulin-producing hybridoma, designated HB125, using oligo dT-cellulose followed by reverse transcription into cDNA. Polymerase chain reaction (PCR) was employed to amplify the immunoglobulin variable heavy and variable light regions using sets of sense and anti-sense oligonucleotide primers specific for murine immunoglobulin gamma and kappa chains. A diagram of the methodology for obtaining immunoglobulin heavy and light chain PCR products from hybridoma cells is shown in figure 2. The results of the PCR amplification for both heavy and light variable regions are shown in figures 3A and 3B. In figure 3A, heavy chain primer set "C" amplified HB125 VH resulting in a PCR product of approximately 450 base pairs (bp). Other weak bands of various sizes on the agarose gel are the result of mis-primings. Figure 3B shows two PCR products for light chain primer sets "B" and "C." Subsequent DNA sequence analysis of the PCR product amplified by primer set "B" indicated that it was the kappa variable region from the fusion partner, Sp2/0-Ag14, used to generate HB125 hybridoma (61). Light chain primer set
"C" amplified the true anti-insulin kappa chain PCR product which can be seen in figure 3B at approximately 430bp.

Figure 2. Strategy for the isolation of immunoglobulin variable regions from hybridoma cells.
Figure 3. PCR amplification of anti-insulin variable heavy and variable light chain genes. Figure 3A shows a 0.9% agarose gel of the variable heavy chain PCRs. Figure 3B shows a 0.9% gel of the variable light chain PCRs. Lanes are designated by the primers sets used in the PCR. The 5' PCR primers for both heavy and light chains hybridize in the leader sequence of the genes, while the 3' primers hybridize with the amino terminus of the first constant regions of the rearranged heavy and light chain genes. Sequences of all primers are listed in Appendix C.

**Nucleotide and Amino Acid Sequence of Heavy and Light Immunoglobulin Variable Regions**

After the heavy and light immunoglobulin variable region PCR products were purified on an agarose gel, they were directly cloned into pT7 plasmid (T-vector) which also served as a sequencing vector. The T-vector enabled direct and efficient cloning of PCR products via an overhanging 3'
adenosine (A) on the PCR product (54) which base-paired with a 5' overhanging thymidine (T) through a modified EcoRV restriction endonuclease site on the T-vector (53). Transformants containing immunoglobulin fragment insert interrupted the β-galactosidase gene and appeared as white colonies on LB agar plates containing both 50μg/ml carbenicillin and 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). Cloning efficiencies using this method ranged from 35% to nearly 90% depending on the amount and quality of insert added to the ligation reaction.

The immunoglobulin fragments were sequenced once they were cloned into the T-vector. Double-stranded sequencing was performed using the Sanger dideoxy-nucleotide termination method. At least two clones were sequenced in the T-vector to ensure that the correct variable region sequence was obtained. In the event of a sequence reading discrepancy, a third clone was sequenced. The nucleotide and amino acid sequences are shown in figures 4A and 4B. According to "Sequences of Proteins of Immunological Interest," the heavy chain of HB125 belongs to a miscellaneous murine heavy chain subgroup, while the kappa light chain belongs to murine kappa subgroup III (62).
Figure 4A. Nucleotide and amino acid sequence of HB125 heavy chain variable region. Frameworks are designated by arrows and CDRs are boxed.
Figure 4B. Nucleotide and amino acid sequence of HB125 kappa light chain variable region. Frameworks are designated by arrows and CDRs are boxed.
Further analysis of the sequences revealed canonical structures based on the findings of Chothia and Lesk (63, 64). Complementarity determining region 1 (CDR1) of the kappa light chain is identical to the J539 Fab (65) canonical sequence subgroup except for the addition of a serine at position 31 and a tyrosine for a serine substitution at position 32; J539 does not have an amino acid at position 31 in CDR1. CDR2 of the light chain is most similar to that of an anti-hen egg lysozyme (HyHEL-5) Fab canonical group (7) except that residue 50 is a serine for HB125 and HyHEL-5 has an aspartic acid at position 50. CDR3 of the light chain does not conform to any defined canonical sequence, however, 3 of the seven canonical residues share identity with HyHEL-5.

The canonical sequence of CDR1 of the heavy chain is identical to an anti-neuraminidase Fab, NC-41, (10) except for an aspartic acid substitution for asparagine at position 31. CDR2 of the heavy chain is most similar to NC-41 canonical structure except that residue 53 of HB125 heavy chain is a glutamic acid (E) while NC-41 is an asparagine (N). No canonical structures have been established for CDR3 of heavy chains because there is a large variation in the length and amino acid composition of the CDR3 loop. A summary of the above comparisons is shown in table 1.
<table>
<thead>
<tr>
<th></th>
<th>HB125 V&lt;sub&gt;k&lt;/sub&gt; CDR1</th>
<th>J539 V&lt;sub&gt;k&lt;/sub&gt; CDR1</th>
<th>HB125 V&lt;sub&gt;k&lt;/sub&gt; CDR2</th>
<th>HyHEL-5 V&lt;sub&gt;k&lt;/sub&gt; CDR2</th>
<th>HB125 V&lt;sub&gt;k&lt;/sub&gt; CDR3</th>
<th>HyHEL-5 V&lt;sub&gt;k&lt;/sub&gt; CDR3</th>
<th>HB125 V&lt;sub&gt;H&lt;/sub&gt; CDR1</th>
<th>NC41 V&lt;sub&gt;H&lt;/sub&gt; CDR1</th>
<th>HB125 V&lt;sub&gt;H&lt;/sub&gt; CDR2</th>
<th>NC41 V&lt;sub&gt;H&lt;/sub&gt; CDR2</th>
<th>HB125 V&lt;sub&gt;H&lt;/sub&gt; CDR3</th>
</tr>
</thead>
</table>

Table 1. Comparison of HB125 variable heavy and light chain sequences of known canonical sequences (63). Order of residues is according to CDR canonical structure. Numbering is according to Kabat et al. (62).

**EXPRESSION AND PURIFICATION OF IMMUNOGLOBULIN VARIABLE REGIONS**

**Bacterial Expression and Purification of Immunoglobulin V<sub>H</sub> and V<sub>k</sub>**

Based on the corresponding sequences, new PCR primers (Appendix C) were designed to amplify the V<sub>H</sub> and V<sub>k</sub> for "in frame" protein expression in the pET expression vector. Since PCR products are difficult to digest with restriction endonucleases, a two-step cloning procedure was performed. In the first step, the immunoglobulin genes were amplified by
PCR with the new primers which contained NcoI and XhoI restriction sites at the 5' and 3' ends of the gene, respectively (Appendix C). These PCR products were directly cloned into the T-vector without modification. In the second step, the immunoglobulin genes were digested out of the T-vector via NcoI and XhoI sites and directly cloned into the pET expression vector so that they were in the correct frame to express the functional immunoglobulin proteins. Once the immunoglobulin fragments were cloned into the pET expression vector, pET22b plasmid was purified and used to transform Novablaue E. coli cells. A diagram of the pET22b bacterial expression vector is shown in figure 5.
Figure 5. Diagram of pET 22b expression vector. Vector was prepared for cloning by double digesting with NcoI and XhoI restriction endonucleases. Immunoglobulin gene inserts were cloned into NcoI and XhoI sites so that expression of the immunoglobulin genes occurred in frame with both the pelB signal peptide and the poly-histidine tag. Figure taken from Novagen technical bulletin #38.
Protein production the pET expression vector system utilizes bacterial host cells, BL21(DE3), which contain a chromosomal copy of the T7 RNA polymerase gene. An important feature of this expression system, is that expression of the immunoglobulin fragments does not occur in bacterial hosts without a source of T7 RNA polymerase. In contrast to other expression systems, only minimal levels of the target gene are expressed in the absence T7 RNA polymerase. This results in more stable propagation of the immunoglobulin gene(s) in the plasmid.

The immunoglobulin fragment-containing expression vector (pET22b) was purified from Novablue cells and super-coiled DNA was used to transform BL21 (DE3) cells. PET22b contains a pel B signal peptide which has been reported to direct proteins toward the periplasmic space in the bacterial cell (66). Individual colonies were picked and grown to mid log in Superbroth and then induced with IPTG from 1 to 4 hours at 32°C. Samples were taken at sequential time points, solublized in SDS-PAGE sample buffer and electrophoresed. The remainder of the cells were processed as stated in Methods. Figure 6 shows a Coomassie blue stained SDS gel of the immunoglobulin fragment producing BL21(DE3) lysates described above. When HB125 VH was cloned into the SacI and XhoI sites, cleavage of the pel B signal peptide did not occur at all. Alternatively, when HB125 VH was cloned into NcoI and XhoI sites, at least 50% of the pel B signal peptide was cleaved, however, lanes 10-13 show that no HB125 VH was secreted from the cells at any of the time points. Expression results
indicated that 2 to 3 hours post IPTG induction was sufficient time for production of the immunoglobulin fragments in bacterial culture.

Figure 6. Expression of HB125 VH from bacterial cultures. Arrows indicate HB125 VH, plus and minus signal peptide.
Lane 1. Molecular weight markers
Lane 2: Five hour cell lysate from NcoI/XhoI VH insert, -IPTG
Lane 3: Five hour cell lysate from SacI/XhoI VH insert, -IPTG
Lane 4: One hour cell lysate from NcoI/XhoI VH insert, +IPTG
Lane 5: One hour cell lysate from SacI/XhoI VH insert, +IPTG
Lane 6: Two hour cell lysate from NcoI/XhoI VH insert, +IPTG
Lane 7: Two hour cell lysate from SacI/XhoI VH insert, +IPTG
Lane 8: Five hour cell lysate from NcoI/XhoI VH insert, +IPTG
Lane 9: Five hour cell lysate from SacI/XhoI VH insert, +IPTG
Lane 10: Five hour cell lysate from pET 22b no insert, +IPTG
Lanes 11-14: Supernatants from cultures in lanes 6-9.
Purification of HB125 VH on a Superose 12 Molecular Weight Sieving Column

Bacterial cell lysates in 6M urea were loaded onto a Superose 12 gel filtration column and fractionated in the presence of 2M urea in PBS. Fractions were collected from the column and electrophoresed in SDS-PAGE to determine which fractions contained the immunoglobulin fragments. Figures 7A and 7B show SDS-PAGE analysis of the fractions from the Superose 12 column. The HB125 VH gene product was eluted between fractions 41 through 52 as shown in figures 7A and 7B. Fractions 41-49 contain mainly HB125 VH plus pel B signal peptide while fractions 50-53 contain mainly HB125 VH without pel B signal peptide.
Figures 7A and 7B. SDS-PAGE analysis of HB125 VH fractions from Superose 12 molecular weight sieving column. Lanes are designated by fraction number. Lane SM is pre-column starting material. MWM indicates the molecular weight markers.
HB125 V\textsubscript{H} Inhibition ELISA

HB125 V\textsubscript{H} inhibition ELISA was performed according to the procedures in Methods. Fractions 47-53 from the Superose 12 columns were dialyzed in PBS and directly coated onto an ELISA plate. Using unlabeled insulin as the inhibitor, it was observed that HB125 V\textsubscript{H} alone, coated onto the ELISA plate, could bind to biotinylated insulin and was inhibited by insulin itself. The results of this inhibition are shown in figure 8. The binding of HB125 V\textsubscript{H} was very weak compared to HB125 parent antibody. The difference in insulin binding capacity by each fraction reflects the different amounts of protein in each fraction. For example, fractions 47-51 contained more protein (Bradford assay) than fractions 52 and 53. Fraction 49 was inhibited by 82% from binding to unlabeled insulin.
Figure 8. HB125 VH insulin inhibition ELISA. The ELISA was performed by coating fractions from the Superose 12 column onto the plate. For the inhibition, 40ug/ml of unlabeled insulin was incubated with the fractions. Then 4ug/ml of biotinylated insulin was added to the wells in the presence of 40ug/ml of unlabeled insulin for one hour and the plate was washed free of excess reagents. Streptavidin-alkaline phosphatase was then added and incubated for one hour and washed out. Substrate was added and the optical density of the plate was quantitated.

HB125 Vk Expression and Purification

The HB125 kappa chain variable region was expressed in BL21(DE3) bacteria using the same procedures as HB125 VH. Interestingly, Vk expression in 100ml of E. coli culture occurred at a very high level. Over 900ug was purified from the cell lysate of a 100ml HB125 Vk-producing
culture. HB125 $V_k$ was purified on a Superose 12 column, as described for HB125 $V_H$. Two milliliter fractions were collected from the column and subjected to SDS-PAGE as shown in figure 9. Total cell lysate (pre-column) showed a predominant band of at approximately 15kD. This band is the HB125 $V_k$ gene product. Fractions 36-38 represent the purified $V_k$ antibody fragment.

![Figure 9. Superose 12 column fractionation of HB125 $V_k$. The arrow points to the purified material. Molecular Weight markers are designated by MWM. Total cell lysate (pre-column) material is designated by P.C. and showed a predominant band at approximately 15kD. This band is the correct size for the $V_k$ gene product. Fractions 36 through 38 represent the purified $V_k$ protein.](image-url)
CONSTRUCTION, CLONING AND EXPRESSION OF HB125 scFv

Single Chain Antibody (scFv) Construction

Primers for construction of a scFv from the variable regions of the heavy and light chain are listed in Appendix C. In brief, HB125 VH was amplified by the PCR using the same 5' primer as before along with a 3' anti-sense primer containing nucleotides coding for one unit of Gly4-Ser linker. The 5' sense primer for the kappa variable region was designed with nucleotides coding for 3 units of Gly4-Ser, while the 3' Vk primer was the same as before. PCR was performed separately for the heavy and light chains using the primers stated above for construction of the scFv. The PCR products containing the 3' and 5' Gly-Ser linkers were combined for PCR single overlap extension (PCR soeing) so that the heavy chain and the light chain were assembled into a scFv separated by a (Gly4-Ser)3 linker. A diagram outlining the construction of the scFv is shown in figure 10. Figures 11A, 11B and 11C show 0.9% agarose gels of PCR products at each stage of the scFv PCR assembly process. Figure 11A shows VH amplification with a 3' Gly4-Ser linker at approximately 390bp. Figure 11B shows Vk amplification with a 5' (Gly4-Ser)3 linker at approximately 390bp. Figure 11C shows the assembled scFv PCR product with VH and Vk flanking a (Gly4-Ser)3 linker.
ASSEMBLY OF HB125 VH AND VL INTO HB125 scFv

Figure 10. Pictorial diagram showing construction of HB125 scFv PCR Assembly.
Figure 11. Agarose gel analysis of scFv PCR assembly. Figure 11A shows HB125 V\textsubscript{H} plus 3' Gly\textsubscript{4}-Ser, 11B shows HB125 V\textsubscript{k} plus 5'(Gly\textsubscript{4}-Ser)\textsubscript{3}, and 11C shows the assembled scFv: V\textsubscript{H}-(Gly\textsubscript{4}-Ser)\textsubscript{3}-V\textsubscript{k}. 
Expression and Purification of HB125 scFv

The assembled scFv PCR product was cloned into the pET 22b vector via the 5' NcoI and the 3' XhoI sites at the ends of the VH andVk, respectively. Then the vector was used to transform BL21(DE3) host cells for expression. Figure 12 shows expression of HB125 scFv from pET 22b in BL21(DE3) cells solublized in urea in lane 2. Urea solublized cell lysates were dialyzed in PBS (lane 3) and loaded onto an insulin-agarose column for purification of the scFv. Running the scFv-containing cell lysates through the insulin-agarose column permitted both purification of the scFv and a check to ensure retention of anti-insulin binding activity. It was observed that the majority of HB125 scFv was eluted in the second fraction with very little in the third fraction, and none in fractions 4 through 6. The SDS-PAGE in figure 12 also shows a doublet at approximately 34kD and 32kD which corresponds to HB125 scFv plus signal peptide and scFv minus signal peptide indicating that the pel B signal peptide did not interfere with the capacity to bind to insulin.
Figure 12. Purification of HB125 scFv on an insulin-agarose column.
Lane 1. Molecular weight markers
Lane 2. Urea solublized HB125 scFv cell lysate
Lane 3. Pre-column starting material: Post PBS dialysis supernatant
Lane 4. HB125 scFv flow through from insulin-agarose column
Lanes 5-10. HB125 scFv eluate, fractions 1 through 6.

Expression of HB125 VH, Vk and scFv in pET 21d

Although the pel B signal peptide did not appear to interfere with the insulin binding activity of HB125 scFv and the VH, the signal peptide was not directing the secretion of the antibody fragments toward the periplasmic space as has been reported (66). It was decided to reclone each antibody fragment into pET 21d which was identical to pET 22b, but without the pel B signal peptide. Figure 13 shows HB125 VH, Vk and scFv expressed from pET 21d in BL21(DE3) cells. There was no detectable difference in binding or activity of the immunoglobulin fragments between
pET 22b and 21d. By using pET21d, however, the possibility of non-specific binding due to the pel B signal peptide was avoided.

Figure 13. pET 21d expression of HB125 VH, Vk and scFv in SDS-PAGE.
Lane1. Molecular weight markers
Lane2. pET21d transformed bacterial cell lysate, no insert.
Lane3. HB125 VH expressed from pET 21d transformed cells.
Lane4. HB125 Vk expressed from pET 21d transformed cells.
Lane5. HB125 scFv expressed from pET 21d transformed cells.

In all subsequent studies, the pET 21d expression vector which does not have a pel B signal peptide was used in place of pET 22b. Expression of immunoglobulin fragments in pET 21d resulted in only one major band of the gene product (without signal peptide). For either expression vector, the immunoglobulin fragments were never secreted so the protein purification scheme remained the same regardless which expression vector was used. Expression levels of immunoglobulin fragments in each of the vectors were
similar, ranging between 2 to 25ug/ml of culture medium for 3 hour cultures, post induction.

**Purification of Recombinant Immunoglobulin V-regions on a Nickel-Agarose column**

Immunoglobulin fragment-producing bacterial cultures were processed as described in Methods. Various commercially available anti-sera were characterized for the ability to bind to HB125 VH, Vk and scFv for affinity purification and detection purposes. No anti-sera were found which had specificity for the variable regions, so it was necessary to obtain an alternative method for purifying the HB125 variable regions. Since the immunoglobulin fragments produced from the pET 21d transformed cells contained a carboxyl (His)6, it was possible to purify them on a nickel-agarose column using immobilized metal affinity chromatography (IMAC) (56). Figures 14A and 14B show SDS-PAGE analysis of the purification of HB125 VH and scFv, respectively. In figure 14A, lanes 2-5, nearly all of HB125 VH was purified from the cell lysate. Lanes 6-9 represent fractions 1-4 from the nickel-agarose column elution profile. A few faint contaminating bands can be seen in lane 6 of figure 14A, but the main VH band is clearly prominent at approximately 17kD. As shown in the gel, all of the VH immunoglobulin fragment was eluted from the column in the first 1ml fraction. Figure 14B shows nickel-agarose column purification of HB125 scFv. The purified scFv can be seen as a distinct band at approximately 31kD in lane 6. Nearly all of the scFv was purified (lane 6) from the cell lysate (lane 2), however, a small amount of the scFv band can
be seen in lane 5 which represents the 60mM imidazole column wash. In addition, another band can be seen in lane 6 just below the main band, at approximately 29kD. This band was not characterized, but it is most likely a degradation product from the scFv. It is probably not a bacterial contaminant because it cannot be seen in the VH and VK nickel-agarose column purifications. HB125 VK could also be purified using the same methodology.

Figure 14A. SDS-PAGE analysis of HB125 VH purification on a nickel-Sepharose column.
Lane 1. Molecular weight marker
Lane 2. HB125 VH cell lysate pre-column starting material
Lane 3. HB125 VH cell lysate nickel column flow through
Lane 4. Nickel column wash #1
Lane 5. Nickel column wash #2
Lane 6. HB125 VH eluate fraction #1
Lane 7. HB125 VH eluate fraction #2
Lane 8. HB125 VH eluate fraction #3
Lane 9. HB125 VH eluate fraction #4
Lane 10. HB125 VH eluate fraction #5
Lane 11. HB125 VH eluate fraction #6
Figure 14B. SDS-PAGE analysis of HB125 scFv purification on a nickel-Sepharose column.
Lane 1. Molecular weight markers
Lane 2. HB125 scFv cell lysate pre-column starting material
Lane 3. HB125 scFv cell lysate nickel column flow through
Lane 4. Nickel column wash #1
Lane 5. Nickel column wash #2
Lane 6. HB125 scFv eluate fraction #1
Lane 7. HB125 scFv eluate fraction #2
Lane 8. HB125 scFv eluate fraction #3

**Peptide inhibition of HB125 Antibody and HB125 scFv**

Using the Selectide process, peptides with different motifs had previously been found which interacted with the hybridoma-produced HB125 antibody (Appendix B). In order to prove that these peptides were indeed specific for the paratope of HB125 antibody, inhibition studies with the free peptides were performed using competitive ELISAs. In the competitive ELISAs, porcine insulin was first immobilized on microtiter ELISA plates. The ability of the peptides to inhibit either parental HB125 antibody or HB125 scFv from binding to the immobilized insulin was tested.
Figure 15A shows the results of the peptide inhibition ELISA with HB125 antibody. Since porcine insulin was the natural ligand for the antibody, it was used as the positive control inhibitor. The IC50 for insulin itself with the parental IgG, HB125, was found to be 10nM. The peptide which best inhibited HB125 antibody from binding to insulin was NSMFWKYGF at an IC50 of 720nM, less than two orders of magnitude from that of the natural ligand itself. The control peptide, ELHWKYGF, which was initially synthesized based upon the sequence of a non-stained, non-reactive bead in the initial screenings, did not inhibit HB125 from binding to insulin. The least effective inhibitory peptide was FDWSNGGG which had an IC50 of 9.5μM.

Once HB125 scFv was constructed, expressed and purified, peptide inhibition ELISAs were performed (figure 15B). The IC50s for HB125 scFv showed the same inhibition pattern as the parental IgG, but the peptides were better inhibitors of the scFv. Insulin inhibited the scFv at an IC50 of 35nM. Peptides NSMFWKYGF and SRQDIWGIGF bound most strongly to the scFv. In most experiments NSMFWKYGF consistently bound strongest to both the scFv and HB125 antibody although SRQDIWGIGF sometimes bound as strongly as NSMFWKYGF. Peptides FDWSNGGG and PQDPR bound most weakly to the scFv. The results of the competitive studies are shown in table 2.
Figures 15A and 15B. Peptide inhibition of HB125 antibody (A) and scFv (B) from binding to insulin. All peptides contain a carboxyl (β-alanine ε-amino caproic acid)2 linker.
Table 2. IC50 values for HB125 antibody and HB125 scFv inhibitory peptides.

**HB125 V\textsubscript{H} Binds to Peptide SRQDIWGIGF**

In order to localize binding of the peptides to either the light chain or the heavy chain, an attempt was made to couple the free peptides to BSA using glutaraldehyde, a homo-bifunctional cross-linker. Only peptide SRQDIWGIGF retained its immunologic reactivity with the antibody fragments when coupled to BSA. The loss of immunologic reactivity for the "WKYGF" motif peptides may be explained by derivatization of the lysine by glutaraldehyde.

Successful coupling of SRQDIWGIGF to BSA meant that the peptide-BSA conjugate could be coated onto an ELISA plate and screened for
reactivity to different antibody fragments. It also permitted competition ELISAs to be performed with the other peptides. Figure 16 shows the results of these studies. The hybridoma-produced HB125 antibody binds to the peptide very strongly. Wild type HB125 scFv does not bind to the peptide as strongly as HB125 antibody, but it still binds quite well. HB125 scFv is monovalent whereas HB125 antibody is divalent. In addition, there might be a difference in the degree of biotinylation between HB125 antibody and the scFv. Although weaker than the scFv, HB125 V_H binds to SRQDIWGIGF. In contrast, HB125 V_K showed no binding activity to this peptide at all. An important conclusion from the figure below is that binding of SRQDIWGIGF peptide is localized to HB125 variable heavy chain.
Figure 16. HB125 antibody fragment reactivity with SRQDIWGIGF peptide. ELISA was performed by incubating serial dilutions of biotinylated antibody fragments with SRQDIWGIGF-BSA coated ELISA plates. Biotinylated antibody fragments were detected with streptavidin-horse radish peroxidase.

**SRQDIWGIGF ELISA: Peptide Inhibition of HB125 scFv**

Since binding of SRQDIWGIGF was localized to HB125 VH, SRQDIWGIGF-BSA inhibition ELISAs were performed to determine which of the other peptides could inhibit SRQDIWGIGF from binding to the scFv. This experiment showed which peptides bound to the same site on HB125 VH. HB125 scFv was used in the SRQDIWGIGF peptide ELISA in place of HB125 VH because HB125 scFv was a stronger binder than HB125 VH to the peptides and gave more reliable results due to higher optical density.
readings in ELISA. The SRQDIWGIGF-BSA inhibition ELISA was performed in an identical manner to the insulin inhibition ELISA. The results from this experiment are shown in figure 17. Peptides NHKGWKYGF, NSMFWKYGF and NHPRWKYGF inhibited HB125 scFv from binding to SRQDIWGIGF, while PQDPR and FDWSNGGG did not. As might be predicted based upon their sequences, the peptides NHKGWKYGF, NSMFWKYGF and NHPRWKYGF bound either to the same site on HB125 VH or to a site in close proximity to the SRQDIWGIGF binding site on HB125 VH. As observed previously in the insulin inhibition ELISA, insulin is a much better inhibitor than any of the peptides, including SRQDIWGIGF. The negative control peptide, ELHWKYGF, does not inhibit HB125 scFv from binding to SRQDIWGIGF-BSA.
Figure 17. Peptide inhibition of HB125 scFv from binding to SRQDIWGIGF-BSA. All peptides contain a carboxyl (β-alanine ε-amino caproic acid)₂ linker.
**Sequential Screening of Progressively Longer Peptide Libraries**

In an attempt to develop synthetic peptides which bound more strongly to HB125 antibody or scFv than the natural ligand, sequential screening of progressively longer peptide libraries was performed by Lam et al. Based upon two different motifs from previously sequenced reactive peptides, four amino acids were randomized onto the N-terminus of each peptide as shown in the quaternary (4°) randomization screening in figure 18. In addition, the third and eighth positions, respectively were randomized in NH_GWKYGF and SRQDIWG_GF peptides (figure 18).
Figure 18. Flow diagram of sequential peptide motifs selected from random peptide libraries by HB125 antibody. Data from Lam et al.
The 4° library was then screened with the HB125 antibody. Reactive beads were subjected to peptide microsequencing and consensus sequences (motifs) were obtained as shown in figure 18. Free peptides were then synthesized based upon the peptide motifs and tested for the ability to inhibit HB125 scFv from binding to insulin in the inhibition ELISA. The results of the inhibition ELISA are shown in figure 19. Peptide QSSVNHPGWKYG was the best peptide at inhibiting HB125 from binding to insulin with an IC50 of 37nM, but no peptide inhibited better than porcine insulin itself.
Figure 19. Peptide inhibition of HB125 scFv using longer peptides selected from the 4° library.
SITE-DIRECTED MUTAGENESIS OF HB125 scFv

Mutational Analysis of Complementarity Determining Region 3 (CDR3) of HB125 scFv Heavy Chain Variable Region

As diagrammed in methods, PCR single overlap extension was employed in CDR mutagenesis studies. Figure 20 shows a 0.9% agarose gel of the PCR assembly process for PCR-based, site-directed mutagenesis.

Figure 20. Agarose gel of PCR-based site-directed mutagenesis in CDR3. For a nucleotide diagram of PCR-based site directed mutagenesis, see figure 1 in Methods section. Lane 1 shows the 5' section of the mutant. Lane 2 shows the 3' section of the mutant. Lane 3 shows the assembled scFv CDR3 mutant. Lane 4 shows the molecular weight markers; the brighter band in the middle of the markers is 600bp.

Initially, 3 amino acid mutations were made in the heavy chain of CDR3 in HB125 scFv: Y96 to F96, K98 to E98, and G99 to V99 (CDR3 mutant #1, Table
3). After cloning and expression of the scFv CDR3 mutant, it was tested for the ability to bind to insulin. Based upon initial analysis, the mutations made in the V_H of CDR3 of HB125 scFv resulted in a complete loss of the ability to bind to insulin as shown in figure 21. However, when V_99 was restored to G_99 through the PCR mutagenesis process, (the two other mutations, F_96 and E_98, were preserved) complete binding activity to insulin was restored.

Figure 22 shows peptide inhibition results of the restoration of V_99 to G_99 in CDR3 of the heavy chain. There was no significant difference in the IC50s of insulin between wild type scFv (figure 15B) and the CDR3 mutant, V_99 to G_99 restoration.

At this point, questions about the nature of position 99 in CDR3 of the heavy chain of HB125 scFv arose. It was decided to generate two more HB125 scFv mutants and substitute alanine and threonine separately at position 99 in CDR3. Alanine was chosen because it is close in size to glycine, but has a hydrophobic character like valine. Threonine was chosen because it is very close in size to valine, but has a relatively hydrophilic character like glycine. A summary of the results of HB125 scFv variable heavy CDR3 mutagenesis is shown in table 3. It was observed that the A_99 mutant was able to bind to insulin, but the T_99 mutant was not able to bind to insulin. This suggests that a small amino acid is necessary in position 99 in CDR3 of the heavy chain of HB125. However, more extensive mutagenesis should be performed to test the effect of a charged residue at position 99.
Figure 21. Insulin binding capacities of HB125 scFv heavy chain CDR3 mutants. Biotinylated CDR3 mutants were incubated with insulin-coated ELISA plates. The mutants were detected with streptavidin-horse radish peroxidase.
Figure 22. Peptide inhibition of HB125 scFv $V_{99}$ to $G_{99}$ heavy chain CDR3 mutant.
Table 3. HB125 scFv CDR3 mutagenesis. Mutations were made in CDR3 in the heavy chain of HB125 scFv. Binding was characterized by an insulin inhibition ELISA. Mutant amino acids are underlined.

<table>
<thead>
<tr>
<th>HB125 scFv mutants</th>
<th>CDR3 mutations</th>
<th>Binding to insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDR3 wild type</td>
<td>G Y G K G Y F D V</td>
<td>IC50 = 35nM</td>
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<tr>
<td>CDR3 mutant #1</td>
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<td>Loss of binding</td>
</tr>
<tr>
<td>CDR3 mutant #2</td>
<td>G F G E G Y F D V</td>
<td>IC50 = 20nM</td>
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<tr>
<td>CDR3 mutant #3</td>
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<tr>
<td>CDR3 mutant #4</td>
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<td>Loss of binding</td>
</tr>
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**HB125 scFv CDR1 and CDR2 mutagenesis in HB125 scFv**

As controls for the CDR3 mutants, CDR1 and CDR2 were mutagenized. Three positions out of the five total residues in CDR1 of the heavy chain were changed. Aspartic acid at position 31 (D31) was changed to a lysine (K31), serine 33 (S33) was changed to glycine (G33) and histidine 35 (H35) was changed to an asparagine (N35). Since the amino acid sequence of CDR2 aligned very closely with many other antibody sequences in the Kabat database, only a glutamic acid at position 53 was changed to an asparagine, making CDR2 identical to CDR2 in the heavy chain of NC-41, an anti-neuraminidase antibody which has been crystallized. The mutagenesis was performed in the same manner as before. Both HB125 scFv CDR1 and CDR2 mutants bound to insulin as shown in figure 23, but the binding of the CDR1 mutant was weak. Figure 23B. shows the results of HB125 scFv CDR1 mutant binding to insulin. Again, binding was weak.
The degree of biotinylation of the CDR1 mutant was tested, but it showed that the biotinylation was equal to that of HB125 scFv wild type (data not shown). Because of this weak binding to insulin, peptide inhibition curves could not be generated for the CDR1 mutant. Peptide inhibition results for the CDR2 HB125 scFv mutant is shown in figure 24. A summary of the binding properties of HB125 antibody fragments is shown in table 4.

![Figure 23A](image)

**Figure 23A.** Insulin binding capacities of HB125 scFv heavy chain CDR1 and CDR2 mutants. Biotinylated mutants were incubated with insulin-coated ELISA plates. The mutants were detected with streptavidin-alkaline phosphatase.
Figure 23B. Reactivity of HB125 scFv CDR1 mutant in SRQDIWGIGF-BSA ELISA. Biotinylated scFvs were incubated with insulin-coated ELISA plates. The mutants were detected with streptavidin-alkaline phosphatase.
Figure 24. Peptide inhibition of HB125 scFv CDR2 mutant.
<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>INSULIN</th>
<th>SRQDIWGIGF</th>
<th>NSMPWKYGF</th>
<th>NHPWKYG</th>
<th>QSSVNHGWGKYGF</th>
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<tr>
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<td>1500nM</td>
<td>720nM</td>
<td>9500nM</td>
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<td>15nM</td>
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<td>37nM</td>
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<td>+, weak</td>
<td>+, weak</td>
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<td>N.D.</td>
<td>N.D.</td>
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<td>N.D.</td>
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<td>N.D.</td>
<td>N.D.</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HB125 Vk</td>
<td>-</td>
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</tbody>
</table>

Table 4. HB125 antibody fragment binding summary. IC50 values are shown. "+" and "-" indicate qualitative binding by the antibody fragment. ScFv CDR1 mut. contains the following mutations (wild type to mutant): D31 to K31, S33 to G33, and H35 to N35. ScFv CDR2 mut. has E53 changed to N53. ScFv CDR3 mut. contains the following mutations (wild type to mutant): Y96 to F96, K98 to E98, and G99 to V99. ScFv CDR3 V->G restores V99 mutant to G99 wild type; scFv V->A changes V99 mutant to an A99 mutant; scFv CDR3 V->T changes V99 mutant to a T99 mutant. The two other mutations in CDR3, K98 to E98, and G99 to V99 remained present while different amino acids were substituted in position 99. N.D. indicates not determined.
CHAPTER 5

DISCUSSION

Rescue of Immunoglobulin Genes

PCR primer sets can easily be designed to amplify immunoglobulin genes for expression in bacteria. Many groups have cloned antibody genes and expressed them in bacteria (18-20, 66-68). Several groups have also expressed antibody variable regions on coat proteins of filamentous bacteriophage (29, 34, 36).

In the work presented in this dissertation, messenger RNA was obtained from either hybridoma cells or lymphoma cells, reverse transcribed into cDNA and subjected to PCR. Oligonucleotide primers were designed to amplify the variable regions of human and mouse immunoglobulin genes (1, 51, 62). Degenerate sense and anti-sense immunoglobulin-specific primers designed for PCR were successfully used for OCILY8 and HB125 immunoglobulin amplification. Rarely did a PCR fail to amplify immunoglobulin template, even from cDNA. The primer set used to amplify HB125 VH corresponded to a miscellaneous murine subgroup (62). The primer set used to amplify HB125 VK corresponded murine subgroup III.

Interestingly, the VK primer set "B" amplified an aberrantly rearranged (VJ junction) kappa chain transcript from the HB125 fusion partner, Sp2/0-Ag14 (figure 3B). This mRNA transcript has been reported to be present in all fusion partners derived from the original MOPC
myeloma tumor cells (61). It was not investigated whether Sp2/0-Ag14 fusion partner contained only rearranged kappa transcript or if the cells contained kappa protein as well. Fortunately, DNA sequence analysis distinguished the fusion partner derived kappa chain from the anti-insulin B cell derived kappa chain.

Since digestion of PCR products with restriction endonucleases is difficult, a two-step cloning protocol described in the methods chapter was necessary for expression of immunoglobulin variable regions. This method worked very well. It allowed rapid and efficient cloning of PCR products without modification into the T-vector via overhanging 3' adenosines (A) on the ends of the double stranded PCR product. From initial amplification to expression, the two-step cloning protocol actually consisted of eight discrete steps: (i) initial amplification of antibody genes, (ii) cloning of genes into T-vector, (iii) sequencing of antibody genes, (iv) design of immunoglobulin-specific primer sets for in frame expression, (v) amplification of antibody variable regions using new primers for expression, (vi) cloning of PCR product into T-vector, (vii) cloning of antibody genes into expression vector, (viii) expression of immunoglobulin variable regions from E.coli. Although this process might be considered tedious, it was the most efficient method found to amplify, sequence, clone and express antibody genes from bacteria.

HB125 variable region gene sequences were categorized into canonical structure subgroups based upon structures of known Fabs (64). Overall, the HB125 light chain variable region is most similar to Fabs J539 and HyHEL-5 while the heavy chain is most like the anti-neuraminidase Fab (NC-41). Interestingly, CDR3 of the HB125 light chain was most
difficult to categorize. CDR3 of the light chain shares sequence length with canonical structure 1, but shares more amino acid similarity with canonical structure 3 which contains the HyHEL-5 Fab. Based upon canonical structure subgroups, length is probably more important than sequence similarity. HB125 light chain CDR1 is most similar in amino acid composition to J539 Fab, belonging to canonical structure 1, however, it is most similar in length to canonical structure 2. HB125 light chain CDR2 belongs to canonical structure 1 and is most similar in sequence and length to HyHEL-5. CDR3 of heavy chains are too variable in length and residue composition to obtain canonical structures. HB125 CDR1 and CDR2 are most similar in both amino acid composition and length to NC-41 and belong to canonical structures 1 and 2, respectively. Further studies with HB125 scFv might include identifying peptide motifs which interact with only one individual CDR. Then those peptides could be tested with other antibodies having an identical CDR or the same canonical structure.

**Construction of HB125 scFv**

PCR assembly was used to construct HB125 scFv. This technique was surprisingly successful and simple to perform. Since HB125 VH contains 16 amino acids from CH1, there were doubts as to whether the scFv would retain the ability to bind to insulin with the (Gly4-Ser)3 linker. As reported (21), there is only a minimum length of 10 or 12 residues for a scFv linker. From analysis of crystal structures (69), a proposed linker must span at least 35Å between the carboxyl and amino ends of the variable regions in order not to distort native Fv conformations. The glycine and
serine residues in (Gly4-Ser)3 would confer maximum flexibility and hydrophilicity so as not to constrain the scFv from binding to its antigen in an induced fit type of interaction. After PCR assembly of HB125 scFv, a band at the predicted molecular weight was observed, strongly suggesting that the new gene was assembled such that the (Gly4-Ser)3 linker was flanked on both sides by VH and VK. Subsequent cloning, sequence analysis and protein expression proved that HB125 scFv was assembled correctly.

If one were to perform computer modeling of HB125 scFv, the most accurate prediction of the conformations of the heavy and light chain variable regions could be obtained by superimposition of HB125 light chain onto HyHEL-5 and superimposition of HB125 heavy chain onto NC-41. Amino acid substitutions, replacing HyHEL-5 and NC-41 residues with HB125 amino acids could then be performed followed by energy minimization of the entire molecule. A crystal structure of a scFv is not yet available so the (Gly4-Ser)3 linker between the heavy and light variable regions would have to be modeled between the carboxyl terminus of the VH and the amino terminus of the VL. Since the linker is rather long (15 residues), flexible, and spatially distal to the CDRs, it probably does not constrain the free movement of the scFv. At the same time, however, the linker will keep the variable regions in close proximity to each other.

Another advantage of a single chain Fv is that it is transcribed and translated into a single polypeptide. The pCOMB3 phagemid expression system utilized two lacZ promoters, one for the heavy chain Fd and one for the light chain Fd. It was observed that an excess of light chain was
produced in this system because the heavy chain promoter also acted on the 
light chain, causing more light chain to be transcribed. Since there was 
not a 1:1 stoichiometry between the heavy and light chains to form a Fab in 
the bacteria, light chain dimers formed. In addition, one could not be sure 
that the heavy and light chains would associate in the reducing 
environment of bacterial cytoplasm. These problems are conveniently 
avoided in scFv construction.

**Expression of HB125 Antibody Fragments from *E. coli***

Once HB125 scFv, VH or Vk was cloned into the pET expression 
vector in BL21(DE3), antibody genes were ready for expression. Timing of 
induction for the expression of the antibody genes in cultures was crucial. 
BL21(DE3) cells, freshly transformed with plasmid containing antibody 
genes, proved to be the best producers of the antibody fragments. Once a 
culture was started, it was important to induce the bacteria to produce 
antibody fragments when the culture was in early log phase and to harvest 
the culture between three and five hours post induction. If a culture was 
induced after early log phase, production of the antibody fragments was 
sporadic; there was no consistency for expression. Several preliminary 
experiments were performed in order to obtain optimal conditions for 
expression of the antibody fragments, but it was extremely difficult to 
reproduce results from culture to culture.

One possible reason for erratic expression might be plasmid 
instability (the number of cells harboring plasmid). The manufacturers of 
the expression vector have commented (70) that plasmids can easily be lost
from bacterial host cells producing antibody fragments. Another explanation for the loss of antibody gene-producing plasmids from bacterial cell is that antibody fragments might be toxic to the cells. In addition BL21(DE3) cells are recA positive which means that they have the ability to remove portions of genes that may put them at a selective growth disadvantage (i.e. over-production of antibody genes). Although this study did not investigate all antibody expression systems, transfection of the scFv genes into eukaryotic cells such as Chinese hamster ovary cells (CHO cells) may give the best and most stable production of correctly assembled scFv. However, bacterial and phage antibody expression systems offer more options for protein engineering and mutagenesis studies.

The amount of antibody produced in a bacterial cultures ranged from less than 0.1ug/ml to over 900ug/ml, depending on the antibody fragment produced. For example, over 1.8mg of HB125 V_k was consistently produced from 100ml cultures, while 10 to 100-fold less was produced in an HB125 scFv culture. In some cases, the amount of induced protein produced from side-by-side cultures of the same antibody fragment was extremely variable even when all outside conditions (medium, temperature, IPTG, antibiotic) were identical. One must conclude that plasmid instability is the cause of the wide ranges of production of the antibody fragments. This conclusion is supported by the observations that colonies freshly transformed with antibody gene containing plasmids produced the most protein upon subsequent culture and induction. The observation is especially valid when compared with antibody production from stock cultures which were very low and, many times, non-producing.
Purification of HB125 Antibody Fragments

Several different methodologies were evaluated for purification of the antibody fragments (21, 71, 72). Antibody fragments expressed from the pET22b vector (plus pel B signal peptide) were expected to be found in the periplasmic space of bacteria. However, upon treatment of the cells with lysozyme, no antibodies were found in the supernatant solution. Even lysozyme treatment followed by sonication could not separate the antibodies from the cells. Only when the cells were sonicated in the presence of 6M urea, were antibody fragments located and purified. These results strongly suggest that the antibody fragments were sequestered into inclusion bodies as has been reported by others (21, 67).

Purification of HB125 antibody fragments consisted of solublizing the bacterial cell pellet in 6M urea followed by sonication to liberate the immunoglobulin variable regions from the bacterial inclusion bodies. Since commercially available anti-sera could not be found which bound specifically to the murine immunoglobulin variable regions, other methods of purification were employed. The antibody fragment-containing lysate was either run through a Superose 12 molecular weight sieving column or run through a nickel-Sepharose column. Both purification strategies purified the proteins of interest, however, the nickel-Sepharose column gave cleaner purifications by SDS-PAGE analysis (figures 14A and 14B).

Figure 12 in the results chapter shows purification of HB125 scFv on an insulin-agarose column. "Wild type" HB125 scFv or scFv mutants which retained the ability to bind to insulin could be purified in this manner. Interestingly, even though HB125 VH bound to insulin, it could
not be purified on the insulin-agarose column. One might speculate that HB125 VH binding to insulin was too weak for purification purposes, but in ELISA the VH was able to bind to insulin. This discrepancy in binding ability by the VH was not further investigated since it could easily be purified via a carboxyl histidine (His)₆ tag from a nickel-Sepharose column so that other characterization studies could be performed.

Upon elution of over 200ug of scFv from the insulin-agarose column, it was observed that precipitation of the pure scFv occurred in the fraction containing the scFv protein. Precipitation occurred both from scFv produced with and without pel B signal peptide. This observation is curious because the scFv bound to the insulin column in a soluble state, but precipitated upon elution. Precipitation of scFvs is usually associated with improper re-folding after the scFv has been subjected to reducing conditions (21). Neither the scFv nor the insulin column was exposed to denaturing or reducing agents. Huston et al. (21) have reported that under the reducing conditions of bacterial cytoplasm, scFvs may not re-fold correctly after in vitro oxidation. They suggest two incorrect scFv folding conformations and only one correct conformation based upon disulfide bond formation between domains (incorrect) and interdomain (correct) disulfide bonding. Glockshuber et al. (73) reported that proper oxidation of disulfide bonds for scFv re-folding is mandatory for binding activity to antigen. Interestingly, HB125 scFv was able to bind to an insulin column for purification, but precipitated upon elution from the column, suggesting incomplete oxidation or free sulfhydryl groups. Although it was not pursued, disulfide
analysis could have been performed To determine whether free thiol groups existed in the scFv (74).

After purification of HB125 scFv from the nickel-Superose column under denaturing conditions, the scFv was dialyzed stepwise against PBS-urea until the urea was completely exchanged for PBS. Only once in several nickel-Sepharose purifications was precipitation of HB125 scFv observed. HB125 VH and VK expressed alone never precipitated after purification, however, significant amounts of non-pure, cell lysate associated VH and VK precipitated during and after dialysis in PBS. As mentioned above, this precipitation problem was circumvented by purification on the nickel column in the presence of 6M urea.

Since each of the expressed proteins contained a carboxyl histidine tag (His)6, purification on a nickel-Sepharose column was not difficult, especially when denaturing conditions could be used to avoid incorrect folding of HB125 scFv. It can be concluded that IMAC chromatography is a simple and convenient method of purifying recombinant proteins.

**Binding Properties of HB125 Antibody Fragments**

Inhibition ELISAs were performed to confirm that peptides found using the Selectide process were specific for the paratope of HB125 antibody. The sequences of all peptides were compared to the primary sequence of porcine insulin. No significant sequence similarity was found, indicating that the peptides were truly mimotopes for the binding site of HB125 antibody on insulin. All peptides contained a carboxyl βeβe. Initially it was hypothesized that peptides could be found that bound to HB125 antibody
more strongly than to insulin itself. None were found that inhibited the parental HB125 antibody or HB125 scFv from binding to insulin more strongly than porcine insulin, the natural ligand. However, peptides S(R/K)QDIWGIGF and QSSVNHPGWKYGF demonstrated the ability to bind to HB125 scFv within an order of magnitude as strong as insulin.

Unsuccessful attempts were made to directly coat free peptides onto ELISA plates so that their individual reactivities toward \( V_H \) and \( V_K \) could be measured. Subsequently, peptides were coupled to BSA using glutaraldehyde. Only SRQDIWGIGF peptide retained immunologic reactivity toward HB125 scFv. The ELISA performed using antibody fragments in figure 16 showed that the binding of peptide SRQDIWGIGF was localized to HB125 \( V_H \). Although HB125\( V_K \) showed no binding activity toward SRQDIWGIGF peptide, HB125 \( V_H \) bound much more strongly to SRQDIWGIGF when associated with \( V_K \). Interestingly, HB125 \( V_K \) also did not exhibit any binding activity toward insulin.

One possibility why the other peptides did not retain immunologic reactivity is that free amino groups involved in binding to the antibody were derivatized by glutaraldehyde during conjugation to BSA. This explanation seems probable because peptides with the "WKYGF" motif only have one amino acid (K) with a free amino group toward the carboxyl end of the peptide. SRQDIWGIGF, on the other hand, has a free \( \alpha \)-amino group at the amino terminus of the peptide. Peptide motifs, "WGIGF-\( \beta_e\beta_e\epsilon \)" and "WKYGF-\( \beta_e\beta_e\epsilon \)" suggest that HB125 binds toward the carboxyl terminus of the peptide, while the amino terminus may provide additional contact residues, thus increasing the affinity of the peptide for the antibody.
Evidence that the amino terminus influences peptide binding is provided by the control peptide, ELHWKYGF-βεβε. This control peptide contains the "WKYGF-βεβε" motif, but does not bind to the antibody fragments or inhibit them from binding to insulin. In addition, peptides with different amino terminal residues, but with the same "WKYGF-βεβε" motif at the carboxyl end demonstrated different affinities for HB125 antibody. It is interesting to note that the control peptide, whose sequence was obtained from a non-stained bead in the initial library screening, has the consensus motif, "WKYGF-βεβε." One might question how many other peptides in the random library contain the same motif.

In the insulin-based inhibition ELISAs, the peptides were able to inhibit HB125 scFv better than HB125 parental antibody. There are several possible reasons for this. One reason is that the HB125 antibody used was intact and, therefore, bivalent. In such a situation, a bivalent antibody has an advantage in competition or inhibition assays over a monovalent scFv. Several attempts were made to generate a Fab from HB125 antibody but they were unsuccessful. Another reason why the peptides were better inhibitors for the scFv might be that the antigen combining site of a scFv is more flexible than an intact antibody and can better accommodate antigen or small peptides. Other groups have found very similar binding constants for scFvs when compared with the parent immunoglobulin (67, 72). It is also possible that HB125 antibody was more thoroughly biotinylated than the scFv, resulting a higher signal after binding to insulin. However, care was taken to keep the number of biotin molecules per molecule of antibody or scFv constant.
Monoclonal antibodies directed against the variable regions of heavy and light chains would be extremely useful reagents for detecting and purifying scFvs and antibody fragments. Anti-immunoglobulin variable region monoclonal antibodies would eliminate the need to label the antibody fragments prior to detection. Other groups have used a c-myc carboxyl terminal tag for which monoclonal antibodies exist for detection and purification (39, 68). Attempts were made to couple horse radish peroxidase to nickel so that the antibody fragments containing a poly-histidine tag could be detected, but they were unsuccessful.

Sequential screening of progressively longer libraries was performed based upon peptide sequencing results from prior screenings (figure 18). Positions observed as variable were randomized along with four new positions on the amino terminus of the peptide. The objective was to generate peptides that could bind to the antibody receptor more strongly than to insulin. Unfortunately, none were found that bound more strongly than insulin to HB125. However, when the new peptides were tested in the insulin-based inhibition ELISA, the binding affinity of peptide QSSVNHPGWKYGF was only 2.5-fold weaker than that of insulin. Randomization of the amino terminal residues on peptides containing the SRQDIWGRGF sequence did not bind as well to the scFv as peptides containing the NHPGKWYGF motif did. Generally it was observed that longer peptides (10 amino acids or more) bound to HB125 more strongly than shorter peptides. This observation supports what others have found concerning contact residues between antigen and antibody (10, 12). It is also reasonable to assume that if more residues are available to interact
with the surface area of a 600-700Å receptor molecule the stronger the binding should be.

**Mutagenesis of HB125 scFv**

Since CDR3 has been reported to be the most important region involved in binding to antigen (7, 75) and since the results had already implicated HB125 VH in binding to both insulin and peptides, CDR3 of the heavy chain from HB125 scFv was chosen for mutagenesis. Residues Y96, K98 and G99 were changed to F96, E98 and V99, respectively. The remainder of CDR3, Y100, F100A, D101 and V102 was remarkably similar to several other antibodies in the Kabat database and was left unchanged. Upon testing CDR3 mutant #1 for activity (table 3), it was observed that the ability to bind to insulin was completely lost. In order to restore binding to insulin it was decided to restore the mutated amino acids one by one to the original (wild type) starting with V99 until binding to insulin was regained. Restoration of V99 to G99 completely restored binding by HB125 scFv to insulin without having to restore the other two mutated amino acids. This result suggests that a glycine at position 99 in CDR3 of the heavy chain is important for binding to insulin. The other two mutations, Y96 to F96 and K98 to E98 did not appear to affect binding to insulin because the peptide IC50s for the CDR3 V99 to G99 mutants were restored to those of the wild type scFv.

There is precedent for one amino acid mutation causing loss of binding activity to an antigen (76). Interestingly in the study by Hasemann and Capra, mutation of position 99 in CDR3 of the heavy chain caused a loss
of binding by an anti-arsonate antibody. In addition, they found that mutation of position 96 in the light chain abrogated binding to arsonate.

By mutagenizing position 99 in CDR3, heavy chain of HB125 scFv to alanine (A) and threonine (T) the effects of amino acid hydrophobicity and size could be investigated in binding assays for position 99. The HB125 scFv A99 mutant demonstrated the ability to bind to insulin while the T99 mutant was not able to bind. This suggests that residue size, not hydrophobicity, is an important factor for HB125 scFv binding. The molecular weight of glycine and alanine are 75 and 89 daltons, respectively, versus valine and threonine which are 117 and 119, respectively. It cannot be concluded that size is the most important determinant for position 99 because V99 may also have caused a collapse of the CDR loop. However, it is unlikely that V99 would have been buried back into the flanking frameworks, because position 99 is exactly in the center of CDR3 loop according to Kabat et al (62). More extensive mutagenesis of position 99 using different sized, charged residues should be performed as well as computer modeling of the mutants. Although there are no canonical models for the structure of heavy chain CDR3, position 99 may be a necessary structural position for this particular antibody.

As controls for the CDR3 mutagenesis, CDR1 and CDR2 were mutated in the heavy chain of the scFv. CDR1 was mutated heavily; three of the five amino acids were changed: D31 to K31, S33 to G33 and H35 to N35. As a result of CDR1 mutagenesis, the ability of the scFv to bind to insulin was decreased (figure 23A). The mutation at position 31 was not a canonical structure change, but it was a non-conserved amino acid change
which may have seriously affected insulin binding capability. Inhibition ELISAs could not successfully be performed with the CDR1 mutant because the binding both to insulin and to SRQDIWGIGF-BSA (figure 23B) were too weak. One might conclude that the residues in CDR1 are involved in binding to insulin, but do not play as large a role as CDR3 residues. An alternative conclusion might be that the CDR1 mutations affect the conformation of the scFv so that insulin does not fit well into the binding site of HB125 scFv. The same conclusion could be true for CDR3 as well.

Due to the sequence similarity of CDR2 to several other antibodies' CDR2s, only one amino acid was mutated in heavy chain CDR2 of HB125 scFv: E53 to N53, making it an identical CDR2 to that of NC-41, an anti-neuraminidase antibody for which the crystal structure is known. There was no measurable difference between the CDR2 mutant and the wild type scFv in binding to insulin, or to the peptides. Unless every residue in CDR2 is mutagenized, one cannot be certain that it is not involved in binding to insulin. Even though one particular CDR may be identical to other CDRs from different antibodies, it can still be involved in binding to antigen because 5 other CDRs (in an intact antibody) influence binding to antigen which may have an indirect effect on the CDR in question. It is this concept that contributes to the vast diversity of immunoglobulin specificity.

In future studies it would be interesting to screen random peptide libraries with HB125 scFv, VH and Vk. This would allow one to compare the peptide sequences selected with the recombinant antibody fragments with the peptide sequences selected with the parental HB125 antibody. In addition, a peptide ligand for the kappa chain would probably be obtained; a
peptide ligand was not identified by screening with the intact HB125 antibody.

In conclusion, peptide mimotopes were found which bind to the paratope of an anti-insulin monoclonal antibody using random peptide library technology. These peptides inhibit insulin from binding to the anti-insulin antibody, indicating that they share at least part of the paratope for insulin. The immunoglobulin variable region genes of the anti-insulin antibody were rescued and cloned independently as $V_H$ and $V_K$ and assembled into a single polypeptide called a single chain Fv (scFv). The scFv demonstrated similar, but not identical binding properties to the parental anti-insulin antibody. Binding of four of six peptide mimotopes was localized to the variable region of the anti-insulin heavy chain. The heavy chain alone was also able to bind insulin. None of the peptides bound to the variable region of the kappa light chain. Mutagenesis studies indicated that CDR1 and CDR3 of the heavy chain were important for binding to insulin and to the peptides. Position 99 in CDR3 of the heavy chain was found to be most critical for binding to insulin. Mutational analysis of position 99 revealed that residue size, not hydrophobicity determined whether the antibody could bind to insulin.
APPENDIX A:

CLONING, SEQUENCING AND EXPRESSION OF B CELL LYMPHOMA IMMUNOGLOBULIN GENES

Figure A1. Nucleotide and Amino Acid Sequence of OCILY8 B cell Lymphoma Heavy Chain Variable Region. Frameworks are designated by arrows and CDRs are boxed.
Figure A2. Nucleotide and Amino Acid Sequence of OCILY8 B cell Lymphoma Light Chain Variable Region. Frameworks are designated by arrows and CDRs are boxed.
Surface display phagemid pComb 3

1) Nhe I, Spe I digest -663 bp gIII fragment

2) ligate

Soluble Fab expressing phagemid

Figure A3. Diagram of pComb3 Phagemid Expression Vector shown in surface display and soluble Fab-producing constructs. For soluble Fab production, gene III is removed by digestion with SpeI and NheI and self-ligation resulting in heavy and light chain production driven from two lacZ promoters.
Figure A4. Western blot analysis of OCILY8 Fab construct expression from pComb3 phagemid. The left blot is stained with an anti-lambda chain antibody. The right blot is stained with anti-sera (B67) produced against an IgM myeloma.

**Anti-λ blot:**
- Lane 1. Normal human IgM (polyclonal)
- Lane 2. no insert (cell lysate)
- Lane 3. µ-λ insert expression (cell lysate) 37°C
- Lane 4. µ-λ insert expression (supernatant) 37°C
- Lane 5. µ-λ insert expression (cell lysate) 30°C
- Lane 6. µ-λ insert expression (supernatant) 30°C

**Anti-μ blot:**
- Lane 1. µ-λ insert expression (cell lysate) 37°C
- Lane 2. µ-λ insert expression (supernatant) 37°C
- Lane 3. µ-λ insert expression (cell lysate) 30°C
- Lane 4. µ-λ insert expression (supernatant) 30°C
- Lane 5. Normal human IgM
Figure A5. Binding characteristics of recombinant OCILY8 antibodies. A microtiter plate was coated with 1µg/ml of anti-idiotype and blocked. "Rescue fusion" antibody, recombinant OCILY8 lambda chain and recombinant OCILY8 mu-lambda heterodimer were serially diluted and incubated in the anti-idiotype coated plate. Antibodies were detected with anti-human lambda chain-alkaline phosphatase anti-sera.
APPENDIX B:

PRELIMINARY CHARACTERIZATION OF HB125 ANTI-INSULIN MURINE IgG

<table>
<thead>
<tr>
<th>HB125 antibody</th>
<th>Species</th>
<th>Isotype</th>
<th>Specificity</th>
<th>K_d</th>
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<tr>
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<td>murine</td>
<td>IgG1, kappa</td>
<td>Insulin</td>
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Table B1. HB125, anti-insulin antibody characteristics.

<table>
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<tr>
<th>Peptide Library</th>
<th>Sequence</th>
<th>Motifs</th>
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<tr>
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<td></td>
</tr>
<tr>
<td>Penta</td>
<td>FNW_ _</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FDW_ _</td>
<td></td>
</tr>
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<td></td>
<td>_QDPR</td>
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</tr>
<tr>
<td>Nonan</td>
<td>_ <em>GF</em> _GF</td>
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</tbody>
</table>

Table B2. Peptide motif identified for HB125, anti-insulin antibody using the Selectide Process. (Data from Lam et al., personal communication)
APPENDIX C:

OLIGONUCLEOTIDE PCR PRIMERS FOR AMPLIFICATION OF IMMUNOGLOBULIN VARIABLE REGIONS

Oligonucleotide primer sets for initial amplification of OCILY8 lymphoma immunoglobulin variable regions from cDNA.

5' human VH leader sequences (sense):
5'-GGGAATTCATGGACTGGACCT GGAGG(AG)TC(CT)TCT(GT)C-3'
5'-GGGAATTCATGGAG(CT)TTGGGC TGA(CG)CTGG(CG)TTT(CT)T-3'
5'-GGGAATTCATGGACATG(AG)(AC)(AC)(AT)ACT(GT)TG(GT)(AT)(CGT)C(AT)(CT)(CG)CT(CT)CTG-3'

3' human N-terminal portion of the µ-constant region (anti-sense):
5'-CCAAGCTTAGACGAGGGGG AAAAGGGTT-3'.

5' human Vλ leader sequence (sense):
5' -GGGAA TTCATG(AG )CCTG( CG )(AT)C( CT)CCTCTC( CT)T( CT)CT(CG)(AT)(CT)C-3'

3' human N-terminal portion of the λ constant region (anti-sense):
5'-CCAAGCTTGAA GCTCCTCAGAGGAGGG-3'.

Oligonucleotide PCR primer sets for initial amplification of HB125 immunoglobulin variable regions from cDNA.

5' murine VH leader sequence (sense):
5'- ACTAGTCGACATGGCTTGGGTGTGGAACTTGCTATTCCTG -3' (-20 to -11)

3' murine VH sequence (anti-sense):
5'- GGTRGICARATAGGKRACCRGGGACCTTCGAACCC-3' (119 to 126)
5' murine Vk leader sequences (sense):
5'- ACTAGTCGACATGAAGTTGCCTGTTAGGCTGTTGGTGCTG -3' (-19 to -10)
5'- ACTAGTCGACATGGAGWCAGACACACTCTCTGTYTATGGGT -3' (-20 to -11)
5'- ACTAGTCGACATGGATTTWCAGGTGCAGATTWTCAGCTTC -3' (-22 to -13)

3' murine Vk sequence (anti-sense):
5'- AGGTAGAAGGGTGGTAGGTCATTCGAACCC -3' (116 to 122)

Oligonucleotide PCR primers for amplification of immunoglobulin variable region fragments and subsequent cloning into pET expression vectors.

5' primer for HB125 VH with NcoI site (sense):
5'- CGATCCATGGCACAGATCCAGTTG -3'

3' primer for HB125 VH with XhoI site (anti-sense):
5'- CGATCTCGAGGCTTCCAGGGGCCAA -3'

5' primer for HB125 Vk with NcoI site (sense):
5'- CCATGGAAATTGTTCTCACCCAG -3'

3' primer for HB125 Vk with XhoI site (anti-sense):
5'- CTCGAGTGCAGCATCAGCCCG -3'

Oligonucleotide PCR primers for single chain antibody (scFv) construction and PCR-based mutagenesis.

3' primer for HB125 VH with Gly4-Ser linker for PCR assembly of scFv (anti-sense):
5'- TGATCCGCCTCGCCGCGCTTCCAGGGGCCAA -3'
5' primer for HB125 Vk with (Gly4-Ser)3 linker for PCR assembly of scFv (sense):
5' - GCCGGAGGCGTGATCAGGAGGAGGATCAGGCGGAGGAGGATCACAATGGTTTCTCACCCAG -3'

Sense primer for HB125 scFv CDR1 (VH) mutagenesis:
5' - AAATATGGCATGAACCTGGGTGAAGCAGGCT -3'

Anti-sense primer for HB125 scFv CDR1 (VH) mutagenesis:
5' - GTTCATGCCATATTTTGTAAGGTATAACC -3'

Sense primer for HB125 scFv CDR2 (VH) mutagenesis:
5' - AACACTGGTGTCGCCAACA -3'

Anti-sense primer for HB125 scFv CDR2 (VH) mutagenesis:
5' - GTTAGGTGTTATCCAGTCC -3'

Sense primer for HB125 scFv CDR3 (VH) mutagenesis:
5' - TTTGGAGAGGTTTACTTCGATGTCTGG -3'

Anti-sense primer for HB125 scFv CDR3 (VH) mutagenesis:
5' - AACCTCTCCAAACCCTCTAGTACAGAA -3'

Sense primer for HB125 scFv CDR3 (VH) mutagenesis, restoring valine mutation to glycine wild type:
5' - TTTGGAGAGGGGTACTTCGATGTCTGG -3'

Anti-sense primer for HB125 scFv CDR3 (VH) mutagenesis, restoring valine mutation to glycine wild type:
5' - CCCCTCTCCAAACCCTCTAGTACAGAA -3'
Nomenclature:

I = inosine
R = A or G
Y = C or T
S = C or G
M = A or C
W = A or T
D = A or G or T
V = A or C or G
B = C or G or T
APPENDIX D:

PEPTIDE INHIBITION CONTROL EXPERIMENT

Some of the peptides which inhibited HB125 antibody from binding to insulin had a "WKYGF-βεβε" motif. Since "WKYGF-βεβε" resembles a CDR3-FR4 antibody loop sequence, FDYWG, a control experiment was performed to ensure that the peptides were specific for HB125 antibody and were not generic, cross-reactive peptides for murine variable heavy chains. The experiment was performed as described in methods.

The results shown in the figure below demonstrated that all peptides found with HB125 antibody were indeed specific for HB125 antibody. As shown in figure D1, only 2,4-Dinitrobenzene-BSA (DNB-BSA) was able to inhibit MOPC315 from binding to its natural antigen. If peptides with the "WKYGF-βεβε" motif had been able to inhibit MOPC315 from binding to DNB-BSA, the results would have suggested that peptides had been found which react with a conserved region on murine immunoglobulin heavy chains. Previously, Marchalonis et al. (77) were able to perform antigenic mapping of immunoglobulin lambda light chains with synthetic peptides, but no one has mapped heavy chains using synthetic peptides.
Figure D1. Dinitrobenzene Inhibition ELISA. Experiment was performed by pre-incubating MOPC315 with dinitrobenzene-BSA or peptides for 1 hour. The mixture was then added to an ELISA plate coated with dinitrobenzene-BSA. MOPC315 was detected with anti-mouse IgA. Percent inhibition was calculated as follows:
Percent inhibition of MOPC315 = 1-(O.D.450 peptide + MOPC315)/(O.D.450 dinitrobenzene-BSA + MOPC315) * 100%.
APPENDIX E:

BUFFERS AND MEDIA

**Phosphate-buffered saline (PBS):** 136mM NaCl, 10mM Na$_2$HPO$_4$, 2.7mM KCl, 1.8mM KH$_2$PO$_4$ in dH$_2$O.

**PBS-Tween-gelatin (PBSTw-gel):** 0.1% gelatin (300 bloom), 0.05% Tween-20 in PBS.

**Tris-buffered saline (TBS):** 136mM NaCl, 2.7mM KCl, 24.8mM Tris-base in dH$_2$O, pH 8.0.

**Carbonate buffer:** 35mM NaHCO$_3$, 15mM Na$_2$CO$_3$, pH 9.6.

**Superbroth (SB):** 30 grams Tryptone, 20 grams Yeast extract, 10 grams MOPS, pH 7.0 in 1 liter dH$_2$O; autoclave for sterility.

**Luria Bertoni medium (LB):** 10 grams Tryptone, 5 grams Yeast extract, 5 grams NaCl, 1ml 1N NaOH in 1 liter dH$_2$O; autoclave for sterility.

**Luria Bertoni Agar:** 10 grams Tryptone, 5 grams Yeast extract, 5 grams NaCl, 15 grams agar, 1ml 1N NaOH in 1 liter dH$_2$O; autoclave to dissolve agar and for sterility. Carbenicillin may be added when temperature cools to 50-55°C at a final concentration of 50ug/ml.

**5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal):** reagent may be added to agar for blue/white colony screening at a final concentration of 20ug/ml.

**Isopropyl β-D-thiogalactopyranoside (IPTG):** reagent was added to mid log phase cultures at 1mM to induce to cultures to produce the desired protein.

**3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMBZ):** Horse Radish Peroxidase substrate: Two solutions (A and B) are mixed 1:1 prior to use. Solution A: 0.225g TMBZ dissolved in 500ml ddH$_2$O adjusted to pH2 with HCl. Solution B: 300ml of 0.1M Na$_2$HPO$_4$, 200ml 0.1M Citric acid, adjust pH to 4.3 and add 275ul of 30% H$_2$O$_2$. 


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2. de la Paz P., Sutton B.J., Darsley M.J., Rees A.R. Modelling of the combining sites of three antilysozyme monoclonal antibodies and of the complex between one of the antibodies and its epitope. EMBO J. 1986;5:514-.


