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Isolation of An Anti-CD20 Single Chain Variable Fragment From a Naive Human Phage-ScFv Library

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ISOLATION OF AN ANTI-CD20 SINGLE CHAIN VARIABLE FRAGMENT FROM A NAÏVE HUMAN PHAGE-SCFV LIBRARY

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ABSTRACT

CD20 is a non-glycosylated transmembrane protein expressed on normal B cells, malignant B cells, and plasma cells, but not their stem cell precursors. It is an ideal target for antibody therapy because it is not significantly shed or internalized, and CD20 expression is generally not lost after antibody binding. Depleted B cells can also be regenerated after antibody therapy. Rituximab, a chimeric monoclonal antibody against CD20, was the first monoclonal antibody to be approved by the FDA for lymphoma. Results from clinical trials have shown that anti-CD20 monoclonal antibodies, which can be used unchanged or as carriers for radionuclides or toxins, have significant therapeutic effects and can be administered with minimal side effects. Several studies have also shown anti-CD20 monoclonal antibodies to be useful against autoimmune diseases such as rheumatoid arthritis.

Phage display is an effective tool in genomics and drug development. The ability to obtain high affinity binders for any antigen with decreased cost and time expenditure has been useful in the development of therapeutics. Single chain variable fragments (scfvs) are the smallest antibody fragments which maintain the specificity and the affinity of the parent antibody. ScFvs can be conjugated to radioisotopes, toxins, drugs, highly effective Fc region isotypes, and gene delivery vectors, which can be employed in diagnosis and therapy.

Studies have shown that chimeric and humanized forms of anti-CD20 demonstrate improved clinical efficacy, as compared with murine anti-CD20 monoclonal antibody, as well as a decrease in HAMA (human anti-mouse antibody) response, which

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is frequently observed with murine antibodies. Although chimerization of murine antibodies through protein engineering can retain the affinity and specificity of the parental molecule, this strategy is time-consuming and does not yield the benefit of fully human antibodies.

To further improve the efficacy of anti-CD20 antibodies, a naïve human scFv phage library (Tomlinson I+J) was used in the present study to screen for human antibodies against CD20. A cell based screening strategy was employed in this study by establishing a tetracycline inducible CD20 CHO cell line, to better mimic the microenvironment where CD20 and anti-CD20 antibody interaction would occur. Panning of the phage library against the CD20 transfected cell lines yielded clones with affinity for CD20 antigen. PCR analysis showed the expected 935bp scFv band, and monoclonal ELISA showed an affinity for CD20 antigen. Periplasmic protein was extracted from the clone and subjected to dot blot analysis, showing that in its native form, Clone 3 had an affinity for CD20. A fusion gene with the CD20 extracellular domain and an in vivo biotinylation domain was constructed for the isolation and characterization of antibody fragments. A functional biotinylated CD20 fusion protein was obtained from bacterial production and western blot analysis under denaturing conditions yielded a 20kDa band as expected.

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CHAPTER 1

OBJECTIVES

The objective of this project was to isolate and characterize anti-CD20 antibody fragments by screening a naïve human scFv phage library against cell lines transfected with the target antigen CD20. A biotinylated CD20 fusion protein expressed in *E. coli* was constructed to provide large quantities of fusion protein for the identification and characterization of the selected antibody fragments.

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CHAPTER 2

REVIEW OF LITERATURE

2.1 Lymphoma and Autoimmune Diseases

Lymphomas are cancers that start in lymphoid tissue. They are classified as Hodgkin's Lymphoma or non-Hodgkin's Lymphoma (NHL) based on their cellular morphology (American Cancer Society (ACS), 2006). Hodgkin's Lymphoma is characterized by Reed-Sternberg cells. Reed Sternberg cells are multinucleate cells mostly derived from germinal or post-germinal center B cells (Diehl et al., 2003). All other lymphomas are classified under the NHL category. Non-Hodgkin's Lymphomas can be further classified as B-cell NHL, T-cell NHL and natural killer (NK) cell NHL. B-cell NHLs include diffuse large B-cell, follicular lymphomas, marginal zone of mucosa-associated lymphoid tissue (MALT), chronic lymphocytic leukemia (B-CLL), and mantle cell lymphoma. The two most common types of B-cell NHL: diffuse large B-cell lymphoma and follicular lymphoma account for about 33% and 14% of NHL cases respectively. T-cell lymphoma subtypes include lymphoblastic, anaplastic and cutaneous lymphomas (ACS, 2006). B-cell lymphoma accounts for 85% of all non-Hodgkins lymphoma cases (Harris et al., 1995 as cited in Multani et al., 2001). The American Cancer Society estimated 58,870 NHL cases in the United States in 2006 and 18,840 deaths from it (ACS, 2006). B cells are also implicated in the pathogenesis of autoimmune diseases like lupus and rheumatoid arthritis. Systemic Lupus Erythematosus (SLE) is an autoimmune disease distinguished by autoreactive T cells, and plasma cells which produce autoantibodies (Tokunaga et al., 2005). In addition to being precursors to

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autoantibody-secreting plasma cells, B cells play a role in the development of lymphoid tissue and secrete cytokines, chemokines and growth factors (Sabahi & Anolik, 2006). Autoantibodies, secreted by plasma cells are also implicated in rheumatoid arthritis, an autoimmune disease typified by inflammation of the synovium (Edwards & Cambridge, 2001).

2.2 Conventional Therapies

Prior to the FDA approval of monoclonal antibodies, therapy for non-Hodgkin's lymphoma consisted of radiation therapy, chemotherapy and bone marrow transplantation. Radiation therapy is the first line of treatment for patients with stage I and indolent disease, and is used in combination with chemotherapy for later stage or more aggressive cancers (NCI, 2007). The head, neck and under the arms, known as the mantle field, are usually irradiated in NHL. A serious side effect associated with radiation therapy is the potential for new cancers to arise in the radiation field. The CHOP regimen—cyclophosphamide, doxorubicin, vincristine and prednisone, is the most common chemotherapy regimen administered to intermediate grade NHL patients. This regimen targets rapidly dividing cells, both normal and malignant, leading to a large number of side effects including a decrease in white blood cell counts, peripheral neuropathy, and infertility in women (ACS, 2006, Franchi-Rezgui et al., 2003). Peripheral blood stem cell transplantation (SCT) is used mostly in patients with relapsed intermediate to high grade lymphomas, or patients who are refractory to conventional therapy (Multani et al., 2001). In SCT, stem cells are collected from the blood of the patient (autologous) or from a

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matched donor (allogeneic). The patient is subjected to high doses of chemotherapy or radiation therapy to kill all cancer cells, depleting normal blood and bone marrow cells in the process. The stem cells are then infused back into the patient to replenish depleted cells. SCT may lead to the development of leukemia, graft-versus-host disease, and to death in some patients (ACS 2006, Multani et al., 2001). Most side effects associated with conventional therapies are due to the non-specific nature of the treatment. Thus targeted therapy offers ways to improve clinical response while decreasing toxicity.

2.3 Targeted Therapy

Targeted therapy in cancer involves the administration of a substance which specifically interacts with a molecule which may be directly or indirectly involved in oncogenesis (Sledge, 2005). These include tumor-associated antigens expressed on the cell surface, soluble factors, extracellular matrix proteins and proteins associated with vascularization of tumors. Carter et al., (2004) outline four steps in the identification of tumor-associated cell surface antigens, and the properties an ideal target should possess. The first is the procurement of tumor tissue and similar non-tumor cells. Differences between the tumor and non-tumor tissue are observed on a DNA, mRNA, protein, or antibody reactivity level to identify the potential target. Antibodies are generated in the next step to characterize the expression of the target in both tumor and non-tumor tissue. It is important to validate that the antigen is established on the plasma membrane and its extracellular components can bind antibody. The last step is in vitro and in vivo screening of generated antibodies to select clones with desired anti-tumor properties (Carter et al.,

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2004). There are several characteristics an antigen should possess before it can be deemed an appropriate target for therapy. The expression of the antigen should ideally be limited to only cancerous cells to decrease any side effects which may result from targeting of normal cells. This ideal is hardly ever met, thus markers expressed on nonvital normal cells, or cells which have a means of being replenished are employed. Presence of the antigen during all stages of cancer broadens the spectrum of cancer patients treatable with its target substance. Antigens involved in pathogenesis are most preferred because they are least likely to be down-regulated by the tumor, thus providing a stable antigen for targeting. Antigens which are not shed are desirable targets for therapy. Shed antigens may cause antibody clearance before any positive effect is achieved, and the antibody complexes formed may cause harmful side effects (Carter et al., 2004). Different types of targeted therapies involve epidermal growth factor receptor inhibitors, enzyme inhibitors, proteasome inhibitors, angiogenesis inhibitors and monoclonal antibodies (ASCO, 2006).

2.4 Antibodies

Antibodies are soluble glycoproteins which are secreted by B lymphocytes (plasma cells) in response to encountered antigen. Antibodies have a molecular mass of approximately 150kDa and consist of two pairs of light chains (25kDa each) and 2 pairs of heavy chains (50Kda each). Light chains, which may be kappa or lambda isotype, consist of one variable domain and one constant domain. A single immunoglobulin contains only one isotype of light chain, even though no functional difference has been

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found between both isotypes. Each heavy chain consists of one variable domain and three or four constant domains (C_H1-4) depending on the isotype. C_H2 domains contain an N-linked glycosylation site. C_H2 and C_H3 domains have sequences which are involved in binding to effector cells and fixation of complement (Vaughan et al., 1998). The class or overall isotype of an antibody, as well as its effector functions depends on its heavy chains. These effector functions are performed through the carboxy-terminal of the heavy chain. Heavy chains can be of an M, A, D, G, and E isotype or class (Janeway et al., 2001). Heavy chain domains are formed by two beta sheets linked by a disulfide bridge and interstrand loops (Morea et al., 2000).

At the amino terminal of each chain lies a region of about 110 amino acids which greatly varies in different antibodies. This is the variable domain which functions in antigen binding. It comprises the framework regions whose sequence and main conformation are conserved, and the hypervariable regions which bind specific epitopes of an antigen. The hypervariable regions or complementarity determining regions (CDR) comprise six loops: three per variable region. Five of the six loops take on a small number of main chain conformations called canonical structures. These structures are dependent upon the length of the loops and key residues which determine the conformation of the loops (Morea et al., 2000). The information acquired from canonical structures is useful in the manipulation and modification of antibodies.

Antibodies are an attractive mode of therapy because of their ability to bind specifically and with high affinity to a vast array of antigens, their stability, and the relative ease with which they can be engineered or manipulated (Kretzschmar & von

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Ruden, 2002). Antigens are substances which elicit an immune response. An epitope or antigenic determinant is a portion of the antigen which is recognized by a specific antibody. After the introduction of an antigenic substance into the body, plasma cells which recognize its epitopes are activated. The resulting group of antibodies is polyclonal because it recognizes a multitude of epitopes. Isolating a single monoclonal antibody to a specific epitope from serum proved difficult. The mortality of B lymphocytes reduced their usefulness as a source of monoclonal antibodies (Janeway et al., 2001). Georges Kohler and Cesar Milstein (1975) came up with the hybridoma technique of generating monoclonal antibodies. A hybridoma is a cell resulting from the fusion of a B lymphocyte and a myeloma cell. B lymphocytes are isolated from the spleen of mice injected with the antigen of choice. The B lymphocyte thus provides the ability to synthesize antibodies to the specific antigen, and the myeloma cells provide immortality.

While this hybridoma technique allowed the isolation and use of countless monoclonal antibodies, their murine origins limited in vivo use. Clinical murine monoclonal antibodies stimulated a response known as HAMA (Human Anti Mouse Antibodies). The body recognizes the murine antibodies as foreign and clears them from the circulation, reducing their efficacy and causing side effects in the patient. Their murine origins also mean reduced ability to recruit human effector cell functions, and inferior pharmacokinetics (Link, 2005). However, because the domain structure of antibodies is conserved across species, protein engineering enables the manipulation of antibodies to maintain high binding affinity, decrease immunogenicity and improve effector cell recruitment (Vaughan et al., 1998).

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Chimeric antibodies were generated to decrease human anti-mouse response. Chimeric antibodies consist of murine variable regions leaving the antigen binding site intact, and human constant regions. While immunogenicity is decreased in vivo, some patients produce human anti-chimeric antibodies which invariably decrease the efficiency of the chimeric antibody (Pavlou & Belsey, 2005). Both immune responses also decrease the effectiveness of the drug with increasing number of administrations as the body builds up a defense against it.

Humanized antibodies sought to decrease further the murine percentage of the therapeutic antibody, restricting murine portions to only the complementarity determining regions of the antibody. Both humanized and human antibodies show minimal or no immunogenicity and the latter allow smaller doses and less frequent administration due to increased serum half-lives (Pavlou & Belsey, 2005). Different manipulations, such as pepsin and papain digestion, and sequence manipulation, provided several antibody fragment types including Fab fragments, Fc regions and scFvs, which can be employed in therapy.

2.5 Single Chain Variable Fragments

Single chain variable fragments (ScFvs) are the smallest antibody fragments which retain the complete antigen binding site of the parent antibody (Leath et al., 2004). An ScFv is expressed as one polypeptide chain, encoded by a single gene (Blazek & Celer, 2003). They are composed of the variable regions from heavy (V_H) and light chains (V_L), linked covalently by a flexible peptide which prevents dissociation (Holliger

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& Hudson, 2005). This covalent bond enhances the association of the two domains after folding. The carboxy terminal of the V_H is linked to the amino terminal of the V_L, allowing the same molar concentration of both variable fragments to be expressed. Various types of linkers have been designed to connect the two domains. Some were designed to have minimal interaction with the variable fragment, while another set consisting of alternating serine and glycine residues were designed to fit into a groove in the back of the fragment (Bird et al., 1988). ScFvs are usually obtained from immunoglobulin G and retain not only its specificity, but also the affinity with which it binds antigen (Holliger & Hudson, 2005). Prior to the use of in vitro display techniques to isolate antibodies and antibody fragments, mRNA was isolated from a hybridoma, reverse transcribed and the antibody gene segments amplified via PCR (Blazek & Celer, 2003). The introduction of phage and ribosomal display libraries decreased the time involved in the generation of highly specific single chain fragments.

Antibodies and their fragments are employed in the identification, isolation, and characterization of cell surface molecules, from which new targets for treatment can be identified. In diagnostics, antibodies can be used to determine different cell types present in serum, and their cell counts (Zola, 2006). ScFvs can also bind and neutralize viruses and toxins. ScFvs with neutralizing activity against human cytomegalovirus glycoproteins and HIV-1 Tat antigen have been isolated and characterized (Blazek & Celer, 2003, Moreau et al., 2003). The quick tumor penetration, rapid clearance from circulation, and increased tumor-to-blood ratio of antibody fragments makes them great candidates for the diagnosis and therapy of cancer (Verhaar et al., 1996). Radionuclides

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like Technetium-99 have been coupled to tumor antigen specific scFvs to aid in tumor imaging and dosimetry (Verhaar et al., 1996, Blazek & Celer, 2003). Antibody fragments can also be equipped to deliver toxins to cancer cells. The antibody target should however internalize after binding, and the enzyme fragment of the toxin should be able to translocate to the cytosol. Toxins usually employed include *Pseudomonas* exotoxin (PE) and truncated diphtheria toxin (Kreitman, 2006). Phase I clinical trials with SS1P an anti-mesothelin-PE-conjugate, in mesothelioma, ovarian cancer, and pancreatic cancer patients showed improved response (Hassan et al., 2004). ScFvs have been used to improve specificity and to decrease immunogenicity of gene therapy vectors. An adenoviral vector including a gene for an anti-erbB2 scFv administered intraperitoneally to ovarian cancer patients, showed no vector-related toxicity and downgraded 38% of patients in the study from recurrent to stable disease (Alvarez et al., 2000 as cited in Leath et al., 2004).

2.6 Phage Display

In vitro display techniques are a cheaper and considerably faster means of obtaining a vast array of human antibodies and antibody fragments. The most prevalent in vitro selection technique is phage display. The basic principle in display technology is the direct link between phenotype and genotype (Holt et al., 2000). The genes encoding the fusion protein and its display are packaged into the phage particle as a single stranded DNA molecule, during the assembly of phage particles. This ensures that all phage from a single bacterial colony are identical (Paschke, 2005).

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The displayed proteins are the result of fusion between a phage capsid protein and the protein or antibody fragment of choice. Phage libraries contain large numbers of phage exhibiting different proteins on their surface. Phage display allows the isolation of high affinity, fully human antibodies in a short time and with high throughput (Vaughan et al., 1998, Kretzschmar & von Ruden, 2002). The most common phage used in phage display is the filamentous non-lytic M13, where the antibody fragment is fused to its minor capsid protein pIII, or major capsid protein pVIII (Kretzschmar & von Ruden, 2002, Paschke, 2005). There are about 2700 molecules of the 5kDa pVIII expressed to form a cylinder which encases the phage genome. Three to five molecules of pIII, which has a molecular weight of about 42kDa, are expressed at one end of a phage particle. pIII consists of three domains: CT at the carboxyl terminal, and N1 and N2 at the amino terminal. N2 binds to the F pilus to start the infection process, while CT may play a role in the translocation of DNA (Webster, in Kay et al., 1996, Paschke, 2005).

Phage display systems can be divided into two groups based on the vectors used to acquire phage. Ff phage (M13, fl and fd) are 98% homologous filamentous phage which are capable of infecting gram negative bacteria bearing the F conjugative plasmid (Webster, in Kay et al., 1996, Paschke, 2005). The first group of phage vectors is derived from the genome of Ff phage, and contains all the genetic information required for replication and phage particle assembly. The fusion protein can be produced by the modification of the capsid protein or by inserting a gene cassette which encodes for the fusion protein. In the latter example, the phage displays both wildtype and fusion coat proteins. The second group involves the use of phagemids, and generally gives rise to

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phage which express both wildtype and fusion coat proteins (Paschke, 2005). A phagemid is a plasmid which contains an origin of replication and a packaging signal from a filamentous phage, as well as an origin of replication and gene expression systems from a bacterial plasmid. A phagemid is able to maintain itself as a plasmid in bacteria, in the absence of phage infection. The phage origin of replication is activated when the host bacteria is infected by helper phage (Webster, in Kay et al., 1996). In this system, phagemids make the fusion protein. However, all the proteins needed for other functions including replication and packaging are provided by a helper phage, which co-infects the same bacterial cell. The helper phage then packages the single stranded phagemid DNA. However, helper phage is also capable of packaging its own genome, resulting in phage particles which do not express the fusion protein. Helper phages with defective origins of replication or defective packaging signals can be employed in order to encourage packaging of phagemid DNA over helper phage genome (Russel et al., 1996; Vieira & Messing, 1987, as cited in Paschke, 2005). Ff phage display three to five copies of pIII on their surface, limiting the amount of fusion protein expressed. It has however been shown that during phage assembly, wild type pIII is packaged over the fusion product. While this aids in avoiding the effects of avidity, the diversity of the system is reduced. This can be overcome by engineering the helper phage to package only the pIII fusion protein. Another option was to eliminate the infectivity of packaged phage particles which expressed wildtype pIII by introducing a protease restriction site between the N1 and N2 domains. Thus, only phage particles which express the fusion protein are capable of

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infection (Rondot et al., 2001; Kirsch et al., 2005; Kristensen & Winter, 1998; as cited in Paschke, 2005).

Phage libraries can be naïve, immune or synthetic. Naïve libraries are generated from antibodies which have yet to encounter antigen. Immune libraries are specific for a particular antigen. Naïve libraries are employed in the search for antibodies against any antigen. They are also a better source of antibodies to cell surface markers compared to immune libraries. Self reactive antibodies removed by tolerance mechanisms may include antibodies reactive to cell surface markers, thereby reducing the availability of these antibodies to immune libraries (Kretzschmar & von Ruden, 2002, Roovers et al., 2001). Holt et al., (2000) define naïve libraries as natural, synthetic, or targeted. Natural libraries comprise naturally rearranged genes obtained from human B lymphocytes. Synthetic libraries involve the in vitro rearrangement of human germline antibody genes by PCR. Targeted libraries consist of optimized human antibody frameworks, with specific residues in the antigen binding site diversified (Holt et al., 2000).

Phage libraries should possess certain characteristics which would enable the selection of useful antibodies. Kretzschmar and von Ruden (2002) describe these collectively as 'high quality' libraries. First, the library should have a large functional size with great sequence diversity, to enable the selection of antibodies with high affinity. Naïve libraries are usually 10^7 to 10^{10} in size (Holt et al., 2000). The antibody genes need to be stably expressed in order to retain their characteristics during panning and screening processes. The design of the library should also allow rapid manipulation of chosen antibodies (Kretzschmar and von Ruden, 2002).

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2.7 CD20

CD20 is a non-glycosylated transmembrane differentiation antigen that is expressed on all B cells with the exception of pro-B cells and activated plasma cells. The CD20 molecule is encoded by a 16kb single copy gene, which comprises eight exons (Tedder et al., 1989). Due to differences in phosphorylation of serine and threonine residues, CD20 is expressed in three forms: 33, 35 and 37Kda (Tedder et al., 1988, 1993 as cited in Tedder & Engel, 1994). CD20 is a member of the MS4A (membrane spanning 4 domains, subfamily A) gene family, along with the β chain of the IgE receptor, FcεRI. FcεRI β and CD20 are similar in structure and the genes for both molecules are found on chromosome 11q (Cragg et al., 2005). CD20 comprises a long hydrophobic chain which passes through the cell membrane four times, creating two extracellular fragments. The larger of these two fragments has an Ala X Pro motif which is important for antibody binding (Cragg et al., 2005). Both the amino and carboxy terminals of CD20 are in the cytoplasm (Tedder & Engel, 1994, Tedder et al., 1989). Cross-linking and immunoprecipitation experiments have shown that CD20 complexes to form homodimers and homotetramers. CD20 also associates with CD40, MHCII and B-Cell receptor (BCR) molecules on the cell surface (Tedder & Engel, 1994, Leveille et al., 1999, Petrie & Deans, 2002, as cited in Cragg et al., 2005).

The structure of CD20, along with its similarities to FcεRI β, suggested that CD20 may form a membrane channel (Cragg et al., 2005). Bubien et al., (1993) showed that CD20 may play a role in calcium conductance. CD20-negative cell lines which were transfected with CD20 showed an increase in the basal level of cytosolic calcium. They

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also demonstrated that calcium uptake was higher in CD20 transfectants than equivalent controls, after the re-introduction of calcium to calcium-depleted cells (Bubien et al., 1993, Tedder & Engel, 1994). B1, a murine IgG2a anti-CD20 monoclonal antibody, causes improved progression from cell cycle phase G0 to G1. However, 1F5, a different anti-CD20 monoclonal antibody of the same isotype, blocked the passage from G1 into S, G2 and M phase (Golay et al., 1985, Tedder et al., 1986, as cited in Cragg et al., 2005). Experiments on Daudi cells showed that B1 induced a slow increase in cytoplasmic calcium upon binding, while 1F5 induced a rapid calcium flux. These results suggest that the differences in effect on cell cycle progression may be enabled through their effects on calcium levels (Cragg et al., 2005). CD20 knockout mice had reduced B cell IgM expression and CD19-induced intracellular calcium responses were reduced. However, other B cell characteristics and functions were normal. This suggests that CD20 is not ultimately vital to the survival of B cells. Alternatively, other MS4A genes may contribute to calcium ion transport, and despite their 73% amino acid homology, mouse and human CD20 may be regulated differently (Uchida et al., 2004, Cragg et al., 2005). Though CD20 has no known natural ligand, it is capable of generating transmembrane signals when bound by a CD20 specific monoclonal antibody (Cragg et al., 2005). However, the type of response depends on the specific antibody. Cragg and Glennie (2004a) classified anti-CD20 monoclonal antibodies into 2 groups based on their ability to eliminate lymphoma xenografts: type I monoclonal antibodies which use complement and Type II which do not.

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Several sets of experimental data support the idea that complement-dependent mechanisms are a major factor in the therapeutic effect of type I anti-CD20 monoclonal antibodies. It has been shown that complement is depleted during treatment with rituximab, a type I antibody (van der Kolk et al., 2001 as cited in Cragg et al., 2005). The consumption of complement by cobra venom factor reduced the efficiency of rituximab and 1F5 in lymphoma xenograft models, but not of B1, a type II monoclonal antibody. Rituximab and 1F5 were also shown to bind C1q, which initiates the classical complement pathway, and deposit C3b (Cragg & Glennie, 2004a). Some cells remaining after anti-CD20 therapy have increased levels of CD59, a molecule which prevents the formation of the membrane attack complex during the classical complement pathway. This suggests that these cells have been subject to complement during antibody therapy (Bannerji et al., 2000, Treon et al., 2001, as cited in Cragg & Glennie, 2004b). While both antibody types have comparable association rates, the maximum level of Type II binding, is approximately half the maximum level of Type I monoclonal antibodies (Chan et al., 2003, Teeling et al., 2004). Other differences between the two groups have been demonstrated. Type I monoclonal antibodies like 1F5 cause the redistribution of CD20 into Triton X-100-insoluble lipid rafts. Type II monoclonal antibodies like B1 do not cause the redistribution of CD20, but are effective at inducing apoptosis and homotypic adhesion (Chan et al., 2003, Cragg et al., 2005).

Shan et al., (1998) demonstrated that crosslinking of CD20 with murine anti-CD20 monoclonal antibodies in the presence of Fc-receptor bearing cells, inhibits B-cell proliferation, causes fragmentation of nuclear DNA, and leads to apoptosis. Apoptosis

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was however obstructed by the addition of calcium ion chelating agents. This suggested that the binding of the antibodies may induce signal transduction events which ultimately lead to apoptosis (Shan et al., 1998). Comprehensive depletion of CD20-positive cells in B-CLL patients treated with rituximab was shown to correspond to apoptosis, as evidenced by the blood levels of apoptotic by-products (Byrd et al., 2002, as cited in Chan et al., 2003). Bannerji et al., (2003) also showed that high levels of complement dependent cytotoxicity (CDC) and apoptosis regulatory proteins in CLL patients corresponded with resistance to rituximab treatment. Experiments with Fab fragments however showed that apoptosis could occur independent of the Fc portion. The levels of apoptosis observed were comparable to levels obtained with complete antibodies. In agreement with Shan et al., (1998), crosslinking was required, and was achieved through the use of bivalent antibody fragments (Shan et al., 1998, Chan et al., 2003, Ghetie et al., 2001).

Both type I and type II antibodies induce similar antibody dependent cell-mediated cytotoxicity (ADCC) (Teeling et al., 2004, Cragg et al., 2005). An IgG4 variant of rituximab, which is less effective at recruiting effector cells than the IgG1 original, was unable to deplete B cells (Anderson et al., 1997, as cited in Cragg & Glennie, 2004a). This was one of the first hints that anti-CD20 antibodies used Fc-dependent mechanisms. $F(ab)^2$ fragments derived from type I anti-CD20 monoclonal antibodies had no therapeutic effect in Burkitt's lymphoma xenograft models, further supporting Fc-dependent mechanisms as a factor in anti-CD20 antibody therapy (Cragg & Glennie, 2004b). Non-Hodgkin's lymphoma patients expressing the 158V allotype of Fc-gamma-

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RIIIa, a higher affinity variant of Fc receptor, showed better response rates to rituximab, than patients with the low affinity 158F allotype. While this lends more support to the importance of Fc receptor mediated mechanisms in anti-CD20 therapy in non-Hodgkin's lymphoma, Farag et al., (2004) showed that allotype is not an indicator of response in chronic lymphocytic leukemia (Cartron et al., 2002, Farag et al., 2004 as cited in Cragg & Glennie, 2004b). Binding of most anti-CD20 monoclonal antibodies leads to increased CD20 phosphorylation, increased expression of MHCII molecules, the downregulation of interleukin-10, and the activation of src-kinases, phospholipases, and caspases (Tedder et al., 1988, Clark et al., 1987, White et al., 1991 as cited in Tedder & Engel, 1994, Johnson & Glennie, 2003). In summary, in vivo and in vitro evidence point to ADCC, CDC and apoptosis as the major mechanisms through which anti-CD20 monoclonal antibodies achieve their therapeutic effect (Cragg et al., 2005).

CD20 is a great target for antibody therapy for a number of reasons. It is expressed on about 95% of all B cell lymphomas (Davis et al., 1999), and its expression is limited to cells of B lineage, allowing for increased tumor specificity. Among these cells, pro-B cells, hematopoietic stem cells and plasma cells do not express CD20. The first two allow for recovery after treatment, and the last helps maintain immunoglobulin levels (Cragg et al., 2005). CD20 has a high antigenic density: there are 100,000-400,000 CD20 molecules per lymphoma B cell. This allows for improved targeting, and increased effector cell response (Cragg et al., 2005, Press et al., 1989). CD20 is not shed after binding, nor is it internalized, which means improved ADCC and CDC, and also makes CD20 a good target for radioimmunoconjugates. Targets which are internalized upon

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binding lead to quicker break down and removal of the radioisotope, decreasing the effectiveness of the conjugate (Glennie & van der Winkel, 2003). The occurrence of CD20-negative variants after treatment is generally not common, allowing the repeat use of antibody therapy (Cragg et al., 2005).

2.8 Current Anti-CD20 Monoclonal Antibodies

Rituximab, a type I chimeric monoclonal antibody was the first monoclonal antibody to be FDA approved for the treatment of cancer. It was approved in 1997 for the treatment of low-grade B-cell Non-Hodgkin's Lymphoma (Cartron et al., 2007). Rituximab is also used in combination with chemotherapy for diffuse large B cell lymphoma and follicular lymphoma. In a study of elderly diffuse large B-cell lymphoma patients, the addition of rituximab to CHOP regimen increased the complete response rate and overall survival of the patients without increased toxicity (Coiffier et al., 2002). Similar results were observed when rituximab was added to CVP (cyclophosphamide, vincristine, and prednisone) for follicular lymphoma (Imrie et al., 2005).

Rituximab is a chimeric antibody comprising light and heavy variable chains from the murine anti-CD20 antibody 2B8, human IgG1 heavy chain, and human kappa light chain (Reff et al., 1994). Due to its ability to deplete B cells, rituximab has proven useful in therapy of a number of hematological diseases. Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease where platelets are destroyed by being opsonized by autoantibodies (Cines et al., 2002, as cited in Braendstrup et al., 2005). Braendstrup et al., (2005) and others have shown a 50% response rate in ITP patients treated with rituximab.

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In a study, rheumatoid arthritis patients treated with rituximab, cyclophosphamide and prednisolone, showed improvements which lasted beyond six months (Edwards & Cambridge 2001). Rituximab has since been FDA-approved in combination with methotrexate to decrease symptoms in adult rheumatoid arthritis patients who had insubstantial response to tumor necrosis factor (TNF) antagonists (Biogen Idec & Genentech, 2007). In a small study with refractory SLE patients, rituximab was shown to reduce B-cell numbers and improve the condition of patients with SLE-associated ailments. Patients did not relapse even after B-cell counts returned to normal (Tokunaga et al., 2005). This could be explained by results obtained by Anolik et al., (2004) where rituximab was shown to settle deviations in SLE patients exhibiting aberrant B cell homeostasis.

Despite its successes, a significant group of NHL patients do not respond to rituximab. This may be due to CD20 being expressed heterogeneously, or the impairment of ADCC and CDC mechanisms which play a role in the efficacy of rituximab. Even with responsive patients, there is a chance of relapse (Schaefer-Cutillo et al., 2007). In some SLE patients, rituximab was shown to cause reactivation of JC virus, a polyoma virus implicated in progressive multifocal leukoencephalopathy (PML), a viral infection of the brain (FDA, 2006).

Zevalin, an anti-CD20 immunoconjugate was FDA-approved as part of a regimen including rituximab for the treatment of relapsed or refractory follicular, low-grade, or B-cell NHL in 2002. It was also approved for treatment of rituximab-refractory follicular NHL (FDA, 2002). It is a murine IgG1 kappa monoclonal antibody, ibritumomab, linked

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to yttrium-90 for therapy, or indium-111, for imaging and to evaluate the biodistribution of ibritumomab, via tiuxetan (Spies, 2004). Tiuxetan is a high affinity linker and chelator for yttrium-90 and indium-111 which is covalently bound to the monoclonal antibody (Krasner & Joyce, 2001, Wiseman et al., 2002, Biogen Idec, 2006, Witzig, 2001). Yttrium-90 is a pure beta-emitter with a half-life of 64hrs. The path length for which greater than 90% of the energy emitted is absorbed is 5.3mm. These characteristics enable efficient delivery of radiation to cancer cells (Knox et al., 1996, Biogen-Idec, 2006). In a randomized study, 30% of B-cell NHL patients treated with Zevalin and rituximab had a complete response, as assessed by an independent panel, versus 16% of patients treated with rituximab alone (Witzig et al., 2002).

Bexxar is an immunoconjugate comprising a murine IgG2a lambda monoclonal antibody tositumomab, covalently linked to Iodine-121. In 2003, tositumomab and Bexxar were FDA approved for treatment of CD20 positive follicular NHL, which was rituximab-refractory or had relapsed after chemotherapy (FDA, 2007). Iodine-131 emits beta and gamma rays, with a path length of 0.8mm, and a half life of about 8 days. In addition to antibody mediated destruction of cells, radiation emitted by Iodine-131 kills tumor cells in contact with the antibody as well as tumor and normal cells nearby via 'bystander effect'. Bexxar and Zevalin have the best results when used as single agents for their approved malignancies in comparison to other anti-CD20 antibodies (Schaefer-Cutillo et al., 2007).

Humax-CD20 (Ofatumumab) is a fully human anti-CD20 IgG1 monoclonal antibody 2F2, currently in Phase III trials for the treatment of NHL, CLL, and rheumatoid

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arthritis (Genmab, 2007). 2F2 is a type I monoclonal antibody, which is excellent in CDC and is capable of lysing rituximab-resistant CLL cells. In comparison to rituximab, 2F2 had significantly lower dissociation rates, which could account for its increased CDC potency, and makes it a good choice for the construction of radioconjugates (Teeling et al., 2004). A human anti-CD20 monoclonal antibody, engineered to have a faster dissociation rate than rituximab was still comparatively potent in CDC, suggesting that CDC potency was not completely dependent on dissociation rate (Teeling et al., 2006). Clinical studies showed favorable response in NHL patients who were refractory to rituximab and patients who had previously responded to rituximab treatment. No dose limiting toxicity was reported for Humax-CD20 during the study. Studies in patients with CLL and rheumatoid arthritis also showed high response rates (Genmab, 2007). Teeling et al., (2006) developed a panel of completely human anti-CD20 antibodies which do not rely on the AxP motif in the CD20 extracellular fragment for binding. A 15 amino acid segment N-terminal of the AxP motif was shown to be the binding site. These antibodies did not need high CD20 cell surface density to cause cell lysis in comparison to rituximab (Teeling et al., 2006).

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CHAPTER 3

MATERIALS AND METHODS

3.1 Cell Lines

A Tetracycline-regulated Expression (T-Rex™) CHO cell line stably expressing the tetracycline (Tet) repressor and designed for use with the T-REx™ System was obtained from Invitrogen. 293T/17 and Raji cells were obtained from ATCC (ATCC# CRL-11268, and CCL-86 respectively). 293T/CD20 cells were a gift from Dr. P. Wang (University of Southern California). All cells were cultured according to the suppliers' protocols.

3.2 Construction, Expression, and Refolding of a Biotinylated Fusion Protein Containing the Extracellular Domain of CD20

RNA was extracted from Raji Cells and reverse transcribed into cDNA. Primers CD20Foward (5'-TTTGAGAGCAAAATGACAACACCCAGA-3') and CD20Reverse (5'-AGAAGAAATCACTTAAGGAGAGCTGTC-3') were designed to amplify the 894bp CD20 ORF. The resulting fragment was ligated into PCR 2.1 vector (Invitrogen). Competent Top 10 *E coli* cells were transformed with vector which had CD20 ORF in the correct orientation. The extracellular fragment of CD20 which falls between nucleotides 418 to 571 in the CD20 ORF was then amplified using primers CD20-EC Forward (5'- TCGCGAAAATATTAAAAT TTCCCATTTTTTAAAAATG-3') and CD20-EC Reverse (5'-TTAGAACAGAGATTGTATGCTGTAACAGTA-3'), designed to amplify the extracellular fragment, as well as introduce an NruI restriction site and a TAA stop codon. This cDNA fragment was ligated into the Pinpoint Xa-1

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Vector (Promega, Madison, WI) in frame with the in vivo biotinylation fragment to form a fusion gene. Rosetta *E. coli* cells (Novagen) were transformed with the ligation reaction and cultured on LB plates overnight. A single colony was picked and cultured overnight in LB medium with biotin and induced with IPTG (100µM) at 37°C for 4hrs. The cultures were centrifuged and pellets resuspended in PBS. After sonication with a Branson Sonifier (VWR Scientific), the cells were centrifuged and the supernatant discarded. The pellet was resuspended in wash solution (0.5% Triton X100, 50mM tris-HCL pH8, 100mM NaCl and 0.1% sodium azide) using a homogenizer on ice. The homogenized sample was centrifuged at high speed for 10min and the supernatant discarded. This wash was repeated five times. The resulting pellet was resuspended in 30ml buffer (8M Urea, 50mM Tris pH 8, 100mM NaCl, 10mM EDTA, and 10mM DTT) and placed on a rocker at room temperature overnight. The sample was centrifuged and the precipitate discarded. The protein was refolded by dialyzing against refolding buffers (50mM Tris pH 8, 100mM NaCl, 10mM EDTA, and 10mM DTT) with decreasing concentrations of urea (8M, 6M, 4M, 2M, 1M, 0.5M) at room temperature for 4 hours at each urea concentration. The sample was finally dialyzed against water at 4°C overnight. This refolding protocol was adapted from Rudolph and Lilie (1996).

3.3 Construction of a Tetracycline Inducible CD20 Expression Vector

The CD20 cDNA sequence from the Ultimate™ ORF plasmid was obtained in the pENTR™221 vector (Invitrogen) and then transferred into the pT-Rex-DEST30 vector

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through an LR recombination reaction with the Gateway® LR Clonase™ Enzyme Mix (Invitrogen) according to the manufacturer's instructions.

3.4 Establishing a CHO Cell Line with Tetracycline Inducible CD20 Expression

A T-75 flask of T-REx CHO cells at 60% confluency was trypsinized and suspended in media. A small volume (1.2ml) was made up to 12 ml with media. Two milliliters was added to each well of a six well plate, and left to grow overnight at 37°C in a 5% CO_2 incubator. CD20 plasmid DNA (1µg, 2µg) was diluted in Opti-MEM® I reduced serum media (50µl, Invitrogen). Lipofectamine™ (2.5µl, 5µl, Invitrogen) was diluted with Opti-MEM® I medium. This was incubated for 5min at room temperature. The diluted DNA and diluted Lipofectamine™ were mixed and incubated at room temperature for 20min. The complex formed (100µl) was added to each well. The cells were incubated overnight at 37°C in a 5% CO2 incubator. The media was replaced the following day.

Two days following transfection of the T-REx CHO cells, the wells were incubated with media containing Geneticin (2mg/ml, 4mg/ml, Gibco). The geneticin resistant cells were transferred to a T-25 flask and cultured in 2mg/ml geneticin. At about 60% confluency, tetracycline hydrochloride (Sigma) was added to each flask to a final concentration of 1µg/ml to induce CD20 protein expression. Twenty-four hours later, the cells were harvested and protein was extracted for Western blot analysis.

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3.5 Western Blots

Protein was extracted from T-REx CHO cells, CD20 transfected T-REx CHO cells, and tetracycline induced CD20 transfected T-REx CHO cells. A western blot was done under denaturing condition using a 1:250 dilution of mouse anti-CD20 antibody (Abcam) as the primary antibody, and a 1:2000 dilution of goat anti-mouse HRP conjugate (Bio-Rad) as the secondary antibody. Complexes were visualized using ECL Detection reagents (Amersham, Piscataway, NJ).

3.6 Phage Library

Human single fold scFv libraries I + J (Tomlinson I + J) were obtained from MRC Laboratories (Cambridge, UK). The libraries consist of over 10^8 different scFv fragments cloned into an ampicillin-resistant phagemid vector, and transformed into TG1 *E coli* cells. KM13, a protease sensitive helper phage to aid in the propagation of phage, and HB2151, a non-amber suppressor strain of *E Coli* for the production of soluble antibody fragments were included (Geneservice, Cambridge, UK).

3.7 Phage Propagation:

KM13 and Libraries I and J were propagated according to manufacturer's instructions (Geneservice, Cambridge, UK). A library stock (500µl) aliquot was added to 200ml of 2xTy with 100µg/ml Ampicillin and 1% glucose and incubated with shaking at 37°C until the O.D. 600 was 0.4. 50 ml of this culture was infected with 2 x 10^{11} KM13 in a 37°C water bath for 30min. After centrifugation at 3,000g for 10min, the pellet was

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resuspended in 100ml 2xTY with 100µg/ml Ampicillin, 50µg/ml Kanamycin, and 0.1% glucose, and the culture was incubated with shaking at 30°C overnight. The overnight culture was spun at 3,300g for 30min. A 20 ml solution of 20% Polyethylene glycol 6000 and 2.5M NaCl was mixed with 80ml supernatant and left on ice for 1hr. The mixture was spun at 3,300g for 30min. The pellet was resuspended in 4ml PBS and spun at 11,600g for 10min. The phage-containing supernatant was recovered and kept at -80^{O}C. To titer the recovered phages, 1µl of the phage stock was serially diluted into 100µl PBS six times. Each dilution was mixed with 900µl of TG1 *E coli* cells cultured until an O.D. 600 of 0.4 and incubated for 30min in a 37°C water bath. 10µl from each dilution was then spotted onto TYE plates and incubated overnight at 37°C.

3.8 Cell-based Phage Selection Procedure

This protocol was adapted from Heitner et al., (2006). Briefly, 293T/17 cells (1 x 10^9) were spun down and resuspended in 5ml blocking buffer (2% BSA, 10% FBS in PBS) and incubated at 4°C with rotation for an hour. The cells were divided into 3 tubes. $1x10^{10}$ pfu of phage from the original library (I+J) in blocking buffer were incubated sequentially with the three tubes of cells for a half hour each time, with rotation. The screened phages were incubated with a pellet of 293T/CD20 for 2hrs, which had been previously blocked, for 1hr at 4°C. The cells were washed with blocking buffer 10 times (1000rpm, 5min). The binding phages were then eluted with 0.5ml 0.1M triethanolamine and neutralized with 1ml 1M Tris-HCL pH 7.5. Log phase TG1 (OD600 of 0.4) was infected with 250µl of the eluted phage. The phage was then amplified, and titered.

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This protocol was repeated using eluted phage from the previous step. The resultant phage was used in the same protocol, with T-REx CHO cells as the depleting cell line, and CD20 transfected T REx CHO cells which had been induced for 24hrs as the selective cell line. This was repeated with decreasing incubation time in subsequent rounds from 2hrs to 1hr and to 30min, while increasing the number of washes from 10 to 15 and to 20.

3.9 Polyclonal Phage ELISA

A 96 well plate was coated overnight at 4°C with 100µl per well of 10µg/ml recombinant GST tagged CD20 protein (Abnova, Taiwan). The wells were washed and blocked with blocking buffer (2% non-fat dry milk in PBS) for 2hrs with rotation at room temperature. Phage from each round (1 $x10^{10}$) in blocking buffer (100µl) was added to the wells for 1hr. After washing with 0.1% Tween in PBS, a 1:5000 dilution of HRP conjugated Anti-M13 antibody (Pharmacia) in blocking buffer was added and incubated for 1hr. Media (2xTY) and library phage were used as controls.

3.10 Monoclonal Phage ELISA

Colonies were picked from titer plates and grown overnight in 2xTY (100µg/ml Ampicillin, 1% glucose). An inoculum was transferred to 200µl of media and grown for 2hrs. The culture was infected with 1 $x10^{10}$ helper phage for 1hr. The resulting pellet was resuspended in media (2xTY, 100µg/ml Ampicillin, 50µg/ml Kanamycin) and grown at

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30°C overnight. The supernatant after centrifugation was used in ELISA as described above, using HRP-Anti M13 (Pharmacia) as the secondary antibody.

3.11 PCR Screening:

Colonies were directly screened by PCR to determine the presence of scFv inserts. The primers LMB3 and pHEN (Geneservice, Cambridge, UK) were used under the following conditions:

Denaturation: 94°C for 9min

30 cycles of: Denaturation: 94°C for 45sec

Annealing: 55°C for 45sec

Elongation: 72°C for 45sec

Final Elongation: 72°C for 5min.

This was adapted from MRC (Geneservice, Cambridge, UK).

The PCR products were run on a 1% agarose gel.

3.12 Extraction of Periplasmic Protein

Phage was obtained from chosen clones using the phage propagation protocol. Log phase HB2151 was infected with the phage in a 37°C water bath for 30min. Several dilutions were plated out. HB2151 clones were then grown overnight and used as an inoculum for a larger culture (350ml) which was grown to an O.D. 600 of 0.9. This was induced with 1mM IPTG overnight at 30°C. Periplasmic protein was extracted using a protocol adapted from Springer Lab Manual (2001). The overnight culture was

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centrifuged at 5000g for 30min at 4°C. The pellet was resuspended in 35ml of cold spheroblast solution (50mM Tris-HCL pH 8.0, 20% w/v sucrose and 1mM EDTA pH 8.0). This was incubated on ice for 20min with occasional shaking. The solution was spun at 6200g for 15min at 4°C. The supernatant was collected and spun at 30,000g for 30min at 4°C to remove any remaining debris. The supernatant containing the periplasmic extract was saved.

3.13 Dot Blot

A nitrocellulose membrane was blotted with 2µl of CD20 antigen at different concentrations (200µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml, and 1.25µg/ml) and allowed to dry. The membrane was blocked with 5% BSA in PBS for 30min. The membrane was incubated with the periplasmic extract for 30min, then washed with TBST. A 1:1000 dilution of Protein L-HRP conjugate (Pierce, Rockford, IL) was incubated with the blot for 30min. The membrane was washed and incubated with ECL reagents (Amersham, Piscataway, NJ), and developed. A second dot blot was performed using the biotinylated fusion protein obtained as antigen, and Clone 3 as the primary antibody.

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CHAPTER 4

RESULTS

4.1 Fusion Protein Data

RNA obtained from Raji cells was reverse transcribed into cDNA. The sequence of CD20 was obtained from NCBI (Accession number: NM-021950) and the open reading frame (ORF) found using ORF Finder (NCBI). Primers were designed to amplify the 894bp ORF. Figure 4.1 shows the expected CD20 ORF band.

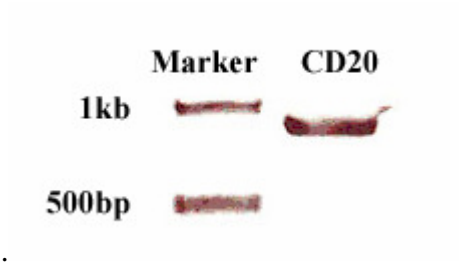

Figure 4.1 CD20 ORF obtained after RT-PCR of Raji cell RNA using CD20 ORF primers.

The cDNA obtained was ligated into PCR 2.1 (Invitrogen). Competent Top 10 *E coli* cells were transformed with the ligation reaction. DNA obtained from resulting clones was restricted with KpnI to determine which clone had the insert in the correct orientation. A 302bp band was expected if the orientation was correct, and a 688bp band if wrong. Figure 4.2 shows Clone 10 (PCR2.1-CD20) with the correct orientation.

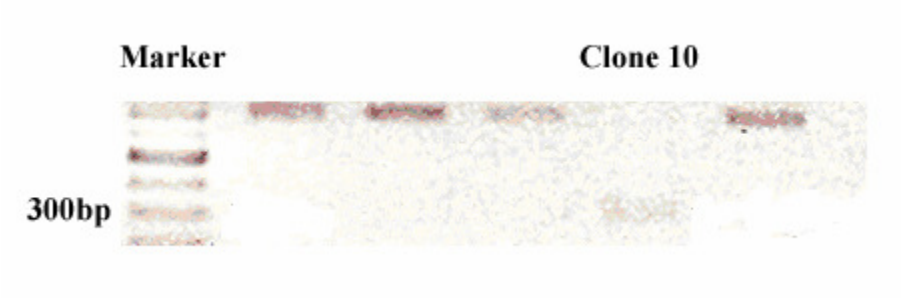

Figure 4.2 Kpn I restriction digest of PCR2.1-CD20 clones

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The extracellular fragment of CD20 (CD20-EC) was predicted to be between nucleotides 426 to 555 (NCBI NM_021950). Primers designed to amplify a larger fragment (nucleotides 418 to 571) were used in PCR. A 150bp band was expected after electrophoresis of PCR products. The primers were also designed to add an NruI site at the 5' end, a stop codon and an extra base at the 3' end, to maintain the reading frame of the original sequence.

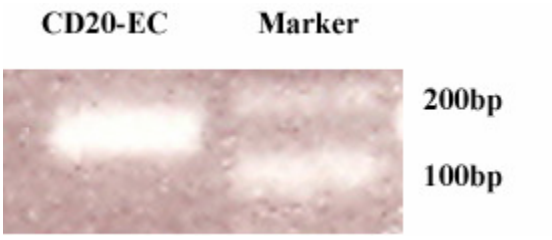

Figure 4.3 Amplification of the CD20 extracellular fragment (CD20-EC) from PCR2.1-CD20.

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The CD20-EC fragment obtained was ligated into PCR2.1 and restricted with Sac I to find the correct clone. A 95bp was expected if the insert was in the correct orientation. In Figure 4.4, Samples 8 and 12 appeared to have the right size bands and thus were sequenced.

Figure 4.4 SacI restriction digest of PCR2.1-CD20-EC

PCR2.1-CD20-EC Samples 8 and 12 were sequenced using CD20-EC forward and reverse primers. The results obtained from sequencing PCR2.1-CD20-EC were aligned with the expected sequence of the CD20 extracellular fragment, NruI restriction site and the TAA stop codon (Table 1). PCR2-1-CD20-EC #12 had the insert in the correct orientation.

TCGCGAAAATATTAAAATTTCCCATTTTTTAAAAATGGAGAGTCTGAATTTTA TTAGAGCTCACACACCATATATTAACATATACAACTGTGAACCAGCTAATCCC TCTGAGAAAAACTCCCCATCTACCCAATACTGTTACAGCATACAATCTCTGTT **CTAA**

Table 1: Expected DNA sequence of CD20-EC fragment with preceding NruI site, and stop codon.

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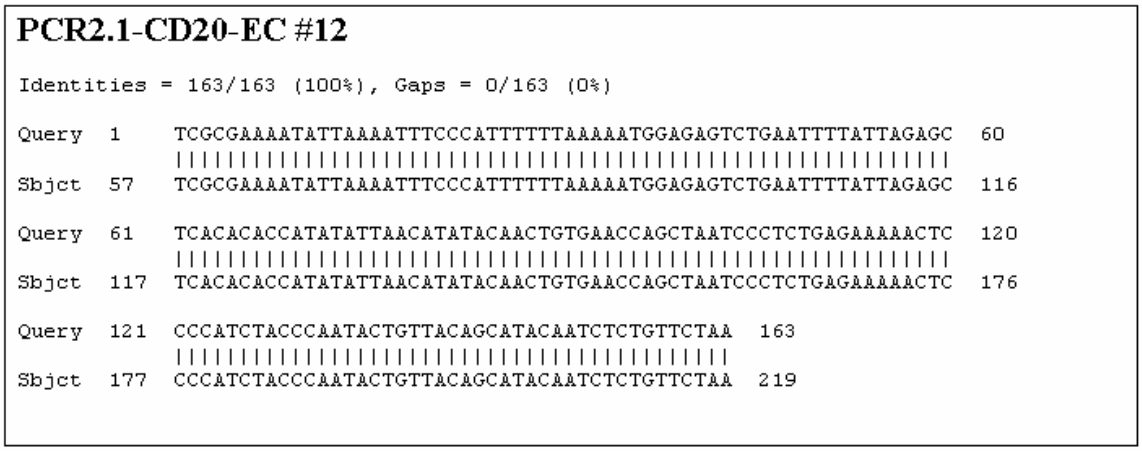

PCR2.1-CD20-EC #12

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Identities = 163/163 (100%), Gaps = 0/163 (0%)

Query  1    TCGCGAAAATATTAAAATTTCCCATTTTTTAAAAATGGAGAGTCTGAATTTTATTAGAGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57   TCGCGAAAATATTAAAATTTCCCATTTTTTAAAAATGGAGAGTCTGAATTTTATTAGAGC  116

Query  61   TCACACACCATATATTAACATATACAACTGTGAACCAGCTAATCCCTCTGAGAAAAACTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  117  TCACACACCATATATTAACATATACAACTGTGAACCAGCTAATCCCTCTGAGAAAAACTC  176

Query  121  CCCATCTACCCAATACTGTTACAGCATACAATCTCTGTTCTAA  163
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  177  CCCATCTACCCAATACTGTTACAGCATACAATCTCTGTTCTAA  219
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Figure 4.5 Alignment of the obtained PCR2.1-CD20-EC sequence with the known CD20-EC insert sequence.

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The CD20-EC portion was cut out of PCR2.1-CD20-EC #12 using restriction enzymes NruI and NotI. Pinpoint Xa Vector (Invitrogen), a protein expression vector, was restricted with the same enzymes to create an insertion point in its multiple cloning site for the insert. The two fragments were ligated to form PXCD20-EC. Rosetta *E coli* cells (Invitrogen) were transformed with the ligation reaction. Miniprep DNA isolated from different clones was restricted with NruI and NotI to confirm the presence of the insert.

Figure 4.6 NruI and NotI Digest of PXCD20-EC to verify the presence of the CD20-EC insert.

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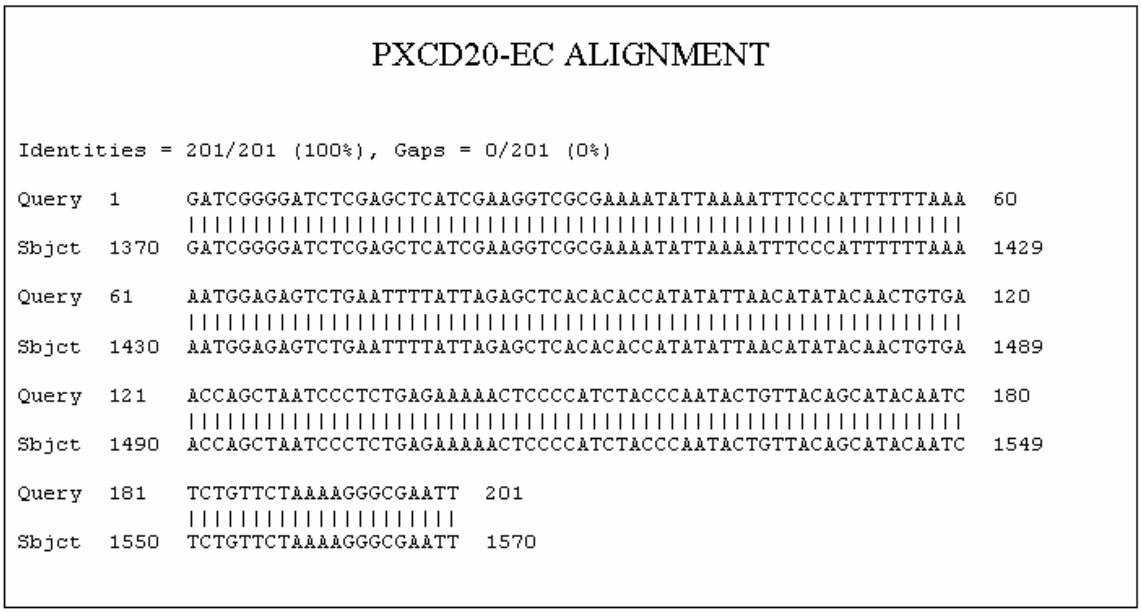
Miniprep DNA from a sample was sequenced using SP6 Promoter and Pinpoint Vector sequencing primers (Invitrogen). The expected insert was present in the correct orientation.



PXCD20-EC ALIGNMENT

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Identities = 201/201 (100%), Gaps = 0/201 (0%)

Query  1     GATCGGGGATCTCGAGCTCATCGAAGGTCGCGAAAATATTAAAATTTCCCATTTTTTAAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1370  GATCGGGGATCTCGAGCTCATCGAAGGTCGCGAAAATATTAAAATTTCCCATTTTTTAAA  1429

Query  61    AATGGAGAGTCTGAATTTTATTAGAGCTCACACACCATATATTAACATATACAACTGTGA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1430  AATGGAGAGTCTGAATTTTATTAGAGCTCACACACCATATATTAACATATACAACTGTGA  1489

Query  121   ACCAGCTAATCCCTCTGAGAAAAACTCCCCATCTACCCAATACTGTTACAGCATACAATC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1490  ACCAGCTAATCCCTCTGAGAAAAACTCCCCATCTACCCAATACTGTTACAGCATACAATC  1549

Query  181   TCTGTTCTAAAAGGGCGAATT  201
             |||||||||||||||||||||
Sbjct  1550  TCTGTTCTAAAAGGGCGAATT  1570
```

Figure 4.7 Alignment of PXCD20-EC sequence with the expected insert sequence

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Rosetta *E. coli* cells transformed with the constructed vector, and expressing the biotinylated fusion protein were cultured. Bacterial pellets were saved before and after induction of protein expression using IPTG. A western blot comparing protein expression before and after induction using a Streptavidin-HRP conjugate was performed. A 20kDa fusion protein band was expected. The expected fusion protein band was more prominent after induction.

Figure 4.8 Western Blot Analysis of Protein Expression before and after induction.

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Protein in inclusion body form was extracted from the bacterial pellets obtained after induction. The proteins were folded in vitro to form their native active state (Rudolph & Lilie, 1996). A protein gel showed the expected 20kDa fusion protein band.

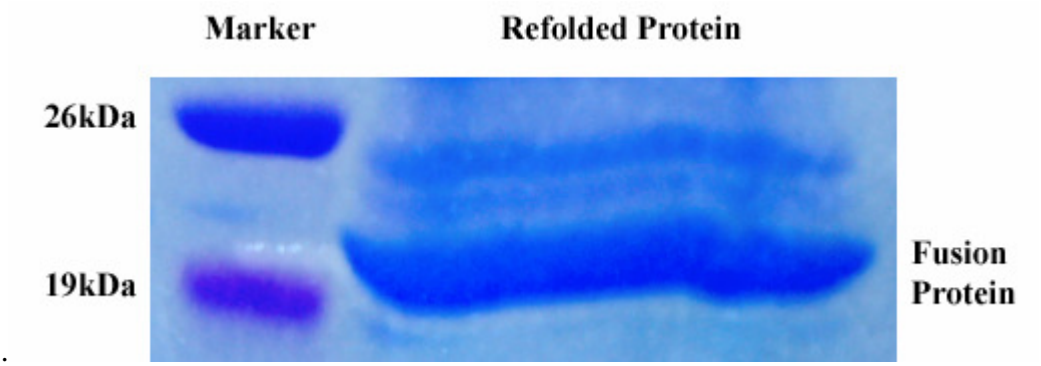

Figure 4.9 SDS PAGE of refolded fusion protein.

Western blot analysis of the re-folded protein using HRP-conjugated Streptavidin was performed. The expected 20kDa band is shown.

Figure 4.10 Western blot of refolded fusion protein.

4.2 ScFv Isolation Data

Western blot under denaturing conditions of protein extracted from 293T/CD20, 293T/17, and Raji cells using a 1: 250 dilution of mouse anti-CD20 monoclonal antibody and a 1:2000 dilution of goat anti-mouse HRP-conjugated antibody was performed. 293T/17 which does not express CD20 showed no bands, while the transfected cell line, 293T/CD20, and Raji cells which express CD20 normally showed the expected 33kDa CD20 band.

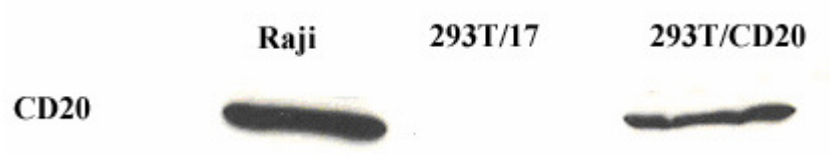

Figure 4.11 Western blot of protein extracted from the 293T/CD20, 293T17 and Raji cell lines using anti-CD20 monoclonal antibody.

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Protein extracted from the CD20 transfected-T-REx CHO cell line (CD20/T-REXCHO), after 24hrs of induction was compared with protein extracted from the same number of cells from the remaining cell lines. As expected, Raji, 293T/CD20 and CD20/T-REXCHO expressed CD20 while the parental cell lines 293T/17 and T-REXCHO did not. The lower lever of CD20 expression on CD20/T-REXCHO could be due to low plasmid copy number.

Figure 4.12 Western blot comparing protein extracted from all cell lines using anti-CD20 antibody.

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Phage eluted after selection was titered before and after amplification.

Averaged Titer Data		
	Eluate Titer (pfu/ml)	Amplified Phage Titer (pfu/ml)
R1	3.10×10^{05}	3.7×10^{12}
R2	2.80×10^{06}	4.75×10^{11}
R3	2.49×10^{04}	4.00×10^{11}

Table 2: Titers of selection obtained phage before and after amplification

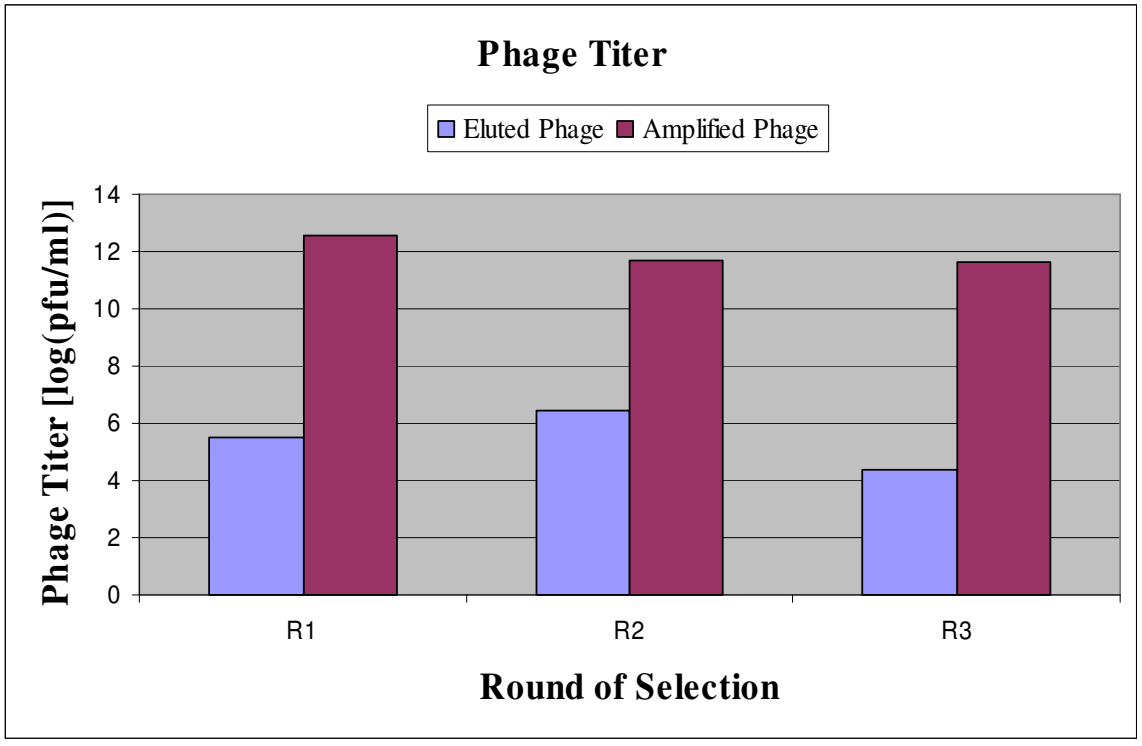

Figure 4.13 Titer of eluted and amplified phage

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Polyclonal ELISA in duplicate using 10µg/ml of CD20 antigen to detect the binding of 10µl of eluted phage from each round of selection. The secondary antibody used was HRP-anti-M13 (Pharmacia). TMB reagents (Pierce, Rockford, IL) were used to detect the reaction. Helper phage (KM13) and blocking buffer (BSA in PBS) were used as controls.

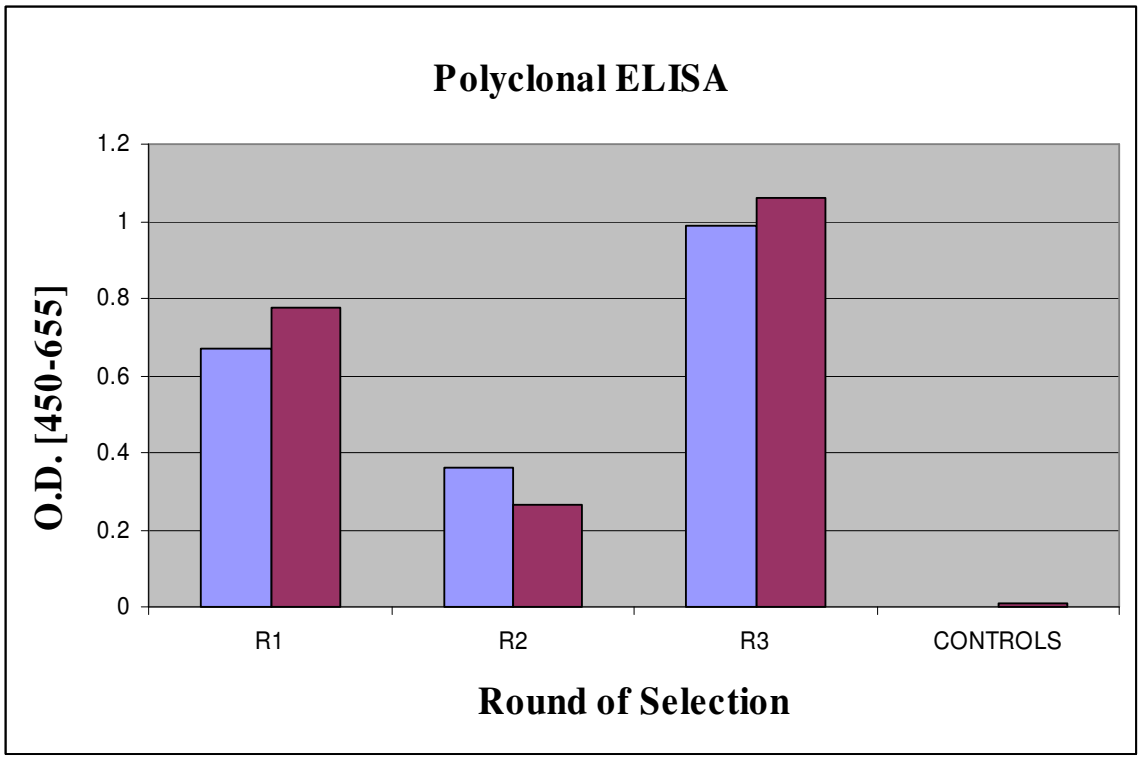

Figure 4.14 Polyclonal ELISA in duplicate of eluted phage from the first three rounds of selection.

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PCR analysis to amplify the 934bp scFv insert, was performed on four random clones from each round of screening. The positive control C is an anti-ubiquitin scFv clone provided with the library (Geneservice, Cambridge, UK). Clones from Rounds 1 to 3 of selection showed the expected band, while none of the Round 4 clones displayed any inserts.

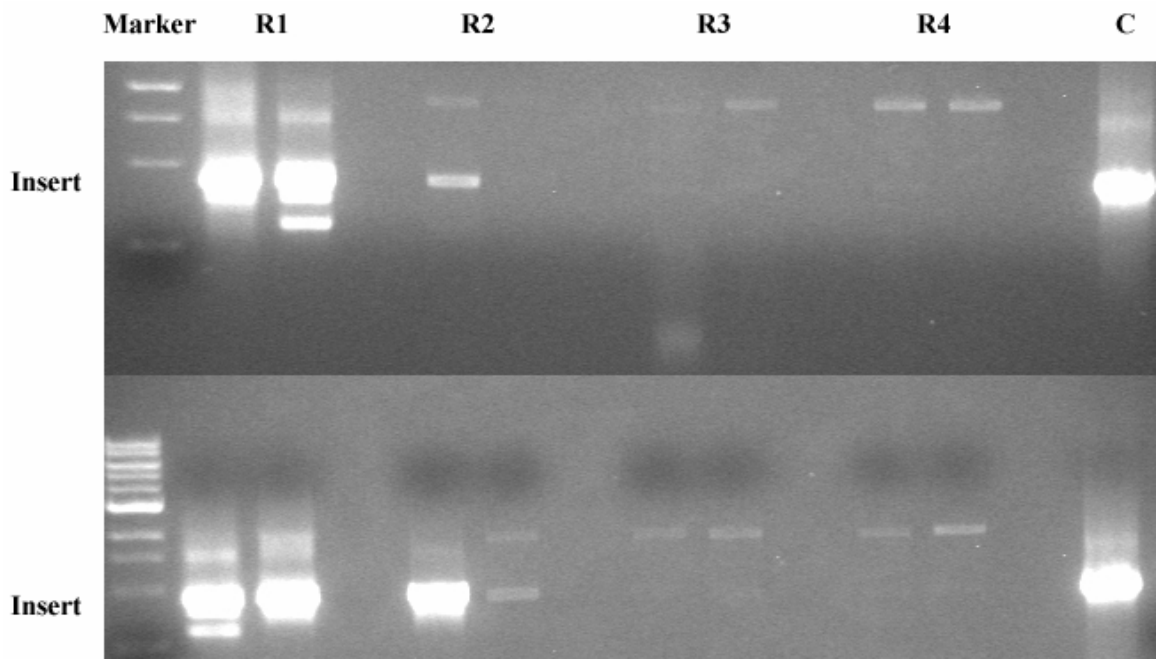

Figure 4.15 PCR analyses of clones from four rounds of selection to determine the presence of the scFv insert.

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More clones from Rounds 2 and 3 of selection were chosen for further PCR analysis. Most clones screened displayed the expected scFv band. The anti-ubiquitin scFv clone was used as a positive control. Clone #3 from Figure 4.17 was chosen for further analysis.

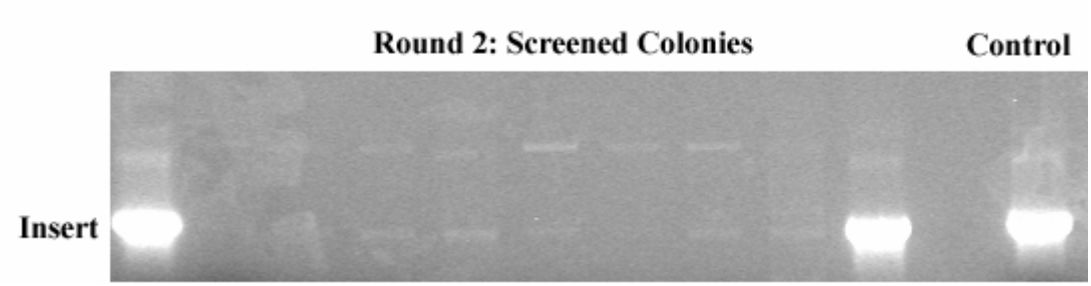

Figure 4.16 PCR analyses of ten clones from Round 2 of selection.

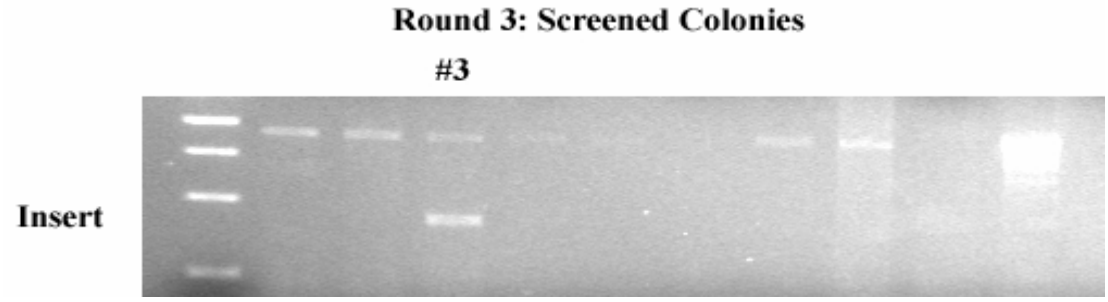

Figure 4.17 PCR analyses of ten clones from Round 3 of selection.

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ScFv inserts from 4 random clones from the third round of selection, were cut out of the phagemid vector using NotI and NcoI restriction enzymes. HaeIII digest of the inserts showed an identical pattern.

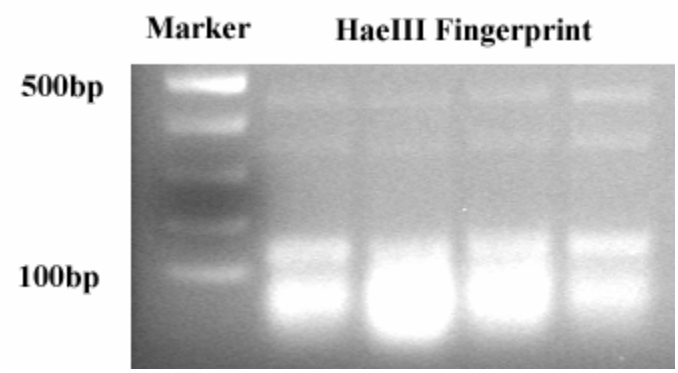

Figure 4.18 HaeIII digest of scFv inserts.

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Phage obtained from the third round of selection was titered to obtain individual colonies. The colonies were cultured and co-infected with helper phage, to enable propagation of phage particles. Monoclonal ELISA was done on these cultures to determine their CD20 binding using 5µg/ml CD20 antigen, and HRP-conjugated Anti-M13 (Pharmacia) as the secondary antibody.

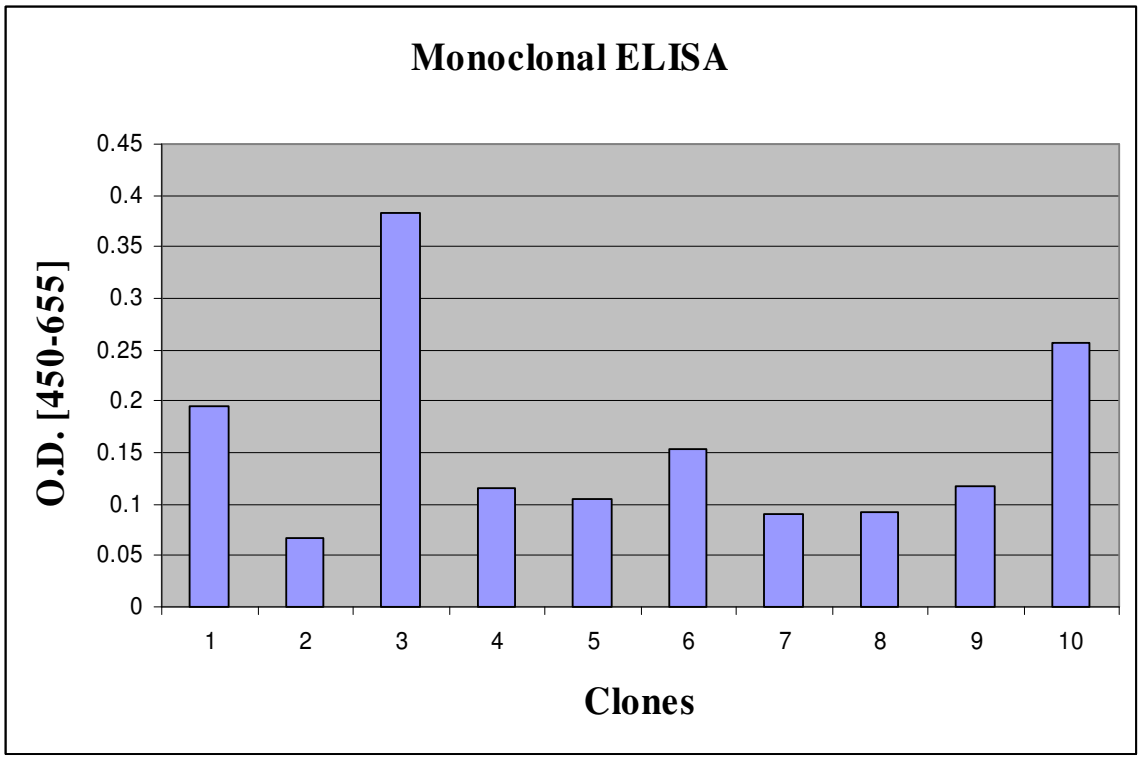

Figure 4.19 Monoclonal ELISA on clones from Round 3 of selection.

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Based on ELISA and PCR results, Clone #3 was selected for further study. Clone #3 phage was used to infect HB2151, a non-suppressor *E. coli* strain, allowing the scFv protein produced to be targeted to the periplasmic space of the bacteria. A dot blot was performed on the periplasmic protein extracted from Clone #3 using varying concentrations of CD20 antigen to determine its binding to CD20. HRP-conjugated protein L was used as the secondary antibody, and binding was visualized using ECL reagents. Binding was evident for the first three dilutions shown.

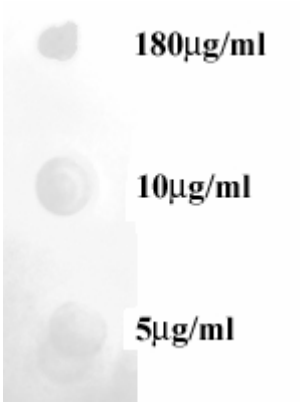

Figure 4.20 Dot blot showing the binding of Clone #3 scFv to CD20 antigen.

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The biotinylated fusion protein created in the first study was blotted onto a nitrocellulose membrane to determine if Clone #3 bound to it. CD20 antigen (180µg/ml) was blotted as a positive control, as Figure 4.20 shows that Clone #3 does bind to it. Figure 4.21 shows that Clone #3 periplasmic protein does bind to the created fusion protein.

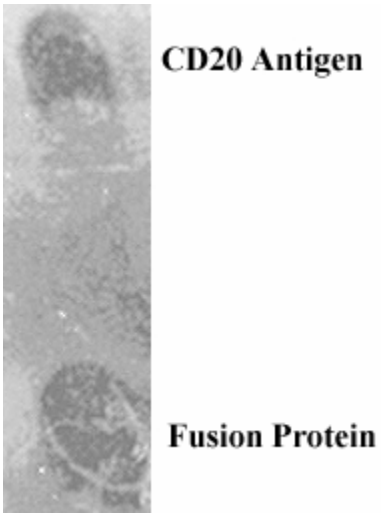

Figure 4.21 Dot blot of the biotinylated fusion protein and Clone #3 scFv-containing periplasmic protein.

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CHAPTER 5

DISCUSSION

The first objective of this project was to create a CD20 fusion protein for phage antibody selection and characterization. By fusing the extracellular fragment of CD20 with the bacterial in vivo biotinylation tag, the recombinant fusion protein produced in *E. coli* could be conveniently coated onto magnetic streptavidin beads or streptavidin coated microplates for phage selection or phage ELISA. Sequencing analysis of the plasmid construct confirmed correct fusion between the two open reading frames. Western blot analysis of induced bacterial pellets, and the protein obtained after the refolding protocol adapted from Rudolph and Lilie (1996), using Streptavidin-HRP conjugate, confirmed the creation of a functional biotinylated fusion protein. To purify the refolded recombinant fusion protein, the SoftLink™ Soft Release Avidin Resin (Promega, Madison, WI) was used for batch processing.

The next objective was to screen for anti-CD20 scFvs from a naïve human library. The scFvs generated would be true human sequences, drastically decreasing immunogenicity associated with murine and chimeric antibodies when used in vivo. ScFvs, like their parent antibodies and other antibody fragments, can be exploited in various ways in diagnosis and therapy of cancer and non-cancerous conditions. High affinity scFvs can be coupled to high affinity Fc variants to obtain a highly specific and potent monoclonal antibody against a target of choice. Target-specific antibody fragments can be labeled and employed in tumor imaging in vivo (Hamdy et al., 2005). ScFvs can be conjugated to molecules which they then deliver to the target cells. These

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molecules could be therapeutic drugs which allow proper functioning of the target cell, or toxins which kill the neoplastic target cell.

In the present study, a cell based procedure was used for phage selection. Under this condition, the interaction of phage particles with CD20 antigen would occur at the cell membrane microenvironment, which mimics in vivo conditions much better than other methods. Though there are tumor cell lines known to over-express CD20 proteins such as Raji, two CD20 cDNA transfected cell lines, 293T/CD20 and CD20/T-Rex CHO, were used in the present study to have a better negative control for depletion selections. The first round of selection was performed on 293T/CD20 cells, and the remaining three rounds were performed on CD20 transfected T-Rex™ CHO cells. The 293T/CD20 cell line was created through the transduction of a lentivector plasmid FUW, into which human CD20 cDNA had been cloned. The vector was pseudotyped with vesicular stomatitis virus (VSVG) before transduction of 293T cells (Yang et al., 2006). Stable expression of CD20 was confirmed by Western blot analysis of proteins extracted from the cell lines before each round of selection.

The second cell line used for selection was a tetracycline-regulated expression CHO cell line, stably transfected with an expression vector into which the CD20 ORF had been cloned. Selection was done 24hrs after induction. The expression of CD20 was low compared to that of 293T/CD20 as observed by Western blot analysis. Alternating the two different cell lines for selections would not only increase the depletion of phage which bound non-specifically to different antigens on the surface of the previous cell line (Heitner et al., 2006), but this would also increase selection stringency when the second

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cell line used has less target antigen expression, as is the case in the current study. The sharp decrease in phage titer after selection on the second cell line, as well as the decrease in polyclonal ELISA signal, suggests that use of the second cell line did deplete some of the non-specific binding phages. As mentioned previously, the CD20/T-Rex CHO cell line had a lower CD20 antigenic density in comparison to 293T/CD20. Using the lower density cell line, also allows the depletion of low affinity antibodies in favor of high affinity ones. The decreasing selection time and increasing stringency of washes allowed for the selection of phage which bound quickly and tightly to the CD20 target.

PCR analysis of the phage should yield a 935bp band if there is an scFv insert present (Geneservice, Cambridge, UK). PCR analysis of the eluted phage showed that a very high percentage of phages from the first to the third rounds of selection showed the antibody fragment inserts, while this fragment appeared to have been lost in a lot of phages from the fourth round of selection. This suggests that while increased rounds of selection may increase the specificity of the phages obtained, it also increases the chance of the insert-carrying phage being lost. Stop codons and frame shifts originated during construction of the library lead to clones which do not express the scFv-pIII fusion protein. Bacteria carrying these insert-less phage have increased growth rates and produce more phage, thereby decreasing the population of phage expressing scFv-pIII with sequential growth passages (Kramer et al., 2003). In addition, insert-free phages are also likely to over-take phage cultures with consecutive rounds of amplification and selection (Trepel et al., 2001). Thus clones were chosen from the second and third rounds of selection for analysis. Most colonies from rounds two and three contained the expected

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935bp band as well as an approximately 2kb band which could be due to multiple inserts in some cases, and more likely due to non-specific amplification.

Comparing monoclonal ELISA data with PCR data, two clones: #3 and #21, were chosen for the production of soluble antibody fragments. Periplasmic protein was extracted from cultures and its affinity for CD20 antigen tested via a dot blot, using HRP-conjugated Protein L as the secondary antibody, which binds to the variable domain of the kappa light chain (Nilson et al., 1992). Clone 3 showed significant binding at decreasing concentrations of CD20 antigen, while Clone 21 did not show significant binding.

The next steps would be further screening of clones from the second and third rounds to select insert-bearing phages and test their affinity for CD20. While PCR screening, monoclonal ELISA and dot blot data prove Clone 3 binds to CD20 antigen, DNA sequencing analysis would further confirm the presence of both a heavy chain and a kappa light chain. For better expression, the insert can be transferred to a protein expression vector, such as pET22b, to expedite production and purification of the antibody fragment. Flow cytometry or Surface Plasmon Resonance (SPR) could be used to elucidate the binding affinity and binding kinetics of the scFv. Cytotoxicity and apoptosis assays can be performed to determine the effect of the scFv on CD20 expressing cells both in vitro and in vivo.

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CHAPTER 6

CONCLUSIONS

Humax-CD20, the fully human anti-CD20 monoclonal antibody currently in clinical trials, shows the advantages human antibodies possess over murine and chimeric ones in the treatment of lymphoma, arthritis, SLE and other ailments (Genmab, 2007). The smaller size of scFvs allows them to be great candidates for imaging tumors when conjugated to radionuclides. The risk of immunogenicity is minimal, reducing side effects when administered to patients. The addition of genes encoding scFvs to viral and non-viral vectors enhances specificity without any added toxicity. ScFvs are also capable of neutralizing viruses and toxins (Blazek & Celer, 2003).

The aim of this study was to isolate a human scFv specific for CD20 antigen. Cell lines provided the antigen in a state more comparable to its native state. The use of two different cell lines as the sources of antigen decreased the chances of isolating clones which bound non-specifically to other surface antigens (Heitner et al., 2006). Clones selected from the second and third rounds of selection maintained their inserts and showed some specific binding to CD20 antigen, while clones from the fourth round of screening did not appear to have any inserts. This agrees with previous findings which suggest that there is a limit on the number of selection cycles a phage library can be put through before insert-free phages overtake set-up cultures (Trepel et al., 2001).

A clone was isolated (Clone 3) which bound to CD20 as observed by ELISA and dot blot analysis. Future work would characterize this clone and screen more clones from the second and third rounds of selection.

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