Engineering Antibodies for Use in Leukemia and Lymphoma Therapy

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Summary

Cancer is one of the major causes of death in the Western world. The treatments currently in place fall short of ideal and this has necessitated the continued search for new, more effective remedies. One of the most promising of these new therapies has been the development of monoclonal antibodies to target treatment to a specific cell type or disease. Monoclonal antibodies were developed in 1975 by Kohler and Milstein and have been used in applications which have allowed detailed studies of the immune system and other systems in the human body. The use of these antibodies in humans has raised some interesting problems which need to be addressed before further treatment. The biggest concern has been the production of human anti mouse antibody (HAMA) responses to therapeutic antibodies of murine origin. This has led to many developments in the antibody engineering field, including the production of chimeric antibodies, humanisation of antibodies by CDR grafting or antibody resurfacing, and the production of antibody fragments by removing the regions not necessary for antibody binding. This thesis describes some of the methods used for the production of therapeutic antibodies, including phage display technology and the production of both anti CD20 scFv and Fab fragments from an anti CD20 hybridoma.

A phage display antibody library was derived from tonsil lymphocytes and a library of small size was produced. This library was not used for antigen panning. The two libraries, "Einstein" Kappa and Lambda, used for screening against CD20 peptide did not yield antibodies to CD20.

The aim was to then produce a library from the bone marrow of a patient in remission with a leukemia or lymphoma. To test the hypothesis that patients with a cancer will produce autoantibodies to the antigens on the surface of the cancerous cells, serum and plasma from cancer patients and patients with auto immune disease were tested for the presence of anti-CD19 or anti-CD20 antibodies. This work showed that autoantibodies to these antigens are not exclusively produced by patients with disease and not all patients produce high titres of these antibodies. These results suggest that if library construction is to be biased towards a particular antigen then screening of individuals serum or plasma, not just those with cancer, should be carried out.

Libraries of antibodies are most commonly screened against pure antigen. The CD20 antigen was produced by synthesising the 45aa loop which protrudes from the cell surface. An ELISA was developed which showed that many CD20 antibodies bind to the synthetic peptide.

Attempts were made to produce the extracellular region of the CD19 antigen in a mammalian expression system. Using a number of methods, soluble recombinant CD19 antigen could not be produced effectively. As an alternative the full length CD19 sequence was cloned into pIRES1neo and expressed in Chinese Hamster Ovary (CHO-K1) cells as a membrane bound protein. A variety of commercial antibodies bound to the CD19 transfected cell line but not to untransfected CHO-K1 cells in flow cytometry.

The anti-CD20 hybridoma, HB13d, was used to produce a single chain Fv fragment. The scFv was shown to bind strongly to CD20 in flow cytometry, ELISA and plasmon resonance. Preliminary experiments described a Fab fragment from the CD20 hybridoma.

The CD20 antigen has been shown to be a suitable target for the therapy of B lymphocyte disease with at least one antibody, Rituximab (an anti-CD20 chimeric antibody), approved for use in the treatment of Non Hodgkin's lymphoma. This shows potential for the anti-CD20 scFv in the treatment of B cell disease. The studies presented in this thesis could provide the basis for second-generation therapeutic agents.

Declaration

This work contains no material that has been accepted for the award of any degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Penelope Jane Adamson

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Publications Arising From This Thesis

- Taylor, B.J., Belch, A.R., Zola, H., **Adamson, P.J.** and Pilarski, L.M. (1999). Differential Expression of CD19 Epitopes Among B Cells in Patients with Multiple Myeloma. Blood. **94:** 121a.

- Hohmann, A.W., Drew, K.J., Mulhern, T.D., **Adamson, P.J.** and Zola, H. (1999). Two Routes to Site-Directed Mutation of a Single-Chain Antibody: Splice Overlap Extension and Whole-Plasmid PCR. Journal of Immunological Methods. Submitted.

- **Adamson, P.J.**, Zola, H., Nicholson, I.C., Pilkington, G. and Hohmann, A. (2000). Antibody Against CD19 and CD20 in Patients with B Cell Malignancy. Submitted.

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Abbreviations

aa	Amino Acid
Ab2	Anti-Idiotype Antibody
Ab3	Anti-Anti-Idiotype Antibody
ADEPT	Antibody - Directed Enzyme - Prodrug Therapy
bp	Base Pairs
BSA	Bovine Serum Albumin
BST-1	Bone Marrow Stromal Cell Antigen - 1
C	Constant Region Gene
°C	Degrees Celsius
cDNA	Complementary DNA
CDR	Complementarity Determining Region
CDRH3	CDR 3 on the heavy chain
CH	Constant Heavy Domains
CH1	First constant domain of the heavy chain
CH2	Second constant domain of the heavy chain
CH3	Third constant domain of the heavy chain
CHRI	Child Health Research Institute, SA, Australia
CL	Constant Light Domain
CLL	Chronic Lymphocytic Leukemia
cm	Centimetre
D	Diversity Region Gene
Da	Dalton
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside 5'-Triphosphate
dsFv	Disulphide Stabilised Fv
DT	Diphtheria Toxin
DTT	Dithiothreitol
EBV	Epstein Barr Virus
E. coli	*Escherichia coli*
ECMV	Encephalomyocarditis Virus
ELISA	Enzyme Linked Immunosorbent Assay
ESB	Electrophoresis Sample Buffer
FBS	Foetal Bovine Serum
FDA	Federal Drug Administration, USA
FACS	Fluorescent Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
FMC	Flinders Medical Centre, SA, Australia
g	Gram
g	Units of Gravity

G418	Geneticin
GFP	Green Fluorescent Protein
Gly	Glycine
HAMA	Human Anti-Mouse Antibody
HIS	Histidine
HIV-1	Human Immunodeficiency Virus Type 1
hr	Hour
HRP	Horse Radish Peroxidase
HSV-1	Herpes Simplex Virus Type 1
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMAC	Immobilised Metal Affinity Chromatography
IMVS	Institute of Medical and Veterinary Science, SA, Australia
IPTG	Isopropyl-ß-Thiogalactopyranoside
IRES	Internal Ribosome Entry Site
J	Joining Region Gene
kb	Kilobase
kDa	Kilodalton
KLH	Keyhole Limpet Haemocyanin
kV	KiloVolt
L	Litre
M	Mole
Mb	Megabase
MFI	Mean Fluorescence Intensity
µg	Microgram
mg	Milligram
min	Minute
µl	Microlitre
ml	Millilitre
µM	MicroMole
mm	Millimetre
mM	MilliMole
mRNA	Messenger RNA
ng	Nanogram
NHL	Non Hodgkin's Lymphoma
Ni NTA	Nickel Nitrilotriacetic Acid
nM	NanoMole
NP40	Nonidet P40
OD	Optical Density
PBL	Peripheral Blood Lymphocytes
PBL-SCID	Peripheral Blood Lymphocyte Reconstituted SCID Mice

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE (flow cytometry)	Phycoerythrin
PE (immunotoxin)	*Pseudomonas* Exotoxin
PEG	Polyethylene Glycol
pM	PicoMole
PN	Control Plasma Samples
PS	Patient Plasma Samples
PSG	Penicillin/Streptomycin/Glutamine
QEH	Queen Elizabeth Hospital, SA, Australia
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RT	Reverse Transcriptase
SAPE	Streptavidin Phycoerythrin
SB	Super Broth
SCID	Severe Combined Immunodeficient
sec	Second
Ser	Serine
scFv	Single Chain Fv
SN	Control Serum Samples
SOE	Splice Overlap Extension
SS	Patient Serum Samples
TB	Terrific Broth
TCR	T Cell Receptor
TCS	Thrombin Cleavage Site
TEMED	N,N,N',N'-Tetraethylethylenediamine
TNF	Tumour Necrosis Factor
U	Units
V	Variable Region Gene
V	Volts
VH	Variable Heavy Region
V_κ	Kappa Light Chain Variable Region
VL	Variable Light Region
V_λ	Lambda Light Chain Variable Region
YAC	Yeast Artificial Chromosome

Chapter One

Introduction

Introduction

1.1 *Summary*

This thesis describes the production of potential reagents for use in B cell leukemia and lymphoma therapy. The introduction chapter explains the background of antibody engineering techniques used throughout this thesis and some more recent developments in this field. Also described are the antigen targets, CD19 and CD20, and methods used to enhance the effects of antibody reagents in therapy.

1.2 *B Cell Malignancy*

Cancer is one of the leading causes of death in the Western world, second only to heart disease. In 1995 cancer was the most common cause of death in Australia, accounting for over 25% of all deaths (NSW Health, 1999). Of new cases diagnosed, leukemia accounts for approximately 5% of cancers in both men and women. At approximately 30% of all cases leukemia is the most common children's cancer.

The conventional therapies for cancers include chemotherapy, radiotherapy and surgery although more alternatives are now being trialed.

Surgical excision is useful in the treatment of solid tumours. It is not effective for all tumour types, and often is not adequate for metastasised tumours or for the treatment of haematological disease.

Radiation therapy is useful in a number of different malignancies. About 60% of all cancer patients require radiation therapy during some phase of their cancer treatment. It involves the use of electron beams or X-rays, brachytherapy or implant therapy (the temporary or permanent placement of radioactive sources) to deliver treatment to a specific site in the body.

Chemotherapy is administered in a number of ways and is useful in a variety of tumours. Many chemotherapeutic drugs target cells which are rapidly proliferating and tumours which are well vascularised. Many act non specifically and have been shown to cause damage to cells and

tissues which are not malignant (Adamson, 1995; Sanderson and Shield, 1996). The mutations caused can catalyse the production of secondary malignancies years after the original therapy.

Current therapies are far from perfect and cure rates are low. As well as effectiveness of the treatment, patient "quality of life" should be taken into consideration. A treatment with a remission rate no better than another but providing less side effects would be preferred. Although current therapies can be successful in some cases, the development of new, more effective reagents is needed. Some of the newer treatments involve the production of antibodies or fragments of them which can be used to target drugs, radioisotopes or toxins to malignant cells via a cell surface molecule which is unique to that cell type. In the case of B cell leukemias and lymphomas there are many antigens which can be used as targets for antibody therapy. Some include CD19, CD20, CD22 and CD23 (Jennings and Foon, 1997). CD19 and CD20 are the antigens chosen as the targets for this thesis.

1.3 *Antibodies*

Production of antibodies usually occurs in response to invasion of the body with foreign substances, such as bacteria or viruses, although there is considerable antibody production in the absence of infection. Antibodies can be found in the serum of the blood and other bodily fluids such as saliva or milk.

There are 5 antibody classes, IgG, IgM, IgA, IgE and IgD, which are all different based on their immunological properties. IgG antibodies have a molecular weight of approximately 150kDa, are the major circulating antibody and are found in extracellular fluid, blood and lymph fluid and they cross the placenta (Brock and Madigan, 1991). The IgG class has been divided into the subclasses IgG_1, IgG_2, IgG_3 and IgG_4.

Figure 1.1 shows a typical immunoglobulin G molecule. It consists of 2 heavy chains and 2 light chains. The heavy chains are made up of 3 constant domains, CH1, CH2 and CH3 and a variable domain, VH. The light chains have a variable domain, VL, but only one constant domain. Each variable domain is made up of 4 framework regions bordering 3 complementarity determining regions (CDR). The framework regions and the constant domains have relatively conserved sequences. The CDRs are regions with highly variable

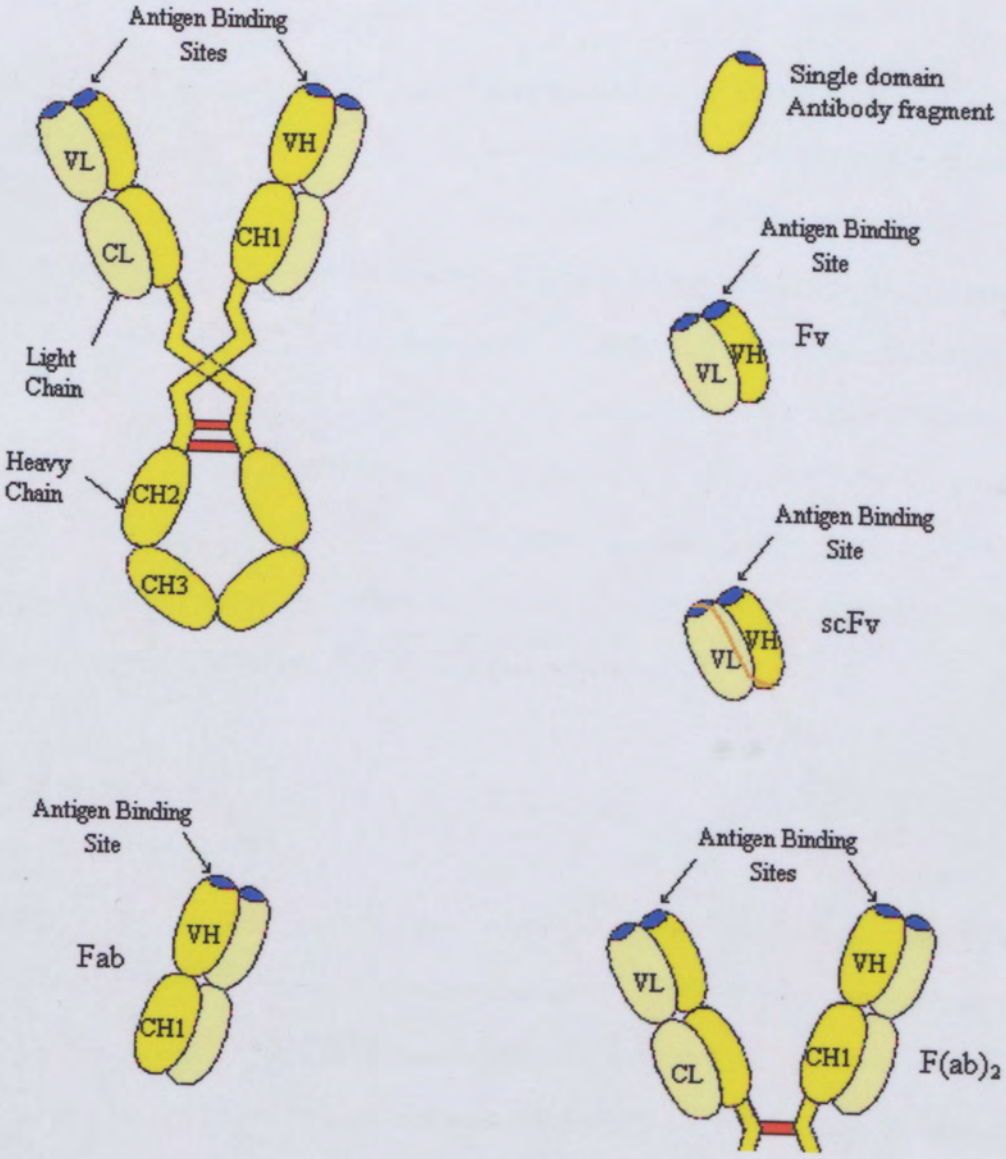


Figure 1.1: Immunoglobulin G Molecule and Fragments of Antibodies.

sequence. It is these residues which form the antigen binding site. The IgG molecule is dimeric, having two identical regions which bind antigen. The variable regions of antibodies are those which are involved in antigen binding and are subject to affinity maturation which is a process which mutates the binding region of an antibody to improve the binding to its target antigen.

The Fc region of an antibody is involved in complement activation and binding to Fc receptors on the surface of leucocytes. These processes are recruited by the Fc region to disable the antigen, cell or virus.

The structure of an antibody molecule reflects the structure of the immunoglobulin gene. In cells which produce immunoglobulin the immunoglobulin genes are rearranged as compared to the genes found in cells which do not produce Ig. A rearranged heavy chain consists of a variable (V) gene, a diversity (D) gene, a joining (J) gene and a constant region (C) gene. A rearranged light chain consists of a V gene, a J gene and a C gene. There is great diversity in the repertoire of antibodies found in the human body which can be explained in part by the number of possible combinations of the immunoglobulin genes.

1.4 *The Antibody Response*

The antibody response elicited by an infection is determined by the nature of the foreign substance. Typically a protein antigen will cause an antibody response which is dependent on cognate interactions between B and T lymphocytes and involves affinity maturation, the production of a memory response (on exposure to an antigen for the second time memory cells will produce a much faster and larger response to the antigen) and immunoglobulin class switching to produce IgG antibodies. A non-protein antigen (such as carbohydrate antigens) will normally cause the production of low affinity IgM antibodies in a response that elicits no T cell help and produces little if any memory response.

Antibodies recognise foreign antigens. At some stage during the development of the immune system discrimination between self and foreign antigens occurs. After the immune system becomes competent in recognising foreign molecules it can produce an appropriate response to disease. Sometimes a defective or inappropriate response is produced by the immune system and pathogenic autoantibodies can be formed. Autoimmune diseases resulting from the

formation of autoantibodies are not uncommon and some include Sjogren's Syndrome, Hashimoto's Thyroiditis, Rheumatoid Arthritis and Systemic Lupus Erythematosus.

1.5 *Hybridoma Technology and Monoclonal Antibodies*

The development of antibodies for use in human disease therapy has been the object of research for many years. When in 1975 Kohler and Milstein introduced a fusion technique that allowed the production of monoclonal antibodies from the fusion of B lymphocytes to mouse myeloma cells, a vision for antibody therapy was recognised. Until this stage polyclonal antibodies, a mixture of many different antibodies, were the only source of antibody reagents. Although there is still a market for polyclonal antibodies as reagents for certain assays, monoclonal antibodies have replaced them in many applications.

The production of hybridomas stemmed from experiments carried out by Cotton and Milstein in 1973 which involved the production of rat and mouse myeloma cell hybrids. The extension of this work by Kohler and Milstein in 1975 involved fusion of mouse myeloma cells with spleen cells from mice immunised with sheep red cells in a reaction assisted by Sendai virus. Cloned cell lines secreting anti-sheep erythrocyte antibodies were produced. These hybridomas were capable of forming tumours when injected into mice and although some variation of the methods has taken place, this revolutionary experimentation is now commonly used by many researchers to produce monoclonal antibodies targeted against a wide range of antigens.

Cells which produce monoclonal antibodies can originate from any animal which produces an immune response but those most commonly used are of murine origin. Although these antibodies are effective for use in laboratory work and *in vitro* diagnostic work they can cause significant problems in *in vivo* diagnostic and therapeutic applications. The main problems associated with murine antibodies involve the production of a HAMA (Human Anti Mouse Antibody) response in humans and the increasing push to reduce the numbers of experimental animals used. This has created a need to look for alternative methods of producing antibodies for diagnostic, therapeutic or research purposes.

1.6 *Limitations of Murine Hybridoma Antibodies*

There are some limitations in using murine monoclonal antibodies for therapy.

One is large scale production of antibodies. These proteins are produced in mammalian culture systems which are more expensive than some other expression systems as they require the use of specific culture media and CO_2 incubators. For clinical trial of monoclonal antibodies several grams of the antibody may be needed (Wendling *et al.*, 1998; Nguyen *et al.*, 1999). Using this system producing such quantities can be difficult and expensive.

The large size of monoclonal antibodies restricts their utility in some therapeutic situations. Penetration of whole antibodies into solid, poorly vascularised tumours is limited but the production of smaller fragments of antibodies using genetic engineering is now overcoming this problem (Yokota *et al.*, 1992; Colcher *et al.*, 1990; Lane *et al.*, 1994).

There are few truly tumour specific antigens, a problem which exists throughout all antibody therapy. Targets for therapy would ideally be molecules which are unique to a tumour cell type. When cancer cells are treated with antibody, the antibodies can target other non malignant cells of the same type. This can be a problem especially if they are stem cells. CD19 and CD20 make good targets for antibody therapy because they are not present on the surface of bone marrow stem cells which replenish the population of the depleted B lymphocyte cells.

Another limiting factor in using whole murine monoclonal antibodies is the inability of some mouse antibodies to effectively recruit human effector functions. This has initiated a search for new methods to produce humanised or fully human antibodies which can induce these responses for therapy (Ono *et al.*, 1999; Lee *et al.*, 1999; Nakamura *et al.*, 1999).

The use of murine antibodies in humans has been effective but in many cases these antibodies are recognised by the human immune system as foreign molecules. This causes a HAMA response.

1.7 *Human Anti Mouse Antibody (HAMA) Response*

The murine monoclonal antibodies produced by hybridoma technology have been used very successfully in research, diagnostics and therapeutics but some problems have been encountered when they have been used in humans. Some trialed in clinical situations (Foon *et*

al., 1984; Khazaeli *et al.*, 1994; Stone *et al.*, 1996), although effective, have induced the production of an antibody response in patients. This antibody response can occur even though patients are usually immunosuppressed (Grossbard *et al.*, 1992). Side effects as a result of the HAMA response are commonly reported after the second or subsequent administrations of the murine antibody. The reactions that have been described include enhanced clearance of monoclonal antibody from the circulation, formation of antibody:antigen complexes which result in organ damage and the neutralisation of the antibody's effects, facial palsy, hypertension and transient acute tubular necrosis (Meeker *et al.*, 1985; Grossbard *et al.*, 1992). However, Rankin *et al.* (1985) found no HAMA response occurred upon the administration of their murine anti-idiotype antibody. The absence of a HAMA response in a group of patients is relatively rare, with some groups reporting up to 100% of patients having an anti-murine response (Grossbard *et al.*, 1992 ; Khazaeli *et al.*, 1994; Stone *et al.*, 1996). To reduce the frequency of patients experiencing these side effects much time has been spent investigating for alternative strategies for the production of therapeutically active antibodies. It is believed that the production of humanised monoclonal antibodies will overcome the problems of immunogenicity to some extent.

However in some cases, HAMA is beneficial, and in fact is the reason for the therapeutic effectiveness of the antibodies. This is due to induction of the idiotype network (Jerne, 1974). A HAMA response against an anti-idiotype antibody (Ab2) may induce an immune response directed against tumour antigens through the elicitation of an antibody (Ab3 - anti anti-idiotype antibody) against Ab2 (Koprowski *et al.*, 1984). This type of response has been applied to the production of antibodies for vaccination against various diseases (Yamamoto *et al.*, 1990; Mittelman *et al.*, 1990) and provides a different approach to treatment of disease.

Most monoclonal antibodies produced are of murine origin and although there have been efforts to adapt human cells to the hybridoma technology it has so far proven relatively ineffective. In an effort to avoid the HAMA response other technologies such as EBV transformation have been developed.

1.8 *Epstein Barr Virus Transformation*

Hybridomas are usually produced by fusing cells isolated from the immune organs of immunised mice to a mouse myeloma partner. These organs provide a large number of lymphocytes for fusion. In humans ethical problems arise as the result of immunising humans to produce specific antibody-secreting cells or removing the immune organs without good reason. B lymphocytes can be isolated from human peripheral blood but the frequency of antigen specific cells is very low. The number of antibody producing PBLs can be improved by culturing the cells with antigen, mitogens or cytokines (James and Bell, 1987; Danielsson *et al.*, 1987; Steenbakkers *et al.*, 1992; Kwekkeboom *et al.*, 1993). Another problem with producing human antibodies by hybridoma technology is that the human myeloma fusion partners which are available are inferior to their murine counterparts (Hohmann *et al.*, 1995).

Epstein-Barr Virus (EBV) is a lymphotropic herpesvirus which preferentially infects B lymphocytes (Steinitz *et al.*, 1977) causing diseases such as infectious mononucleosis and is present in all African patients with Burkitt's lymphoma (Chapel and Haeney, 1984). Infection of B-lymphocytes with this virus can transform them if the viral genome is integrated into the cell's genome. Although the cell is then infected with a virus, the ability of the infected cell to secrete antibody remains unchanged (Steinitz *et al.*, 1977). This presents an alternative to the hybridoma methodology for production of human monoclonal antibodies. Steinitz's group (1977) showed that this method could be used to produce a specific antibody-forming cell line by selecting B lymphocytes which secreted antibody with a high affinity for a specific antigen for transformation with EBV.

Unfortunately immortalisation of human B lymphocytes with EBV is not without problems. In culture the cells have a tendency to proliferate slowly, often they are unstable and secrete only a small amount of antibody (Hohmann *et al.*, 1995). EBV transformed cells can be improved using hybridoma technology. Kozbor *et al.* (1982) found that transforming B lymphocytes prior to fusion with a myeloma cell can eliminate some of these problems. Fusion of EBV transformed lymphocytes can increase the incidence of fusions from 1 in 10^5 - 10^6 to 1 in 500 (Shammah *et al.*, 1993) compared to direct B cell - myeloma fusions. Still this technology has not really evolved as an efficient way to produce antibodies for use in human disease therapy.

1.9 *Chimeric Monoclonal Antibodies*

The most effective ways to bypass the immunogenicity problems of murine antibodies is to either produce human antibodies or to engineer murine antibodies to make them more human. Chimeric molecules have been constructed which consist of the murine variable regions joined to human constant regions. These antibodies retain specificity for the target while gaining more effective activation of human effector functions.

This technology is relatively easy to carry out and the replacement of the murine Fc portion has been shown to significantly reduce the immunogenicity of some chimeric molecules (LoBuglio *et al.*, 1989; Brüggemann *et al.*, 1989). However others have shown an antiglobulin response after multiple doses with chimeric antibodies (Khazaeli *et al.*, 1991; Elliott *et al.*, 1994).

The major immune response raised against chimeric antibodies is directed against the murine portion of the antibody (LoBuglio *et al.*, 1989). Brüggemann *et al.* (1989) showed that while a bigger response is formed against constant regions of whole murine antibodies, approximately 10% of the response is directed against the variable region.

There have been many chimeric antibodies described including two anti CD20 chimeric antibodies (2H7, Liu *et al.*, 1987 and IDEC-C2B8, Reff *et al.*, 1994). The Rituximab (IDEC-C2B8) antibody first described by Reff *et al.* in 1994, has gone through phase 1 clinical trial successfully (Maloney *et al.*, 1994; Anderson *et al.*, 1997; Maloney *et al.*, 1997), phase II clinical trial (Coiffier *et al.*, 1998) and has recently been approved for use in the treatment of low-grade Non Hodgkin's Lymphoma (NHL). Therapeutically this antibody has performed well. It has high affinity for human CD20 and has the effector functions of a human antibody which promote tumour cell lysis in patients with NHL. This antibody may work synergistically with chemotherapeutic agents for better treatment of patients with resistant aggressive B lymphomas (Demiden *et al.*, 1997). As with most cancer treatments there have been side effects described in some of the patients treated with this antibody. These included fever, nausea, rigor, orthostatic hypotension, bronchospasm, thrombocytopenia. A human anti chimeric antibody response occurred in one patient (Anderson *et al.*, 1997) and some severe initial infusion-related reactions occurred in patients with a high number of tumour cells in their blood (Byrd *et al.*, 1999). Most interesting was the loss of CD20 antigen expression on the surface of B lymphoma tumour cells in one individual (Davis *et al.*, 1999).

1.10 *CDR Grafting and Humanised Monoclonal Antibodies*

Although chimeric antibodies have shown reduced immunogenicity in comparison to whole murine antibodies, in most instances the murine variable regions still cause some immunogenicity. Alternatives to the use of murine antibodies which supersede chimeric antibodies have been described.

The complementarity determining regions (CDRs) of murine antibodies, which interact with the antigen, can be grafted to human antibody framework regions, replacing the human CDRs. Jones *et al.* (1986) showed that this method was effective, by grafting the CDRs from the heavy chain variable region of a mouse antibody, which bound a hapten, into the corresponding CDR sites of a human myeloma protein. The antibodies produced showed a similar affinity for antigen as the mouse parent and competed effectively for binding to the antigen. Rat antibody binding sites, directed against CAMPATH-1 (an antigen on the surface of human lymphocytes), have also been transplanted onto human antibodies. This antibody was as effective as the parent rat antibody in complement mediated lysis and was more effective in cell-mediated lysis of human lymphocytes (Riechmann *et al.*, 1988).

Not all CDR grafted antibodies work this well as the structure of the antigen binding site is often dependent on the underlying framework region. CDR grafting is a difficult technique which relies upon fitting the CDR loops to the human framework regions while still maintaining the antigen binding site. This work can result in a loss of antibody affinity and if this occurs it is necessary to reintroduce some of the lost murine framework structure to restore the binding properties of the original antibody (Queen *et al.*, 1989; Riechmann *et al.*, 1988). Computer modelling has been used to determine which framework amino acids should be retained to maximise antibody binding (Queen *et al.*, 1989).

The grafting of mouse CDRs to human framework regions may introduce new antigenic epitopes on the humanised antibody, defeating the purpose of CDR grafting. To reduce this effect, attempts have been made to resurface the murine antibody by removing only surface residues from the murine antibody which are believed likely to be immunogenic (Padlan, 1991). These residues are identified by using X-ray crystallographic coordinates (Novotny *et al.*, 1986) or by analysing the relative solvent accessibility distributions of both human and mouse variable domains (Pedersen *et al.*, 1994; Roguska *et al.*, 1994). This resurfacing

process leaves the remainder of the antibody untouched thereby preserving much of the antibody's affinity (Roguska *et al.*, 1994).

The only way to totally avoid an anti mouse immune response is to produce truly human antibodies. An approach which allows for the sequential replacement of the heavy and light chains of a murine antibody specific for a particular target with human antibody chains has been described (Jespers *et al.*, 1994). This technique, termed guided selection, involves the display of a murine VH chain on the surface of bacteriophage which is selected against antigen in unison with VL domains from a human library. This technique relies on a compatible human VL pairing with the murine VH. The second step involves the human VL/phage being selected against antigen simultaneously with a human VH amplified from a library. This results in the conversion of the murine antibody into a fully human antibody.

Even humanised antibodies are not without limitations. Detail about antibody structure is required and no matter how good the technique there is often a loss of affinity associated with reducing the immunogenicity of the molecule. None of the methods described here are above causing a reaction if used in therapy. Techniques such as chimerisation, humanisation by CDR grafting and EBV transformation have potential but still the search for an ideal therapy continues. Other modifications of whole antibodies through genetic engineering have been described and include the construction of fragments of antibodies and their production in different expression systems.

1.11 *Single Domain Antibody Fragments*

Up until a few years ago it was believed that the smallest fragment of an antibody that could bind to an antigen was the Fv fragment consisting of variable light and variable heavy chains. Recently with the discovery of a class of antibodies found in camelids (Hamers-Casterman *et al.*, 1993), the engineering of fragments which consist solely of the variable heavy chain has become a possibility. However, engineering of single domain antibody fragments had been investigated prior to this discovery. Variable heavy chain libraries were cloned from mice immunised with lysozyme or keyhole - limpet haemocyanin (Ward *et al.*, 1989). Antibodies to both antigens were isolated from these libraries, some with quite high affinities. Others have

tried to analyse the structure of engineered single domain fragments (Davies and Riechmann, 1994) but encountered problems with aggregation through the surface normally in contact with the light chain.

Camel antibodies are homodimers consisting only of the heavy chain (Hamers-Casterman *et al.*, 1993) and sequence analysis of these antibodies show similarities to human and mouse V_H regions. The differences between camel antibodies and the engineered single domains are that the mouse V_H regions by themselves are "sticky" (Ward *et al.*, 1989; Davies and Riechmann, 1994) and the CDRH3 region in camelid antibodies is much longer, approximately 14 residues, than the average human or mouse CDRH3 loop. The solubility of the camel antibodies may be due to differences in amino acids along the surface of the fragments. Improving the solubility of these engineered fragments by mutating the CDRs to make them more similar to the camelid antibodies (Davies and Riechmann, 1995) may increase the potential for application of both human and murine V_H fragments.

Advantages of single domain antibody fragments are their small size, approximately 12 - 14kDa, allowing these antibody fragments (figure 1.1) better penetration into solid tumours than Fv or scFv antibodies. They would be expected to be more stable than the other engineered antibody fragments and have a reduced risk of immunogenicity.

1.12 *Fv Antibody Fragments*

The smallest fragment of an antibody that retains its binding properties (before the camel VH domain was identified) is the Fv fragment (figure 1.1; Givol, 1991; Fan *et al.*, 1992; Padlan, 1994). It consists of variable light and variable heavy chains associated together in an alignment which is conducive to antigen binding. These non covalently linked fragments are readily produced in bacterial expression systems, and are active as glycosylation of these fragments is not required for antigen binding (Skerra and Pluckthun, 1988; Skerra, 1993). Due to the absence of a linking bond these fragments tend to dissociate into VL and VH domains at low concentrations (Glockshuber *et al.*, 1990). To prevent this dissociation, variations of the Fv fragment have been engineered which involve joining the two domains either with a polypeptide linker (Huston *et al.*, 1988) or disulphide bonds (Brinkmann *et al.*, 1993). The disulphide bond is stable but the engineering of these fragments can affect the

protein folding and hence the binding of the antigen. The more commonly used linker consists of a short flexible polypeptide chain which is situated between the VL and VH chains producing single chain Fvs.

1.13 *Single Chain Fv Antibody Fragments*

Figure 1.1 shows a scFv fragment, consisting of the variable light and heavy domains joined by a short flexible polypeptide linker. The VL and VH domains can be orientated in either VL-linker-VH or VH-linker-VL conformations.

Linkers have been described as a combination of glycines and serines (Gly_4Ser) (Huston *et al.*, 1988) in a different number of repetitions, most commonly 3 or 4. Serine rich linkers (Dorai *et al.*, 1994) and the use of threonine, lysine and glutamic acid (Filpula *et al.*, 1996) can increase the solubility of the linker and therefore the scFv. The inclusion of these amino acids in the linker can reduce the immunogenicity of the linker due to the lack of a well organised 3 dimensional structure.

The use of single-chain Fv antibody fragments as an alternative to whole murine monoclonal antibodies has advantages in some therapeutic and diagnostic situations. Their small size, approximately 28kDa, allows better solid tumour penetration (Yokota *et al.*, 1992; Yokota *et al.*, 1993; Adams *et al.*, 1993) and they rapidly infiltrate tumours (Yokota *et al.*, 1992; Yokota *et al.*, 1993; Adams *et al.*, 1993). Because of the small size of the scFv, the molecules have a short half life in circulation (Colcher *et al.*, 1990; Begent *et al.*, 1996) and they do not persist as long in non tumour tissue. These molecules are easily produced in bacterial expression systems which are much cheaper and faster than the production of whole antibodies in mammalian expression systems. The absence of the constant regions from these molecules and also the rapid clearance of the scFvs from the system means that they are less likely to cause a HAMA.

1.14 *Fab Antibody Fragments*

The Fab antibody fragment (figure 1.1) consists of the variable and constant regions of the light chain and variable and CH1 regions of the heavy chain joined together by a disulphide bond.

Fab antibodies can be produced by enzymatically cleaving the Fab region from the whole antibody, but are now commonly engineered using PCR and a bacterial expression system. The light and heavy chains are produced independently of each other and transported to the periplasm where they associate and disulphide bonds are formed. Fab fragments are expressed into the culture supernatant but can also be isolated from the periplasm of the bacteria.

Fab antibodies, like scFvs, have advantages over whole antibodies for use in therapeutic situations rather than diagnostics, to which scFvs are quite suited. The most obvious difference is their size, being smaller than a whole antibody they show better tumour penetration (Yokota *et al.*, 1992), less serum retention time, and remain in the body longer than scFv fragments (Yokota *et al.*, 1992; Colcher *et al.*, 1990; Milenic *et al.*, 1991). Fab fragments are less immunogenic than whole murine antibodies due to the lack of the Fc domain (LoBuglio *et al.*, 1989; Brüggemann *et al.*, 1989) but will be more immunogenic than scFvs due to the presence of the CH1 and CL domains and their longer retention time in the circulation. This immunogenicity may be reduced by constructing chimeric Fab molecules replacing murine constant regions with human domains.

1.15 *F(ab)$_2$ Antibody Fragments*

$F(ab)_2$ antibody fragments (figure 1.1) consist of 2 Fab fragments joined in the constant regions by a flexible hinge. The only difference between a monospecific $F(ab)_2$ antibody and a whole antibody is the absence of the Fc portion and the functions that the Fc region elicit upon antigen binding (Carayannopoulos and Capra, 1993). The $F(ab)_2$ fragment was originally produced by digestion of whole antibody with pepsin (Carayannopoulos and Capra, 1993). The Fab' molecule can be produced by mild reduction of the $F(ab)_2$ fragment and a Fab molecule is produced by digestion with papain. These methods can be expensive so the engineering of these fragments and the use of bacteria to express the fragments is an attractive alternative.

Bispecific $F(ab)_2$ molecules can be engineered by crosslinking two F(ab') fragments in the hinge region (Inouye *et al.*, 1997; Segal *et al.*, 1999). They could be used for targeting two different cell types to bring them into close proximity so that one can exert specific functions on the other (Brandl *et al.*, 1999).

The advantages of using these antibody fragments range from having similar binding properties to whole antibodies with reduced immunogenicity (Brüggemann *et al.*, 1989; Seccamani *et al.*, 1989; Wahl *et al.*, 1983) to having a longer serum half life than a smaller molecule such as the Fab (Milenic *et al.*, 1991; Yokota *et al.*, 1993).

1.16 *Multimeric Antibodies and Bispecific Antibody Fragments*

The major disadvantage in the use of antibody fragments (except $F(ab)_2$ fragments) over whole monoclonal antibodies is their monovalency. The fragments lack the avidity of whole immunoglobulin molecules due to the presence of only one binding site. Two terms often used to describe antibody binding are affinity and avidity. Affinity is a measure of the strength of the binding of an antibody binding site to its epitope on the antigen, and is determined by the relative rates of association and dissociation of the antibody from the antigen. When a monovalent construct, such as a scFv or a Fab, binds to an epitope on an antigen, the dissociation step results in loss of the fragment from the cell surface. If the construct is bivalent or multivalent the result of one binding domain dissociating from a site on the cell will not necessarily mean the detachment of the antibody from the cell. It is likely that upon reassociation the antibody domain will rebind the same surface. The strength of multivalent binding is called avidity or functional affinity. Avidity is significantly higher than affinity, but only if the frequency of antigen on the surface of the cell is high enough for both antibody binding sites to find a target.

Engineering of antibody fragments, such as scFv and Fabs, has now been extended to include the production of dimers and trimers of these fragments in an effort to recreate the avidity of a whole antibody. There are a number of ways to produce these modified fragments. The use of shortened linkers, between 0 - 10 amino acids, in scFv can force the association of the VL and VH domains of one scFv to another molecule because the length of the linker is not enough to allow two domains from the same antibody fragment to associate together. This effect can cause the formation of dimers (Holliger *et al.*, 1993) and abolishing the linker altogether can cause the formation of stable trimers of the scFv (Iliades *et al.*, 1997). Reducing the linker length to 1aa can cause the formation of tetramers which have been shown to have prolonged association with cell bound antigens (Le Gall *et al.*, 1999). Dimerised scFvs can also be

formed by incorporation of the fragments into dimerisation vectors (Krebber *et al.*, 1997) or fusion of the C terminal of two scFvs to a helix turn helix motif (Pack *et al.*, 1993) or a leucine zipper (de Kruif and Logtenberg, 1996). The formation of a four helix bundle produces miniantibodies which have been reported as having significantly higher avidity than leucine zipper containing constructs (Pack *et al.*, 1993).

These multivalent antibodies can be useful for a number of applications. It is thought that trimeric and tetrameric scFv fragments would be ideal reagents for tumour imaging. Dimeric antibodies promise to be useful in both diagnostic and therapeutic situations (Beresford *et al.*, 1999) but the larger multimers may be better alternatives.

New approaches to tumour targeting have required the production of bispecific antibodies (Renner and Pfreundschuh, 1995). These fragments can be used to link two cell types to produce an immune response through the recruitment of cytotoxic T cells (Demanet *et al.*, 1996; Bauer *et al.*, 1999), macrophages (Valone *et al.*, 1995; Ely *et al.*, 1996; Pullarkat *et al.*, 1999) or to target viruses to cells for use in gene therapy (Wickham *et al.*, 1997). This method can also be used to target two different epitopes on the same antigen (Robert *et al.*, 1999). These antibodies can be used as carriers of conventional conjugates or could be fused to a second functional domain.

Dimeric scFv fragments will be of similar size to a monovalent Fab fragment and will have similar properties in terms of tissue penetration and circulation retention time but will have increased avidity if the target molecules are both expressed on the same surface.

1.17 *Phage Display Technology*

Phage display technology is based on the early work of Smith (1985) who used bacteriophage to display small peptides. One of the most important uses for this technology is the display of antibody fragments on the surface of phage in the search for new therapeutic reagents. This technology is unique as it allows the display of not only murine antibodies on the surface of phage but also human antibodies from libraries constructed from human B lymphocytes.

The phage display method, which has already proved successful in the production of diverse antibody repertoires, involves the construction of a library of antibodies, in scFv or Fab form, displayed on the surface of bacteriophage. The display is facilitated through the fusion of the

recombinant protein to either coat protein III on the tip of the phage (3 copies), which is most commonly used, or coat protein VIII which is a protein which covers most of the body of the phage (2800 copies; Chiswell and McCafferty, 1992).

A generalised method for library construction involves extraction of mRNA from cultured cells or B lymphocytes, which have been isolated from peripheral blood, spleen, tonsils or bone marrow. cDNA is generated and this is used in the PCR amplification of the antibody genes. Light and heavy chains are then cloned into a suitable vector and this construct is transformed into competent *Escherichia coli*. Transformed *E. coli* are infected with bacteriophage which package the phagemid, containing the antibody genes, into its particle and displays the antibody fragment on its surface due to the attachment of one chain, generally the heavy chain, to one of the coat proteins. The library is then complete and it can be used to screen against a target antigen.

One of the original phage display antibody libraries was produced from the spleen of mice immunised with KLH coupled p-nitrophenyl phosphonamidate antigen 1 (Huse *et al.*, 1989). This was a library of Fab fragments in which the light and heavy chains were brought together by producing a light chain library and a heavy chain library and randomly combining the light and heavy chain vectors to produce light and heavy chain pairing. Using this library the group were able to isolate Fab antibodies which bound the immunogen.

Random combinatorial human libraries have been produced from the peripheral blood lymphocytes of unimmunised donors (Marks *et al.*, 1991). Four libraries were constructed with IgM or IgG heavy chains and kappa or lambda light chains. Antibodies to antigens that the donor individuals had not encountered before, turkey egg-white lysozyme, BSA or 2-phenyloxazol, were retrieved from the libraries after four rounds of panning. No high affinity antibodies were retrieved from these libraries.

The documentation of human germline V_H (Tomlinson *et al.*, 1992), V_λ (Williams and Winter, 1993) and V_κ (Cox *et al.*, 1994) sequences has allowed the production of synthetic antibody libraries. These libraries usually have engineered CDRH3 regions. The heavy chain CDR3 is a popular target for the production of synthetic libraries as it is a highly variable CDR. The CDRs can be altered in length and variability of individual residues. Human antibodies have been selected against haptens from a library containing human germline V_H gene segments

which have CDR3 regions altered in vitro to produce synthetic CDRH3s of 5 or 8 residues (Hoogenboom and Winter, 1992). de Kruif *et al.* (1995) also targeted the CDRH3 regions in the library they constructed, again using 49 germline V_H sequences, and combining these heavy chains with seven light chains. In this case the CDRH3 regions varied between 6 and 15 residues. Nissim *et al.* (1994) extended the Hoogenboom and Winter (1992) library by increasing the variation in the CDRH3 to between 4 - 12 residues. From this library the group successfully isolated antibodies to 18 antigens even though the library was reasonably small compared to standards set more recently. Griffiths *et al.* (1994) enlarged the library from 10^8 to 6.5 x 10^{10} Fab fragments in the synthetic repertoire making it one of the largest libraries constructed to date.

Originally phage display libraries were screened against pure antigen but recent developments have allowed the selection of antibodies against surfaces displaying a wide range of antigens. Phage display libraries have been panned against whole cells (Watters *et al.*, 1997; Schmidt *et al.*, 1999), tissue sections (Tordsson *et al.*, 1997) and whole organs *in vivo* (Pasqualini and Ruoslahti, 1996) successfully isolating antibodies and peptides against antigens such as CD20, antigens on the surface of colorectal carcinoma tissue and the brain and kidney of mice.

Until recently it was not common to produce phage display antibody libraries of a size large enough to resemble the human antibody repertoire. Now the size of antibody libraries has expanded from 10^7 - 10^8 to 10^{10} - 10^{11} (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996; de Haard *et al.*, 1999). This has allowed the isolation of a number of antibodies with good affinity for their target antigen.

One of the major advantages the development of phage display technology has brought to antibody construction is the ability to isolate human antibodies to different diseases without human immunisation. The successful isolation of antibodies against HIV-1, targeted to the Rev and Tat regulatory proteins (Pilkington *et al.*, 1996) and gp120 (Burton *et al.* 1991; Burton and Barbas, 1993; Barbas *et al.*, 1993), Respiratory Syncytial Virus (Barbas *et al.*, 1992b), Hepatitis B (Zebedee *et al.*, 1992), Tetanus Toxoid (Mullinax *et al.*, 1990) and many more have been described.

It is also possible to select antibodies against self antigens. Antibodies against human antigens, thyroid peroxidase (Portolano *et al.*, 1993), antigens expressed on cancer cells (Ridgway *et al.*, 1999), human chorionic gonadotropin, human leuteinizing hormone and human follicle

stimulating hormone (de Haard *et al.*, 1999), have been isolated from human phage display antibody libraries. Human antibodies have also been isolated against B lymphocyte cell surface antigens (personal correspondence, Pilkington, G., Intracel Corporation) which may be useful in the treatment of B cell disease.

Other applications for phage display include chain shuffling (Jespers *et al.*, 1994) for humanisation of antibody fragments and affinity maturation. Several different approaches have been used to increase the affinity of phage displayed antibodies. Non proof reading DNA polymerases such as Taq polymerase or the use of mutant strains of *Escherichia coli*, like MutD5, which has a defective 3' - 5' exonuclease function and does not allow correction of mutations in DNA sequence during replication (Fowler *et al.*, 1986) can induce random mutations into antibody sequence. Other methods of increasing affinity include site directed mutagenesis and chain shuffling.

1.18 *Ribosomal Protein Display*

Methods which link protein to its mRNA via a ribosome, ribosomal protein display, for use in both prokaryotic (Mattheakis *et al.*, 1994) and eukaryotic (He and Taussig, 1997) systems have been described. This involves the synthesis of proteins which remain attached to their ribosome and allows the screening of large numbers of proteins, comparable with phage display. Screening is carried out, non specific binders dissociated and whole specific complexes eluted with antigen. Isolated mRNA is then reverse transcribed to cDNA and PCR amplified. The genes coding for protein can then be transformed into bacteria and soluble protein expressed.

Hanes and Pluckthun (1997) have developed the prokaryotic system to produce ribosomally displayed scFv fragments. This system has been used to screen antibody libraries developed from immunised mice (Hanes *et al.*, 1998). In 3 rounds of ribosome display this group selected a number of different scFvs, one of which showed high affinity for antigen.

1.19 *Bacterial Protein Display*

A protein surface display system has been described in which the proteins of interest are displayed on the surface of bacteria as an alternative to bacteriophage or ribosomes.

Proteins such as ß-lactamase, can be transported and anchored to the external surface of *Escherichia coli* using outer membrane proteins, such as OmpA (Francisco *et al.*, 1992). This has been extended to the display of recombinant proteins. The fragments displayed on the cell surface can be detected using methods such as whole cell ELISA, fluorescence microscopy and flow cytometry (Francisco *et al.*, 1993). Several different membrane proteins have been used for the display of recombinant proteins on the surface of *E. coli* from the display of small peptides on outer membrane proteins such as LamB (Charbit *et al.*, 1988), PhoE (Agterberg *et al.*, 1990) and Lpp-OmpA (Francisco *et al.*, 1992) to display on other surface appendages of bacteria such as the flagellin FliC (Lu *et al.*, 1995) and pili FimH (Pallesen *et al.*, 1995) and PapA (Steidler *et al.*, 1993).

As described in phage and ribosomal display this method can be used to screen libraries of proteins (Georgiou *et al.*, 1997). Antibody fragments with high affinity for their target can be isolated from these libraries with the use of flow cytometry and cell sorting over a number of rounds of screening and amplified for the production of soluble protein (Daugherty *et al.*, 1998).

1.20 *Human Antibody Repertoires in SCID Mice*

An advance in the production of human immunoglobulins has been the repopulation of severe combined immunodeficient (SCID) mice with human lymphoid cells (Mosier *et al.*, 1988). These PBL-SCID mice are a unique source of human monoclonal antibodies and have been used to investigate some of the properties of the human immune response. When these mice were immunised with antigens the donors had not encountered in many years (hepatitis B core antigen and tetanus toxoid) and had low antibody serum titres for, they produced an immune response at levels 50 - 450 fold higher than the non immunised mice (Duchosal *et al.*, 1992). Lymphocytes from these mice were used to construct a Fab phage display library from a donor

who had been immunised with tetanus toxoid 17 years previously. A number of human Fabs against tetanus toxoid were isolated from the library.

Investigation into the extent of the reconstitution of the immune function in these mice showed that production of immunoglobulin isotypes and specific antibody were comparable to that found in the human donors (Nonoyama *et al.*, 1993), indicating that the memory cells survive well during and after transfer into the mice.

Sandhu *et al.* (1994) examined the primary immune response of mice repopulated with human PBLs. They argue that the method used for grafting human PBLs into mice (using the Mosier *et al.* (1988) approach) is ineffective at transferring large numbers of human PBLs into various SCID mouse tissue. They modified a method described by Shpitz *et al.* (1994) by pretreating the mice prior to grafting with anti-asialo GM-1 antiserum and radiation and found higher levels, 385-fold for IgM and 13-fold for IgG, of primary antibody production to that previously cited. $CD4^+$ cells were important in the production of this response but $CD8^+$ cells were not.

A different approach to the expression of human immunoglobulins in mice has been described (Jakobovits, 1995). This approach involves two manipulations. Firstly the inactivation of the endogenous mouse immunoglobulin genes and secondly the transfer of large portions of unrearranged human immunoglobulin gene segments into these mice. Considering the number of immunoglobulin genes and the amount of DNA this involves, not all the genes can be transferred simultaneously. Large amounts of sequence have been transferred into mice using two approaches; Minigenes (Brüggemann *et al.*, 1991; Tuaillon *et al.*, 1993; Lonberg *et al.*, 1994), about 100kb of a limited number of cloned genes can be transferred, or the use of yeast artificial chromosomes (YACs; Jakobovits *et al.*, 1993; Green *et al.*, 1994) in which approximately 1Mb of contiguous immunoglobulin sequence can be incorporated (Mendez *et al.*, 1997).

If the murine immunoglobulin genes are still functional when the human genes are transferred the circulating immunoglobulin will be of both human and murine origin. To gain maximal expression of the human antibody genes the murine immunoglobulin function must be silenced. Green *et al.* (1994) accomplished this by fusing yeast spheroplasts, containing the human immunoglobulin YACs, with mouse embryonic stem cells. This fusion yielded a high frequency of embryonic stem cells which carried intact YAC sequence. The mice arising from this were bred with mice which were incapable of producing mouse antibodies to generate a

strain of mice which only produce human antibodies. This has resulted in serum levels of human immunoglobulin reaching 800μg/ml (Mendez *et al.*, 1997).

The progression from minigene constructs to the use of YACs to incorporate human genes into mice has greatly improved the quality of the antibody response. Mendez *et al.* (1997) produced a YAC construct which contained large fragments of the human heavy and kappa light chain in nearly germline configuration. The variety of the antibodies produced in a system which has more antibody genes will be much more diverse than one with a more restricted repertoire. Recently Nicholson *et al.* (1999) described mice which contained immunoglobulin heavy genes and both kappa and lambda light chains. The addition of human lambda light chains to the repertoire is important as approximately 40% of human antibodies have lambda light chains.

The advantage of this approach is that a large proportion of the human immunoglobulin genes are present and using the murine immunological machinery the response to antigen is not dependent on the repertoire of antibodies provided by the donor of the immunoglobulin producing cells.

1.21 *Antibody Conjugates*

Where conventional therapies fail opportunities arise for alternative treatment methods. Already described are methods of construction and selection of antibodies which can target antigens found on the surface of malignant cells. For some applications these antibodies and fragments made from them have different therapeutic agents attached to them. These include immunotoxins (Kreitman, 1999), radioisotopes (DeNardo *et al.*, 1999), chemotherapeutic drugs (Trail and Bianchi, 1999) and enzyme treatments (Bagshawe *et al.*, 1999).

Immunotoxins

Immunotoxins are composed of a potent protein toxin conjugated to a carrier protein, antibody or growth factor, which directs the toxin to its target (Pastan and FitzGerald, 1991). A target antigen which internalises upon ligation with antigen best delivers the antibody toxin conjugate into the cytoplasm of the cell where the toxin can inhibit protein synthesis (Pastan and FitzGerald, 1991; Kreitman, 1999).

Immunotoxins can be produced either by chemically linking the toxin to the antibody (Ghetie *et al.*, 1990; Vitetta *et al.*, 1987; Pastan *et al.*, 1986) or by genetically engineering modified toxin genes fused to growth factors or antibodies in bacterial expression systems (Pastan and FitzGerald, 1989; Chaudhary *et al.*, 1989). The toxins can be used in their natural form but derivatives of the toxins are also commonly used (Kreitman, 1999). Advances in the understanding of toxin structure has allowed modification of the natural toxins. Examples of this include chemically linking only the A chain of ricin to an antibody (Blythman *et al.*, 1981) or using the whole ricin molecule but blocking the B chain (Thorpe *et al.*, 1984; Lambert *et al.*, 1991), deleting the domain 1 (binding domain) of PE and linking the other domains to antibody (Kondo *et al.*, 1988) and construction of a mutant form of DT with reduced binding activity and linking that to antibody (Greenfield *et al.*, 1987; Colombatti *et al.*, 1986). These changes reduce the nonspecific binding of the toxins by removing or inactivating the natural binding domains of the toxins so entry into the targeted cell only occurs through antibody ligating to the cell. Chemical conjugation can be expensive as large amounts of the toxin and antibody are required (Pastan and FitzGerald, 1991). Genetically engineered recombinant toxins have been produced as an alternative to this method. Versions of PE (Reiter and Pastan, 1996; Reiter *et al.*, 1994), DT (LeMaistre *et al.*, 1998) and Ricin immunotoxins have been engineered (Kreitman, 1999).

There are limitations to treatment with immunotoxins. They have dose limiting toxicities (Pastan *et al.*, 1992; Vitetta *et al.*, 1992; Byers et al., 1989) and are highly immunogenic if used in therapy without immunosuppressive agents, with neutralising antibodies developed in approximately 10 days (Pai *et al.*, 1991; Durrant *et al.*, 1989; Byers *et al.*, 1989). If conjugated to whole antibody they display poor tumour penetration which can be overcome with the use of antibody fragments.

Radioimmunotherapy

Radioisotopes are alternative agents for conjugation to monoclonal antibodies. Radioimmunotherapy delivers substantial doses of radiation to tumours while minimising normal tissue exposure (DeNardo *et al.*, 1999). It has displayed good results in the treatment of heamatologic malignancies (Knox *et al.*, 1996; Kaminski *et al.*, 1996) such as Non

Hodgkin's Lymphoma in patients relapsed after conventional treatments. The ability of these reagents to cause damage to tumours without internalisation gives advantages over the use of immunotoxins and chemotherapeutic drugs. Although this indicates that these agents would be useful in solid tumour therapy this work has so far shown limited results due to lack of sensitivity of these cells to radioisotopes (DeNardo *et al.*, 1999). However there have been some success in breast cancer (DeNardo *et al.*, 1999).

Drug Conjugates

A problem associated with the use of chemotherapeutic drug therapy is the effect of non specific activity of the drugs (Sanderson and Shield, 1996). Directed therapies, fusing the drugs to monoclonal antibodies, have the potential to improve the efficacy of the drugs by allowing higher doses to be administered and minimising exposure of non malignant tissue (Trail and Bianchi, 1999). It has allowed the use of very toxic drugs such as the enediynes which are usually excluded from use due their low therapeutic index (Trail and Bianchi, 1999). Encouraging results have been gained in treatment of patients with lymphoma (Sievers *et al.*, 1999), colorectal cancer after tumour resection (Riethmüller *et al.*, 1998) and breast cancer as a combination of HER-2/neu and cisplatin (Hancock *et al.*, 1991; Pietras *et al.*, 1994; Pegram *et al.*, 1998) but drug conjugated monoclonal antibodies are not as effective when used as a single agent in patients with advanced disease (Trail and Bianchi, 1999).

Other Conjugates

Other functional domains can be fused to antibody fragments rather than using drugs, toxins or isotopes. One alternative is the use of immunoliposomes (Ahmad *et al.*, 1993). The liposomes consist of various derivatives of polyethylene glycol and act as carriers of substances which are toxic to cells. The linkage of the liposome to an antibody fragment provides an excellent method of targeting a treatment, such as doxorubicin (Tseng *et al.*, 1999), specifically to diseased cells without causing the side effects often seen in non malignant tissue.

Another development has seen the introduction of antibody - directed enzyme - prodrug therapy (ADEPT) which allows the transport of a prodrug to a target site at which point the enzyme

fusion protein catalyses the activation of the prodrug to a cytotoxic agent (Haisma *et al.*, 1998). Recombinant antibodies have been used as vaccines (Timmerman and Levy, 1999; Biragyn *et al.*, 1999). They have also been fused to the coat proteins of viruses, for gene therapy (Somia *et al.*, 1995; Marin *et al.*, 1996; Poul and Marks, 1999; Martin *et al.*, 1999) and to cytokines for stimulation and proliferation of T cells at the site of tumours (Harvill *et al.*, 1996; Lode *et al.*, 1998; Melani *et al.*, 1998; Peng *et al.*, 1999).

1.22 *Cell Surface Antigens*

For antibody therapy it is important to have an understanding of what structures are present on the surface of the cells and how their functions affect the cell. Cell surface antigens are present on all normal and malignant cells. Antibody therapy against antigens on neoplastic cells can also affect non malignant cells carrying the same cell surface markers. Although some tumour specific antigens have been described (Türeci *et al.*, 1997) identification of these antigens on all tumour types has not yet become reality so focus remains on those antigens known to be present on the cell surface.

There is a range of B cell surface antigens identified (Uckun, 1990) many of which can be used for the therapy of B cell disease. The antigens chosen for this thesis, CD19 and CD20, are suitable targets for the therapy of B cell leukemias and lymphomas as their expression is restricted to B lymphocytes.

1.23 *CD19*

CD19 is a 95kDa glycoprotein which was first described as the B4, B cell-specific antigen (Nadler *et al.*, 1983). This protein is expressed exclusively on normal and neoplastic B cells. It is first expressed by the B progenitor cells in the bone marrow, either at the late pro-B or early pre-B stages, persists during all stages of B-cell maturation and is lost upon terminal differentiation to plasma cells (Nadler *et al.*, 1983). CD19 has also been reported to be expressed by follicular dendritic cells and the use of a monoclonal antibody to CD19 showed staining of the mantle-zone and the germinal-centre lymphoid cells (Zhou and Tedder, 1995a).

It has not, however, been detected on normal, activated or malignant cells of T or myeloid origin (Nadler *et al.*, 1983).

CD19 is expressed on neoplastic B cells including most non-T cell acute lymphocytic leukemia, B-chronic lymphocytic leukemia (Zhou and Tedder, 1995a; Anderson *et al.*, 1984) and B cell lymphoma (Nadler *et al.*, 1983).

The predicted amino acid sequence of CD19 has been published (Tedder and Isaacs, 1989). The CD19 protein has a molecular weight of 95kDa, glycosylated, but has been identified as ranging from 75kDa to 95kDa depending on the cell source. These differing weights have been attributed to differences in glycosylation or differences in phosphorylation between cell types (Tedder and Isaacs, 1989).

Figure 1.2 shows the hypothetical structure of CD19 (adapted from Tedder *et al.*, 1989a). It is a glycoprotein which spans the cell membrane once, with an extracellular amino terminus and the carboxyl terminus cytoplasmic. It has 5 sites of N-linked glycosylation on the long extracellular portion of the molecule as well as 2 immunoglobulin-like IgC2 domains (Tedder and Isaacs, 1989).

It has a third smaller domain which as yet has not been identified as being similar to any other recognised immunoglobulin-like domains (Tedder and Isaacs, 1989).

CD19 is relatively conserved between species with the sequence for the putative cytoplasmic domain of the murine version of the protein being 79% homologous to the human protein, and the overall sequence and length of the two showing 75% homology (Tedder and Isaacs, 1989).

Tedder *et al.* (1989a) have reported at least two antibody-binding sites on CD19, with most antibodies tested binding to the same site and one antibody which bound a unique epitope.

The human CD19 gene has been mapped to band p11.2 on the proximal short arm of chromosome 16 and has been shown to be composed of 15 exons (Zhou and Tedder, 1995a).

Pezzutto *et al.* (1987) evaluated the function of the CD19 antigen by using the CD19 monoclonal antibody HD37. Binding of the monoclonal antibody to B cells inhibited the proliferative response to anti-Ig and had an effect on the early phase in B cell activation by reducing the number of cells that left G_0 and entered the G_1 phase of the cell cycle upon triggering with anti-μ antibody. CD19 crosslinking with anti-CD19 antibodies induces cell cycle arrest at G_1 (Ghetie *et al.*, 1994) and production of dimerised anti-CD19 antibodies show improved crosslinking which does not require the Fc region of the antibody to be effective

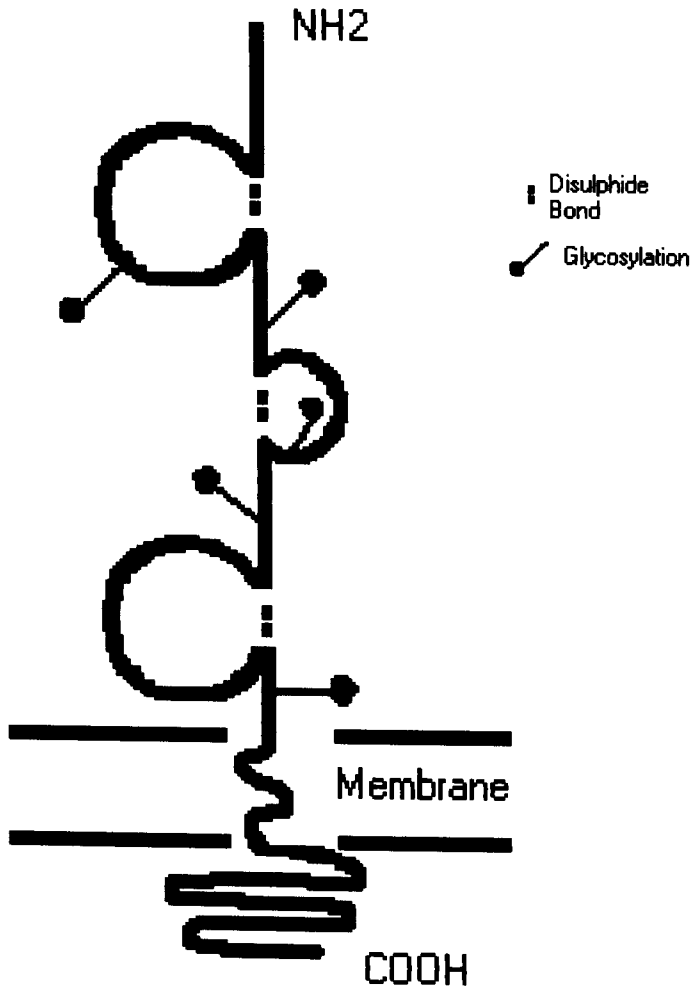


Figure 1.2: CD19 Antigen.

(Ghetie *et al.*, 1997). These antibodies could make potent anti-tumour agents. In 1989, Tedder and Isaacs postulated that their predicted CD19 structure suggested a role in cell surface recognition and generation of transmembrane signals through its long cytoplasmic tail and concluded that it may be a receptor important for B cell function. More recently with the use of transgenic mice which are either deficient in CD19 or reconstituted with human CD19 a better insight into the function of CD19 has been allowed. Over-expression of human CD19 in the transgenic mice severely affected the development of immature B cells in the bone marrow and also the number of B cells found throughout the body (Zhou *et al.*, 1994; Engel *et al.*, 1995). Mice deficient in CD19 showed decreased B cell proliferation in response to mitogens and decreases in serum immunoglobulin levels (Engel *et al.*, 1995). These results indicate that CD19 plays a role during early B cell development and also functions to define signalling thresholds for receptors which regulate B cell development and activation. CD19 also appears important in the development of B-1 lymphocytes (Rickert *et al.*, 1995; Sato *et al.*, 1996; Krop *et al.*, 1996) and the development of autoantibodies (Sato *et al.*, 1996).

1.24 *CD20*

CD20 is a cell surface antigen whose expression is solely restricted to the B cell lineage (Stashenko *et al.*, 1980). It is a 33kDa integral membrane phosphoprotein which can also be found in 35 and 37kDa forms. This is believed to be due to differential phosphorylation (Tedder and Schlossman, 1988; Tedder *et al.*, 1988a).

Initially described as the B1 molecule it was a cell surface antigen discovered just prior to the discovery of CD19 (B4; Nadler *et al.*, 1983). The hypothetical structure of CD20 can be seen in figure 1.3 (adapted from Tedder *et al.*, 1989b). It is a molecule that passes through the membrane four times and contains three extensive hydrophobic regions. Two of these regions are long enough to pass through the membrane once and the other is believed to traverse the membrane twice. Both the carboxyl and amino terminal ends are located within the cytoplasm with only a minor portion of the molecule exposed to the extracellular environment (Tedder *et al.*, 1988b). It is not believed to be glycosylated or phosphorylated in resting B cells although upon mitogen stimulation it becomes heavily phosphorylated (Valentine *et al.*, 1987; Tedder and Schlossman, 1988). It may be a component of a multimeric surface receptor complex

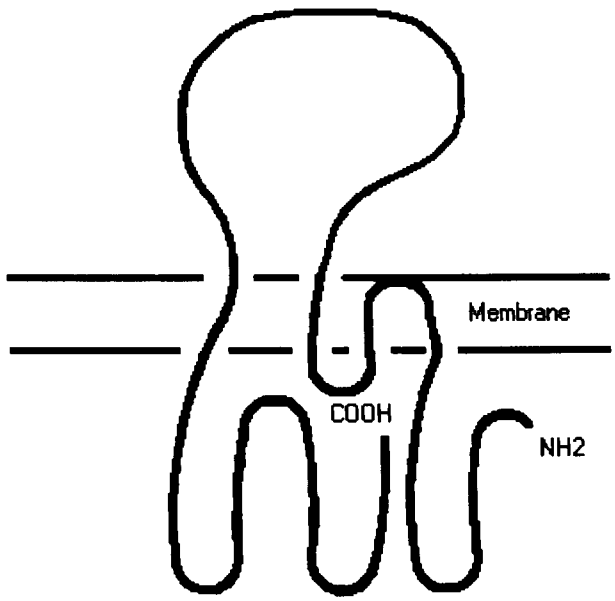


Figure 1.3: CD20 Antigen.

(Tedder *et al.*, 1988c). CD20 is expressed on both normal and neoplastic B cells. In normal cells it is expressed from the pre-B cell stage after immunoglobulin heavy chain rearrangement, persists throughout all stages of B cell maturation but is lost upon terminal differentiation into plasma cells (Stashenko *et al.*, 1980). In neoplastic cells it is expressed by the majority of B-cell lineage malignancies including 50% of acute lymphoblastic leukemias. Like CD19 it has also been identified in mantle zone and germinal centre cells (Zhou and Tedder, 1995c).

The human CD20 gene has been mapped to band q12-q13.1 on the proximal long arm of chromosome 11 (Tedder *et al.*, 1989a).

Like CD19, CD20 is relatively conserved across different species showing 73% homology in amino acid sequence to the murine version (Tedder *et al.*, 1988a).

Monoclonal antibodies to CD20 show binding to the same or a closely related epitope although some data suggests that they might bind to slightly different regions of CD20 (Zhou and Tedder, 1995c).

The nucleotide and amino acid sequence of CD20 was published in 1988 (Tedder *et al.*, 1988b) and in 1989 the structure of the CD20 gene was determined. The gene is 16kb and consists of 8 exons (Tedder *et al.*, 1989b).

Binding of a monoclonal antibody to the external segment of CD20 inhibits B-lymphocyte entry into the S/G2 + M stages of the cell cycle after mitogen stimulation and also blocks cell differentiation (Tedder *et al.*, 1985). Monoclonal antibody binding to CD20 has been shown to generate a transmembrane signal that enhances phosphorylation of CD20 (Tedder and Schlossman, 1988), initiation of tyrosine kinase activity (Kansas and Tedder, 1991), and induction of c-myc oncogene expression (Smeland *et al.*, 1985) indicating that CD20 function includes processes such as B-lymphocyte activation, proliferation and differentiation.

CD20 may be a representative of a unique family of proteins that function either directly as ligand-gated ion channels or as essential components of such channels (Zhou and Tedder, 1995c). It is known to act as a calcium channel by forming a complex of maybe four CD20 molecules (Bubien *et al.*, 1993). This calcium ion channel is critical for cell cycle progression. This molecule appears to form part of an important complex which is involved in regulating B cell function in terms of activation, proliferation and differentiation.

1.25 *Thesis Plan*

- The overall aim of this project is to produce antibody fragments which can be used in the therapy of B cell leukemias and lymphomas. Two cell surface antigens, CD19 and CD20, have been identified as suitable targets for these antibodies.
- The first aim of this project is to produce the cell surface antigens, CD19 and CD20, for screening potential antibody candidates. The CD20 protein has a relatively small portion of the molecule exposed on the outer surface of the cell. It has been established that most antibodies against CD20 bind in this area. This part of the protein was manufactured synthetically and provided by Intracel.
- Ideally the CD19 antigen will also be produced as a pure protein. It is unrealistic to produce the CD19 antigen in a similar manner to the CD20 as the CD19 molecule is large with the majority of the protein being extracellular. A transfected cell line which produces CD19 solubly by secreting the protein into the cell culture medium will be produced using PCR techniques and a mammalian expression system. If this proves too difficult a transfected cell line producing membrane bound CD19 will be constructed.
- It has been postulated that phage display libraries constructed from patients in remission with B cell malignancy would produce high titres of antibody against the cell surface antigens on the tumour due to the excessive proliferation of those cells. To test this theory, plasma and serum samples from patients with malignancy and also those with autoimmune disease will be assayed for antibodies against CD19 and CD20.
- The second aim of the project is to produce human Fab antibody fragments against CD19 and CD20 from phage display libraries constructed from human tonsil lymphocytes and human bone marrow lymphocytes.
- The third aim is to produce murine scFv and Fab antibodies from an anti-CD20 hybridoma, HB13d. Once produced the characteristics of the scFv will be investigated thoroughly and the Fab antibody will be used to spike a Fab phage display library.

Although this thesis has a general methods section there are also materials and methods sections in all the results chapters. The nature of some of the research has required that in some chapters the methods have been combined with the results section to improve the clarity of the work.

Chapter Two

General Methods

Chapter 2

General Methods

2.1 *Summary*

This chapter will describe the general methods used throughout the course of the project. Many of these methods are generally well known and commonly used throughout molecular biology laboratories. Cell culture media and techniques and protein assay methods are also described.

2.2 *Materials and Methods*

2.2.1 *General Media and Solutions*

PBS (10X)

80g NaCl (Merck)

2g KCl (Merck)

11.5g Na_2HPO_4 (BDH Chemicals)

2g KH_2PO_4 (Merck)

1L MilliQ water and autoclave

2.2.2 *Bacterial Culture Media, Solutions and Techniques*

SB

30g Bacto Tryptone (Oxoid)

20g Yeast Extract (Oxoid)

10g MOPS(4-Morpholinepropanesulfonic acid; Boehringer Mannheim)

900ml MilliQ water

pH adjusted to 7.0

Volume adjusted to 1L and autoclave

2YT

16g Bacto Tryptone

10g Yeast Extract

5g NaCl

1L MilliQ water and autoclave

LB

10g Bacto Tryptone

5g Yeast Extract

10g NaCl

1L MilliQ water and autoclave

Agar

16g Agar (Sigma) per 1L media and autoclave

SOC (SOB + Glucose + $MgCl_2$)

SOB

20g Bacto Tryptone

5g Yeast Extract

0.5g NaCl

950ml MilliQ water

10ml 25nM KCl

pH adjusted to 7.0 with 5M NaOH (AJAX Laboratory Chemicals)

Adjust to 1L and autoclave

SOC 100ml

To 100ml SOB add 2ml 2M Glucose (AJAX Laboratory Chemicals; filter sterilised through a 0.2μM filter (Sartorius))

Add 5ml 2M $MgCl_2$ (BDH Chemicals; autoclaved)

Periplasmic Extraction Buffer (Borate Buffer)

200mM $Na_2B_4O_7$ (BDH Chemicals)

100mM NaCl

100mM EDTA (BDH Chemicals)

1L MilliQ water

pH 8.0

Hepes Extraction Buffer

20mM Hepes

0.5M NaCl

pH 7.0 with HCl

Kanamycin

Kanamycin was purchased as a powder from Sigma and was stored at room temperature. It was reconstituted in Sterile Water for Injection (Astra) at 10mg/ml and filter sterilised through a 0.2μM filter. It was aliquoted and stored at -20°C.

Carbenicillin

Carbenicillin was purchased as a powder from Sigma and stored at -20°C. It was reconstituted in sterile water for injection at 100mg/ml and filter sterilised through a 0.2μM filter. It was aliquoted and stored at -20°C.

Tetracycline

Tetracycline was purchased as a powder from Sigma and stored at -20°C. It was reconstituted in ethanol at 5mg/ml and filter sterilised through a 0.2μM filter. It was aliquoted and stored at -20°C.

Ampicillin

Ampicillin was purchased as a powder from Sigma and stored at 4°C. It was reconstituted in sterile water for injection at 100mg/ml and filter sterilised through a 0.2μM filter. It was aliquoted and stored at -20°C.

Bacterial lines

The bacterial cell lines used for the cloning work were *Escherichia coli* XL1-blue (Stratagene; Bullock *et al.*, 1987; *rec* A1, *end* A1, *gyr* As6, *thi*, *hso* R17 (*rk*, *mk*+)*sup* E44, *rel* A1, λ-, *lac*-, [F', *pro* AB, *lacI*^{q}ZΔM15, Tn10(tet)]) and *Escherichia coli* HB2151 (Carter *et al.*, 1985; K12, *ara* D(*lac-pro* AB), *thi* [F' *pro* AB *lacI*^{q}ZΔM15]). Culture conditions for growth were 37°C in 2YT or SB media or on an LB plate with the appropriate antibiotics.

Electrocompetent Cells

To produce electrocompetent *E. coli* XL1-blue or HB2151 cells a single colony from a LB plate or approximately 10µl from a glycerol stock (-70°C) was inoculated into 10ml of SB/10µg/ml tetracycline and incubated overnight at 37°C with shaking. This culture was used to inoculate 1L of SB/10ml 2M glucose and incubated at 37°C for approximately 5hr with shaking until the OD_{600nm} = 1.0 (log phase).

The culture was chilled to 4°C for 30min and centrifuged at 3000rpm (1458g) in a Beckman JA14 for 20min at 4°C. The culture pellet was washed three times in decreasing volumes (500ml, 400ml and 200ml) of 10% glycerol, centrifuging in between at 3000rpm (1458g) in a Beckman JA14 for 20min at 4°C.

The culture was resuspended in 3ml 10% glycerol, aliquoted in volumes of 200µl and snap frozen in liquid nitrogen and stored at -70°C until use.

Transformation efficiency of the electrocompetent cells was determined by transforming them with 0.1ng pUC19 (Boehringer Mannheim) to determine the number transformed by 1µg of this plasmid.

Transformation into *E. coli*

A proportion of the ligation mix and control ligations were transformed into electrocompetent XL1-Blue or HB2151 *E. coli*. The transformation mixture containing the ligation mix, 200µl *E. coli* and water in a volume of 300µl was electroporated at 2.5kV in a *E. coli* Pulser Transformation Apparatus (Biorad). Directly following electroporation the cuvettes (Gene Pulser/*E.coli* Pulser Cuvette, 0.2cm electrode gap) were flushed with 3ml SOC and incubated with vigorous mixing for 1hr at 37°C. The cultures were diluted with 10ml SB and plated in 1, 10 and 100µl volumes on LB/100µg/ml carbenicillin plates and incubated at 37°C overnight. The 10ml culture was grown for 1hr at 37°C with mixing and then used to inoculate 100ml SB/50µg/ml carbenicillin and incubated at 37°C overnight with mixing.

Recovery of Plasmid DNA from Bacteria

Plasmid was recovered from bacteria by centrifuging the culture at 4000rpm (2600g) for 20min at 4°C. The plasmid was then extracted from the bacterial pellet using the Wizard *Plus* Midiprep Purification System (Promega).

2.2.3 *Tissue Culture Media, Solutions and Techniques*

Hams F12 Medium

Sachets of powdered Hams F12 medium (Life Technologies) were stored at 4°C. 1 sachet was resuspended in 1L milliQ water, 1.176g of sodium hydrogen carbonate was added and pH adjusted to 7.2 (for a final pH of 7.4 after filter sterilisation). The medium was filter sterilised using 0.2μM filter and stored at 4°C. Before use the medium was supplemented with 10% foetal bovine serum (FBS) and penicillin/streptomycin/glutamine(PSG) and stored at 4°C between use.

RF10 Medium

RPMI medium was purchased in liquid form from the Department of Clinical Immunology, Flinders Medical Centre, SA, Australia or from the Department of Paediatrics, Women's and Children's Hospital, SA, Australia. The medium was stored at 4°C until used. Before use the medium was supplemented with 10% FBS and PSG and stored as above.

Foetal Bovine Serum

Foetal Bovine Serum (FBS; Life Technologies) was stored at -20°C until needed and was then aliquoted into working volumes of 50ml. These aliquots were stored at -20°C and thawed immediately before use. FBS was either purchased heat inactivated or heat inactivated before aliquoting by incubating at 56°C for 30min.

Stock Penicillin - Streptomycin - Glutamine (PSG)

Penicillin-G was purchased from Sigma as Benzylpenicillin (potassium salt) and was stored at room temperature. Penicillin-G had an activity of 1600U/mg.

Streptomycin Sulphate was purchased from Sigma and stored at 4°C.

L-Glutamine was purchased from Sigma and stored at room temperature.

Stock Penicillin - Streptomycin - Glutamine (100X) was prepared by dissolving 3.125g of penicillin, 5g streptomycin and 14.615g of L-glutamine in 500ml of milliQ water. The solution was sterilised by filtration through a 0.2μM filter and was stored at -20°C in 5ml aliquots. The addition of 5ml of this solution to 500ml of cell culture growth medium resulted in final concentrations of 100U/ml penicillin, 100μg/ml streptomycin and 292.3μg/ml L-Glutamine.

Geneticin

Geneticin (Life Technologies) was purchased as a powder and stored at room temperature. Stock geneticin (500mg/ml) was prepared by dissolving 1g of geneticin in 2ml of water. The solution was sterilised by filtration through a 0.2μM filter and was stored at 4°C. The addition of 2.4μl of this solution to 1ml of cell culture media gave a working concentration of 1.2mg/ml. Geneticin is an aminoglycoside which is related to gentamicin. Geneticin, otherwise identified as G418 sulphate, is a potent inhibitor of protein synthesis (Jiminez and Davies, 1980). It is toxic to both prokaryotic and eukaryotic cells and is used as a selective agent in molecular genetics experiments.

Trypsin/EDTA

Trypsin/EDTA was purchased from Sigma in liquid form and stored at -20°C. The stock solution was 50mg/ml porcine trypsin and 20mg/ml EDTA in 0.9% NaCl solution. This was diluted 1/10 in sterile water. Trypsin/EDTA acts to break down the extracellular matrix formed by cells which adhere to the plastic surface of the flasks and therefore releases them from the surface.

Cell Line Maintenance

Cell lines were cultured in the appropriate medium and incubated at 37°C and 5% CO_2. Once the cell lines were confluent or approximately 10^6 cells/ml they were split into new flasks.

After washing the cells with PBS to remove residual serum, which inhibits the action of trypsin/EDTA, adherent cell monolayers were removed from the tissue culture flask by adding trypsin/EDTA to detach the cells from the plastic. When trypsinising cells, enough to cover the bottom of the flask was used. Typically 1ml for small ($25cm^2$) and 3ml for large ($75cm^2$) flasks (Nunc). The flasks were incubated at 37°C for 2min, because trypsin is active at 37°C, and were checked for detachment of the cells. Cells were resuspended in the appropriate medium and the cells split 1/10 into a flask of fresh medium.

Passaging the non-adherent cell lines involved splitting the cells 1/10 or 1/30 into a flask of fresh medium or removing 90% of the culture from the flask and replacing it with pre-warmed medium.

Transfection

LipofectAMINE (Life Technologies), a lipid based DNA carrier reagent, was stored at 4°C. Cells were grown to 50 - 70% confluence in $25cm^2$ tissue culture flasks. 17.5µl of LipofectAMINE and 5µg DNA was added to 1.5ml of serum free Hams F12 medium. The control for the transfection involved adding an equal volume of water instead of DNA. The DNA/LipofectAMINE mix was incubated for 45min at room temperature. Cells were washed twice in sterile PBS and once in serum free Hams F12. The cells were overlayed with the LipofectAMINE - DNA mixture and incubated at 37°C and 5% CO_2 for 5hr. 10ml of complete Hams F12 medium was added to the cells. If after 3 days the cells had grown they were split and geneticin was added to a final concentration of 1.2mg/ml for selection against untransfected cells.

Preparation of Cell Lysates

Cells were collected and centrifuged at 1500rpm (380g) for 10min. The cell pellet was resuspended in 1ml PBS/0.5% Nonidet P40 (NP40, BDH Chemicals). This was incubated on ice for 1hr and centrifuged at 13000rpm (17000g) for 10min at 4°C. The supernatant was collected and stored at -80°C.

2.2.4 *Molecular Biology Solutions and Techniques*

TBE (10X)

108g Tris Base (Research Organics)

55g Boric Acid (Merck)

9.3g EDTA

1L MilliQ water and autoclave

TAE (50X)

242g Tris Base

57.1ml Glacial Acetic Acid (AJAX Chemicals)

100ml 0.5M EDTA

pH 8.0

1L MilliQ water and autoclave

Agarose Gel Loading Buffer

10mg Bromophenol blue (0.1% final concentration; Sigma)

2ml Glycerol (BDH Chemicals)

Adjust to 10ml with dH_2O and boil for 30min

(ESB) Electrophoresis Sample Buffer (Reducing Buffer)

1.54g Tris Base

2g SDS (or use 20ml 10% SDS; BDH Chemicals)

20ml Glycerol

1.54g Dithiothreitol (DTT; Sigma)

Adjust to 100ml with water

Few mg of Bromophenol Blue as a dye

Store in aliquots at -20°C

Coomassie Blue Stain 0.5%

2.5g Coomassie Brilliant Blue G (Sigma)

500ml Methanol (Merck)

Destain

200ml Methanol

100ml Glacial Acetic Acid

700ml water

10X SDS-PAGE Running Buffer

30.275mg Tris

144.13mg Glycine

1L MilliQ water

1X SDS-PAGE Running Buffer

100ml 10X Running Buffer

900ml MilliQ water

10ml 10% SDS

Tonsil Material and Lymphocyte Isolation

Tonsil samples were collected from the operating theatres after routine tonsillectomies were carried out at the Women's and Children's Hospital (North Adelaide, South Australia).
Tonsil lymphocytes were isolated from tonsil tissue by mashing small pieces of the tissue through a metal sieve into RF10 media. The lymphocytes were separated from the other cells by spinning tonsil material on a ficoll gradient using Lymphoprep (Nycomed) at 2000rpm (715g) for 20min. The lymphocyte layer, identified as the white band between the lymphoprep and the RF10 media, was collected. Lymphocytes were washed in PBS and then spun at 2000rpm (715g) for 10min. The cells were washed again and then resuspended in 1ml PBS and counted using a haemocytometer. Aliquots containing 1 X 10^7 cells were frozen and stored at -80°C.

mRNA Extraction (Using RNAzol)

Cells were collected, centrifuged at 1500rpm (400g) for 5min and resuspended at 1 x 10^7 cells in 2ml RNAzol (Biotecx). The cells were incubated on ice for 5min and then 200µl chloroform was added and shaken vigorously for 15sec and incubated on ice for 10min. Tubes were centrifuged at 13000rpm (17000g) for 15min at 4°C. The upper (aqueous) layer was collected and an equal volume of isopropanol was added and incubated at -20°C overnight (or 1hr minimum). The tubes were centrifuged at 13000rpm (17000g) for 10min at 4°C. The pellets were washed in 1ml 75% ethanol and recentrifuged as above. mRNA pellets were air dried in a laminar flow cabinet and then resuspended in 25µl DEPC treated water. The mRNA was not left to dry completely as it would be difficult to reconstitute.

Generation of cDNA

The mRNA extracted from cells was used to generate cDNA. A reaction containing 10µg mRNA, 2µg OligodT (Promega) and water in a volume of 54µl was heated at 70°C for 10min and 4°C for 5min. To this was added 160U RNAsin (Promega), 5X RT buffer (Life Technologies), 1.2mM dNTPs (Promega), 0.1M DTT (Promega), water and 400U SUPERSCRIPT™ RNase H Reverse Transcriptase (Life Technologies) in a volume of 100µl. This was incubated at room temperature for 10min, 42°C for 50min, 90°C for 5min and 4°C for 10min. 2U RNase H (Life Technologies) was added and incubated at 37°C for 20min.

DNA Extraction

Cells were collected, centrifuged at 1500rpm (380g) for 5min and resuspended at 1 x 10^6 cells/ml and 10µl (1 x 10^4 cells) used for a DNA extraction using the Dynabeads DNA extraction kit (Dynal). DNA pellets were resuspended in 20µl TE buffer (Dynal) and stored at -20°C.

PCR

PCR reactions were carried out with 0.4µM or 50ng of each primer, 10X buffer (Promega), 2mM $MgCl_2$ (Promega), 0.2mM dNTPs, 0.5U Taq polymerase (Promega), 2µl template and water in a volume of 25µl. This was amplified with 35 cycles of 95°C for 15sec, 52°C for 50sec, 72°C for 1min 30sec and 1 cycle of 72°C for 10min in a FTS-960 Thermal Sequencer (Corbett Research). PCR products were analysed on an agarose gel.

Agarose Gel Electrophoresis

Agarose gels were used to analyse DNA from PCR or restriction digestion. The percentage agarose in the gel depended on the expected size of the product. Any product between 400 - 1000bp was analysed on a 2% agarose gel and 1000 - 6000bp was analysed on a 1% agarose gel. Normal agarose was purchased from Scientifix Scientific and stored at room temperature.

Low melting point agarose was purchased from FMC Bioproducts and stored at room temperature. Normal agarose gels were made up and run in 1/2X TBE buffer. Low melting point agarose gels were made up and run in 1X TAE buffer. Gels were photographed under ultraviolet light using the Imagemaster VDS (Pharmacia Biotech).

Sequencing

There were two sequencing methods carried out at different times during the project. The sequencing reactions were either carried out in the laboratory and then sent to the IMVS (Institute of Medical and Veterinary Science) Sequencing Centre (Adelaide, South Australia) for analysis or the primers and DNA were sent to Flinders Sequencing Centre (Bedford Park, South Australia) and the whole reaction carried out there.

Sequencing for the IMVS analysis was carried out using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The reactions were carried out using 8µl terminator ready reaction mix, 200 - 500ng double stranded plasmid DNA, 3.2pmol of primer and made up to a final volume of 20µl with water. As a control to determine that the sequencing reaction was working the control reaction from the Big Dye kit was used, plasmid pGEM?-3Zf(+) and the -21 M13 primer. The sequencing mixture was overlayed with mineral oil. The sequencing reaction was carried out in the DNA Thermal Cycler 480 (Perkin Elmer). The reaction consisted of 25 cycles of 96°C for 30sec, 50°C for 15sec and 60°C for 4min and then was held at 4°C until use. Sequencing products were purified using the ethanol precipitation steps described in the manual for the Big Dye kit which involved adding ethanol to the sequencing reaction mix to 60 +/- 3%, precipitating extension products at room temperature for at least 15min, centrifuging products at 13200rpm (17500g) for 20min and washing in 70% ethanol and drying products at 90°C for 1min. Sequencing was then sent to the IMVS sequencing centre for processing. Returned sequences were analysed using MacVector software.

Sequencing was also carried out at Flinders University of South Australia DNA Sequencing Core Facility. 0.3pM purified PCR product and 3.2pM/µl primers were sent for the sequencing reaction. Returned sequences were analysed using MacVector software.

DNA Quantitation

Knowing the amount of DNA present in a sample is vital for its use in other manipulations such as digestions and ligations. DNA measurements for this work was carried out initially using ethidium bromide agarose plates. This involved spot dropping 1 - 2µl onto the surface of an ethidium bromide plate and comparing the brightness of the sample spot to the DNA standards of 100, 50, 25, 12, 6 and 3µg/ml and making an estimation.

For more accurate measurements the GeneQuant RNA/DNA Calculator (Pharmacia Biotech) was used. Absorbance at OD_{260nm} and OD_{280nm} was determined and DNA concentration was calculated based on the equation $1OD_{260nm}$ = 50µg DNA/ml for double stranded DNA.

DNA Purification

DNA from PCR, extracted from gels or from digestions needed to be cleaned up before it was be used in other manipulations. Two methods were used for this: phenol chloroform extraction and DNA clean up kits (Promega and QIAGEN).

For PCR products the Wizard PCR preps purification system was used, for DNA other than PCR products the Wizard *Plus* DNA purification system was used and for 10 - 100ml plasmid preparations from bacteria the Wizard *Plus* Midiprep DNA purification system was used. QIAGEN Plasmid Mini Kits were also used for plasmid recovery from bacterial preparations.

DNA was also purified using phenol/chloroform extraction. This involved making the volume of the impure DNA solution to 100µl with water. 100µl phenol/chloroform was added, mixed and then centrifuged at 13200rpm (17500g) for 5min in a microfuge (MicroMax, IEC). The aqueous layer was collected and the DNA precipitated with 1/3 volume 7.5M ammonium acetate and 2.5 volumes 100% ethanol and incubated on ice for 30min. The DNA mixture was centrifuged at 13200rpm (17500g) for 15min. The DNA pellet was washed once with 1ml

70% ethanol and centrifuged at 13200rpm (17500g) for 15min. The DNA pellet was air dried and resuspended in water (usually 20µl).

SDS-Polyacrylamide Gel Electrophoresis

Protein samples were analysed using SDS - Polyacrylamide gel electrophoresis. The glass plates of the minigel were cleaned with ethanol and the gel apparatus assembled. The 11% resolving gel was made by mixing 2.8ml 40% Acrylamide/Bis Solution, 37.5:1 (Biorad), 4.4ml water, 2.7ml 1.5M Tris (pH 8.8), 100µl 10% SDS, 15µl 40% ammonium persulphate (Sigma), and 15µl TEMED (N,N,N',N'-tetracethylethylenediamine; Merck). The ammonium persulphate and TEMED both work to set the gel so the gel must be poured immediately after adding these reagents. Once the gel was poured it was overlayed with water and left until set. The 4% stacking gel was made by adding 1ml 40% bis acrylamide, 6.4ml water, 2.5ml 0.5M Tris (pH 6.7), 100µl 10% SDS, 15µl 40% ammonium persulphate, and 15µl TEMED. This gel was poured on top of the resolving gel. The samples and markers were added to ESB 1:1 and were boiled at 90°C for 5min. The gel was run in 1X running buffer at 200V until the gel front reached the bottom of the gel.

The gel was destained for 30min and stained with 0.1% Coomassie Brilliant Blue stain for approximately 2hr with shaking. The gel was then destained overnight with shaking. The gel was photographed under white light using the Imagemaster VDS.

2.2.5 *Protein Screening Assays and Solutions*

6HIS Buffer

50mM NaH_2PO_4

300mM NaCl

20mM Imidazole

pH 8.0

Elution Buffer for Phage Display Panning

1.6ml 12M HCl

150ml MilliQ water

Adjust to pH 2.2 with solid glycine

Adjust to 200ml with water

Check pH

ELISA Coating Buffer (Carbonate/Bicarbonate Buffer)

80ml 0.2M $NaCO_3$ (Merck)

170ml 0.2M $NaHCO_3$ (Merck)

pH adjusted to 9.6 by using one of the buffers

Make up to 1L with water and add 1ml of 20% NaN_3 (Sigma)

Store at 4°C

Alkaline Phosphatase Substrate Buffer (Diethanolamine Buffer)

48.5ml Diethanolamine (Sigma)

400ml water

pH adjusted to 9.8

Volume to 500ml with water

Add 0.5ml 0.5M $MgCl_2$

Store at 4°C - discard after 1 month

Western Blot Transfer Buffer 1X

100ml 10X SDS-PAGE Running Buffer

700ml MilliQ water

200ml Methanol

1X HBS Buffer

0.01M Hepes

0.15M NaCl

3.4mM EDTA

0.05% tween 20

pH 7.4

FACS Fix

1% Paraformaldehyde

PBS

pH 7.2 - 7.4

Keep in the dark at 4°C for 1 week

ELISA

ELISA polysorp strips (Nunc) were coated with 100µl 10µg/ml CD20 peptide (Chapter 3) in bicarbonate buffer and incubated at 4°C overnight. Plates were blocked with 100µl 3% BSA/PBS/0.05% tween 20 and incubated at 37°C for 1 hour with mixing. Plates were washed 3 times with PBS/0.05% tween 20. Plates were either stored at -20°C or used immediately. 100µl samples and controls were added to the wells and 1/3 serial dilutions made down the plate. PBS was used as a blank in well A1. Samples were incubated in the plates at 37°C for 2hr with shaking. Plates were washed 6 times with PBS/0.05% tween 20 and then incubated with either 100µl 1/1000 alkaline phosphatase conjugated anti-human IgG (Silenus) or 100µl 1/1000 alkaline phosphatase conjugated anti-mouse Ig (Silenus), depending on the primary antibody, for 2hr at 37°C with shaking. Plates were washed as above and then developed. The developing reagent was 1 p-nitrophenyl phosphate tablet (Sigma) in 5ml diethanolamine substrate buffer. 100µl of the alkaline phosphatase substrate buffer was added to each well of the plates and the optical density of the wells measured at 410nm at 5, 10, 20 and 40 minute time points on a Dynatech MR7000 plate reader (Dynatech).

Slot Blot Assay

The slot blot assay was used to determine production of soluble protein by transfected mammalian cells or transformed bacterial cells. Before blotting, 1ml of the supernatant was transferred to a 1.5ml eppendorf tube and centrifuged at 13200rpm (17500g) in a microfuge for 2min to remove any cells or cellular debris from the sample. Slot blot apparatus was set up with a thickness of 4 pieces of 3MM Chr chromatography paper (Whatman) wetted with PBS and a single nitrocellulose filter (Biorad) placed on top of this prewetted with PBS. Once the blotting apparatus had been set up, 100 - 200µl samples and controls were loaded onto the nitrocellulose using suction.

The nitrocellulose filter was blocked for 30min in 3% skim milk/PBS/0.05% tween 20 and then incubated with primary antibody. Dilutions of primary and secondary antibodies were usually carried out in 3% skim milk/PBS/0.05% tween 20. The primary antibody was incubated for 2hr at room temperature with mixing or overnight at 4°C. The secondary antibody, horseradish peroxidase (HRP) conjugated anti-mouse Ig (Amrad) or HRP conjugated anti-human Immunoglobulin (Silenus), diluted 1/1000 in 3% skim milk/PBS/0.05% tween 20 was added and incubated for 2hr at room temperature with mixing. In between incubations with antibodies the blots were washed with PBS/0.05% tween 20 with gentle mixing. The blot was developed using ECL western blotting detection reagents (RPN 2106; Amersham Pharmacia Biotech) by blotting the nitrocellulose dry and incubating it in 10ml of a 1:1 mixture of ECL reagents 1 and 2 for 1min. The nitrocellulose was again blotted dry and placed in a light-proof cassette and medical X-ray film (100NIF 18x24cm; Fuji Photo Film) exposed to it for 10sec, 1min and 5min. Films were developed in a KODAK RP X-OMAT Processor, Model M6B.

Western Blot

For a western blot the SDS-polyacrylamide gel was taken directly after electrophoresis and set up in a semi-dry western blotting apparatus (Pharmacia LKB Novablot Multiphor II, Pharmacia). 4 pieces of Whatman paper and the nitrocellulose were soaked in transfer buffer. The transfer was carried out at 100milliamps for 1.5hr. The blot was treated with 1ml 0.1% ponceau red/1% acetic acid and the markers were marked on the nitrocellulose with a ball point

pen so that they could be seen after the antibody treatments. The blot was blocked with 3% skim milk/PBS/0.05% tween 20 for 30min at room temperature. The nitrocellulose was incubated with the primary antibody followed by the secondary antibody. All incubations were 2hr at room temperature with mixing or overnight at 4°C. Between incubations with antibodies the blots were washed with PBS/0.05% tween 20. The nitrocellulose was developed in the same manner as for slot blot assays.

Flow Cytometry

Flow cytometry was used to detect expression of membrane bound proteins on cells or antibody binding to cell surface antigen. One method used was the high sensitivity immunofluorescence method (Zola *et al.*, 1990). Mammalian cells were counted and resuspended in PBS/0.01% azide at 1 X 10^7 cells/ml. 50µl cells were added to flow cytometry tubes and to each was added 50µl PBS or control antibodies or primary antibody. All incubations were for 30min on ice. In between the incubations were washes with PBS/0.01% azide and tubes were centrifuged at 1500rpm (450g) at 4°C for 5min. The cells were incubated with 50µl 1/50 biotinylated anti-mouse IgG (Vector) and then 50µl 1/50 SAPE (Sigma). The cells were analysed for fluorescence on the cell sorter (Coulter Elite) or preserved with 50µl FACS fix and kept at 4°C until analysis.

The other method involved the use of a directly conjugated secondary antibody such as FITC conjugated anti-mouse Ig (Silenus) instead of the biotinylated anti-mouse IgG and the SAPE (Sigma). Apart from this change the assays are the same The cells were analysed for fluorescence on the cell sorter or preserved with 50µl FACS fix and kept at 4°C until analysis.

The Coulter Elite flow cytometer has excitation at 488nm with an Argon ion laser (15mW). For analysis of samples "standard" filter sets were used. These were a 525 band pass for analysis of FITC labelled samples, 575 band pass for PE labelled samples and 610 band pass for CyChrome (PE/Cy5) labelled samples. Electronic Compensation was used to correct for spectral overlap.

2.2.6 *List of Suppliers*

Supplier	Location
AJAX Laboratory Chemicals	Sydney, NSW, Australia
Amersham Pharmacia Biotech	Sydney, NSW, Australia
Amicon	Danvers, MH, USA
Amrad	Melbourne, Vic, Australia
Astra Pharmaceuticals	Sydney, NSW, Australia
BAbCO	Richmond, California, USA
BDH Chemicals	As Merck
Beckman Instruments (Now Beckman Coulter)	Miami, Florida, USA
Becton Dickinson	San Jose, CA, USA
Biorad	Hercules, CA, USA
Biotecx	East Houston, TX, USA
Boehringer Mannheim (Now Roche Molecular Biochemicals)	Sydney, NSW, Australia
Bresatec (Now Geneworks)	Adelaide, SA, Australia
Caltag	San Fransisco, CA, USA
Clontech	Palo Alto, CA, USA
Corbett Research	Sydney, NSW, Australia
Coulter (Now Beckman Coulter)	Miami, Florida, USA
Dynal	Oslo, Norway
Dynatech	Chantilly, VA, USA
Flinders Medical Centre	Adelaide, SA, Australia
FMC Bioproducts	Rockland, ME, USA
Fuji	Dusseldorf, Germany
IEC	Needham Hts, MD, USA
Institute of Medical and Veterinary Science	Adelaide, SA, Australia
Intracel Corporation	Rockville, MD, USA

Kodak	Rochester, NY, USA
Life Technologies	Rockville, MD, USA
Merck	Poole, Dorset, England
New England Biolabs	Beverly, MA, USA
Nunc	Roskilde, Denmark
Nycomed	Little Chalfont, Buckinghamshire, UK
Oxoid	Basingstoke, Hampshire, England
PE Applied Biosystems	Foster City, CA, USA
Pharmingen	San Diego, CA, USA
Promega	Madison, WI, USA
QIAGEN	Hilden, Germany
Research Organics	Cleveland, Ohio, USA
Sartorius	Gottingen, Germany
Scientifix Scientific	Sydney, NSW, Australia
Sigma	St. Louis, Missouri, USA
Silenus	As for Amrad
Stratagene	La Jolla, CA, USA
Synpep Corporation	Dublin, CA, USA
Vector	Burlingame, CA, USA
Whatman	San Centre, Singapore

Chapter 3

Construction of a Transfected Cell Line Expressing Soluble Human CD19 Protein and Production of a Synthetic Peptide of Human CD20

Chapter 3

Construction of a Transfected Cell Line Expressing Soluble Human CD19 Protein and Production of a Synthetic Peptide of Human CD20

3.1 *Summary*

This chapter describes the characterisation of a synthetic peptide of the human CD20 cell surface antigen. Although only a small portion of the whole CD20 antigen, the extracellular region, anti-CD20 antibodies are shown to bind to the peptide in ELISA. Also described are the efforts made to produce a transfected cell line which would express recombinant soluble human CD19. Mammalian expression was used for the production of CD19 due to the cells' ability to synthesise glycosylated proteins. This system was preferred since it should yield native protein.

Two primer pairs were designed to amplify the CD19 extracellular region and two vectors were used for the expression of the CD19 protein. Two cell types were used for the transfection, CHO-K1 and COS-1. Although the CD19 insert could be PCR amplified from DNA extractions and cDNA generated from mRNA extractions from the transfected CHO-K1 and COS-1 cells, no detectable CD19 protein was expressed by the geneticin resistant transfected cells.

Another approach to produce recombinant CD19 protein involved fusing the CD19 extracellular region to the Fc domain of murine immunoglobulin. CHO-K1 cells transfected with the CD19MuIgG/pIRES1neo construct were shown to be producing mouse immunoglobulin. When the geneticin resistant transfected cells were grown in Hams F12/FBS with depleted IgG or serum free Hams F12 both CD19 and mouse Fc protein was detected by slot blot assay. Western blot analysis of the supernatants was inconclusive although mouse Ig was identified in the CD19MuIgG culture supernatant. PCR analysis of the cDNA of these cells showed the presence of both the CD19 and MuIgG fragments but the CD19MuIgG insert could not be amplified as a whole product.

3.2 *Introduction*

CD20 antigen is expressed only on B lymphocytes. This 33kDa protein is expressed from the early pre-B stage of B cell development and is lost upon differentiation into plasma cells (Stashenko *et al.*, 1980). CD20 makes a suitable target for antibody therapy as its expression is restricted to B cells.

CD19 is a 95kDa glycoprotein which is expressed on B lymphocytes from the late pro-B stage and is lost upon differentiation to plasma cells (Nadler *et al.*, 1983). As CD19 expression is exclusively restricted to B lymphocytes it is also an attractive target for therapy of B lymphocyte cancers.

Currently there are at least 4 scFvs (Haisma *et al.*, 1998; Jensen *et al.*, 1998; Schmidt *et al.*, 1999; Shan *et al.*, 1999) and a chimeric antibody (Reff *et al.*, 1994) described which bind CD20 antigen and at least 5 anti-CD19 scFvs (Bejcek *et al.*, 1995; Kipriyanov *et al.*, 1996; Nicholson *et al.*, 1997) and a chimeric CD19 antibody (Zola *et al.*, 1991) described. Production of soluble recombinant CD19 and CD20 antigen will allow further characterisation and improvement of these engineered antibodies.

CD19 is not expressed as a soluble molecule *in vivo*. At least until November 1999 expression of soluble human CD19 protein had not been described in the literature. However, Tedder and Isaacs (1989) have transfected COS cells with full length CD19 cDNA producing a transfected cell line which expresses membrane bound human CD19 antigen. Chapter 4 also describes the construction of a transfected cell line which expresses human CD19 as a membrane bound protein. CD19 is useful in this form for experiments which use whole cells or cell extracts, such as in flow cytometry, microscopy or cell bound ELISA. For other studies purified soluble CD19 is more desirable. The intended use for the recombinant soluble CD19 protein was for the screening of a phage display antibody library and more recently for use as the ligand in experiments to be carried out in a BIAcore biosensor.

Such difficulty was experienced in expressing recombinant soluble CD19 that other methods were investigated to assist in the expression of CD19 as a soluble protein. The Fc regions of immunoglobulin can secrete in the absence of light chains (Seligmann *et al.*, 1979). This observation has allowed construction, soluble expression and further characterisation of recombinant fusion proteins for the production of new therapeutic reagents. A soluble CD4

molecule which circulates in the body for extended periods of time could inhibit the binding of the HIV-1 virus to the CD4 cell surface molecule for initiation of infection. Fusion of human immunoglobulin IgG1 constant regions (CH1 - CH2 - CH3) to CD4 have extended the circulation time of the soluble CD4 protein and have incorporated in the recombinant protein the ability to recruit effector functions (Capon *et al.*, 1989). The use of other immunoglobulin classes for fusion to CD4 can increase the activity of the recombinant protein at least 1000-fold (Traunecker *et al.*, 1989). Fusion of the Fc region of immunoglobulin cell surface molecules has given a better insight into the function and interactions of many cell surface antigens including CD44 (Aruffo *et al.*, 1990), CD62 (Aruffo *et al.*, 1991), B7 (Linsley *et al.*, 1991a), CD28 (Linsley *et al.*, 1991a) and CTLA-4 (Linsley *et al.*, 1991b).

One of the major advantages of fusion of recombinant proteins to Fc regions is the extended circulation time of the protein (Capon *et al.*, 1989; Blazar *et al.*, 1994) but it has also been shown to stabilise the soluble expression of some proteins. Peppel *et al.* (1991) have described the expression of a fusion product between a murine Fc and Tumour Necrosis Factor (TNF) Receptor. Although TNF receptor, lacking transmembrane and cytoplasmic domains still have the ability to interact with TNF, the soluble form of TNF receptor is highly unstable. This group engineered a protein which was a combination of the TNF receptor and the CH2 and CH3 domains of murine IgG1 heavy chain resulting in a functional, stable, chimeric protein which had an extended circulation time. Adding the immunoglobulin region as a partner in a chimeric protein could be a useful strategy for the expression of molecules which are otherwise unstable after expression. This method was adapted for the production of soluble human CD19.

3.3 *Materials and Methods*

3.3.1 *CD20 Peptide*

The CD20 peptide was supplied by Intracel Corporation. The CD20 extracellular region was synthesised by SynPep Corporation. Figure 3.3.1 shows the region of the molecule which was synthetically produced. Amino acid sequence from aa140 -aa184 was used to synthesise the CD20 and biotin was fused to the sequence at aa140. The molecular weight of the synthetic peptide is 5504 Daltons.

3.3.2 *CD20 ELISA*

PBS (well A1) was used as a blank for reading the plate, FMC63 (IgG2a antibody which binds CD19; Zola *et al.*, 1991) and X63 (P3-X63-Ag8.653; Kearney *et al.*, 1979) were used as negative antibody controls and B-Cl (described in the Oxford Human Leucocyte Differentiation Antigen workshop; Ling *et al.*, 1987) was used as the positive control. The secondary antibody was 1/1000 alkaline phosphatase conjugated anti-mouse Ig (Silenus).

3.3.3 *CD19*

The pSP65 plasmid carrying the CD19 cDNA (figure 3.3.2) was supplied by Dr. Thomas F. Tedder (Duke University, NC; Tedder and Isaacs, 1989). The cDNA for the CD19 was cloned from a human tonsil cDNA library (Tedder and Isaacs, 1989). It was stored at -20°C while not in use. Plasmid was expanded by transforming 1µl of the CD19 vector into XL1-Blue cells and colonies were screened by PCR using CD19.SENSE and CD19.ANTI.NOT primers which are described in table 3.3.1. Colonies shown to be carrying the vector were grown in 100ml 2YT/50µg/ml ampicillin at 37°C overnight with shaking and the plasmid recovered using the Wizard *Plus* Midiprep DNA Purification System (Promega).

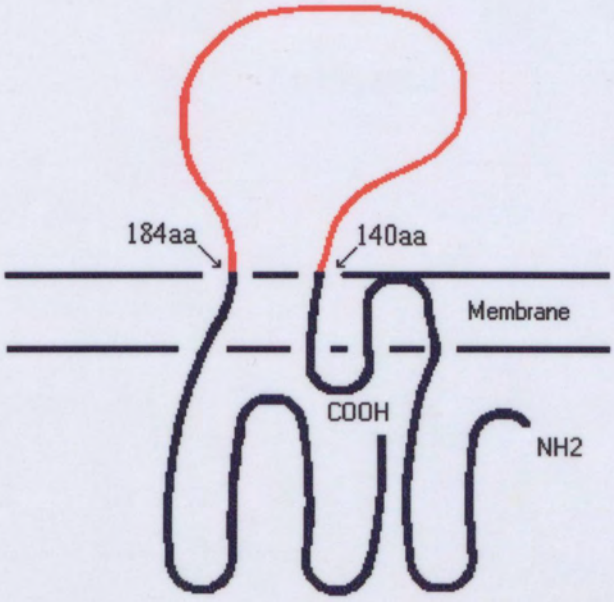


Figure 3.3.1: Region of Synthesised CD20 Peptide. Red section from aa140 to aa184 is the synthetically produced region.

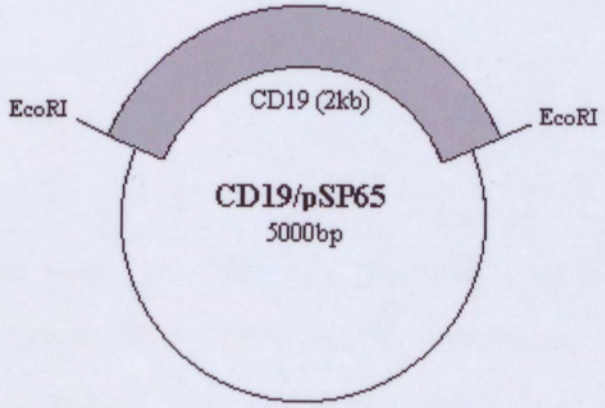


Figure 3.3.2: CD19/pSP65.

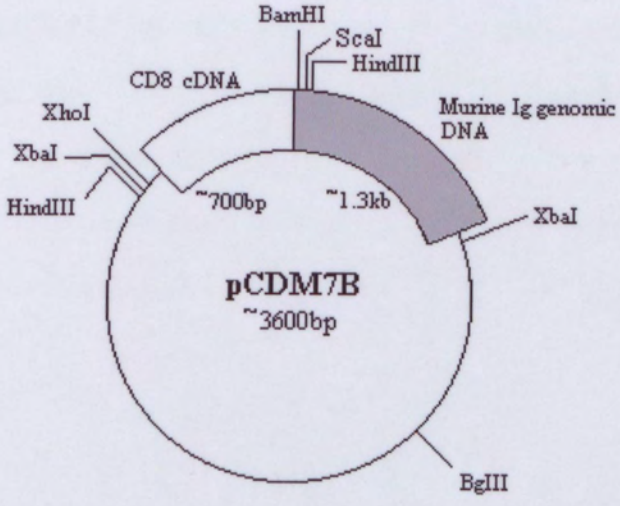


Figure 3.3.3: pCDM7B.

3.3.4 *Murine IgG Plasmid*

The CDM7B-Ig plasmid (figure 3.3.3) was kindly supplied by Dr. M. Sandrin, The Austin Research Institute, Victoria, Australia. It has a CD8 cDNA insert followed by Ig genomic DNA in the multiple cloning site.

3.3.5 *Expression Vectors*

The mammalian expression vectors were pBucHis, kindly provided by Assoc. Prof. David Gordon, Flinders Medical Centre, SA, and pIRES1neo (Clontech). pBucHis (figure 3.3.4) is based on a pBluescript vector and has ampicillin and neomycin resistance, origin of replication in bacteria and bacteriophage, SV40 origin of DNA replication, LacZ promoter, and cloning sites between a XbaI and XhoI site 5' of a 6 histidine tag.

pIRES1neo shown in figure 3.3.5 has a human cytomegalovirus immediate early promoter/enhancer followed by a multiple cloning site with ClaI, EcoR V, NotI, EcoRI, BamHI and BstXI sites. This site is followed by a synthetic intron (IVS) which has been reported to enhance the stability of the mRNA (Huang and Gorman, 1990). The vector was named for the Internal Ribosome Entry Site (IRES) from the encephalomyocarditis virus (ECMV) which allows the translation of two open reading frames from one mRNA which is this vector's main feature. pIRES1neo has a neomycin resistance gene which allows selection of transfected cells with geneticin (G-418 Sulphate).

3.3.6 *Primers*

PCR was carried out to amplify the extracellular region of CD19. The primers which amplify the extracellular region of CD19, CD19.SENSE, CD19.ANTI, CD19.ANTI.NOT, CD19.ECO.ANTI and CD19.ECO.SENSE, were designed to incorporate a restriction enzyme site into the PCR product.

The 3' CD19 primer, CD19-TCS, was modelled on the primer described by Peppel *et al.* (1991) and includes the 3' end of CD19 and the start of the thrombin cleavage site (TCS). The primers used for the amplification of the murine CH2 and CH3, MuIgG-TCS and MuIgG-

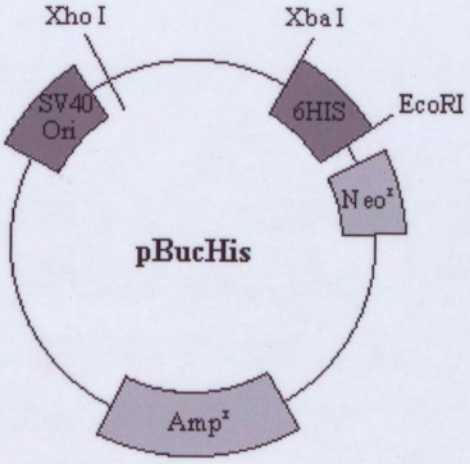


Figure 3.3.4: pBucHis.

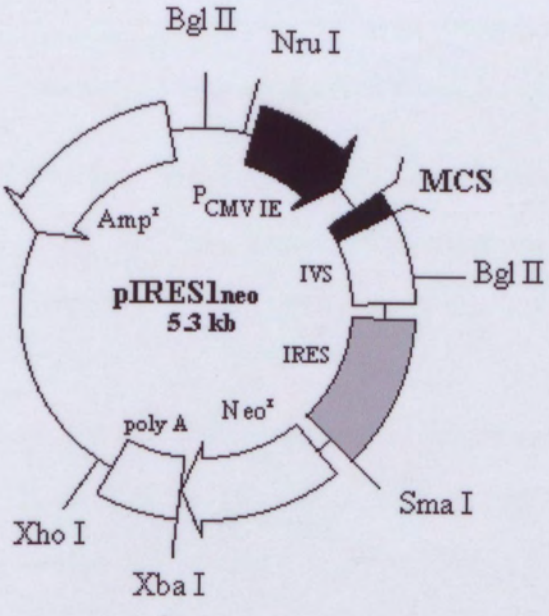


Figure 3.3.5: pIRES1neo.

ECO, were as described in Peppel *et al.* (1991), although an EcoRI site replaced the XbaI site in the 3' primer, MuIgG-ECO. Primer sequences can be seen in table 3.3.1.

pIRES5' was complementary to the region of pIRES1neo directly upstream from the multiple cloning site.

Primer Name	Primer Sequence 5' - 3'	Restriction Site or Unique Site
CD19.SENSE	ggctcgagcaaaaactgaccaccatgccacctcctcg	XhoI
CD19.ANTI	ggtctagacaaagtcacagctgagaccttcca	XbaI
CD19.ANTI.NOT	atgcggccgccttccagccaccagtcctcagcag	NotI
CD19.ECO.ANTI	gcgaattctcacttccagccaccagtcct	EcoRI
CD19.ECO.SENSE	cggaattcctctgaccaccatgccacctcctcgc	EcoRI
CD19ANTI-TCS	atccacgcggaaccagccagccaccagtcctcagc	TCS
MuIgG-TCS	tggttccgcgtggatccgtgcccagggattgtggt	TCS
MuIgG-ECO	atgcgaattctcatttaccaggagagtg	EcoRI
pIRES5'	ctcactatagggagaccc	

Table 3.3.1: Primers Used for the Amplification of the Extracellular Region of CD19, MuIgG and Screening of Clones. Figure 3.3.6 shows the CD19 sequence and the position of the primers in this sequence. TCS is the thrombin cleavage site.

The primers were purchased from Bresatec (now Geneworks) and reconstituted to a stock of 100X (2mM for all except the CD19.ECO.SENSE, CD19.ECO.ANTI, CD19-TCS, MuIgG-TCS and MuIgG-ECO (10X) which were reconstituted at 5μg/μl) using sterile water. Rehydrated primers were stored at -80°C. Stock primers were stored at -20°C. When the rehydrated primers were diluted to working stocks the concentrations of the primers were 20μM or 500ng/μl. The difference in the primer concentration was that the 20μM primer concentration was recommended for one PCR protocol and later the primers were used at 500ng/μl to conform to the CHRI laboratory protocol.

5' CD19 cDNA sequence

EcoRI Signal Peptide

1 10 20 30 40

gaattcCTCT GACCACC ATG CCA CCT CCT CGC CTC CTC TTC TTC CTC

50 60 70 80

CTC TTC CTC ACC CCC ATG GAA GTC AGG CCC GAG GAA CCT CTA

90

GTG.....

CD19.SENSE 9 - 31

CD19.ECO.SENSE 1 - 32

3' CD19 Extracellular Region cDNA sequence

855 860 870 880 890

.....TTA TGG CAC TGG CTG GTG AGG ACT GGT GGC TGG AAG GTC

Transmembrane Region

900 910 920

TCA GCT GTG ACT TTG GCT TAT CTG ATC TTC

CD19.ANTI 885 - 908

CD19.ANTI.NOT 873 - 890

CD19.ECO.ANTI 873 - 890

Figure 3.3.6: Primer Locations in CD19 Sequence. Partial sequences of the full length CD19 cDNA. Primer positions indicated under the sequences.

3.3.7 *Cell Lines and Maintenance*

The cell lines used for transfection were CHO-K1 (Chinese Hamster Ovary) and COS-1 (SV40 transformed kidney cells from an African green monkey). The CHO-K1 cells were maintained in Hams F12 medium. The COS-1 and Raji (Burkitts Lymphoma; Pulvertaft, 1964) were maintained in RF10 medium.

3.3.8 *Transfection*

CHO-K1 and COS-1 were both used for transfection. CHO-K1 were used to produce stable transfectants. This relied upon the transfected construct being integrated into the CHO-K1 genome. COS-1 were used for transient expression of the protein. Transfection of vectors containing the SV40 origin of DNA replication into these cells cause a massive proliferation of the vector and production of recombinant protein for a short period of time before cell death. Transfections were either carried out using LipofectAMINE (Chapter 2) or FuGENE6.

The FuGENE6 was purchased from Boehringer Mannheim and stored at -20°C. In a variation of the manufacturer's directions the cells were grown to 50 - 70% confluence. 6µl FuGENE6 was added to 2 eppendorf tubes and 30µl FuGENE6. All tubes were made to a volume of 100µl with Hams F12. This was incubated at room temperature for 5min. The 6µl FuGENE6:medium mix was added to 2µg CD19MuIgG and the water control. The higher concentration of the FuGENE6 was added to 5µg CD19MuIgG DNA and all tubes were incubated at room temperature for 15min. Medium was removed from the CHO-K1 cells and 2ml fresh Hams F12 medium added to 3 flasks. The FuGENE6/CD19MuIgG mix was added drop wise to the cells and incubated at 37°C and 5% CO_2 for 6hr and after this time 8ml fresh medium was added. After 2 days the transfected cells were split 1/10 and untransfected cells selected against using 1.2mg/ml geneticin.

3.3.9 *Slot Blot Assay*

Cell culture supernatants were collected before passaging cells and either blotted immediately or stored at -20°C. 200µl of samples were loaded onto the nitrocellulose filter. Positive controls for CD19, 1/10, 1/100 and 1/1000 of JVM13 cell extract, were loaded.

The primary antibody was FMC63, an anti-human CD19 monoclonal antibody, diluted 1/2 in Skim milk/PBS/0.05% tween 20. The secondary antibody was HRP conjugated anti-mouse Ig (Amrad), diluted 1/1000 in 3% skim milk/PBS/0.05% tween 20.

3.3.10 *cDNA Generation for Screening of Clones*

12.5µl mRNA was used to generate cDNA in a reaction containing 500ng OligodT in a volume of 54µl which was incubated at 70°C for 10min and 4°C for 5min. To this was added 40U RNasin (Promega), 5X reverse transcriptase buffer (Life Technologies), 1.2mM dNTPs (Promega), 10mM DTT, 20U reverse transcriptase and water in a volume of 100µl. This was incubated at room temperature for 10min, 42°C for 50min, 90°C for 5min and on ice for 10min. 2U RNase H was added and incubated at 37°C for 20min. cDNA was stored at 4°C until it was screened by PCR.

3.3.11 *PCR Screening of DNA and cDNA from mRNA Extractions*

DNA and cDNA generated from mRNA extractions were screened for the presence of the CD19 insert by PCR in a reaction volume of 25µl. CD19.SENSE and CD19.ANTI were used to screen for CD19 and as a positive control 2 primers for β-actin, β-actin sense and β-actin anti (table 3.3.2), were used to determine whether DNA and mRNA had been extracted from the cells.

Primer	Primer Sequence 5' - 3'
β-actin sense	tgacggggtcacccacactgtgcccatcta
β-actin anti	agtcatagtccgcctagaagcatttgcggt

Table 3.3.2: ß-Actin Primers. Primers used to screen DNA and cDNA.

3.4 *Methods and Results*

3.4.1 *Analysis of the CD20 Peptide*

The CD20 peptide was used to coat ELISA plates and an anti-CD20 antibody, B-Cl, bound the peptide in an ELISA (figure 3.4.1).

3.4.2 *Production of Recombinant Soluble CD19 Protein in a Mammalian Expression System (Reaction 1)*

CD19.SENSE and CD19.ANTI were used to amplify the extracellular region of CD19 in a reaction volume of 50µl. 2µg of CD19 product and pBucHis were digested in reactions containing DNA, XhoI (140U for CD19 and 20U for the plasmid), 10X buffer 2 (NEB) and water in a reaction volume of 50µl. These tubes were incubated at 37°C for 3hr. After this time XbaI (140U for CD19 and 20U for the plasmid), 10X buffer 2 (NEB), 100X BSA and water to a volume of 70µl was added. This reaction was incubated at 37°C for 3hr. XhoI/XbaI digested CD19 and pBucHis were ligated together in a molar ration of 2:1. The reaction contained 2µg CD19, 4.5µg pBucHis, 10U T4 DNA Ligase, 5X Ligase buffer (Life Technologies) and water to a volume of 100µl. The ligations were incubated at 16°C overnight. 20% of the ligation mix was electroporated into XL1-Blue *E. coli.* After electroporation the cuvettes were immediately flushed with 1ml SOC and incubated at 37°C for 1hr with vigorous mixing. This was then diluted with 10ml SB and 100, 10 and 1µl volumes were plated on LB/100µg/ml carbenicillin plates and incubated at 37°C overnight. 2.5ml SB/50µg/ml ampicillin aliquots were inoculated with 50 colonies from the plates and incubated at 37°C overnight with shaking. CD19.SENSE and CD19.ANTI primers were used to screen colonies for CD19 in a reaction containing 0.4µM of each primer, 10X buffer (with $MgCl_2$; Promega), 0.2mM dNTPs, 0.02U Taq Polymerase, 2µl of template and water to a reaction volume of 45µl. 8 colonies, CD19 100-6, CD19-6, CD19-13, CD19-16, CD19-18, CD19-21, CD19-30 and CD19-32, were positive. Plasmid was collected from all clones, except CD19-6 and CD19-16, by growing the colonies in 100ml SB/50µg/ml carbenicillin and incubating them at 37°C overnight with shaking. The plasmids were recovered using the Wizard *Plus* Midiprep

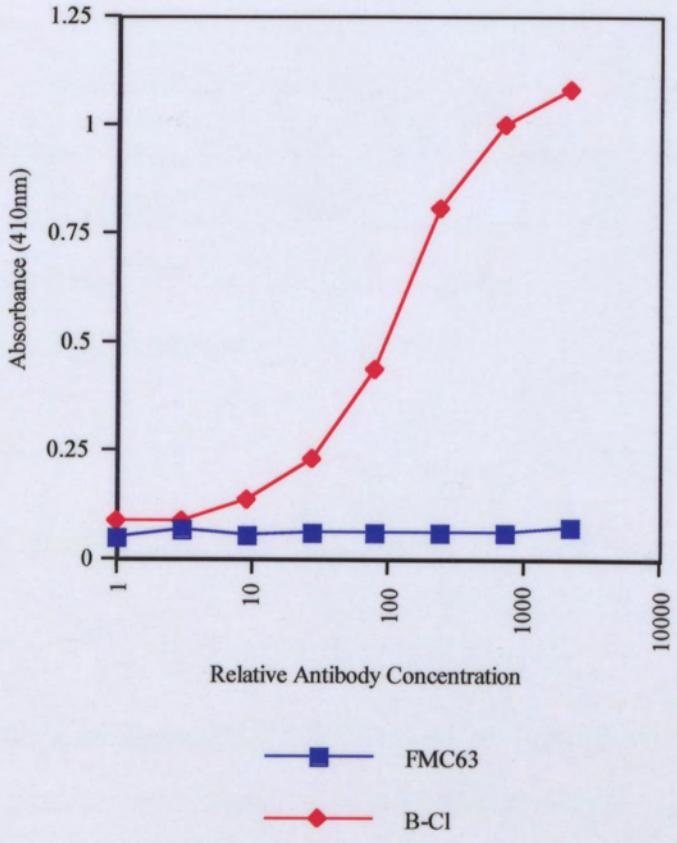


Figure 3.4.1: CD20 Monoclonal Antibody Binding CD20 Peptide. FMC63 is an anti-CD19 antibody and B-Cl is an anti-CD20 antibody.

DNA Purification System (Promega).

CD19 100-6 was transformed into CHO-K1 cells using LipofectAMINE. The supernatant from the transfected cells was screened using a slot blot assay. These results showed that the geneticin resistant CHO-K1 cells were not producing levels of CD19 protein which were detectable using this assay. Slot blot assays, using ECL, are sensitive to picogram amounts of protein but are dependant on optimisation of the protein being blotted and the antibodies used to detect it (ECL manual, Amersham Pharmacia Biotech).

Sequencing of the CD19 insert was carried out at Flinders University of South Australia DNA Sequencing Core Facility. 0.3pM purified CD19 PCR product and 3.2pM/µl primers were used for the sequencing reaction. The sequencing showed that the cloned soluble human CD19 was identical to the published sequence of the extracellular region of human CD19 (Tedder and Isaacs, 1989).

3.4.3 *Production of Recombinant Soluble CD19 Protein in a Mammalian Expression System (Reaction 2)*

The CD19 PCR product described above was religated into pBucHis and transformed into XL1-Blue *E. coli*. 40 colonies were screened (as described in section 3.4.2) for the presence of CD19 and 7, CD19-10, CD19-19, CD19-20, CD19-21, CD19-29, CD19-37 and CD19-38, were shown by PCR to be positive (figure 3.4.2). The plasmids were recovered and all clones were transfected into CHO-K1 cells. The cell culture supernatants were screened for production of CD19. The geneticin resistant cells were not producing CD19 protein at a level which was detectable by slot blot assay.

The CD19.ANTI primer was designed with an 18bp overlap in the transmembrane region of the CD19 gene. Although this overlap should not be extensive enough to anchor the CD19 protein in the cell membrane, lysates of the transfected cells were prepared to ensure this was not the case. Screening the lysates by slot blot showed no detectable amount of CD19 protein in the lysate preparation.

Usually constructs are transfected into COS cells for transient expression of protein before stable transfection is attempted. In this case one protocol being considered (Peppel *et al.*, 1991) described transfection into CHO-K1 cells for stable transfection. When this failed the

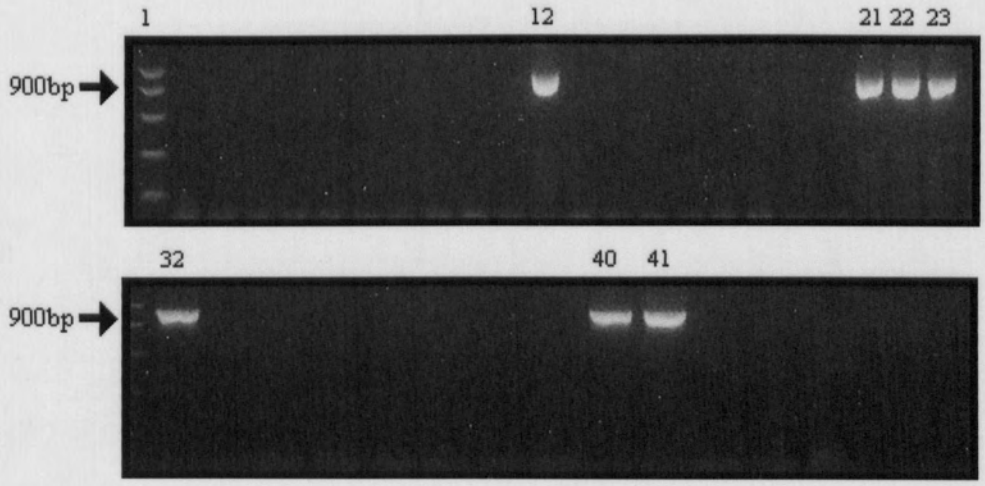


Figure 3.4.2: Screening Colonies Transformed with CD19. Lane 1 and 31 are 100bp DNA ladder.

CD19/pBucHis constructs were transfected into COS-1 cells using LipofectAMINE and were called COS-10, COS-19, COS-20, COS-21, COS-29, COS-37, COS-38 and COS-ve. The supernatants were screened after 24hr for the production of soluble CD19 protein. Assay of the supernatants using the slot blot technique showed no detectable expression of soluble CD19 protein.

DNA and mRNA was extracted from all the CHO-K1/CD19 and COS-1/CD19 clones to determine whether the CD19 construct had been successfully transfected into the cells. PCR was used for screening. The CD19 gene could be amplified from the cDNA (figure 3.4.3) and DNA of all soluble transfectants, CD19-10, 19, 20, 21, 29, 37 and 38, COS-10, 19, 20, 21, 29, 37 and 38.

3.4.4 *Production of Recombinant Soluble CD19 Protein in a Mammalian Expression System (Reaction 3)*

New primers, CD19.ECO.SENSE and CD19.ECO.ANTI, were designed for the amplification of the CD19 extracellular region only and none of the transmembrane region. This PCR was carried out in a volume of 25µl. The CD19 template was neat or diluted 1/10, 1/25 and 1/50. Water was the negative control template. 4µg CD19 PCR product and 5µg pIRES1neo were digested with 24U EcoRI, in 10X buffer H (Promega) and water in reaction volumes of 30µl for the CD19 digest and 20µl for the pIRES1neo digest. These digests were incubated at 37°C overnight. EcoRI digested CD19 and pIRES1neo were ligated together in a molar ratio of 6:1. The reaction contained 1µg CD19, 1µg pIRES1neo, 15U T4 DNA Ligase, 10X ligase buffer (Promega) and water in a reaction volume of 75µl. The ligation and the control which had no CD19 insert, just pIRES1neo plasmid, were incubated at 16°C overnight. 20% of the ligation mix and the ligation control were electroporated into XL1-Blue *E. coli* and the cuvettes flushed with 3ml SOC and incubated at 37°C for 1hr with vigorous mixing. This was diluted with 10ml SB and 100, 10 and 1µl volumes were plated on LB/100µg/ml carbenicillin plates and incubated at 37°C overnight. 1.5ml 2YT/50µg/ml ampicillin aliquots were inoculated with 24 colonies from the plates and incubated at 37°C overnight with shaking. These colonies were screened for the presence of the CD19 insert using CD19.ECO.SENSE and CD19.ECO.ANTI in a reaction volume of 25µl. Water was used as a negative control template and CD19 plasmid

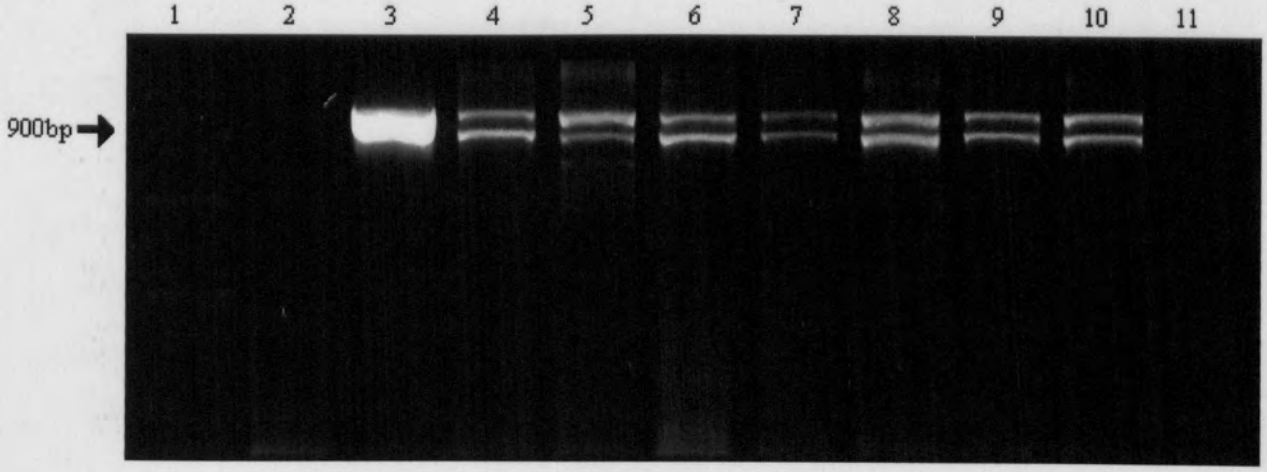


Figure 3.4.3a: PCR Screening cDNA from CD19 Transfected CHO Cells. Lane 1 is 100bp DNA ladder; Lanes 2 & 11 are water controls; Lane 3 is CD19 plasmid positive control; Lanes 4 – 10 are cDNA samples from CD19 transfected CHO cells. Lane 4 is CD19-10; Lane 5 is CD19-19; Lane 6 is CD19-20; Lane 7 is CD19-21; Lane 8 is CD19-29; Lane 9 is CD19-37; Lane 10 is CD19-38. Primers used were CD19.SENSE and CD19.ANTI.

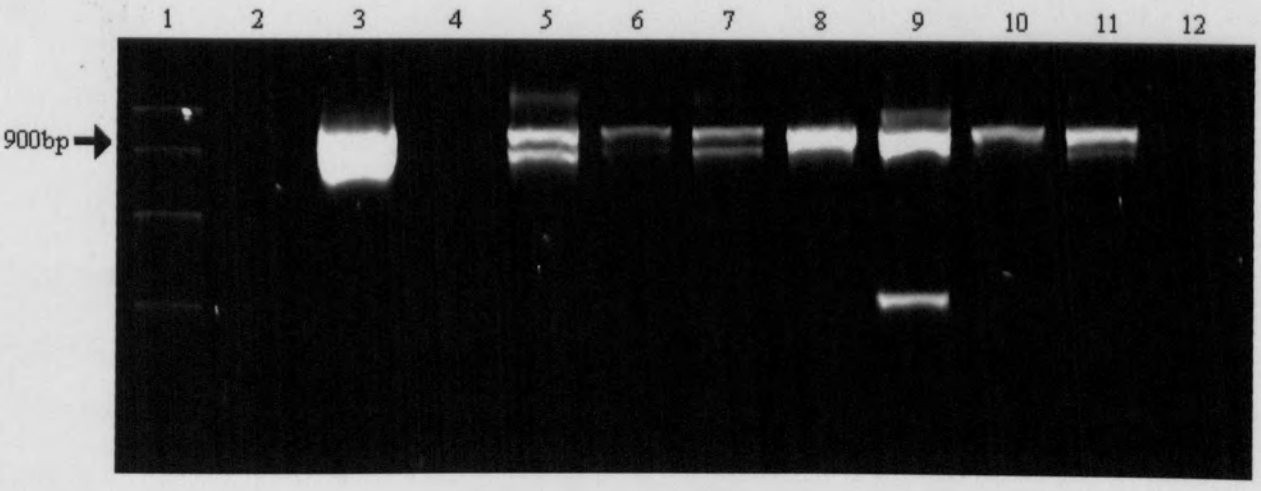


Figure 3.4.3b: PCR Screening cDNA from CD19 Transfected COS Cells. Lane 1 is 100bp DNA ladder; Lanes 2 & 12 are water controls; Lane 3 is CD19 plasmid positive control; Lane 4 is cDNA from untransfected COS cells; Lanes 5 – 11 are cDNA samples from CD19 transfected COS cells. Lane 5 is COS-10; Lane 6 is COS-19; Lane 7 is COS-20; Lane 8 is COS-21; Lane 9 is COS-29; Lane 10 is COS-37; Lane 11 is COS-38. Primers used were CD19.SENSE and CD19.ANTI.

as a positive control template. The 17 positive colonies were screened with primers pIRES5' (table 3.3.1) and CD19.ECO.ANTI to determine the orientation of the CD19 insert in the plasmid in a reaction as described above. 14 of the 17 positives had CD19 inserted into pIRES1neo in the correct orientation. Positive colonies were grown into 100ml SB/50µg/ml carbenicillin and incubated at 37°C overnight with shaking and the plasmids recovered from the bacteria using the Wizard *Plus* Midiprep DNA Purification System. 6 of the positives were chosen (CD19-S4, CD19-S5, CD19-S8, CD19-S17, CD19-S18 and CD19-S21) for transfection into CHO-K1 cells. Slot Blot analysis of the supernatants from the geneticin resistant transfected cells showed no detectable CD19 protein production.

To determine whether just a small proportion of the geneticin resistant cells were producing protein which wasn't being detected by the slot blot, the CD19-S8 and CD19-S17 cells were cloned at 100, 10 and 1 cell/well in 1ml volumes in a 24 well plate (Nunc). The cells plated at 100 cell/well for the CD19-S8 and CD19-S17 all grew and were analysed by slot blot. This showed no detectable soluble CD19 protein produced by these cultures.

Cell lysates of the 6 transfected cell lines were prepared and screened for the expression of the CD19 protein. Detectable levels of CD19 were not being produced by the transfectants (figure 3.4.4).

3.4.5 *Production of Recombinant Soluble CD19 Protein Fused to a Murine Fc Domain*

A different approach to the production of soluble CD19 was taken. Primers were designed to produce a CD19-murine CH2-CH3 IgG fusion protein. CD19.ECO.SENSE and CD19-TCS amplified the CD19 extracellular region and MuIgG-TCS and MuIgG-ECO amplified the murine CH2-CH3 domains in reaction volumes of 25µl. The CD19 plasmid and the murine CH2-CH3 plasmid was diluted 1/10, 1/25 and 1/50. Water was used as a negative control. CD19.ECO.SENSE and MuIgG-ECO were used for the splice overlap extension (SOE) of the CD19 and the MuIgG PCR products in a reaction containing 1µg each of CD19-TCS and MuIgG-TCS or 200ng each of CD19-TCS and MuIgG-TCS. The reactions also contained 5X eLONGase buffer B (Life Technologies), 0.8mM dNTPs, 2U eLONGase (Life Technologies) and water in a volume of 49.6µl. This mix was incubated in a reaction of 92°C for 3min and 2 cycles of 92°C for 1min, 63°C for 30sec, 58°C for 50sec, and 72°C for 1min. 100ng of the

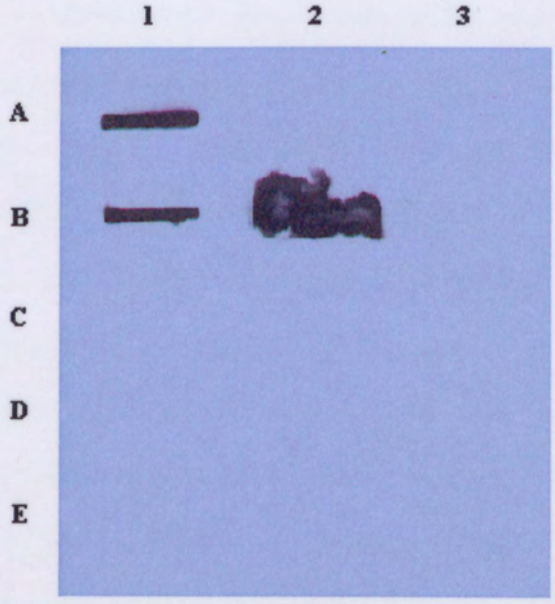


Figure 3.4.4: Slot Blot Screen of Cell Lysates. Slot A1 is 1/10 JVM13 cell lysate; Slot A2 is 1/100 JVM13 cell lysate; Slot B2 is Raji cell lysate; Other slots are cell culture supernatant samples. Nitrocellulose was incubated with ½ FMC63 supernatant and 1/1000 HRP conjugated anti-mouse Ig.

CD19.ECO.SENSE and the MuIgG-ECO was added to each reaction and incubated for 5 cycles of 92°C for 1min, 63°C for 30sec, 58°C for 50sec, 72°C for 1min and 25 cycles of 92°C for 1min, 63°C for 30sec, 72°C for 1min. Water was used as a negative control template. The CD19MuIgG SOE product and pIRES1neo vector were digested with EcoRI (24U for pIRES1neo and 48U EcoRI for CD19MuIgG) in reactions containing 2μg of pIRES1neo and 4μg of CD19MuIgG, 10X buffer H (Promega) and water in volumes of 20μl for pIRES1neo and 30μl for CD19MuIgG and incubating at 37°C overnight. The digested pIRES1neo was alkaline phosphatase treated in a reaction containing the digestion mix, 10X alkaline phosphatase buffer, 3U alkaline phosphatase and water in a volume of 30μl and incubated at 37°C for 30min. The reaction was stopped by adding 10mM EDTA and incubating at 65°C for 10min. 2μg CD19MuIgG insert was ligated into 1.8μg pIRES1neo in a molar ratio of 4:1. The reactions contained the CD19MuIgG and the pIRES1neo, 10X ligase buffer (Promega), 9U T4 DNA Ligase and water in a volume of 70μl. The negative control ligation contained no CD19MuIgG insert. This was incubated at room temperature for 1hr and then 4°C for 2 nights. 1/7 of the ligation mixes were transformed into HB2151 *E. coli*. The reaction contained the ligation mix and 100μl electrocompetent HB2151 *E. coli* in a volume of 110μl. The control was the transformation of 1μg pIRES1neo plasmid. After electroporation the cuvettes were flushed with 1ml 2YT and incubated at 37°C for 1hr with vigorous shaking. The bacteria were centrifuged at 13200rpm (17532g) for 2min and the pellet resuspended in 100μl 2YT and plated on LB/100μg/ml carbenicillin plates. The plates were incubated at 37°C overnight. 40 colonies from the transformation plates were grown in 5ml 2YT/1% glucose/20μg/ml carbenicillin at 37°C overnight with vigorous shaking.

These colonies were PCR screened for the CD19MuIgG construct using CD19.ECO.SENSE and MuIgG-ECO. One colony was positive. To determine that the insert was in the correct orientation the colony was rescreened by PCR using the pIRES5' and MuIgG-ECO primers. The colony was shown to have the construct with the insert in the right way. This colony was grown in 100ml 2YT/1% glucose/50μg/ml carbenicillin at 37°C with shaking overnight. The CD19MuIgG construct was transfected into CHO-K1 cells using FuGENE6. The geneticin resistant transfected cell culture supernatants were screened for production of soluble CD19MuIgG by slot blot. The positive controls for Fc was hybridoma supernatant from X63, an IgG1 murine myeloma protein with no known reactivity with human cells, and for CD19

was FMC63 supernatant and a detergent lysate of the JVM13 cell line. Supernatants screened with 1/1000 HRP conjugated anti-mouse Ig (Amrad) in a slot blot showed that the cells were expressing mouse protein. Screening the supernatants with 1/100 biotinylated anti-human CD19 (Pharmingen) followed by 1/5000 avidin peroxidase conjugated (Sigma) showed no detectable CD19 protein.

The geneticin resistant CD19MuIgG transfected culture was cloned at 0.5, 1 and 2 cells/well in 200µl in a 96 well plate. Two clones, CD19MuIgG 18 and CD19MuIgG 48, were identified as producing mouse protein using the slot blot assay (figure 3.4.5).

The effect of depletions of IgG from FBS and FBS from the medium the transfectant was grown in was investigated. FBS can contain significant amounts of bovine immunoglobulin. This is a problem because of cross reactivity of anti-mouse Ig antibodies with bovine Ig. FBS was run through a GammaBind G Sepharose column (Pharmacia Biotech) to remove excess bovine Ig. The transfectant was grown in medium supplemented with this FBS and also medium which had no FBS added to it for approximately 2 weeks with no change of media. The supernatants from these cells were positive for both mouse protein and CD19 protein by slot blot assay (figure 3.4.6).

The protein in the supernatants was concentrated for SDS-PAGE and western blot analysis. 10ml CD19MuIgG supernatant from cultures grown in Hams F12/FBS with depleted IgG or serum free Hams F12 were incubated with 200µl GammaBind G Sepharose beads for 30min at room temperature with mixing or 4°C overnight. The beads were centrifuged at 13200rpm (17532g) for 2min in a microfuge in 1ml aliquots, washed with 1ml PBS, resuspended in 50 - 200µl ESB and boiled for 5min at 95°C. The tubes were centrifuged at 13200rpm (17532g) for 2min in a microfuge and the supernatant loaded on a SDS-PAGE. The resolving gel was 11% (chapter 2) and the stacking gel was 4% (chapter 2). The samples, including the markers, cell culture supernatant and control hybridoma supernatants and JVM13 extracts, were treated by adding ESB buffer 1:1 and boiling them at 90°C for 5min.

For the western blot the primary antibody was 1/1000 anti-mouse HRP or 1/100 biotinylated anti-human CD19 followed by 1/5000 avidin peroxidase. After western blotting the nitrocellulose filter was treated with 4-chloro-1-napthol. One 30mg tablet of 4-chloro-1-napthol (Sigma) was dissolved in 1ml methanol. This was diluted to 0.5mg/ml in a solution of 0.05M Tris/0.15M NaCl pH 7.4 and 5µl 30% H_2O_2. The nitrocellulose was incubated in this solution

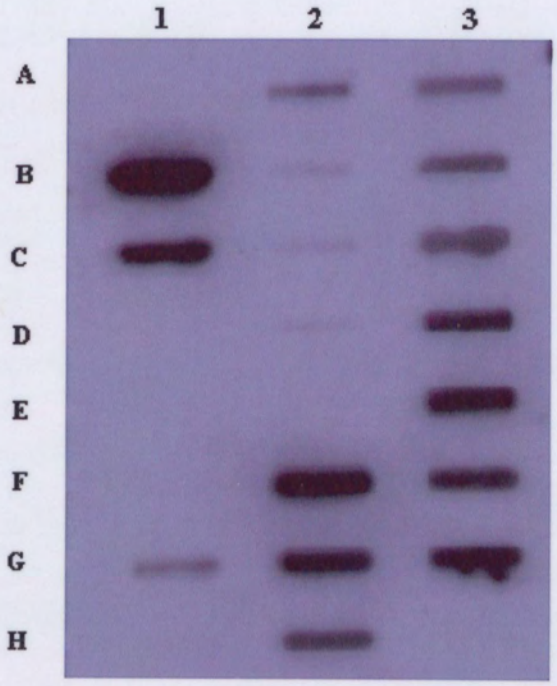


Figure 3.4.5: Slot Blot Screen of CD19MuIgG Clones for Protein Production. Slots B1 and C1 are murine monoclonal antibodies; Slots D1 – G3 are supernatant samples from the transfected CHO culture. The nitrocellulose filter was incubated with 1/1000 HRP conjugated anti-mouse Ig.

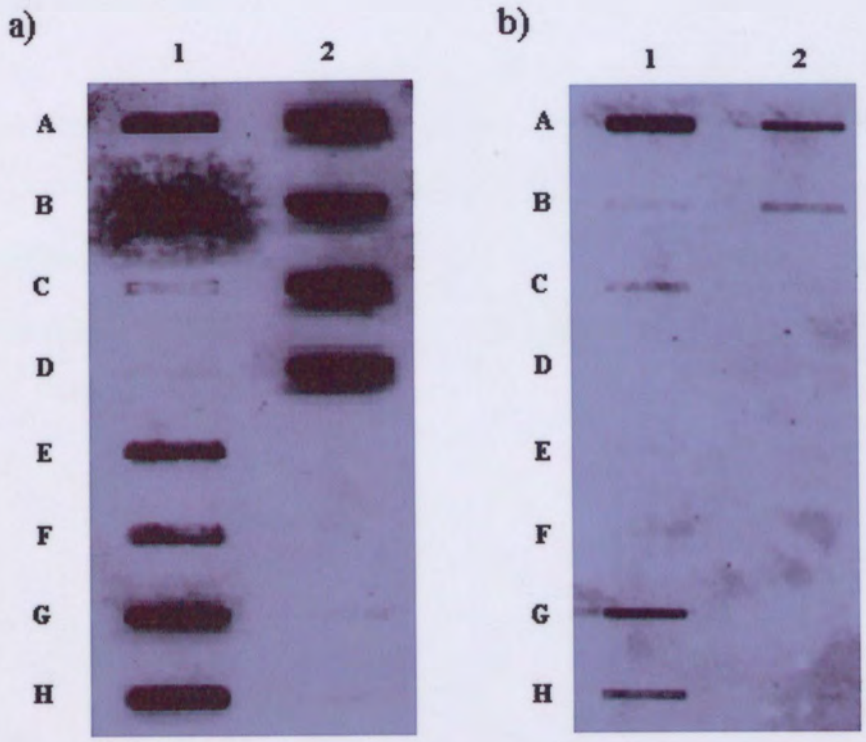


Figure 3.4.6: CD19MuIgG Clones Grown in Modified Media. Slot A1 is 1/10 JVM13 cell lysate; B1 is X63 supernatant; D1 is untransfected CHO cell supernatant; E1 is CD19MuIgG clone 18 grown in medium with FBS; F1 is CD19MuIgG clone 48 grown in medium with FBS; G1 is CD19MuIgG clone 18 grown in medium with FBS with low IgG; H1 is CD19MuIgG clone 48 grown in medium with FBS with low IgG; A2 is CD19MuIgG clone 18 grown in serum free medium; B2 is CD19MuIgG clone 48 grown in serum free medium. The nitrocellulose in a) was incubated with 1/1000 HRP conjugated anti-mouse Ig and nitrocellulose in b) was incubated with 1/100 biotinylated anti-human CD19 (Becton Dickinson) and 1/5000 HRP conjugated avidin.

for 30min at room temperature. The blot was washed with water. The expected size of the fusion protein was approximately 50kDa. The results from the western blot were inconclusive but one blot treated with 4-chloro-1-napthol substrate showed the production of mouse protein by the transfectants, CD19MuIgG 18 and 48 (figure 3.4.7). The size of these bands was ~50kDa.

mRNA was extracted from the CD19MuIgG 18 and 48 cells and CD19.ECO.SENSE, CD19-TCS, MuIgG-TCS and MuIgG-ECO used to screen the cDNA in a reaction volume of 25µl. The PCR screen of the clones showed the presence of the CD19 portion of the insert and the MuIgG portion of the insert separately but the whole fusion product could not be amplified as a single product (figure 3.4.8).

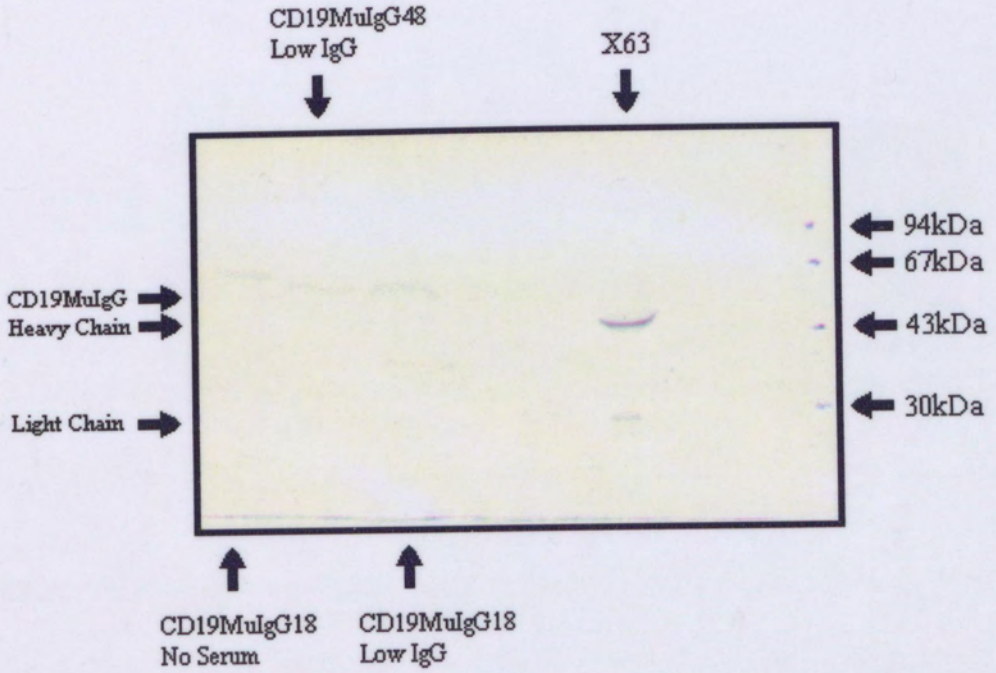


Figure 3.4.7: CD19MuIgG Western Blot with 4-Chloro-1-Napthol.

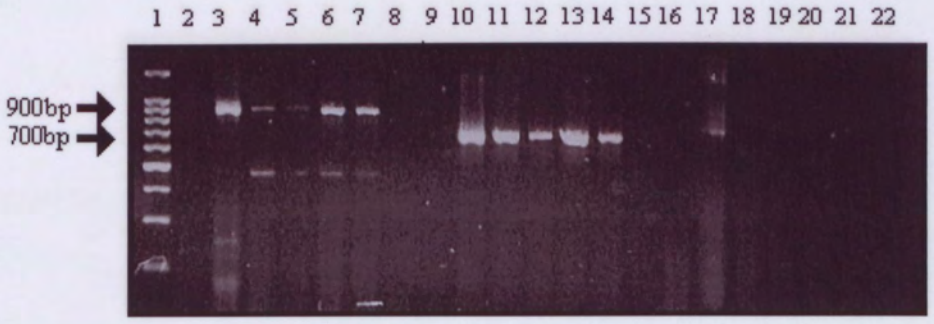


Figure 3.4.8: PCR Screening CD19MuIgG Clones. Lane 1 is the 100bp marker; Lanes 2, 8, 9, 15, 16 & 22 are water controls, Lanes 3, 10 & 17 are CD19MuIgG SOE product positive control; Lanes 4, 5, 11, 12, 18, & 19 are CD19MuIgG clone 18, Lanes 6, 7, 13, 14, 20 & 21 are CD19MuIgG clone 48; Lanes 2 – 8 are amplified with CD19EcoSense & CD19TCS; Lanes 9 – 15 are amplified with MuIgGTCS & MuIgGEco; Lanes 16 – 22 are amplified with CD19EcoSense and MuIgGEco.

3.5 *Discussion*

A synthetic 45aa CD20 peptide was bound by CD20 antibodies in ELISA. This peptide has since been used for ELISA and BIAcore (chapter 7) and in other screening assays by others in the Institute.

Several alternative methods were tried in an attempt to produce recombinant soluble human CD19. A series of primer pairs, vectors and cell lines were used and mammalian expression chosen as it should produce native human CD19.

CD19 is a molecule with an extensive extracellular region. The amplification of this region with CD19.SENSE and CD19.ANTI from reaction 1 incorporated 18bp of the transmembrane region into the recombinant protein. Although the reason the CD19 protein was not expressed in either attempt at the mammalian expression system has not been ascertained it was originally thought that the first attempt failed because the primers were designed poorly and that the molecule may have been remaining in the cell. Further testing with other primer pairs indicated that soluble expression of the protein was not inhibited by the incorporation of the small part of the transmembrane region.

The reason for the absence of soluble CD19 protein could also be explained if only a small number of cells had been transfected and were not producing large enough amounts of protein to be detected in a slot blot assay. To determine efficiency of the transfection green fluorescent protein (GFP) could have been co-transfected with the CD19 construct. Transfection of a GFP construct into mammalian cells causes the cells to fluoresce green light (Yang *et al.*, 1996). This provides an easy detection system for transfected cells. Alternatives to transfection with lipid based reagents are calcium phosphate-DNA precipitation, DEAE dextran and more recently electroporation. Electroporation, which involves exposing the cells to a pulsed electric field which creates pores in the cytoplasmic membrane, has been used successfully for a wide range of cell types in systems which have poor transformation efficiencies (Schwachtgen *et al.*, 1994).

There could be structural reasons why the extracellular region of CD19 would not express solubly. The primers were designed at the 5' end of the published CD19 sequence (Tedder and Isaacs, 1989), which has a hydrophobic signal peptide which is cleaved in the mature protein.

It could be that the protein is remaining inside the cell. This does not seem likely since slot blot of the cell lysates did not detect recombinant CD19 protein.

The approach which fused the CD19 extracellular region to a murine IgG Fc domain showed the most potential. Screening supernatants from geneticin resistant transfected cells showed expression of mouse immunoglobulin. When the cells were grown with modified growth medium human CD19 protein was also detected. The CD19 protein could not be isolated. mRNA extraction and PCR of the cDNA showed the CD19 and the MuIgG inserts to be present, but the whole CD19MuIgG insert was not detected. It is possible that the CD19MuIgG insert was digested during integration into the CHO-K1 genome.

As an alternative to mammalian expression there are a vast number of expression systems now available which can be used for the production of recombinant proteins. Baculovirus vectors (Possee, 1997) are commonly used in insect cells for this purpose but restrictions such as incubation at 30°C, different glycosylation and the discontinuous expression due to the death of the insect host and the need to reinfect the cells for each round of protein synthesis discouraged the use of this system for the production of CD19. However, other cell surface molecules, including BST-1 (Bone Marrow Stromal Cell Antigen-1; Sato *et al.*, 1999), CD45 (Cromlish *et al.*, 1999) and CD40 ligand (Mullins *et al.*, 1998), have been expressed in this system. Bacterial expression is one of the most frequently used systems but lack of glycosylation by bacterial cells is a disadvantage in the production of mammalian proteins. Other systems described for recombinant proteins include production using methylotropic yeast cells such as *H. polymorpha* or *P. pastoris* (Hollenberg and Gellissen, 1997) and viruses, such as poxvirus (Carroll and Moss, 1997) and alphavirus (Lundstrom, 1997), for mammalian expression.

If future work with the CD19MuIgG construct does not yield any positive results other methods of soluble expression of the CD19 protein should be considered. Okada *et al.* (1997) describe a method to replace the transmembrane domains of TCR genes with sequences for phosphatidylinositol-linked surface expression. Once expressed by transfected cells the recombinant TCR was cleaved from the surface of the cells with phospholipase and purified.

CD19 has a very large extracellular region. A different approach for the production of CD19 could include identification of regions of the human CD19 that do not show homology to murine CD19 and synthesis of short peptide sequences of these regions. This would be useful

for screening antibodies or antibody fragments from murine origin in ELISA or BIAcore. However, this method limits the search for antibodies which bind different epitopes of CD19. Although for some applications soluble CD19 protein is ideal, there are many other situations in which membrane bound protein can be just as useful. This is described in more detail in chapter 4.

Chapter 4

Production of a Transfected Cell Line Expressing Membrane Bound Human CD19

Chapter 4

Production of a Transfected Cell Line Expressing Membrane Bound Human CD19

4.1 *Summary*

This chapter describes the production of a transfected cell line which expresses human CD19 as a membrane bound protein. The method of expression involved transformation of a full length CD19 cDNA construct into *E. coli* XL1-blue and subsequent expression in Chinese Hamster Ovary (CHO-K1) cells.

CHO-K1 cells were screened for production of membrane bound CD19 by flow cytometry. FMC63, an anti human CD19 monoclonal antibody, was used to identify cells expressing CD19. A stable cell line was produced which expresses human CD19.

4.2 *Introduction*

CD19 is a molecule found predominantly on the surface of B lymphocytes. This makes it a suitable target for B lymphocyte disease therapy. Antibodies which bind CD19 have been engineered (chapter 3) for use in therapeutic situations. Thorough characterisation of these antibodies is necessary before they can be approved for clinical use and part of the primary analysis of the reagents is testing the specificity of the antibody for its target. A cell line transfected with full length CD19 cDNA would be a very useful reagent to investigate binding of new or mutated antibodies to CD19.

This chapter describes the construction of a transfected cell line which expresses human CD19 as a membrane bound protein. This is not the first description of a human CD19 transfected cell line. In 1989 Tedder and Isaacs isolated the cDNA for CD19. After transfection with the CD19 construct, a large proportion of COS cells expressed a cell surface antigen which was bound by anti-CD19 antibodies. NIH3T3 cells have also been transfected with a construct containing CD19 cDNA. These cells were used for analysis of the anti-CD19 antibodies submitted to the Human Leucocyte Differentiation Antigen workshop (Zhou and Tedder, 1995a).

In this chapter the CD19 transfected CHO-K1 cells were used to confirm that FMC63 is an anti-human CD19 monoclonal antibody. Although it is widely recognised in many laboratories as an anti-CD19 antibody some doubt is still being voiced about FMC63 specificity. This stems from the absence of FMC63, until recently, from the Human Leucocyte Differentiation Antigen workshop for classification.

A CD19 transfected cell line could play a part in many important studies. The transfected cell line has been used in screening samples of blood from patients with diseases such as Chronic Lymphocytic Leukemia, Myeloma, Non Hodgkin's Lymphoma and Sjogren's Syndrome for autoantibodies against CD19 (chapter 5). Site directed mutagenesis was performed on an anti-CD19 single chain Fv in an attempt to improve the affinity of the antibody. The mutated antibody was tested for binding to the CD19 transfected cell (Hohmann *et al.*, 1999). Studies comparing the scFv against CD19, produced from the FMC63 hybridoma (Nicholson *et al.*, 1997), with the mutants derived from it could be carried out with the transfected cell line in flow cytometry.

This chapter describes the construction and expression of a CD19 transfected cell line which has the potential for use in a number of applications.

4.3 *Materials and Methods*

4.3.1 *Cell Lines and Maintenance*

HRIK (B Lymphoblastoid Cell Line; Minowada *et al.*, 1978) cells were cultured in RF10 medium. The cells were maintained as described in chapter 2.

4.3.2 *Digestion of the CD19 Gene*

The CD19 cDNA was excised from the pSP65 (figure 3.3.2) plasmid by digesting with EcoRI. pIRES1neo was also cut with EcoRI in preparation for cloning. The digestion reaction contained 8µg of the CD19 vector or 2.5µg of pIRES1neo, 12U of EcoRI, 10X buffer H (Promega) and water in a volume of 40µl for CD19 and 20µl for pIRES1neo. Digests were incubated at 37°C overnight. The pIRES1neo digest was treated with 3U calf intestinal alkaline phosphatase in 10X alkaline phosphatase buffer at 37°C for 30min. This reaction was stopped with 10mM EDTA and incubation at 65°C for 10min.

4.3.3 *Ligation of CD19 into pIRES1neo*

CD19 was ligated into pIRES1neo in a reaction containing 0.5µg EcoRI-digested CD19 and 1.2µg EcoRI-digested pIRES1neo in a molar ratio of 6:1. The reaction contained the EcoRI-digested insert and vector with 3U T4 Ligase, 10X ligase buffer (Life Technologies) and water in a volume of 50µl. This reaction was incubated at 16°C overnight. 20% of the ligation mixture was transformed into electrocompetent *E. coli* XL1-Blue. 100µl of the 3ml SOC electroporation mixture was plated on LB/100µg/ml ampicillin plates and incubated at 37°C overnight.

4.3.4 *Screening Colonies for CD19*

Colonies were grown in 200µl SB/50µg/ml ampicillin in 96 well plates at 37°C overnight. CD19.SENSE and CD19.ANTI.NOT (table 3.3.1) were used to screen the colonies for the pIRES1neo/CD19 construct in a PCR reaction containing 2µl of culture templates. The positive colonies were screened again with pIRES5' (table 3.3.1) and CD19.ANTI.NOT to determine if the CD19 had been inserted in the correct orientation. The positive control for the PCRs were the CD19/pSP65 plasmid and the negative control was water.

4.3.5 *Flow Cytometry*

Flow cytometry was used to detect expression of the membrane bound protein. The method used was the high sensitivity immunofluorescence method (Zola *et al.*, 1990). FMC63 was used to detect cells expressing CD19. The negative control for this was X63, an IgG1 murine myeloma protein with no known reactivity with human cells. The untransfected CHO cells were the negative cellular control and the Raji cells were the positive control cell line. The secondary antibody was 1/50 biotinylated anti-mouse IgG and the tertiary reagent was 1/50 SAPE.

4.3.6 *Cloning of the Membrane Bound CD19 Transfectant*

To produce a population of cells which uniformly expressed the CD19 protein the CD19 transfected CHO-K1 cells were cloned. Cells were counted and plated in 1ml volumes in 24 well plates (Nunc) at cell concentrations of 100, 10 and 1 cell/well. Once the cloned cells had become confluent single clones from the plates were expanded into 3ml volumes in 6 well plates. When the colonies in these plates were confluent the cells were collected and tested for expression of the CD19 protein by flow cytometry.

4.3.7 *Comparison of Reactivity of FMC63 as a CD19 Antibody*

As FMC63, a monoclonal antibody routinely used in the laboratory as an anti-human CD19 antibody, has only recently been submitted to the Human Leucocyte Differentiation Antigen workshop for analysis it was tested for binding to the CD19 transfectant using flow cytometry. Commercial CD19 positive controls included B4 (Coulter), Leu-12 FITC (Becton Dickinson) and CD19 monoclonal antibody PE conjugated (Caltag). A monoclonal antibody, Sal5 (IgG2a which binds Salmonella antigen; developed by Dr. Leonie Ashman, University of Adelaide, SA, Australia), supernatant was used as a negative isotype control for FMC63. Untransfected CHO cells were used as a negative cellular control and HRIK was used as a positive cellular control. 50µl of cell supernatants were used in the assay and 5µl of the commercial antibodies. Unless treated with an antibody directly conjugated to a fluorescent marker the tubes were blocked with 10µl heat inactivated horse serum, to block nonspecific binding of anti-horse antibodies produced by the cells to the horse anti-mouse Ig (secondary antibody), and incubated at room temperature for 10min after the primary antibody. Tubes for the high sensitivity immunofluorescence method were then incubated with 1/50 biotinylated anti–mouse IgG and 1/50 SAPE.

4.4 *Results*

4.4.1 *Production of Recombinant Membrane Bound CD19 Protein*

Figure 4.4.1 shows the 1% agarose gel of the EcoRI digestion of the pIRES1neo and pSP65/CD19 with a comparison of the digested plasmids to the undigested plasmids. The CD19 cDNA is 2kb, pSP65 3kb and pIRES1neo 5.3Kb.

The CD19 cDNA was ligated into pIRES1neo and electroporated into XL1-Blue *E. coli*. 20 random colonies were picked from the electroporation plates and screened by PCR for the presence of the CD19 gene with CD19.SENSE and CD19.ANTI.NOT. 90% of the colonies screened had been transformed with the CD19 cDNA (figure 4.4.2).

The cultures were then rescreened by PCR to determine if the CD19 cDNA had been inserted into the pIRES1neo vector in the correct orientation using pIRES5' and CD19.ANTI.NOT. 60% of the 20 colonies screened initially had the CD19 cDNA in the correct orientation.

Four cultures, CD19-1, CD19-2, CD19-4 and CD19-6, had the most intense bands in the PCR and were chosen for transfection of mammalian cells using LipofectAMINE. 5µg of each plasmid and a negative control of water were transfected into CHO-K1 cells. The transfected cultures were incubated for 2 - 3 days before geneticin was added to select against untransfected cells. Approximately 2 weeks after transfection the geneticin resistant CD19 transfected population grew to confluence and the cells were screened for expression of CD19.

4.4.2 *Screening CD19 Transfected Cells for CD19 Expression Using Flow Cytometry*

Screening of the transfected cells was carried out using immunofluorescence flow cytometry. To determine if CD19 was cleaved from the surface of the cells by trypsin/EDTA the Raji cell line was treated with an equal volume of trypsin/EDTA as the CHO cells. CD19 is not cleaved from the surface of Raji or CHO cells with the use of trypsin/EDTA (figure 4.4.3). Preliminary screening of the transfectants showed CD19-1 had a population of both positive and negative cells with the majority of the cells being positive (figure 4.4.4). The other three transfected populations, CD19-2, CD19-4 and CD19-6, showed some positive cells but most were negative.

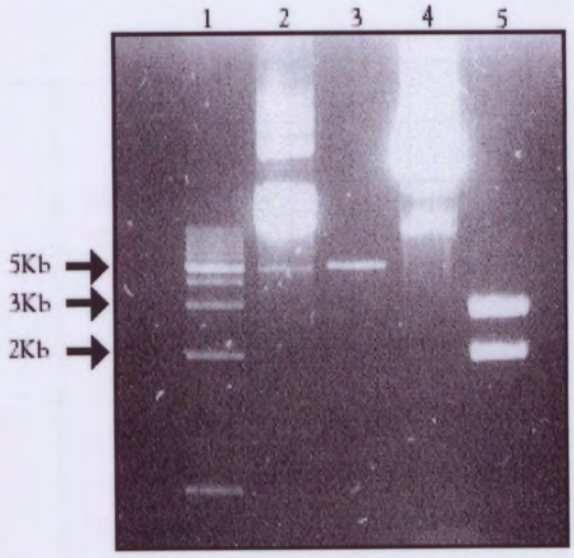


Figure 4.4.1: EcoRI Digestion of pIRES1neo and CD19/pSP65. Lane 1 is 1Kb DNA Ladder; Lane 2 is pIRES1neo; Lane 3 is gel purified EcoRI digested pIRES1neo; Lane 4 is CD19/pSP65; Lane 5 is EcoRI digested CD19/pSP65 (2Kb band CD19, 3Kb band pSP65).

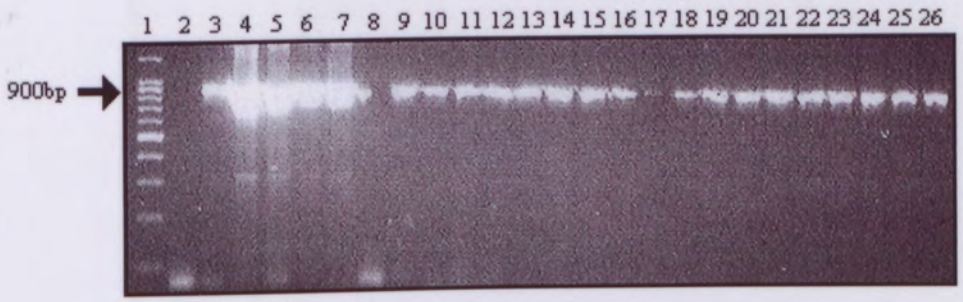


Figure 4.4.2: Colony Screening for the CD19 Insert. CD19.SENSE and CD19.ANTI.NOT were used to screen for the CD19 gene. Lane 1 is 100bp DNA ladder; Lane 2 is water control; Lane 3 is CD19 plasmid control; Lanes 4 – 26 are transformed bacterial colonies. Clones chosen for transfection after orientation PCR were CD19-1 (lane 4), CD19-2 (lane 5), CD19-4 (lane 7) and CD19-6 (lane 9).

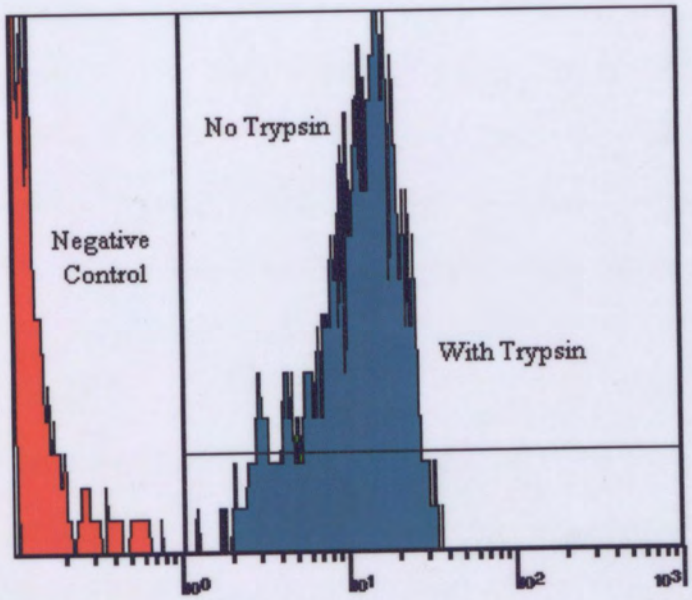


Figure 4.4.3: The Effect of Trypsin on Raji Cells. The negative control is X63. The cells without the trypsin treatment are directly underneath the cells treated with trypsin.

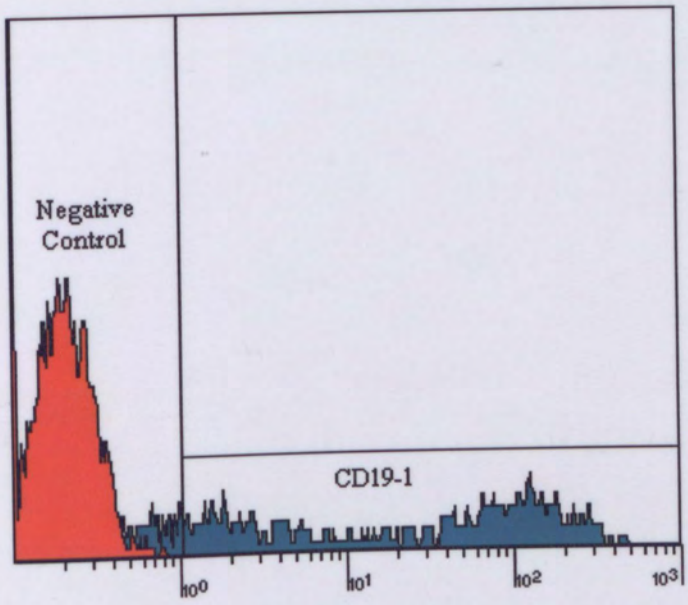


Figure 4.4.4: Preliminary Screen of CD19 Transfected Cells. The negative control is X63.

4.4.3 *Cloning the Transfected Culture Expressing CD19*

CD19-1 was maintained in culture and rescreened for CD19 expression. As the ratio of positive to negative cells in the population remained constant and was not changed by selection with geneticin the culture was cloned. 14 colonies cloned at 1 cell/well in 1ml were transferred, when they were confluent, from the 24 well plate to 3ml of medium in 6 well plates. After 3 days the colonies had grown to confluence and were screened by flow cytometry. The results showed 6 negative clones (43%), 7 wells which had positive and negative populations (50%) and 1 clone, CD19-1.13, positive for CD19 expression (7%).

4.4.4 *Comparison of FMC63 to Commercial Anti Human CD19 Antibodies*

Although many laboratories recognise FMC63 as an anti-CD19 antibody, until recently, it has not been submitted to the Human Leucocyte Differentiation Antigen workshop for classification. To show that FMC63 is an anti-CD19 antibody CD19-1.13 was used to compare FMC63 binding to the binding of other commercial anti-human CD19 antibodies. In this testing all of the commercial CD19 antibodies and FMC63 bound CD19-1.13 in a similar manner (figure 4.4.5). The untransfected CHO-K1 control cells were not bound by any of the anti-CD19 antibodies and Sal5, the negative isotype control, did not bind the CD19 transfected cell line.

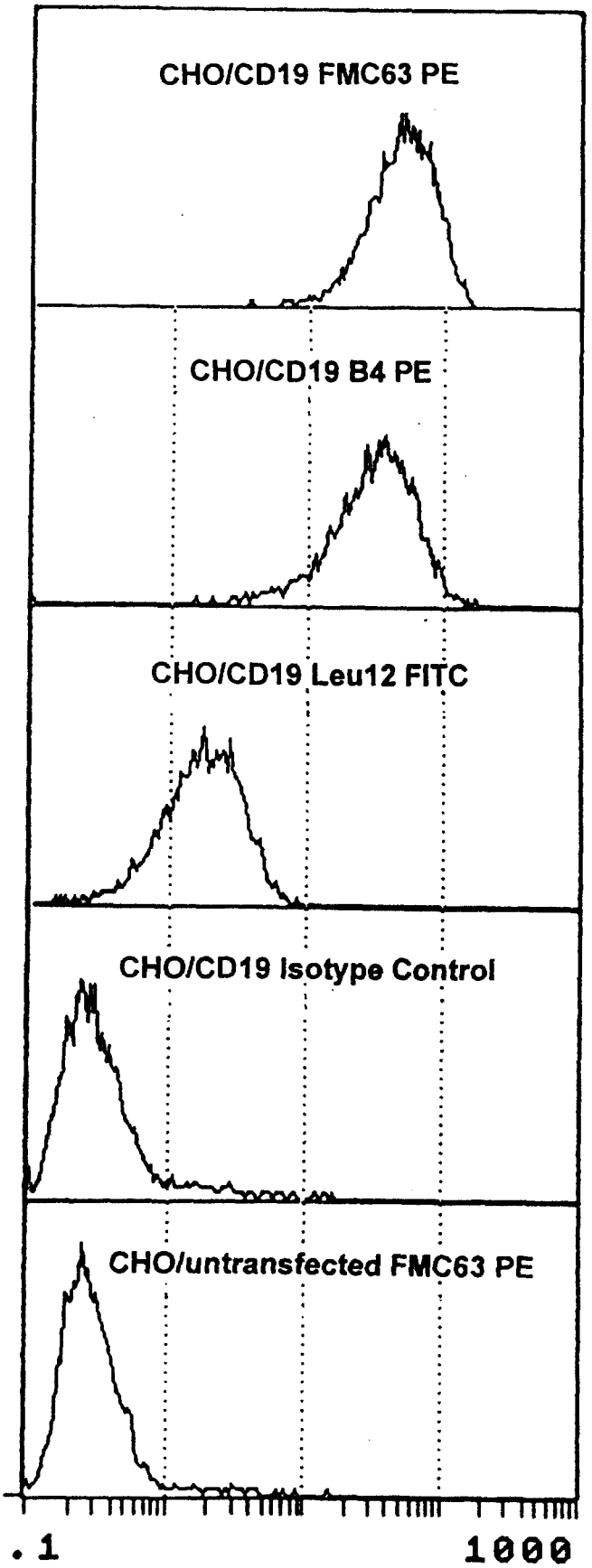


Figure 4.4.5: Binding of Anti CD19 Antibodies to the CD19 Transfected Cell Line. CHO/CD19 is the transfected cell line, FMC63, B4 and Leu-12 are anti-CD19 antibodies and the isotype control is Sal5.

4.5 *Discussion*

Chinese Hamster Ovary (CHO-K1) cells were transfected with full length CD19 cDNA and were shown to be expressing functional human CD19 on their surface by immunofluorescence flow cytometry. Initially the CD19 transfected culture had to be cloned to produce a transfected cell line in which all cells express CD19 on their surface. The cloned culture uniformly expressed CD19 as shown by flow cytometry with FMC63 and commercial anti-CD19 antibodies. Flow cytometry analysis showed that trypsin/EDTA does not cleave the CD19 from the membrane of Raji or CHO cells.

The CD19 transfectant was used in a study comparing FMC63 to commercial anti-human CD19 antibodies. This showed that, even though FMC63 has not previously been submitted to the Human Leucocyte Differentiation Antigen workshop, it is an anti-human CD19 monoclonal antibody.

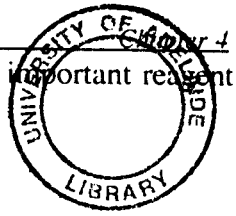
The CD19 transfectant has been useful in the CD19 scFv (Nicholson *et al.*, 1997) site directed mutation studies carried out in 1998 (Drew, 1998; Hohmann *et al.*, 1999). Retention studies comparing binding of scFvs to the CD19 transfected cell line showed that the L94E mutant had 50% reduced binding to CD19 antigen.

The transfected cell line was also used in a study described in chapter 5 in which samples of plasma and serum were screened on the CD19 transfectant for the presence of anti-CD19 antibodies in the blood of patients in different disease states.

The construction of phage display antibody libraries is described in chapter 6 and the CD19 transfected cells were to be used for screening the library. Although the transfected cell line was not used in this case it is a good source of CD19 antigen for panning of libraries for affinity maturation.

An endogenous ligand for CD19 has never been identified and this transfected cell line could be used for some interesting studies in this area. CD77 has been implicated as a possible partner for CD19 as the structure of the CD19 molecule has extensive similarities in sequence to the verotoxin-B-subunits (Maloney and Lingwood, 1994). This and the observation that CD19 and CD77 cocap on Daudi cells (Maloney and Lingwood, 1994) suggest that this is a relationship that should be examined further.

The transfected cell line which expresses membrane bound CD19 has been an important reagent in the laboratory and will be used in many more applications in the future.



Chapter 5

Screening Patient Samples for Antibody Against CD19 and CD20

Chapter 5

Screening Patient Samples for Antibody Against CD19 and CD20

5.1 *Summary*

Autoimmunity is a condition of the immune system in which an immune response is raised against a self antigen. This aberration of the immune system may also be seen in other disease states such as in different types of cancer. It has been postulated that cancer may trigger the production of an immune response against the antigens on the surface of the malignant cells due to the overpopulation of these cells. Also suggested was that if patients with B cell malignancies were producing antibodies against antigens on the surface of their cancer cells then these patients would be good sources of starting material for the production of a phage display antibody library. This could then be used to screen against antigens from the surface of the cancer cells for isolation of antibodies to those molecules.

To test this hypothesis, plasma and serum samples from control individuals and patients with Chronic Lymphocytic Leukemia (CLL), Myeloma, Non Hodgkin's Lymphoma (NHL) and Sjogren's Syndrome were screened against CD19 and CD20 to determine which group of samples showed higher antibody titres against the cell surface antigens. There was no significant difference between the plasma patient and control samples but there was a significant difference between the serum patient and control samples in the CD20 assay.

More importantly some samples from the groups bound the CD19 and CD20 antigens better than the majority of the population. Some of these outliers were tested for specific binding to CD20 and were shown to be binding the antigen and not causing excessive background binding.

The individuals who showed higher antibody titres to cell surface antigens were not restricted to the patient groups. Only a small number of people from the patient and control groups had high levels of anti-CD19 and anti-CD20 antibodies. This suggests that individuals should be screened for high titres of autoantibodies prior to lymphocyte donation for library construction if the library is to be biased toward a particular antigen.

5.2 *Introduction*

The human immune response is a sophisticated mechanism which is responsible for defending the body against disease. Foreign substances which enter the body trigger this mechanism. Cell mediated immunity, involving cells providing a defence against disease, and humoral immunity, which encompasses the production of an antibody response, are two components of this system. Although these two are often referred to separately they are also known to work together to form an appropriate response to antigen. It is the production of antibodies through the humoral immune response that is particularly relevant to this study.

A normal immune response will produce a reaction against virtually any protein, many polysaccharides, nucleoproteins, lipoproteins, synthetic polypeptides and also many small molecules if they are linked to proteins. Therefore it is important for the immune system to distinguish between self antigens and those which are foreign. Before immunocompetence is reached by an infant they must learn to discriminate between "self" and "nonself". Whatever molecules with potential antigenic properties they encounter before the immune system has become competent are then recognised as self. In future no antibody response will be mounted against those antigens. This is known as self tolerance. It is a complex mechanism which involves the apoptosis of immature cells which recognise antigen in environments, particularly the thymus, where they are exposed to self antigens.

The immune system develops during foetal life. Humoral immune responses to some antigens most commonly begin sometime in the first month of life but theoretically the foetus should be able to produce an antibody response during the second trimester of pregnancy although this response is not often required (Kimball, 1990). Not all antigen types are initially recognised by infants and the immune system continues to develop during the first few years of life reaching adult immunoglobulin serum levels in the teenage years (Stiehm and Fudenberg, 1966).

Any antigens encountered by the immune system before it is fully operational are therefore deemed as self and an immune response is not usually mounted against these antigens. A mechanism of self tolerance has been described by Burnet who in 1959 postulated that the immune system deleted self reactive clones from the preimmune repertoire leaving only those clones which were non self reactive to fight infection. This in itself posed problems in that it left only very few clones that were "non-self" oriented. Nossal in 1983 suggested that there

may be an affinity cut off to avoid deleting the majority of the potential repertoire of antibody producing cells. Currently research into resolving how self tolerance is acquired is focussed on tracking individual B and T cells to determine their eventual fate (Goodnow, 1997).

The above describes a normal immune system in a healthy individual, but in some situations excessive, defective or inappropriate response to self antigens can occur. Response against self antigen is described as autoimmunity. Some examples of autoimmune disease are Sjogren's Syndrome, Hashimoto's Thyroiditis, Hyperthyroidism, Systemic Lupus Erythematosis and Rheumatoid Arthritis. Sometimes these diseases are triggered by some underlying cause, for example the production of antibodies to heart muscle protein which can appear after myocardial infarction or cardiac surgery (Eisen, 1990) or a parasitic organism called *Trypanosoma cruzi* which is associated with Chagas' disease (Teixeira *et al.*, 1978). Viral damage may also be a trigger for the initiation of diseases like Multiple Sclerosis (Steinman and Conlon, 1997).

It has been suggested (G. Pilkington, personal communication) that people with cancer would produce an immune response against their malignant cells. However, the immune system has been trained to ignore self antigens. There have been antigens described which are unique to some tumours (these are found on the neoplastic cell type but not on the non malignant counterpart). The identification of tumour associated antigens has been the focus of much research. One strategy, called SEREX (Serological analysis of autologous tumour antigens by recombinant cDNA expression cloning; Türeci *et al.*, 1997), allows the serological identification of these antigens by recombinant expression cloning. This technology involves the construction of cDNA libraries from tumour specimens. These are cloned into λ phage expression vectors, transformed into *E. coli* and the proteins expressed are blotted onto nitrocellulose membranes. These membranes are incubated with patient serum and clones bound by IgG are identified with anti-human antibodies. Once positive clones are identified the nucleotide sequence of the cDNA for the antigen can be determined. The human tumour antigens that have been identified using this method can be divided into different categories. Some have included differentiation antigens which are present on both normal and neoplastic cells of the same type (Boon *et al.*, 1994; Coulie *et al.*, 1994).

Therefore in B-cell leukemia or lymphoma a patient may produce antibodies to CD19 and CD20. This hypothesis is tested in this chapter by screening the plasma and serum of people

who have myeloma, Non Hodgkin's Lymphoma, Chronic Lymphocytic Leukemia and the auto immune disease, Sjogren's Syndrome.

Multiple Myeloma is a cancer of plasma cells. It encompasses a range of diseases including Plasmacytic Myeloma, Plasma Cell Myeloma, Myelomatosis and Kahler's Disease. Multiple myeloma is a common form of lymphoid cancer with most patients being over 40 years of age (Kyle, 1975) and incidence of this disease increases with age. There is a high mortality rate for people with myeloma with approximately 30 - 50% of patients not responding to first line therapy (Buzaid and Durie, 1988). Some circulating plasma cells in myeloma express CD19, in about 5% of patients, (Rawstron *et al.*, 1997) but most myeloma cells do not express CD19 or CD20; It would therefore seem unlikely that patients with this disease would have an excess of antibodies to these antigens in their plasma or serum but it is a good patient control.

Non Hodgkin's Lymphoma describes a large number of distinctly different tumour types. It is found in all age groups but it is more common in adults and the incidence of disease rises with age (Vose *et al.*, 1991). It is recognised as being approximately 8 times as prevalent as Hodgkin's Lymphoma in the United States of America (Barela, 1998). Classification of this disease continues to be ambiguous even following the introduction of the Rappaport classification system in 1956 (Ersbøll *et al.*, 1985) and others since (Working Formulation of Non Hodgkin's Lymphoma, Kiel and Lukes & Collins Classifications). Different cell types and cell development stages are included in this group of diseases. These malignant cells are CD19 and CD20 positive which makes it an interesting sample group for testing against antigen.

B cell Chronic Lymphocytic Leukemia (CLL) is reported to be the most common adult leukemia in the Western World (Cheson, 1989; Faguet, 1994). It comprises 25 - 30% of all leukemia cases. This disease mainly affects people over 60 years of age but it is not uncommon to find it in younger age groups. It has been reported that there is a higher incidence of CLL in males than in females and prevalence of B-cell CLLs in Asian countries is much lower than in America (Rai and Sawitsky, 1991; Faguet, 1994).

Chronic Lymphocytic Leukemia is characterised by proliferation and accumulation of malignant lymphocytes (Rai and Sawitsky, 1991). The malignancy can be in either the B or T lymphocytes but is much more prevalent in cells of the B lymphocyte series which are CD19 and CD20 positive, hence it is an important component of this study. Eventually without

treatment these lymphocytes overpopulate the system and a reduction in red blood cells and platelets can be a result. The prognosis of this disease depends on many factors including age, general health and the stage of the disease upon diagnosis.

Sjogren's Syndrome is a chronic, incurable, autoimmune disorder where the immune system of the affected individual recognise secretory glands as foreign. This results in the patient suffering attacks on the secretory glands including tear ducts and salivary glands resulting in dryness in the eyes, mouth and other sites of secretion. It is a condition first described by Henrik Sjogren, a Swedish ophthalmologist, in 1933. Females are most commonly affected and the mean age of patients is approximately 50. Treatment of this disease is symptomatic. Sjogren's Syndrome patients were chosen for this study as a control group which produce autoantibodies.

This chapter explores the possibility that patients with malignant and autoimmune diseases produce antibodies to CD20. Screening plasma and serum from patient and control donors should indicate if a particular group exhibits higher levels of antibody to this molecule. This would provide valuable information about where source material for engineered antibodies or phage display libraries could originate from. The CD20 peptide and the CD19 transfected CHO-K1 cells provide the tools to investigate these questions.

5.3 *Materials and Methods*

5.3.1 *Patient and Control Plasma and Serum Samples*

CLL plasma samples were collected from the Institute of Medical and Veterinary Science, Adelaide, South Australia and Flinders Medical Centre, Adelaide, South Australia. Sjogren's Syndrome serum samples were collected at the Queen Elizabeth Hospital, Adelaide, South Australia. Non-Hodgkin's Lymphoma and Myeloma serum samples were collected at Flinders Medical Centre. Control serum samples were supplied by Flinders Medical Centre and control plasma samples were collected from healthy individuals in the Child Health Research Institute laboratory, Adelaide, South Australia. Appendix 1 shows the disease state associated with the different serum and plasma samples.

5.3.2 *ELISA*

The serum and plasma samples were diluted 1/20 in 3% BSA/PBS/0.05% tween 20. PBS (well A1) was used as a blank for reading the plate, FMC63 and X63 were used as negative antibody controls and B-Cl was used as a positive control. The secondary antibody was either 1/1000 alkaline phosphatase conjugated anti-human IgG (Silenus) or 1/1000 alkaline phosphatase conjugated anti-mouse Ig (Silenus) depending on the primary antibody.

5.3.3 *Flow Cytometry*

The serum and plasma samples were diluted 1/20 in 3% BSA/PBS. The diluted plasma and serum samples were used in a flow cytometry assay on the CD19 transfected CHO cells (chapter 4). The controls were B-Cl and X63 as negative antibody controls and FMC63 was used as a positive control. The CD19 transfected cell line and untransfected CHO cells were resuspended at 1 x 10^7 cells/ml and 50µl of this suspension added to each tube. 100µl diluted plasma or serum was added and incubated at 4°C for 30min. The secondary antibodies were 50µl 1/50 FITC conjugated anti-human IgG (Silenus) or 50µl 1/50 FITC conjugated anti-mouse Ig (Silenus). Flow cytometry was carried out as described in chapter 2.

5.3.4 *Statistics*

Outliers in the plasma and serum samples were identified using the Inter-Quartile Range (IQR) convention (samples with a value greater than 1.5 x IQR above the third quartile are deemed to be outliers). All statistical equations are shown in appendix 2 and were obtained from Weiss (1999).

To test for statistical differences between the control and patient populations in the ELISA data a Mann-Whitney test was used. To test for statistical differences between groups in the flow cytometry the paired Mann-Whitney test was used. Calculations for this were done using a statistical computer program called InStat.

5.4 *Results*

5.4.1 *ELISA*

Comparison of Patient Samples to Control Samples

19 patient plasma samples, 11 control plasma samples, 21 patient serum samples and 12 control serum samples were screened in an ELISA against the CD20 peptide. Appendix 1 shows a list of the malignancies and autoimmune diseases in these groups. Figure 5.4.1 shows a comparison of the patient plasma samples to the control plasma samples. Figure 5.4.2 shows a comparison of the patient serum samples to the control serum samples. Using the Mann-Whitney test the medians of the patient and control samples in the plasma and serum groups were compared and showed that the difference between the two populations was not significant. The same test was carried out to examine the difference between the patient and control serum samples and the median of the patient serum samples was calculated to be significantly greater than the median of the control serum samples ($p = 0.0453$).

Identification of Outliers

There were some serum and plasma samples that appeared to have higher levels of CD20 than others in their groups. The interquartile range was determined for the plasma and serum sample and control groups (tables 5.4.1 and 5.4.2).

The samples in bold were identified as being outliers. The two patient plasma samples were from individuals with chronic lymphocytic leukemia and the patient serum sample was from an individual with Sjogren's syndrome. There is no information known about the control sample. Some of these high binders and some samples which were not considered high binders were tested in an ELISA to show that they were binding to the CD20 peptide and not binding to the plastic of the plate or showing high background binding to something else used in the ELISA. Two different assays were run in parallel. One set of plates were coated with CD20 peptide and the other set of plates were treated exactly the same except the CD20 peptide was not added to the plate coating mix. Results of this assay can be seen in figure 5.4.3. The high binders were

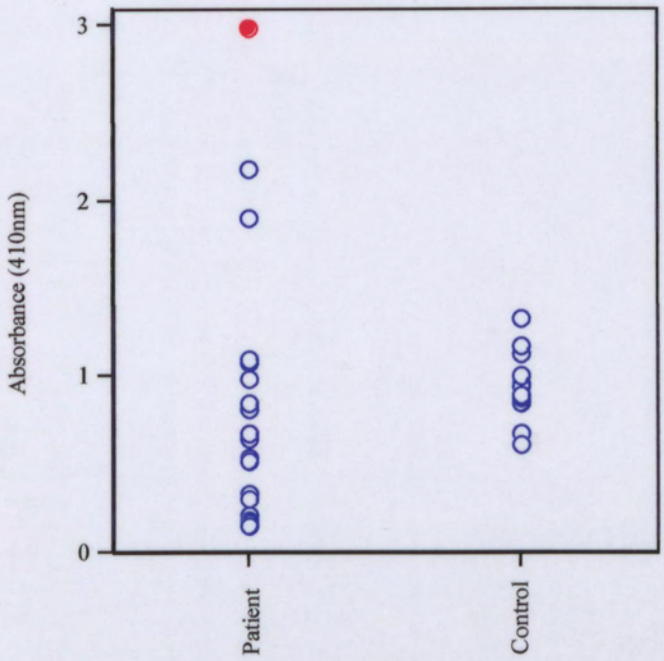


Figure 5.4.2: Serum Patient and Control Samples Against CD20. ELISA with CD20 peptide. Red filled circles are outliers.

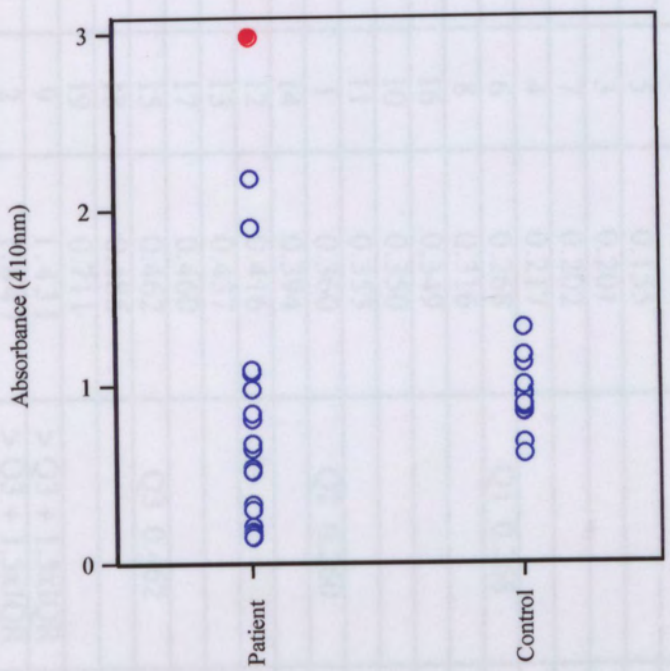


Figure 5.4.2: Serum Patient and Control Samples Against CD20. ELISA with CD20 peptide. Red filled circles are outliers.

Sample Number	Patient Raw Data	SN	Data Rearrangement	Quartiles	Control Raw Data	SN	Data Rearrangement	Quartiles
Mean	0.495				0.569			
SD	0.43				0.26			
1	0.360	5	0.155		0.823	2	0.330	
2	1.847	3	0.201		0.330	3	0.344	
3	0.201	7	0.202		0.344	8	0.395	Q1 0.395
4	0.217	4	0.217		0.608	7	0.404	
5	0.155	6	0.268	Q1 0.268	0.436	5	0.436	
6	0.268	8	0.316		1.190	10	0.466	Q2 0.466
7	0.202	16	0.349		0.404	9	0.585	
8	0.316	10	0.350		0.395	4	0.608	
9	1.431	11	0.353		0.585	11	0.674	Q3 0.674
10	0.350	1	0.360	Q2 0.260	0.466	1	0.823	
11	0.353	14	0.394		0.674	6	**1.190**	> Q3 + 1.5xIQR
12	0.416	12	0.416					
13	0.437	13	0.437					
14	0.394	17	0.460					
15	0.462	15	0.462	Q3 0.462				
16	0.349	18	0.485					
17	0.46	19	0.711					
18	0.485	9	**1.431**	> Q3 + 1.5xIQR				
19	0.711	2	**1.847**	> Q3 + 1.5xIQR				

Table 5.4.1: Plasma Patient and Control Sample Data. Table of raw data and quartiles for each experimental group. SD is standard deviation, SN is the rearranged sample numbers, data rearrangement is an arrangement of the data into increasing order, Q1 is quartile 1, Q2 is quartile 2, Q3 is quartile 3 and IQR is the interquartile range. Outliers were identified as being more than 1.5 IQRs above the third quartile and are shown in bold.

Sample Number	Patient Raw Data	SN	Data Rearrangement	Quartiles	Control Raw Data	SN	Data Rearrangement	Quartiles
Mean	0.823				0.956			
SD	0.73				0.20			
1	0.159	1	0.159		0.626	1	0.626	
2	0.682	5	0.172		0.906	12	0.667	
3	0.178	3	0.178		1.332	7	0.851	Q1 0.860
4	1.106	6	0.192		1.177	8	0.869	
5	0.172	19	0.210	Q1 0.260	0.889	5	0.889	
6	0.192	17	0.305		1.008	2	0.906	Q2 0.922
7	1.889	20	0.345		0.851	9	0.939	
8	2.187	15	0.520		0.869	11	0.967	
9	0.853	14	0.524		0.939	6	1.008	Q3 1.100
10	>2.999	18	0.538		1.131	10	1.131	
11	1.085	16	0.650	Q2 0.650	0.967	4	1.177	
12	0.827	2	0.682		0.677	3	1.332	
13	0.992	12	0.827					
14	0.524	21	0.850					
15	0.520	9	0.853					
16	0.650	13	0.992	Q3 1.040				
17	0.305	11	1.085					
18	0.538	4	1.106					
19	0.210	7	1.889					
20	0.345	8	2.187					
21	0.850	10	**2.999**	> Q3 + 1.5xIQR				

Table 5.4.2: Serum Patient and Control Sample Data. Table of raw data and quartiles for each experimental group. SD is the standard deviation, SN is the rearranged sample numbers, data rearrangement is an arrangement of the data into increasing order, Q1 is quartile 1, Q2 is quartile 2, Q3 is quartile 3 and IQR is the interquartile range. Outliers were identified as being more than 1.5 IQRs above the third quartile and are shown in bold.

shown to bind the CD20 peptide and there was no excessive background binding to the plastic.

5.4.2 *Flow Cytometry*

To determine whether any of the groups had high levels of CD19 antibody some of the samples were screened against the CD19 transfected CHO-K1 cells. The results of the flow cytometric work can be seen in figure 5.4.4. The patient and control samples were compared to each other on both untransfected and CD19 transfected CHO-K1 cells. There was no significant difference between the 4 groups using a paired Mann Whitney test but the results showed that some samples bind better than others to both CD19 transfected CHO-K1 cells and untransfected CHO-K1 cells.

Identification of Outliers

Tables 5.4.3 and 5.4.4 show the statistical analysis of the results of incubating the patient and control samples with the cells to determine if any of the samples were outliers. Using the interquartile range none of the samples were deemed to be outliers.

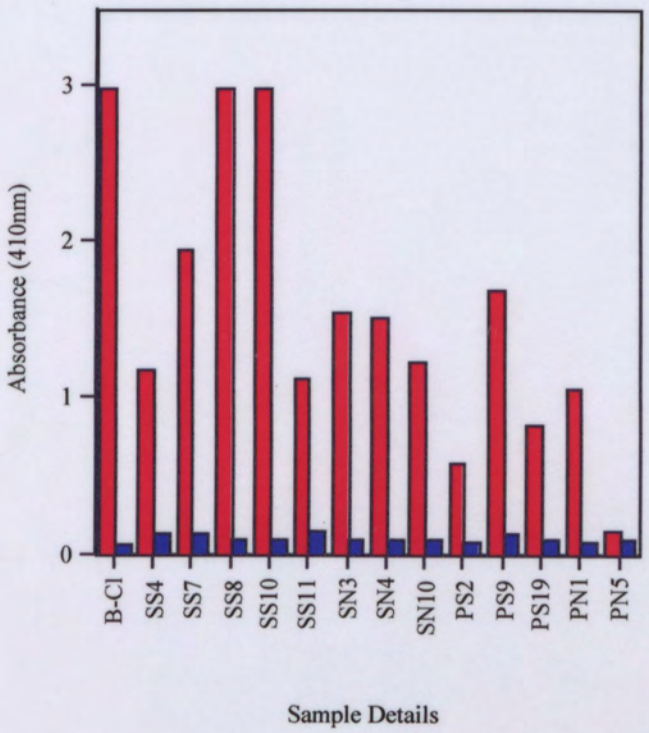


Figure 5.4.3: Screening Plasma and Serum Outliers for Specific Binding to CD20. Highest plasma and serum patient and control samples which bound best against CD20 peptide (red) in preliminary experiments and bicarbonate buffer only (blue). B-Cl is the positive control. SS is serum sample, SN is serum control, PS is plasma sample, PN is plasma control.

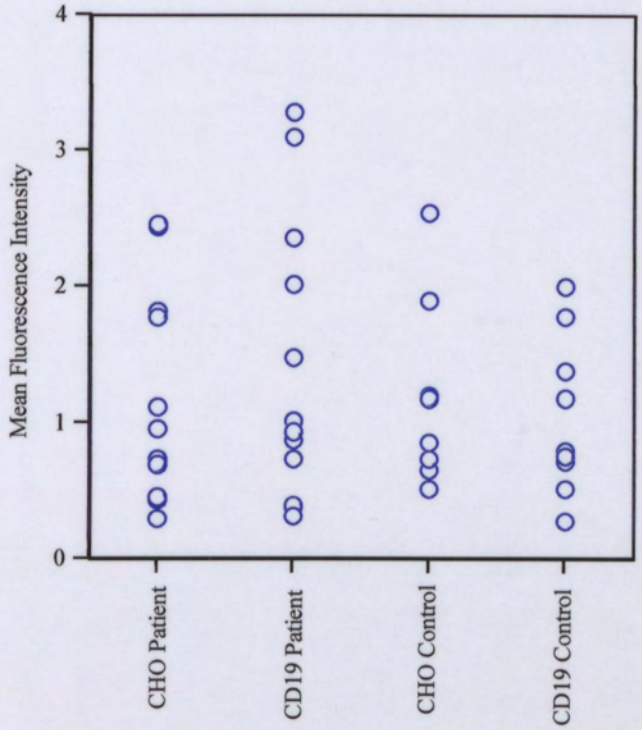


Figure 5.4.4: Plasma Patient and Control Samples Against CD19. Samples were tested in flow cytometry with the CD19 transfectant and untransfected CHO-K1 cells. Red filled circles are outliers.

Sample Number	CHO Cells				CD19 Cells			
	Raw Data	SN	Data Rearrangement	Quartiles	Raw Data	SN	Data Rearrangement	Quartiles
Mean	1.04				1.13			
SD	0.58				0.67			
PN1	0.755	PN5	0.295		0.75	PN5	0.527	
PN2	0.729	PN10	0.5	Q1 0.615	0.675	PN7	0.532	Q1 0.604
PN3	1.3	PN2	0.729		2.5	PN2	0.675	
PN4	2	PN1	0.755		1.9	PN1	0.75	
PN5	0.295	PN11	0.8	Q2 0.8	0.527	PN6	0.85	Q2 0.85
PN6	1.8	PN7	1.19		0.85	PN10	1.2	
PN7	1.19	PN3	1.3	Q3 1.55	0.532	PN11	1.2	Q3 1.55
PN10	0.5	PN6	1.8		1.2	PN4	1.9	
PN11	0.8	PN4	2		1.2	PN3	2.5	

Table 5.4.3: Plasma Control Sample Data from Flow Cytometry. Table of raw data and quartiles for each experimental group. SD is the standard deviation, SN is the rearranged sample numbers, data rearrangement is an arrangement of the data into increasing order, Q1 is quartile 1, Q2 is quartile 2, Q3 is quartile 3 and IQR is the interquartile range. Outliers were identified as being more than 1.5 IQRs above the third quartile and are shown in bold. PN is plasma control samples.

Sample Number	CHO Cells				CD19 Cells			
	Raw Data	SN	Data Rearrangement	Quartiles	Raw Data	SN	Data Rearrangement	Quartiles
Mean	1.1				1.34			
SD	0.77				1.04			
PS1	0.959	PS2	0.307		0.753	PS2	0.326	
PS2	0.307	PS5	0.314		0.326	PS5	0.387	
PS3	0.47	PS4	0.444		0.403	PS12	0.387	
PS4	0.444	PS3	0.47		0.948	PS3	0.403	
PS5	0.314	PS13	0.708		0.387	PS1	0.753	
PS6	2.47	PS15	0.734		2.03	PS15	0.89	
PS11	2.44	PS14	0.742		2.37	PS4	0.948	
PS12	1.79	PS1	0.959		0.387	PS13	1.02	
PS13	0.708	PS16	1.11		1.02	PS14	1.49	
PS14	0.742	PS12	1.79		1.49	PS6	2.03	
PS15	0.734	PS17	1.83		0.89	PS11	2.37	
PS16	1.11	PS11	2.44		3.29	PS17	3.1	
PS17	1.83	PS6	2.47		3.1	PS16	3.29	

Table 5.4.4: Plasma Patient Sample Data from Flow Cytometry. Table of raw data and quartiles for each experimental group. SD is the standard deviation, SN is the rearranged sample numbers, data rearrangement is an arrangement of the data into increasing order, Q1 is quartile 1, Q2 is quartile 2, Q3 is quartile 3 and IQR is the interquartile range. Outliers were identified as being more than 1.5 IQRs above the third quartile and are shown in bold. PS is plasma patient samples.

5.5 *Discussion*

The results of this chapter showed that while there was no significant difference between the plasma patient and control groups using either ELISA or flow cytometry the median of the patient serum samples was significantly higher than the median of the serum control samples using ELISA. That the patient serum samples were higher than the control serum samples may have been influenced by the large number of samples from patients with the autoimmune disease, Sjogren's Syndrome, included in this group. These results were reproducible as the samples, both patient and controls, were tested multiple times and there was little variation in sample results between assays carried out under identical conditions.

In the ELISA a small number of samples, from both the patient and control groups, were shown statistically to be outliers. Those deemed to be high binders were shown to be binding specifically to the CD20 antigen and not to the plastic of the plate or anything in the binding buffer. There seems to be no pattern to those samples which were outliers. Those identified as being outliers were a mix of 2 CLL samples, 1 Sjogren's Syndrome sample and 1 control sample. No patients with Non Hodgkin's lymphoma or Myeloma were identified as having high titres of CD20 antibody.

Although in the flow cytometry some samples appeared to bind with a high mean fluorescence intensity to the CD19 transfected cells and the untransfected CHO-K1 cells no outliers were identified.

A conclusion that can be drawn from these results is that anybody, not just those with malignancies of their B lymphocytes, can have circulating CD19 or CD20 autoantibodies, some with significantly higher levels than others. This indicates that individuals should be screened for antibody before the construction of a phage display library if the library is to be biased against a particular antigen. It has been suggested that there are low levels of anti-'self' antibodies in circulation but they are of a low affinity (Guilbert *et al.*, 1982). The results in this chapter show that there are antibodies in the blood of some individuals which have an affinity for CD19 and CD20. It is not uncommon for an individual to have a high titre of an autoantibody and no disease state associated with it. Guilbert *et al.* (1982) showed the presence of antibody to antigens such as thyroglobulin, actin, albumin and others in serum samples taken from 800 healthy donors.

Excessive proliferation of a cell type in the body, due to cancer for example, could be controlled by autoantibody production. This chapter showed that not all patients with malignancy had high titres of CD19 and CD20 antibodies. This could be because antibody has already bound cells in circulation with the molecule on the surface.

This study showed that 2 patients with CLL, 1 person suffering Sjogren's Syndrome and 1 healthy donor all produced a higher response to CD19 and CD20 than others. It does not appear that patients in remission with a B cell malignancy have more anti CD19 or anti CD20 antibody than healthy individuals. This indicates that starting material should be selected from people screened for antibody prior to donation if the phage display library is to be biased toward a particular antigen.

Chapter 6

Construction of a Phage Display Antibody Library

Chapter 6

Construction of a Phage Display Antibody Library

6.1 *Summary*

This chapter describes the construction of a Fab phage display antibody library from tonsil lymphocytes. A small Fab antibody library with a size of 2.6 x 10^5 members was produced. After determining the percentage of light and heavy inserts the actual library size was determined to be 7.9 x 10^4. In light of the problems experienced further library construction was postponed.

Existing libraries ("Einstein") were used in a panning experiment against CD20. The libraries were panned over four rounds against CD20 peptide immobilised on ELISA plates. Aliquots of the phage were applied to the plates, unbound phage washed away and phage eluted from the plates were titred to determine the average yield. No phage were eluted from the panning plates suggesting either that the libraries did not contain antibodies reacting with the CD20 peptide or the panning procedure was ineffective. It was decided to construct a CD20 Fab from a hybridoma in order to "spike" the library and optimise panning conditions. In the meantime panning of libraries was discontinued.

6.2 *Introduction*

Phage display libraries have been described as being useful for isolating antibody fragments, Fab or scFv, and peptides from large pools of proteins. They have the potential to supersede the use of hybridomas for production of antibodies for therapy as they allow the isolation of human antibodies which has previously been difficult using other methods. Although there has been some success in the production of fully human antibodies, using both the hybridoma method (Kohler and Milstein, 1975) and EBV transformation (Steinitz *et al.*, 1977; Haspel *et al.*, 1985), these technologies have been successful for only a few human antibodies.

Ideally antibodies used in human disease therapy would be of human origin, but the majority of monoclonal and recombinant antibodies produced so far have been of murine descent. The use of murine antibodies in therapy, although proven successful, can produce an adverse reaction in humans known as the HAMA response (chapter 1). This reaction normally precludes the use of the therapeutic antibody in the further treatment of that patient. Other methods have been used to produce humanised or fully human antibodies to reduce the immunogenicity of therapeutic antibodies (reviewed in chapter 1).

Library construction begins with the isolation of lymphocytes from a suitable donor. Often the donor is infected with a disease that elicits an immune response. This increases the possibility that therapeutically active antibodies against that disease will be isolated from the library. Libraries constructed from the lymphocytes of HIV-1 infected individuals have produced antibodies against HIV-1 surface glycoprotein gp120 (Burton *et al.*, 1991; Barbas *et al.*, 1993) and Rev and Tat regulatory proteins (Pilkington *et al.*, 1996) and construction of a library from the lymphocytes of a HSV-1 donor yielded Fab antibodies to HSV (Cattani *et al.*, 1997).

Even if the immune system has not encountered the antigen in many years antibody producing cells can be isolated. A combinatorial library was produced from the bone marrow of an individual who had been immunised against measles in his early child hood (Bender *et al.*, 1994). From this library anti measles Fab antibodies were isolated showing the potential benefits of using bone marrow lymphocytes as a source for the isolation of antibodies which are no longer in circulation.

Lymphocytes from non immunised sources have also been used for library construction (Marks *et al.*, 1991). These libraries can be used to isolate antibodies against antigen the donor has

never encountered before. Antibodies isolated from these libraries have low affinity for antigen but affinity maturation techniques, such as random mutagenesis (Gram *et al.*, 1992), can improve the quality of the antibodies produced.

One of the most recent developments in this field has been the construction of phage display libraries with sequences which have been altered *in vitro* (Hoogenboom and Winter, 1992; de Kruif *et al.*, 1995, Nissim *et al.*, 1994; Griffiths *et al.*, 1994). The production of synthetic libraries has become possible due to the generation of human germline V_H databases. They are produced by fusing synthetic CDR3 regions to collections of cloned V_H genes and combining these with one or more light chains (Hoogenboom and Winter, 1992). The CDRH3 is the region which is commonly focussed on as it is the CDR which shows most variability. The lengths of the synthetic regions can vary from 4 to 15 residues in different libraries. Antibodies against a large range of antigens have been isolated from libraries with these synthetic CDRH3 domains (Nissim *et al.*, 1994; de Kruif *et al.*, 1995).

Other applications for phage display libraries have been developed and include guided selection of human antibodies with murine antibodies as a template (Jespers *et al.*, 1994) and affinity maturation using a variety of methods such as bacterial mutator strains, non-proof reading enzymes and site directed mutagenesis (chapter 1).

The advantage of phage display libraries is they mimic the immune repertoire *in vitro*. Although the libraries are not the same size as a human immune repertoire, antibodies against a wide range of antigens have been isolated from them (Burton *et al.* 1991; Burton and Barbas, 1993; Barbas *et al.*, 1993; Pilkington *et al.*, 1996; Ridgway *et al.*, 1999). However more recently antibody libraries are being created which are very large (de Haard *et al.*, 1999) and antibodies against human antigens have been retrieved from them. The production of antibodies against human cell surface antigens would be of great therapeutic benefit in the treatment of cancers. The construction of phage display antibody libraries that are biased toward human cell surface antigens would more readily yield these antibodies.

The natural production of human antibodies against cell surface antigens was investigated in chapter 5. Although the immune system is trained to ignore self antigens there is evidence to suggest that autoantibodies may be produced in individuals other than those with autoimmune disease (Guilbert *et al.*, 1982; Türeci *et al.*, 1997). This indicates that human B lymphocytes are a potential source for an antibody library which could be probed for antibodies to self

antigens. It has been postulated that patients in remission from B cell leukemia and lymphoma would be appropriate donors for isolation of antibodies against B cell antigens. The results from chapter 5 showed that not all patients with B cell malignancy produce high levels of anti-CD20 antibody. Individuals in other groups produced higher than average levels. If the library is to be biased toward a certain antigen, individuals should be screened for antibody to the antigen before donating lymphocytes for library construction. In this project tonsil lymphocytes from a routine tonsillectomy were used to produce a library before using precious patient samples.

This chapter describes the construction of a Fab phage display antibody library of small size which, although the selection is limited, could yield useful antibodies.

6.3 *Materials and Methods*

6.3.1 *pAbClone Vector*

The pAbClone vector (figure 6.3.1) was provided by Intracel. The design of this plasmid was based on the pUC19 vector and has two cloning sites, one for insertion of the heavy chain of a Fab fragment and the other for insertion of the light chain of a Fab fragment. The heavy chain insertion point is 5' of gene III in the plasmid so that the Fab Fd is fused to gene III for phage display. Both chains are led to the periplasm by a pelB leader sequence and both have a lac promoter. The vector has ampicillin resistance and origin of replication for bacteria and bacteriophage.

6.3.2 *Generation of cDNA*

The total mRNA extracted from the tonsil cells (chapter 2) was used to generate cDNA. The reaction containing mRNA, 2µg OligodT and water in a volume of 54µl was heated at 70°C for 10min and 4°C for 5min. To this was added 160U RNAsin, 5X RT buffer, 0.3mM dNTPs, 10mM DTT, water and 800U reverse transcriptase (Life technologies) in a volume of 100µl. This was incubated at room temperature for 10min, 42°C for 50min, 90°C for 5min and 4°C for 10min. 2U RNase H (Life Technologies) was added and incubated at 37°C for 20min.

6.3.3 *PCR*

PCR amplification of the antibody genes was carried out using a range of primers, which were designed to identify a large number of different human antibody light and heavy chain sequences. Tables 6.3.1 - 6.3.3 shows the primers used to construct a library of Fab antibody fragments. Primers were either provided by Intracel Corporation or purchased from Bresatec and resuspended at a stock concentration of 2mM. Primers were diluted to a working concentration of 20µM.

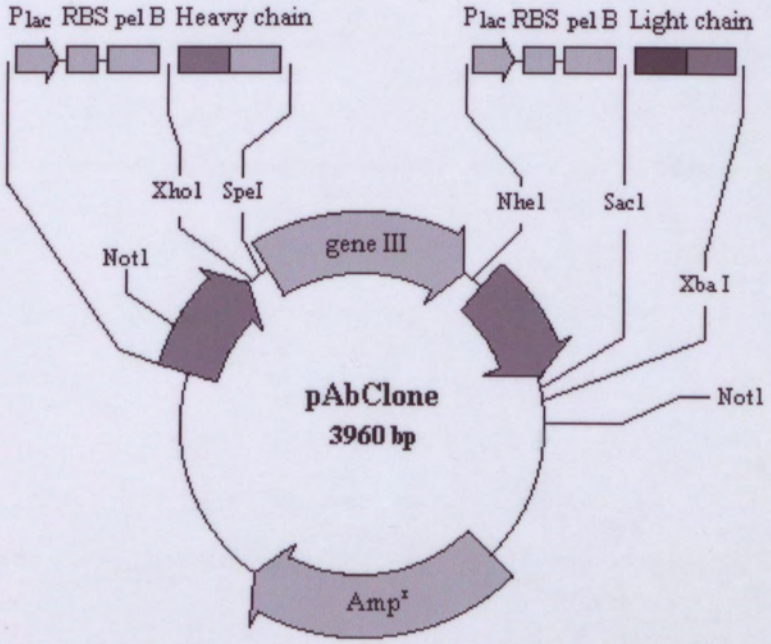


Figure 6.3.1: pAbClone.

Primer	Sequence 5' - 3'	Restriction Site
VK1a	gacatcgagctcacccagtctcca	SacI
VK2a	gatattgagctcactcagtctcca	SacI
VK3a	gaaattgagctcacgcagtctcca	SacI
CK1d	gcgccgtctagaattaacactctcccctgttgaagctctttgtgacggg cgaactcag	XbaI

Table 6.3.1: Human Kappa Light Chain Primers. The first 3 are the 5' primer for the variable domain and the CK1d is the 3' primer for the constant domain.

Primer	Sequence 5' - 3'	Restriction Site
VL1	aattttgagctcactcagccccac	SacI
VL2	tctgccgagctccagcctgcctccgtg	SacI
VL3	tctgtggagctccagccgccctcagtg	SacI
VL4	tctgaagagctccaggaccctgttgtgtctgtg	SacI
VL5	cagtctgagctcacgcagccgccc	SacI
VL6	cagactgagctcactcaggagccc	SacI
VL7	caggttgagctcactcaaccgccc	SacI
CL2	cgccgtctagaactatgaacattctgtagg	XbaI

Table 6.3.2: Human Lambda Light Chain Primers. The first 7 are 5' primers designed in the variable region of the light chain and the CL2 is the 3' primer designed in the constant region of the light chain.

Primer	Sequence 5' - 3'	Restriction Site
VH1a	caggtgcagctcgagcagtctggg	XhoI
VH2f	caggtgcagctactcgagtcggg	XhoI
VH3a	gaggtgcagctcgaggagtctggg	XhoI
VH3f	gaggtgcagctgctcgagtctggg	XhoI
VH4f	caggtgcagctgctcgagtcggg	XhoI
VH6a	caggtacagctcgagcagtcagg	XhoI
CONGa	tccaccaagggcccatcg	None
CG1z	gcatgtactagttttgtcacaagatttggg	SpeI

Table 6.3.3: Human Heavy Chain Primers. The first 6 are the variable domain primers, the CONGa is for the constant domain as a control and all are 5' primers. The last primer CG1z is the 3' primer specific for the heavy chain constant region.

Each table shows a mix of 5' primers with one 3' primer. Each 5' primer from the group was used in a separate PCR reaction with the 3' primer. The reactions contained 0.4µM of each primer and 2µl cDNA template and water in a volume of 25µl. cDNA was replaced with water for a negative control. The reactions were carried out as described in chapter 2 except the denaturing step was 94°C for 15sec instead of 95°C. Light and heavy chain PCR products were pooled together and purified using Wizard PCR Preps Purification System (Promega).

6.3.4 *Digestion of Light Chains and pAbClone Vector*

4µg light chain PCR product and 8µg pAbClone was digested with SacI (140U for the PCR product and 80U for the pAbClone) and XbaI (280U for the PCR product and 80U for the pAbClone) in 10X buffer 4 (NEB), 100X BSA and water in 100µl. Digests were incubated at 37°C overnight. The pAbClone digest was gel purified and the DNA recovered from the agarose using the Wizard *Plus* DNA Purification System (Promega). The light chain digest was purified using the same kit.

6.3.5 *Ligation of Light Chains into Vector*

2µg light chain insert was ligated into 6µg pAbClone in a molar ratio of 2:1. A reaction containing the light chain insert, the pAbClone, 5X buffer (Life Technologies), 10U T4 DNA Ligase and water to a volume of 100µl was incubated at 16°C overnight. The control ligation contained no light chain insert and 1µg pAbClone, 5X buffer, 5U T4 DNA ligase and water in 50µl.

6.3.6 *Transformation into XL1-Blue E. coli*

20% of the ligation mix and control ligation was transformed into electrocompetent XL1-Blue *E. coli.* The transformation was carried out as described in chapter 2.

6.3.7 *Screening of Colonies for Light Chain Inserts*

Colonies were screened by PCR. Using primer pHEN.USP (table 6.3.4) which anneals at the 5' end of the antibody chains and the 3' constant region primers CK1d and CL2.

Primer	Sequence 5' - 3'	Primer Position
pHEN.USP	cagtcataatgaaatacctattgcctac	pelB leader
FWR3.Sense	ctgtcgacacggccgtgtattactg	3' end of framework 3

Table 6.3.4: Primers for Screening Clones. Primers for screening the library for light and heavy chains.

The PCR contained 50ng of the pHEN.USP and 0.4µM of the 3' primer and 2µl template in a reaction volume of 25µl (Chapter 2).

6.3.8 *Digestion of Heavy Chains and Light Chain/pAbClone*

2µg heavy chain and 10µg light chain pAbClone (recovered from the first electroporation) were digested with XhoI (140U for the PCR product and 100U for the light chain pAbClone) and SpeI (34U for the PCR product and 30U for the light chain pAbClone) in a reaction containing 10X buffer 2, 100X BSA and water in a volume of 100µl. The digestions were incubated at 37°C overnight. The light chain/pAbClone was gel purified.

6.3.9 *Ligation of Heavy Chains into Vector*

1µg digested heavy chain product was ligated into 6µg light chain/pAbClone in a molar ratio of 1:1. A reaction containing the insert and the vector, 5X buffer, 10U T4 DNA ligase and water in a volume of 100µl was incubated at 16°C overnight. The control ligation contained no insert, 2µg pAbClone/light chain, 5X buffer, 5U T4 DNA ligase and water in a volume of 50µl.

6.3.10 *Transformation into XL1-Blue E. coli and Screening*

20% of the ligation mix and the control ligation was transformed into XL1-Blue *E. coli* and colonies screened by PCR to determine percentage heavy chain inserts. The reaction contained 50ng FWR3.Sense (table 6.3.4) and 0.4µM CG1z in a reaction volume of 25µl.

6.3.11 *The "Einstein" Library*

The "Einstein" library was provided by Dr. C. Kowal, Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York, NY. The library has two parts, Kappa and Lambda, which are the light chains which are present in each sub-library. This library is derived from a patient with Systemic Lupus Erythematosis.

6.3.12 *Library Preparation*

To two 10ml XL1-Blue *E. coli* log phase cultures was added 10µl Einstein Kappa and Lambda primary stock library and incubated at room temperature for 15min. 10ml SB was added to each culture and incubated at 37°C for 1hr with shaking. 100µg/ml ampicillin was added and incubated at 37°C for 1hr with shaking. 1ml M13KO7 helper phage (Promega; 1 x 10^{11} pfu/ml) and 100ml SB was added and incubated for 1hr at 37°C with shaking. 70µg/ml kanamycin (Sigma) was added and incubated at 30°C overnight with shaking.

The bacterial cells were centrifuged at 6000rpm (5835g) for 15min at 4°C. 20% PEG/2.5M NaCl was added to the supernatants (5ml PEG/20ml supernatant). The phage were precipitated on ice for 30min. Precipitated phage were centrifuged at 9000rpm (13130g) for 20min at 4°C. Phage pellets were resuspended in 2ml water. These were incubated at 37°C for 5min and spun at 13200rpm (17500g) for 5min and the supernatant retained and stored at 4°C.

6.3.13 *Library Panning*

Prepared phage were diluted 1/2 in 3% skim milk/PBS/0.05% tween 20 and 100µl of this added to 1 strip of the CD20 coated ELISA plates (Chapter 2) and incubated at room temperature for 2hr with mixing. Unbound phage solution was removed and retained for titration. Wells were washed once with PBS/0.05% tween 20 and twice with PBS. Phage were eluted from the wells with the addition of 100µl elution buffer (Chapter 2). This was incubated at room temperature for 10min with mixing. This solution was neutralised by adding 2M Tris Base pH 7.4. An aliquot of this was retained for titration.

The library eluates were added to 2ml log phase XL1-Blue *E. coli* and incubated at 37°C for 30min without shaking. 10ml SB/20µg/ml carbenicillin/10µg/ml tetracycline was added and 1 and 0.1µl plated on LB/100µg/ml carbenicillin plates. The plates were incubated at 37°C overnight. The 10ml culture was incubated at 37°C for 1hr with shaking. This culture was added to 100ml SB/50µg/ml carbenicillin/10µg/ml tetracycline and incubated at 37°C for 1hr with shaking. 100µl M13KO7 helper phage (10^{10} pfu) were added and incubated at 37°C for 2hr with shaking. 70µg/ml kanamycin was added and incubated at 30°C overnight with shaking. Phage were recovered as described above. Library panning was repeated 3 times.

After the last panning the eluted phage were used to infect 2ml log phase XL1-Blue *E. coli* at 37°C for 30min with no shaking. To this was added 10ml SB/20µg/ml carbenicillin/10µg/ml tetracycline and 1 and 0.1µl plated on LB/100µg/ml carbenicillin plates. The 10ml culture was incubated at 37°C for 1hr with shaking. This culture was added to 100ml SB/50µg/ml carbenicillin/10µg/ml tetracycline and incubated at 37°C overnight. The phage vectors were recovered by spinning the overnight culture at 5000rpm (4052g) for 10min at 4°C. The plasmids were recovered.

6.3.14 *Phage Titration*

Phage were collected after every step and were stored at 4°C. The phage from the panning was titred to determine phage numbers during the panning experiment. 180µl log phase XL1-Blue was added to wells in a 96 well plate. 20µl phage was added to the bacteria and was incubated at room temperature for 30min. Serial 1/10 dilutions were made of these phage in SB to a dilution of 10^{-12} and 20µl of the dilutions from 10^{-4} to 10^{-10} were spotted onto LB/100µg/ml carbenicillin plates and dried under a bunsen burner. The plates were incubated at 37°C overnight.

6.4 *Results*

6.4.1 *Construction of a Fab Phage Display Library*

The light chains (figure 6.4.1) were digested and inserted into the pAbClone vector. 3.9×10^4 bacterial cells were transformed with light chain library. Colonies from this library were screened for the presence of light chains. Of 30 colonies screened 19 were positive for kappa light chains and 2 were positive for lambda light chains. The light chain library was retransformed to try to increase the library size and these libraries named LC1 and LC2. The LC1 library size was 9.12×10^4 and the LC2 library size was 8.62×10^4. From 10 colonies screened from each library the LC1 library had 80% kappa and lambda chains and LC2 had 70% kappa chains (figure 6.4.2).

The pAbClone/Light chain vector and the heavy chain insert were digested and ligated together. After transformation a library of 2.63×10^5 was produced. These colonies were screened for the presence of the heavy chain. Of 10 colonies screened 60% had heavy chains. To see if the light chain ratio was the same as calculated above the light chains were also screened for. The number of light chains in the library was 50%.

From a library of 2.63×10^5, 60% of colonies screened had heavy chains and 50% of colonies screened had light chains bringing the actual library size to 7.89×10^4. When this library was to be used and transformed into bacteria again no colonies grew on the screening plates.

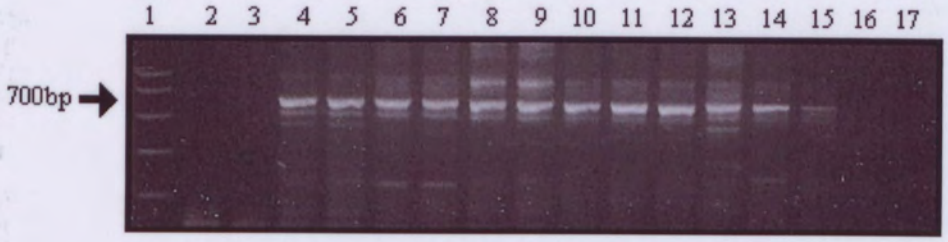
The library was not able to be transformed into bacteria reliably, with adequate numbers of light and heavy chain inserts so it was not used.

6.4.2 *Library Panning*

The "Einstein" kappa and lambda libraries were screened in an effort to isolate antibodies which bound the CD20 peptide. Libraries were panned against the CD20 antigen over 4 rounds and aliquots from each step of library panning collected for phage titration at the completion of the panning.

The phage titration showed that no detectable levels of phage were eluted during any round of panning. This may have been because the dilutions carried out in the phage titration were too

a)



b)

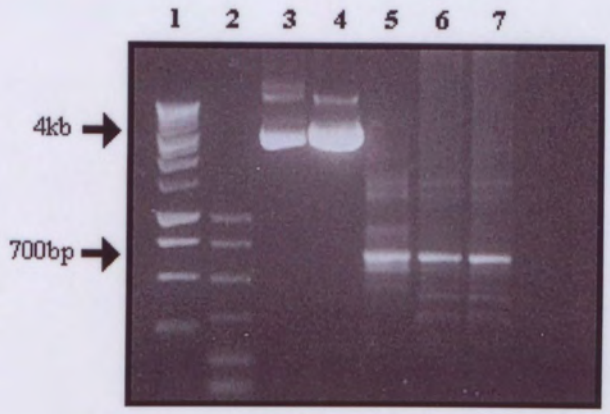


Figure 6.4.1: Generation of Light Chains and Preparation for Library Construction. Figure a) shows the PCR amplification of lambda light chains using the primers in table 6.3.2. Lane 1 is 100bp DNA ladder; Lane 2 water control; Lanes 4 – 17 amplification of tonsil lymphocyte cDNA with the 7 primer pairs in duplicate. Figure b) shows the digestion of pAbClone and pooled kappa and lambda light chains. Lane 1 is 1kb DNA ladder; Lane 2 is 100bp DNA ladder; Lane 3 undigested pAbClone; Lane 4 SacI and XbaI digested pAbClone; Lane 5 undigested pooled light chains; Lanes 6 & 7 SacI and XbaI digested pooled light chains.

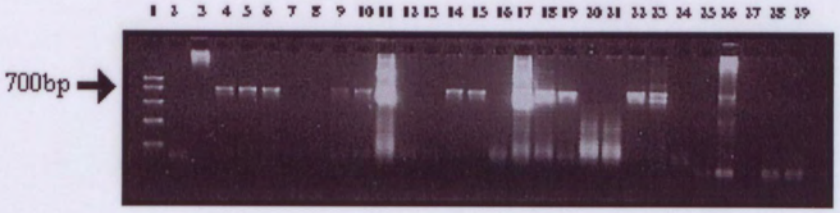


Figure 6.4.2: Screening Colonies for Light Chain Inserts. Library colonies were screened with pHEN.USP and CK1d and CL2 for presence of light chains.

large. There were adequate titres for each round of phage applied to the plates for panning. Since there were no elution values no relative yields could be determined. No CD20 antibodies were isolated from this library.

6.5 *Discussion*

The construction of a Fab antibody phage display library was successful but its small size indicated that its usefulness would be limited. A library with 2.63 x 10^5 clones was produced and was screened for the presence of light and heavy chains. 50% of the colonies in the library had a light chain and 60% had heavy chains and this reduced the actual size of the library to 7.89 x 10^4.

The problems which were encountered in the construction of the phage display library originally stemmed from faulty dNTPs. Once this was discovered and they were changed a dramatic improvement in the quality of the PCR products was observed. In all 1.5 years were spent preparing libraries and despite this effort had to be discontinued.

Problems were also experienced in producing a library of large size. Contributing factors to this could have been the preparation of the electrocompetent cells, limitations of the ligations and sub-optimal DNA concentrations. When electrocompetent *E. coli* were made in the laboratory the transformation efficiency was usually about 5 x 10^8 cells transformed with 1μg pUC19. The transformation efficiency of the *E. coli* will be reduced 100-fold when they are transformed with freshly ligated library construct instead of pUC19. Even if every cell was transformed with an intact construct, with both light and heavy chains present, the library would not be greater than 5 x 10^6 using these cells. Increased transformation efficiency relies on removal of salt from these cells by extensive washing with 10% glycerol, limited handling time and low temperatures during handling (4°C). Some claim that transformation efficiencies of 10^{10} - 10^{11}/μg pUC19 can be reached (Thiel *et al.*, 1999) and this would be conducive to large library sizes. Most companies now sell electrocompetent cells with transformation efficiencies of at least 5 x 10^9.

The size of the phage display libraries described today are at least 10^7 - 10^8 (Barbas *et al.*, 1992a; Meulemans *et al.*, 1994; Bender *et al.*, 1994; de Kruif *et al.*, 1995; Pilkington *et al.*, 1996) and more commonly now libraries of 10^{10} - 10^{11} are being constructed (Griffiths, *et al.*, 1994; Vaughan, *et al.*, 1996; de Haard *et al.*, 1999). Larger libraries increase the possibility of isolating high affinity antibodies against a wider range of antigen as they are becoming closer in size to the human antibody repertoire (approximately 10^{12}).

Panning the kappa and lambda Einstein libraries yielded no CD20 specific antibodies. The phage retained from each step of the panning was titred at the conclusion of the panning and although there were always phage to be applied for each panning step phage were not detected to be binding the CD20 peptide in the eluted aliquots. Spiking a library with an antibody known to bind CD20 would make it possible to determine the efficiency of the phage display library panning technique. Traditionally phage display panning has been carried out on purified antigen bound to plates but more recently new methods of panning have been described. Whole cells (Watters *et al.*, 1997), whole cells fixed to plates (Schmidt *et al.*, 1999), tissue sections (Tordsson *et al.*, 1997) and whole organs *in vivo* (Pasqualini and Ruoslahti, 1996) have all been used successfully as surfaces for panning phage display libraries, showing that the antigen does not need to be purified or in an uniform protein mix even though it would be easier.

Although there have been many antibodies isolated from phage display libraries there are some technical difficulties associated with this technique. 1) Isolation of high affinity antigen-specific phage relies upon selection against antigen bound on a surface. This is dependant on the antigen being bound in a way which allows antibody binding to exposed epitopes and the conditions of the surface on which the interaction is to take place (Francisco and Georgiou, 1994). 2) Enriched phage must infect *E. coli* to be recovered. This infection is prone to contamination with wild-type phage which would grow faster and would be selected over recombinant phage (Francisco and Georgiou, 1994). 3) Specific antigen binding phage must be eluted from the antigen using reagents which can be detrimental to phage viability (Francisco and Georgiou, 1994). 4) Phage are relatively unstable, are inclined to lose the antibody from their surface and are poor at packaging the phagemid DNA.

There are two new methods described that could be used as alternatives to phage display technology. They are bacterial surface display and ribosomal display (reviewed in chapter 1). These may offer advantages in future work on CD19 and CD20.

Chapter 7

Construction and Analysis of a ScFv Antibody Against CD20

Chapter 7

Construction and Analysis of a ScFv Antibody Against CD20

7.1 *Summary*

This chapter describes the construction and characterisation of a single chain Fv antibody fragment which binds to CD20, an antigen on the surface of B lymphocytes. The parent antibody is expressed by the hybridoma, HB13d, and is confirmed to bind Raji, HRIK and tonsil B lymphocytes which all express the target molecule, CD20, on their surface. The method used to construct the scFv was described by Krebber *et al.* (1997) and involves the extraction of mRNA from the hybridoma, generation of cDNA and PCR amplification of heavy and light chain genes using a large panel of primers designed to amplify a wide range of murine V_L and V_H sequences. The products of the PCR are then linked together in a splice overlap extension reaction and cloned using SfiI , a rare cutting enzyme, into one of the 6 specialised vectors provided. The unique qualities of these vectors allow for either phage display, enhanced expression or a variety of purification and detection systems. Initially the scFv was cloned into the most simple expression vector, pAK100, for screening of clones producing single chain Fv protein, but was later transferred to pAK400 and pHB400. pAK400 is specifically designed for increased expression of the scFv and pHB400 is a variation of the pAK400 vector which includes a cassette which allows expression of chaperone proteins in the periplasm, simultaneously with the scFv. The production conditions of the scFv were investigated for efficient scale up from flask cultures to a fermenter. The best conditions for the production of the CD20 scFv were growth without adding glucose to the TB medium. Protein concentration was determined using OD_{280nm}. The amount of scFv produced from the fermentation was approximately 10mg from an initial 11L of culture.

The scFv, HB13d-7, showed strong binding in an ELISA against CD20 peptide. In flow cytometry the scFv bound a range of CD20 expressing cell lines and the B cell population of peripheral blood lymphocytes but not Jurkat cells, a T cell line. The scFv was shown to effectively block a commercial CD20 monoclonal antibody, Leu16, and was blocked in the reverse reaction. It also competed effectively with the monoclonal antibody for binding to cells

expressing CD20. Molecular modelling of the scFv allowed the 3-dimensional structure of the antibody to be predicted from its amino acid sequence. A comparison of the scFv with two mutant scFvs, HB13d-4 and HB13d-11, revealed that some of the mutations were positioned in the region where the antibody would contact antigen. This may explain the different binding properties of the 3 single chains. These differences were assessed by ELISA which showed the HB13d-4 and HB13d-11 to have affinities at least 13-fold and 4-fold less, respectively than the HB13d-7. BIAcore analysis of the HB13d-7 scFv showed binding to the CD20 peptide which was immobilised on a streptavidin sensor chip. The scFv, HB13d-7, described here has been shown to bind well to its target and has the potential to be developed into a useful tool for the therapy or diagnosis of B cell malignancy.

7.2 *Introduction*

7.2.1 *Antibody Fragments in Medicine*

Single chain antibodies have emerged as potentially useful diagnostic and therapeutic reagents for diseases such as AIDS (Marasco, 1995), Hepatitis B (Sanchez *et al.*, 1999), pertussis (Williamson and Matthews, 1999) and malignant tumours like B cell leukemia/lymphoma (Nicholson *et al.*, 1997) and melanoma (Zhang *et al.*, 1995).

Although recently there have been antibodies found in camels in which the variable heavy region is the only chain required for binding (Hamers-Casterman *et al.*, 1993), previously the Fv fragment (Chapter 1) was believed to be the smallest fragment of an antibody with effective binding ability. The non-covalently linked domains do not require glycosylation making them ideal for production in a bacterial expression system, although assembly of these fragments during expression by bacteria is poor and usually results in dissociation of the two chains into the smaller domains. Single chain Fv fragments have now been constructed which consist of the two domains, VH and VL, joined together by a flexible linker of amino acids which is designed to not interfere with the binding site of the fragment (figure 1.1).

Some single chain Fv antibodies previously constructed have had linker lengths of 15aa (Nicholson *et al.*, 1997; Shan *et al.*, 1999) and 20aa (Krebber *et al.*, 1997) which is the size of the linker used for the construction of the CD20 antibody. The effect of linker length on binding of various scFv fragments has been described (Filpula *et al.*, 1996; Shan *et al.*, 1999), with the most appropriate length appearing to depend on the particular scFv. Shan *et al.* (1999) described the construction of one single chain Fv with linker lengths of 0, 5, 10 and 15aa. These scFvs were fused to an immunoglobulin hinge-CH2-CH3 domain and binding comparisons showed the 1F5 scFv, anti-CD20, with a linker of 5aa bound CD20 expressing cells best. Investigation of the effect linker length has on scFv properties has shown that shortened linker lengths often cause scFv dimerisation (Whitlow *et al.*, 1991; Huston *et al.*, 1991; Stemmer *et al.*, 1993; Holliger *et al.*, 1993). Whether fusion to the immunoglobulin region prevented it, the 1F5 scFvs presented only as monomers and any dimerisation was explained by association of the CH3 domain of the Ig tail (Shan *et al.*, 1999). The $(Gly_4Ser)_4$ linker used for the construction of HB13d scFv was initially described by Huston *et al.* (1988),

but the use of serine rich linkers (Dorai *et al.*, 1994) and threonine, lysine and glutamic acid (Filpula *et al.*, 1996) may improve solubility. An alternative to the polypeptide chain as a linker is the use of a disulphide bond which can be engineered between structurally conserved framework regions of the heavy and light chains (Brinkmann *et al.*, 1993). These bonds are more rigid than a scFv polypeptide linker and the positioning of the bond must be determined by structural analysis of the light and heavy chains.

The superior avidity of bivalent molecules over monomers has encouraged the design of bivalent antibody fragments. These can be formed by shortening the normal polypeptide linker so that the two chains cannot associate and must form a partnership with another single chain (Holliger *et al.*, 1993). Trimers can be produced by completely removing the linker so fused products associate in a similar fashion to those described above (Iliades *et al.*, 1997). These molecules have none of the flexibility which is associated with having a linker. Miniantibodies have also been described (Pack and Pluckthun, 1992; Pack *et al.*, 1993) which are joined using flexible helical bundle linkers. These constructs are about the size of Fab fragments and have been shown to have a higher avidity than molecules constructed with a leucine zipper (Pack and Pluckthun, 1992).

There are both advantages and disadvantages in the use of single chain antibodies in diagnostics or therapy over whole antibodies. The advantages of using scFvs are that they have more uniform solid tumour penetration (Yokota *et al.*, 1992) and they have a shorter half life in the circulation (Colcher *et al.*, 1990) which can be useful for imaging work. The scFv short half life means they will not persist in non tumour tissue and damage non malignant tissue which is especially relevant if they have been conjugated to a cell killing agent. These small fragments are easily produced using bacterial expression which can allow larger amounts of protein to be expressed than in mammalian systems. The culture of bacteria and the reagents used to express the protein are also cheaper and faster than mammalian cell culture.

Most monoclonal antibodies are murine. When these antibodies have been used in therapy some have caused an antibody response in patients. This human anti mouse antibody (HAMA) response is responsible for limiting the usefulness of these antibodies for continued therapy of these patients. The development of chimeras and humanised antibodies has improved the potential of these antibodies in therapy but the use of fragments of whole mouse antibodies has been described as an alternative, since the constant regions of antibodies are more

immunogenic. No patients administered with an anti-CEA scFv antibody during clinical trial produced anti-mouse responses against the treatment (Begent *et al.*, 1996). This indicates that variable regions and the linker are better tolerated than the constant regions of the molecule.

7.2.2 *scFv Preparation from Hybridomas*

An effective method for producing scFv fragments from hybridomas has been described by a group from Switzerland headed by Andreas Pluckthun (Krebber *et al.*, 1997). Their method involves the use of a large set of degenerate primers which cover a wide range of the murine antibody repertoire. SfiI, which recognises eight bases interrupted by five non recognised bases, is the enzyme used for cloning. SfiI sites are very rare in antibody sequences which makes it an ideal enzyme for cloning these fragments. This group also designed a number of vectors for use in different applications. Of these pAK100 and pAK400 were used during this chapter. These vectors have a number of qualities including 1) chloramphenicol resistance 2) origin of replication for both bacteriophage and *E. coli* 3) a tetracycline resistance gene to monitor SfiI digestion 4) pelB leader sequence to direct the single chain Fv to the periplasm 5) a truncated version of gene III 6) a strong t_{HP} terminator 7) lacI repressor gene 8) the lac promoter/operator which works with glucose repression of the lac promoter to completely suppress background expression of the protein before induction and 9) an amber stop codon upstream from the gene III sequence in pAK100. There are modifications between the 6 vectors described by Krebber *et al.* (1997) which should be considered before use.
Recently in the CHRI laboratory the pAK400 vector was modified by merging the cloning site of pAK400 with pHB110 (Bothmann and Pluckthun, 1998). The vector was called pHB400 (Mavrangelos *et al.*, in preparation). The skp vector coexpresses chaperone proteins which aid in the folding of the scFv protein. Using this vector, more efficient protein production may be achieved due to a higher percentage of protein being folded correctly.

The target for the scFv is CD20, which is described in detail in chapter 1. A chimeric antibody against CD20, Rituximab (Reff *et al.*, 1994; Coiffier *et al.*, 1998) has recently been approved by the FDA (USA) for use in relapsed, low-grade Non Hodgkin's lymphoma. This antibody has shown good results in clinical trial in spite of being administered to patients relapsed from

drug therapy (Nguyen *et al.*, 1999). Progression to disease after treatment with Rituximab occurs in a median time of 10.2 months (Maloney *et al.*, 1997). This chapter describes the construction and characterisation of a scFv against CD20 which may provide an alternative treatment for B lymphocyte malignancies.

7.3 *Materials*

7.3.1 *pAK Vectors*

The vectors used for cloning the scFv fragment were provided by Dr. Andreas Pluckthun (Biochemisches Institut der Universitat Zurich, Switzerland; Krebber *et al.*, 1997). Figure 7.3.1 shows the pAK vectors, pAK100 and pAK400, which are used in this chapter. pAK100 is the basic cloning vector. pAK400 has been designed by replacing the original Shine Dalgarno sequence with a stronger SDT7g10 (Krebber *et al.*, 1997) for enhanced protein expression and the cmyc tag of the pAK100 has been replaced by a polyhisititidine tag for IMAC purification. Figure 7.3.2 shows the pHB400 vector. This is essentially the same as the pAK400 except for the addition of the skp cassette (Bothmann and Pluckthun, 1998; Mavrangelos *et al.*, in preparation).

7.3.2 *Sequencing Primers*

4 primers were designed for sequencing. Two were designed on either side of the scFv insert, one in the pelB leader sequence, pAK100.USP, and the other in gene III, pAK100.RSP (Table 7.3.1).

Two were designed inside the scFv in the linker region called pAK.LINK.VL and pAK.LINK.VH (Table 7.3.1). Later another primer pAK.LINK.VH2 was designed as the pAK.LINK.VH was not binding specifically to the linker region.

7.3.3 *Cell Lines and Maintenance*

The cell lines used for flow cytometry were Namalwa (Burkitt's Lymphoma cell line), REH (Pre B cell line) and Jurkat (T cell line). All were maintained in RF10 medium.

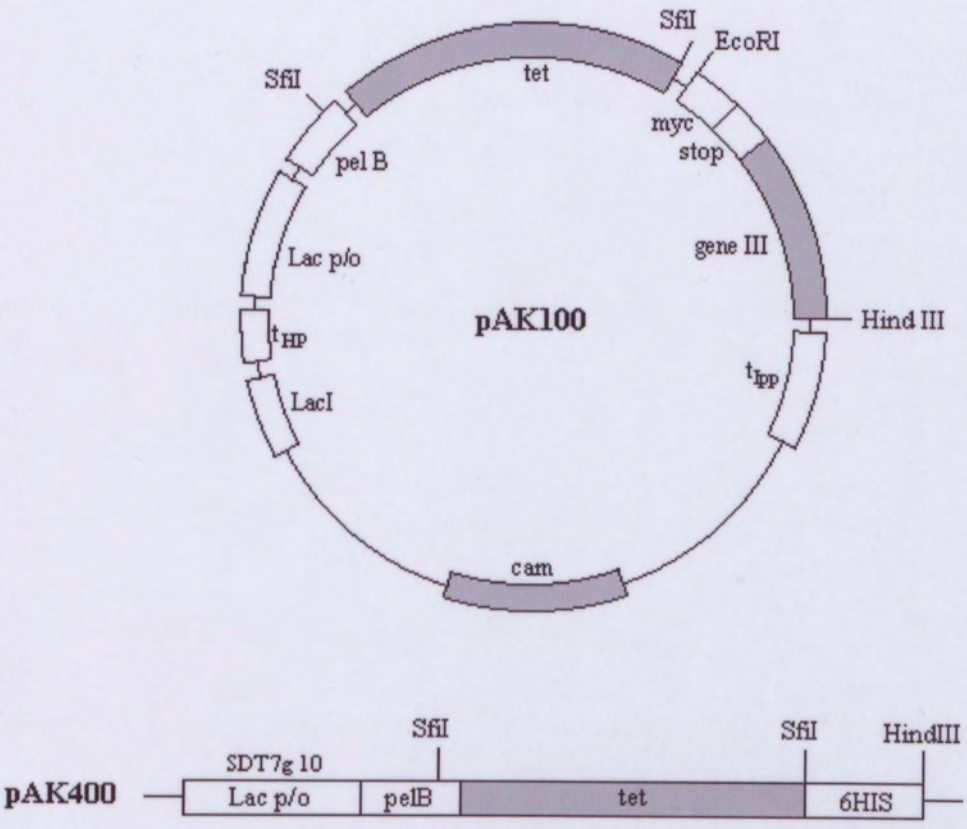


Figure 7.3.1: pAK Vectors.

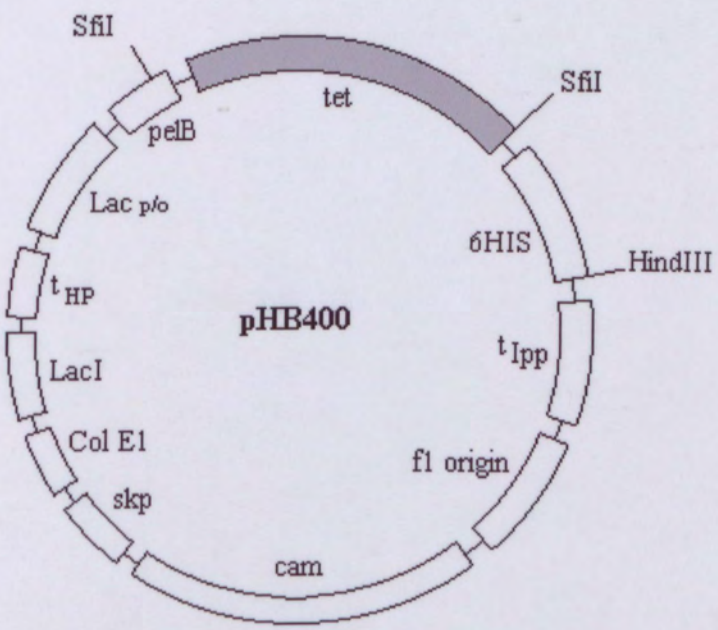


Figure 7.3.2: pHB400 Vector.

Primer Name	Primer Sequence 5' - 3'	Primer Location
pAK100.USP	atgaaatacctattgcct	pAK100, 5' of the scFv insert site, in the pelB leader sequence
pAK100.RSP	ttcataatcaaaatcacc	pAK100, 3' of the scFv insert site, in geneIII
PAK.LINK.VL	accaccaccggagccgcc	Linker
PAK.LINK.VH	ggtggtggtggttctggt	Linker
PAK.LINK.VH2	tctggtggtggtggttctgg cggcggcggctcc	Linker

Table 7.3.1: Sequencing Primers.

7.4 *Methods and Results*

7.4.1 *CD20 Parent Hybridoma*

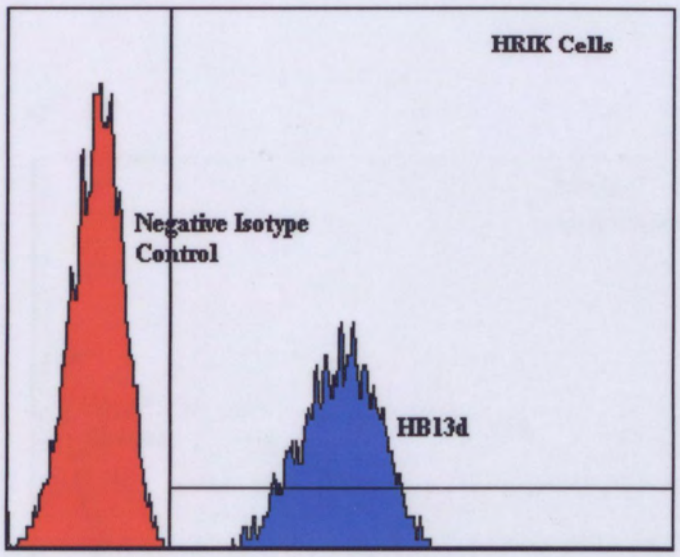
The hybridoma HB13d, provided by Dr. Thomas F. Tedder (Duke University, NC), was used for the construction of the anti-CD20 scFv. At the time of the mRNA extraction the hybridoma was producing antibody which was shown by flow cytometry to bind to Raji, HRIK and freshly prepared tonsil B lymphocytes (Figure 7.4.1), all which are CD20 positive.

7.4.2 *Construction of a ScFv Antibody Against CD20*

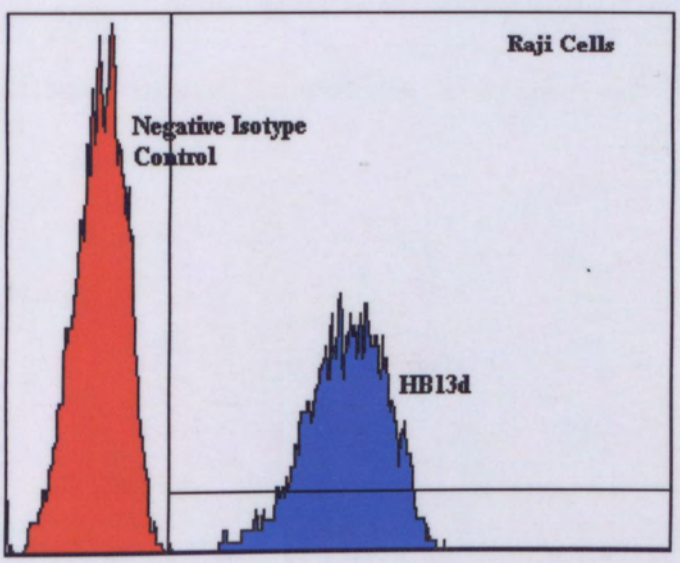
20µg of HB13d mRNA was used to generate cDNA. PCR amplification of cDNA was carried out as described by Krebber *et al.* (1997). The scFv primers used are shown in figure 7.4.2. For amplification of the light chain LB (LB1-LB17+LBλ) and LF (LF1-LF5+LFλ) primers were used and for amplification of the heavy chain HB (HB1-HB19) and HF (HF1-HF4) primers were used. Reactions containing 0.2mM dNTPs, 2µM of either HF and HB primer mix or LF and LB primer mix, 5X ELONGASE buffer B (Life Technologies), 2µl cDNA and water in a volume of 49µl and mineral oil were run in the DNA Thermal Cycler 480 (Perkin Elmer). After 3min denaturation at 92°C, 1U ELONGASE enzyme was added. This was followed by 7 cycles of 92°C for 1min, 63°C for 30sec, 58°C for 50sec and 72°C for 1min. Another 25 cycles of 92°C for 1min, 63°C for 30sec and 72°C for 1min was followed by a 4°C soak file at the completion of the reaction. PCR amplification of variable heavy and variable light chains showed bands of approximately 400bp (figure 7.4.3).

V_L and V_H PCR products were purified by phenol chloroform precipitation (Chapter 2). DNA pellets were resuspended in 20µl of water. 2 SOE reactions were set up. One containing 50ng of both the light and heavy purified PCR products and another containing 125ng of each product. A reaction mix containing the PCR products, 0.2mM dNTPs, 5X ELONGASE buffer B, 2U ELONGASE enzyme and water in a volume of 100µl was incubated in a DNA Thermal Cycler 480 at 92°C for 3min and cycled for 2 cycles of 1min at 92°C, 30sec at 63°C, 50sec at 58°C and 1min at 72°C before adding 1µM of scfor/scback (figure 7.4.2) mix to each tube at a ratio of 1:1. The cycle used for the SOE was 92°C for 2min and then 5 cycles of 1min at 92°C,

a)


HRIK Cells
Negative Isotype Control
HB13d

b)


Raji Cells
Negative Isotype Control
HB13d

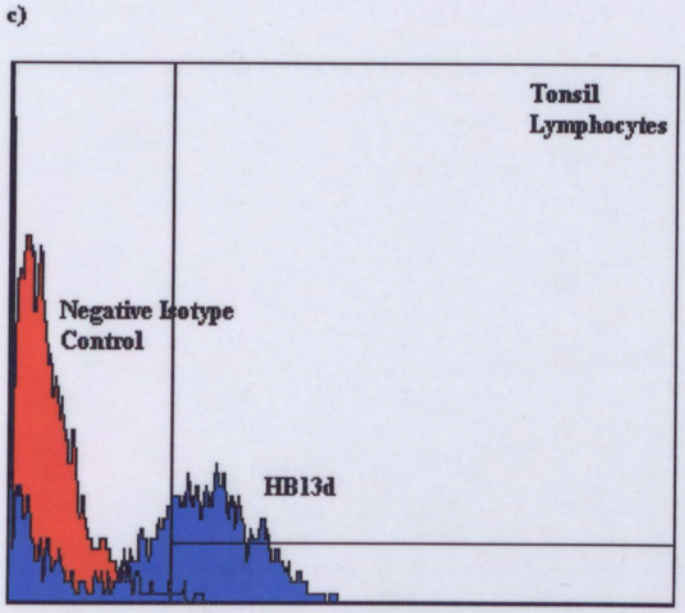


Figure 7.4.1: HB13d Monoclonal Antibody Binding to CD20 Positive Cells.

Primer VL Backwards

	5' Flag VL 3'
scback	ttactcgcggccccagccggccatggcggactacaaaG
	5' Flag VL 3'
LB1	gccatggcggactacaaaGAYATCCAGCTGACTCAGCC
LB2	gccatggcggactacaaaGAYATTGTTCTCWCCCAGTC
LB3	gccatggcggactacaaaGAYATTGTGMTMACTCAGTC
LB4	gccatggcggactacaaaGAYATTGTGYTRACACAGTC
LB5	gccatggcggactacaaaGAYATTGTRATGACMCAGTC
LB6	gccatggcggactacaaaGAYATTMAGATRAMCCAGTC
LB7	gccatggcggactacaaaGAYATTCAGATGAYDCAGTC
LB8	gccatggcggactacaaaGAYATYCAGATGACACAGAC
LB9	gccatggcggactacaaaGAYATTGTTCTCAWCCAGTC
LB10	gccatggcggactacaaaGAYATTGWGCTSACCCAATC
LB11	gccatggcggactacaaaGAYATTSTRATGACCCARTC
LB12	gccatggcggactacaaaGAYRTTKTGATGACCCARAC
LB13	gccatggcggactacaaaGAYATTGTGATGACBCAGKC
LB14	gccatggcggactacaaaGAYATTGTGATAACYCAGGA
LB15	gccatggcggactacaaaGAYATTGTTGATGACCCAGWT
LB16	gccatggcggactacaaaGAYATTGTGATGACACAACC
LB17	gccatggcggactacaaaGAYATTTTGCTGACTCAGTC
LBλ	gccatggcggactacaaaGATGCTGTTGTGACTCAGGAATC

Primer VL forwards

	5' $(Gly_4Ser)_3$-linker VL 3'
LF1	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACGTTTGATTTCCAGCTTGG
LF2	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACGTTTTATTTCCAGCTTGG
LF4	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACGTTTTATTTCCAACTTTG
LF5	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACGTTTCAGCTCCAGCTTGG
LFλ	**ggagccgccgccgcc**(agaaccaccaccacc)$_2$ACCTAGGACAGTCAGTTTGG

Figure 7.4.2a. scFv Light Chain Primers. Adapted from Krebber *et al.* (1997).

Primer VH backwards

	5' (Gly$_4$Ser)$_3$-linker BamH1 VH 3'
HB1	ggcggcggcggctccggtggtggtggatccGAKGTRMAGCTTCAGGAGTC
HB2	ggcggcggcggctccggtggtggtggatccGAGGTBCAGCTBCAGCAGTC
HB3	ggcggcggcggctccggtggtggtggatccCAGGTGCAGCTGAAGSASTC
HB4	ggcggcggcggctccggtggtggtggatccGAGGTCCARCTGCAACARTC
HB5	ggcggcggcggctccggtggtggtggatccCAGGTYCAGCTBCAGCARTC
HB6	ggcggcggcggctccggtggtggtggatccCAGGTYCARCTGCAGCAGTC
HB7	ggcggcggcggctccggtggtggtggatccCAGGTCCACGTGAAGCAGTC
HB8	ggcggcggcggctccggtggtggtggatccGAGGTGAASSTGGTGGAATC
HB9	ggcggcggcggctccggtggtggtggatccGAVGTGAWGYTGGTGGAGTC
HB10	ggcggcggcggctccggtggtggtggatccGAGGTGCAGSKGGTGGAGTC
HB11	ggcggcggcggctccggtggtggtggatccGAKGTGCAMCTGGTGGAGTC
HB12	ggcggcggcggctccggtggtggtggatccGAGGTGAAGCTGATGGARTC
HB13	ggcggcggcggctccggtggtggtggatccGAGGTGCARCTTGTTGAGTC
HB14	ggcggcggcggctccggtggtggtggatccGARGTRAAGCTTCTCGAGTC
HB15	ggcggcggcggctccggtggtggtggatccGAAGTGAARSTTGAGGAGTC
HB16	ggcggcggcggctccggtggtggtggatccCAGGTTACTCTRAAAGWGTSTG
HB17	ggcggcggcggctccggtggtggtggatccCAGGTCCAACTVCAGCARCC
HB18	ggcggcggcggctccggtggtggtggatccGATGTGAACTTGGAAGTGTC
HB19	ggcggcggcggctccggtggtggtggatccGAGGTGAAGGTCATCGAGTC

Primer VH forwards

	5' EcoRI 3'
scfor	ggaattcggccccgag
	5' EcoRI VH 3'
HF1	ggaattcggccccgaggccCGAGGAAACGGTGACCGTGGT
HF2	ggaattcggccccgaggccCGAGGAGACTGTGAGAGTGGT
HF3	ggaattcggccccgaggccCGCAGAGACAGTGACCAGAGT
HF4	ggaattcggccccgaggccCGAGGAGACGGTGACTGAGGT

Figure 7.4.2b. scFv Heavy Chain Primers. Adapted from Krebber *et al.* (1997).

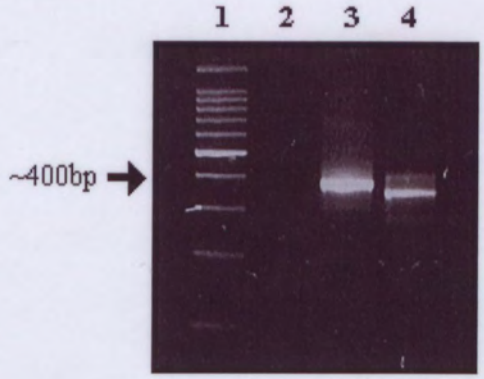


Figure 7.4.3: PCR Amplification of Variable Light and Heavy Chain Genes. Lane 1 is 100bp DNA ladder; Lane 2 is water control; Lane 3 is the light chain; Lane 4 is the heavy chain. Primers were HB and HF mixes for the heavy chain and LB and LF mixes for the light chain.

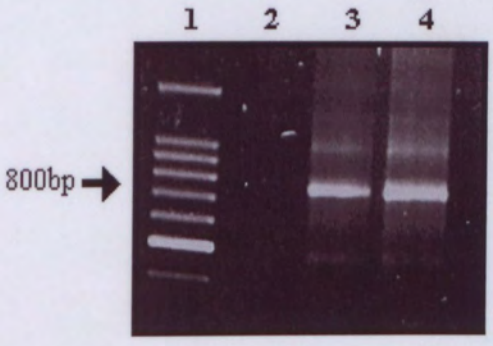


Figure 7.4.4: Splice Overlap Extension PCR of Light and Heavy Chains. Lane 1 is 100bp DNA ladder; Lane 2 is water control; Lane 3 & 4 are the scFv product of the SOE reaction. Primers were scfor/scback mix.

30sec at 63°C, 50sec at 58°C and 1min at 72°C and then 23 cycles of 1min at 92°C, 30sec at 63°C and 1min at 72°C. The SOE reaction produced bands of 800bp (figure 7.4.4). SOE PCR products were gel purified.

2µg SOE product and 1µg pAK100 vector were digested with 20U SfiI (New England Biolabs) in 10X Buffer 2 in a volume of 50µl at 50°C overnight. 1.25µg of SfiI digested SOE product was ligated into 1.2µg of SfiI digested pAK100 in a molar ratio of 1:7. A reaction containing the SOE product and pAK100, using 2U T4 DNA Ligase, 5X ligase buffer (Promega) and water in a volume of 50µl was incubated at 16°C overnight. The control for this experiment was the pAK100 vector with no insert. 10% of the ligation mix was used to transform 70µl electrocompetent *E. coli* HB2151 cells and water in a volume of 275µl. The electroporation mix was incubated in 1ml of SOC media at 37°C for 1hr with shaking. 200µl was plated onto LB/1% glucose/25µg/ml chloramphenicol plates and incubated at 37°C overnight.

Colonies from the transformation were screened for production of scFv. Colonies were picked into 1ml 2YT/1% glucose/25µg/ml chloramphenicol and incubated at 37°C for 5hr. 200µl from each of these cultures was used to inoculate 3ml of 2YT/25µg/ml chloramphenicol and incubated at 37°C for 4hr with shaking. When the colonies reached log phase 1mM IPTG was added and the cultures were incubated at room temperature overnight with shaking. 1ml of the overnight cultures was centrifuged at 13000rpm (17000g) for 5min and 100µl supernatant screened in a slot blot assay. 100µl 1/200 FMC63 scFv concentrate (Nicholson *et al.*, 1997) was used as a positive control. The nitrocellulose membrane was incubated with 1/2 9E10 (Evan *et al.*, 1985), which binds to the cmyc tag, as the primary antibody and 1/1000 HRP conjugated anti mouse Ig (Silenus) as the secondary antibody. From 87 colonies screened, 7 showed production of protein in a slot blot (figure 7.4.5) indicated by 9E10 antibody binding to the cmyc tag on the protein.

The 7 clones were tested by flow cytometry for binding to Raji cells. 100µl scFv supernatant was used as the primary antibody followed by 50µl 9E10 supernatant, 50µl 1/50 biotinylated anti-mouse and 50µl 1/50 SAPE. 2 clones, HB13d-7 and HB13d-11, were shown to bind these cells. The sequences of these 2 scFvs and one scFv, HB13d-4, which didn't bind Raji cells were determined using the BigDye Terminator Cycle Sequencing Ready Reaction Kit with the primers pAK100.USP, pAK100.RSP, pAK.LINK.VL and pAK.LINK.VH2 (figure

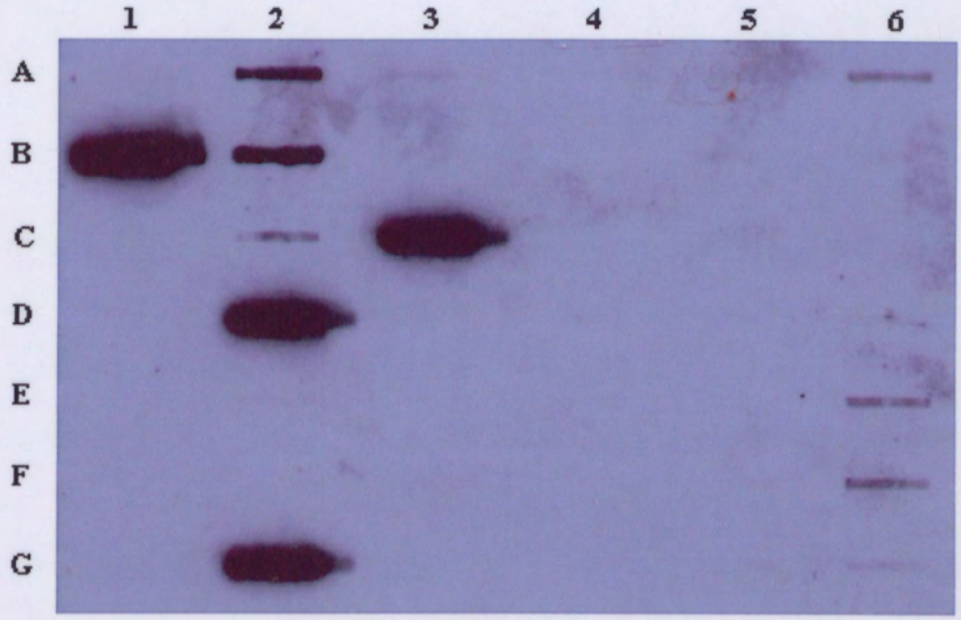


Figure 7.4.5: Slot Blot Screening for Colonies Expressing scFv Protein. Slot B1 is 1/200 positive control scFv, FMC63 scFv; Other slots are bacterial cell supernatants; Slot D2 is HB13d-4; Slot G2 is HB13d-7; Slot C3 is HB13d-11. The nitrocellulose filter was incubated with ½ 9E10 (anti-cmyc) and HRP conjugated anti-mouse Ig.

7.4.6). The translation of the nucleotide sequence to amino acid sequence can be seen in figure 7.4.7. This figure also shows the differences between the three sequences (bold amino acids). Many of the mutations are in the framework regions. The deletion in the linker is interesting but not surprising due to the repetitive nature of the linker. The CDRs H1 and H2 both have mutations that may affect binding.

7.4.3 *ELISA*

Primary antibody, 100µl of neat purified antibody control or bacterial or cell culture supernatant was applied to wells and 100µl 1/3 serial dilutions made for each sample to a 1/2187 dilution. The secondary antibody was 100µl 1/1000 monoclonal anti-polyhistidine clone HIS-1 antibody (Sigma). The tertiary antibody was 100µl 1/1000 alkaline phosphatase conjugated anti mouse Ig (Silenus). The substrate was p-nitrophenyl phosphate (Sigma) in diethanolamine buffer. The HB13d-7 scFv bound the CD20 peptide in an ELISA as shown in figure 7.4.8.

7.4.4 *Cellular Specificity of CD20 scFv*

The CD20 scFv was tested on HRIK, Namalwa, REH and Jurkat cell lines (table 7.4.1). The CD20 scFv bound to HRIK, REH and Namalwa cell lines as demonstrated by the mean fluorescence intensity values for the CD20 scFv being higher than those of X63, the negative control. The T cell line (CD20 negative), Jurkat, was not bound by the CD20 scFv as seen by the MFI value being similar to the negative control, X63. The other cell lines showed CD20 scFv binding at higher levels than the positive control, B-Cl.

Figure 7.4.9 shows CD20 scFv reactivity with the lymphocyte population of peripheral blood lymphocytes. These cells were also stained with CD19, a B cell marker, and CD3, a T cell marker. Figure 7.4.9c shows gating (gate A) on the peripheral blood lymphocytes; figure 7.4.9d shows another gate on the CD19 positive cells (gate J; B lymphocytes). Figure 7.4.9e is the population gated with A and J which shows that almost all cells stained with CD19 are also stained with CD20. This is also the case with the positive control CD20 whole antibody (figure 7.4.9f). Figures 7.4.9g and h show the peripheral blood lymphocytes stained with CD3 FITC and the CD20 monoclonal antibody or the CD20 scFv. These figures show some

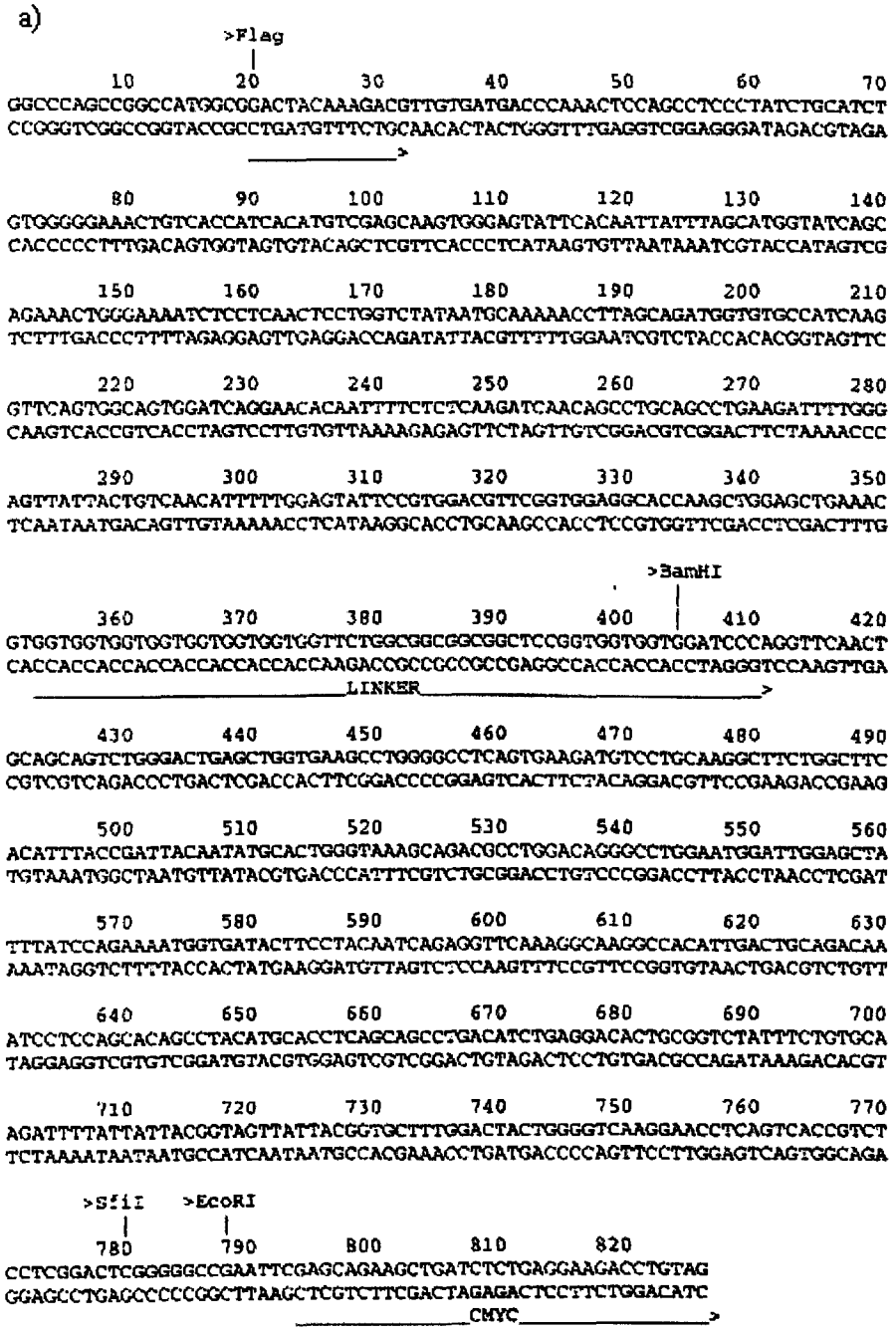


Figure 7.4.6a: HB13d-7 Sequence.

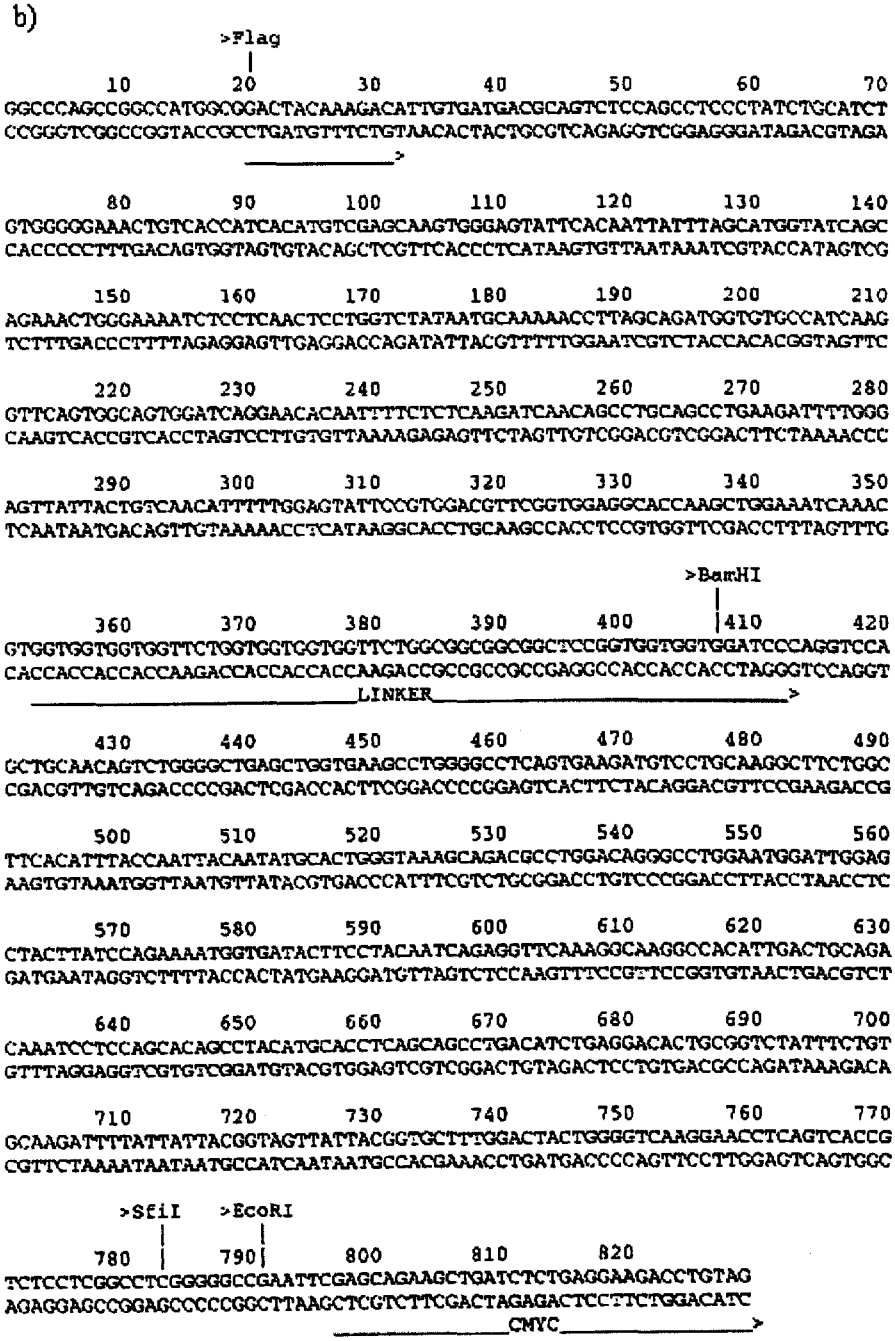


Figure 7.4.6b: HB13d-11 Sequence.

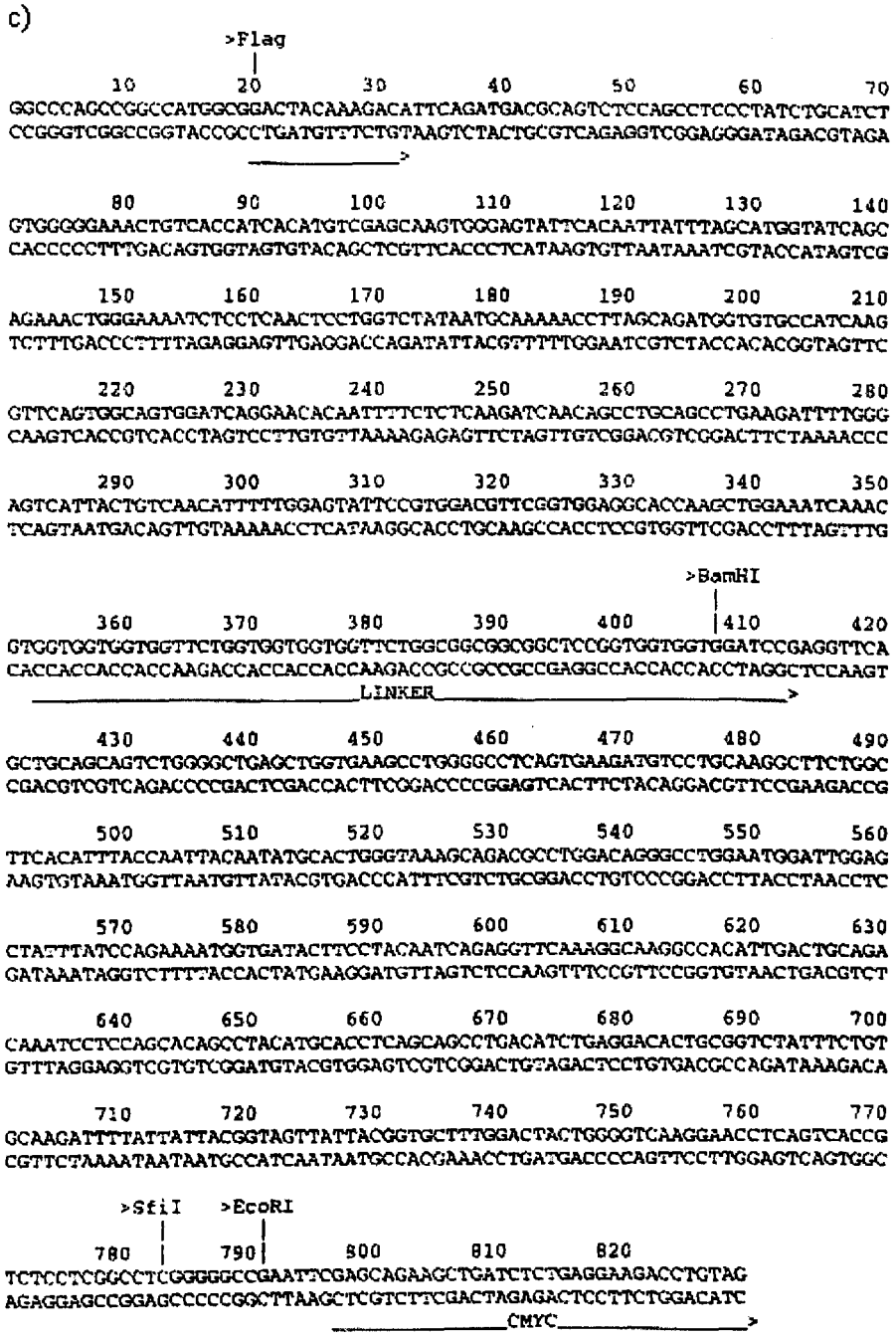


Figure 7.4.6c: HB13d-4 Sequence.

Clone	FWRL1	CDRL1	FWRL2	CDRL2
HB13d-7	MADYKD**VV**MTQ**T**PASLSASVGETVTITC	RASGSIHNYLA	WYQQKLGKSPQLLVY	NAKTLAD
HB13d-11	MADYKD**IV**MTQ**S**PASLSASVGETVTITC	RASGSIHNYLA	WYQQKLGKSPQLLVY	NAKTLAD
HB13d-4	MADYKD**IQ**MTQ**S**PASLSASVGETVTITC	RASGSIHNYLA	WYQQKLGKSPQLLVY	NAKTLAD

Clone	FWRL3	CDRL3	FWRL4	Linker
HB13d-7	GVPSRFSGSGSGTQFSLKINSLQPEDFGS**Y**YC	QHFWSIPWT	FGGGTKLE**L**KR	GGGG-GGGGSGGGGSGGGGS
HB13d-11	GVPSRFSGSGSGTQFSLKINSLQPEDFGS**Y**YC	QHFWSIPWT	FGGGTKLE**I**KR	GGGG**S**GGGGSGGGGSGGGGS
HB13d-4	GVPSRFSGSGSGTQFSLKINSLQPEDFGS**H**YC	QHFWSIPWT	FGGGTKLE**I**KR	GGGG**S**GGGGSGGGGSGGGGS

Clone	FWRH1	CDRH1	FWR2	CDRH2
HB13d-7	**Q**VQLQQSG**T**ELVKPGASVKMSCKAS	GFTFT**D**YNMH	WVKQTPGQGLEWIG	A**I**YPENGDTSYNQRFKG
HB13d-11	**Q**VQLQQSG**A**ELVKPGASVKMSCKAS	GFTFT**N**YNMH	WVKQTPGQGLEWIG	A**T**YPENGDTSYNQRFKG
HB13d-4	**E**VQLQQSG**A**ELVKPGASVKMSCKAS	GFTFT**N**YNMH	WVKQTPGQGLEWIG	A**I**YPENGDTSYNQRFKG

Clone	FWR3	CDRH3	FWR4	CMYC
HB13d-7	KATLTADKSSSTAYMHLSSLTSEDTAVYFCAR	FYYYGSYYGALDY	WGQGTSVTVSS**D**SGAEFE	QKLISEEDL*
HB13d-11	KATLTADKSSSTAYMHLSSLTSEDTAVYFCAR	FYYYGSYYGALDY	WGQGTSVTVSS**A**SGAEFE	QKLISEEDL*
HB13d-4	KATLTADKSSSTAYMHLSSLTSEDTAVYFCAR	FYYYGSYYGALDY	WGQGTSVTVSS**A**SGAEFE	QKLISEEDL*

Figure 7.4.7: Comparison of the Amino Acid Sequences of the 3 CD20 scFvs. Mutations or deletions are shown in bold lettering.

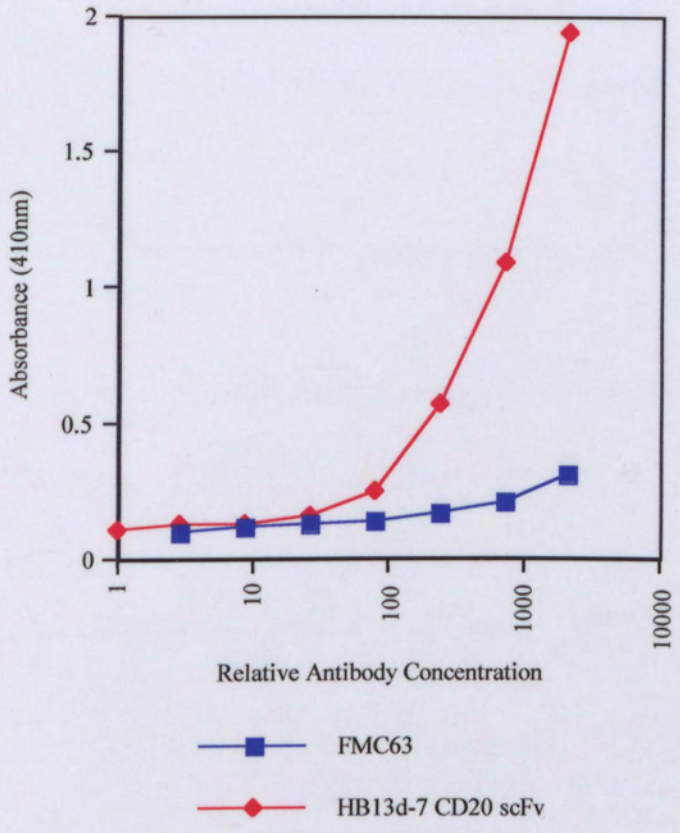


Figure 7.4.8: CD20 scFv Against CD20 Peptide in ELISA.

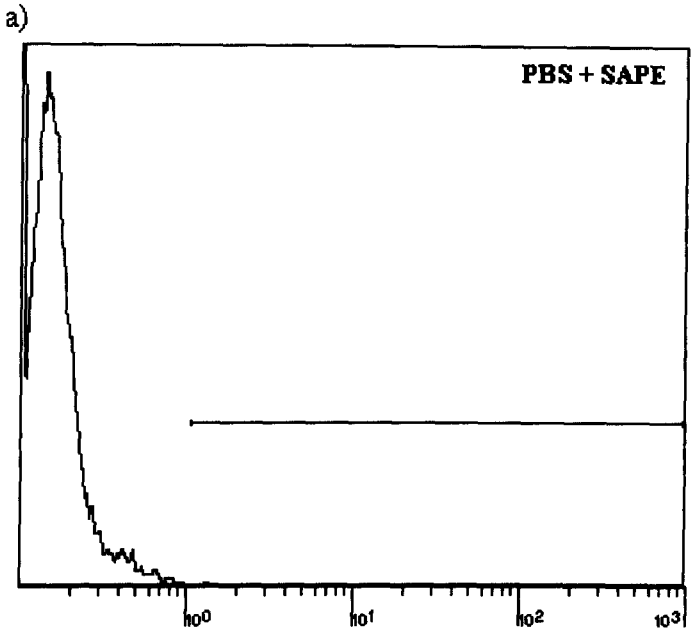


Figure 7.4.9a: Negative Control Staining of Peripheral Blood Lymphocytes. PBS was used as a negative control.

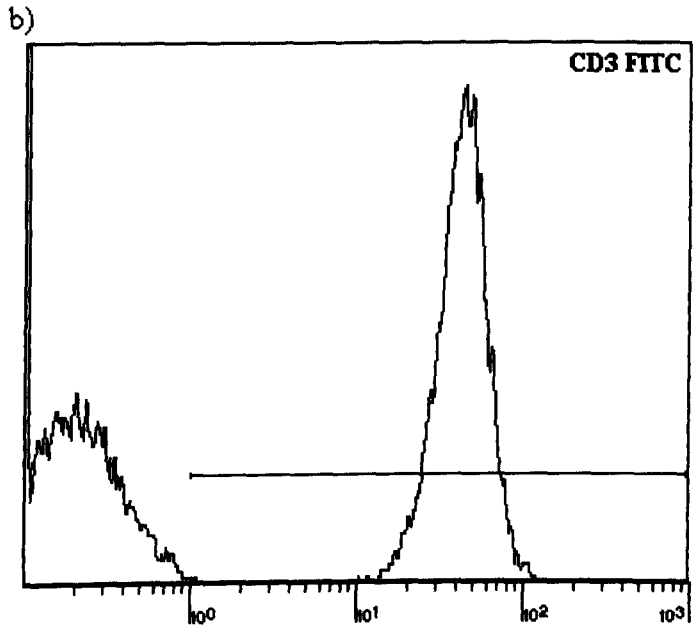


Figure 7.4.9b: CD3 FITC Staining of Peripheral Blood Lymphocytes. CD3 is a T cell surface marker.

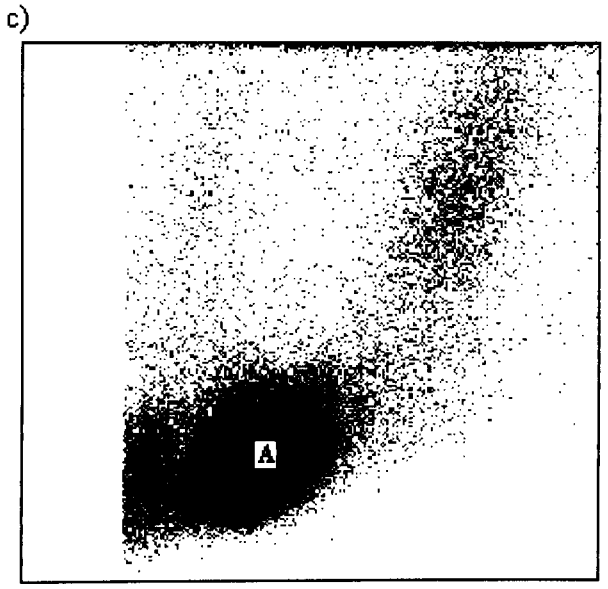


Figure 7.4.9c: Gating Peripheral Blood Lymphocytes After Staining with CD19 CyChrome. A is the gate on the peripheral blood lymphocytes.

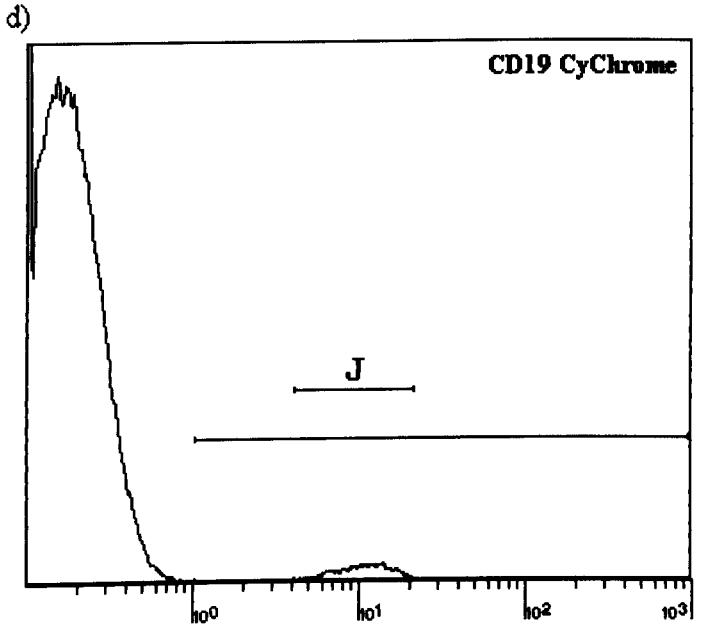


Figure 7.4.9d: CD19 CyChrome Staining of Peripheral Blood Lymphocytes. CD19 is a B cell surface marker. J is a gate on the CD19 positive cells.

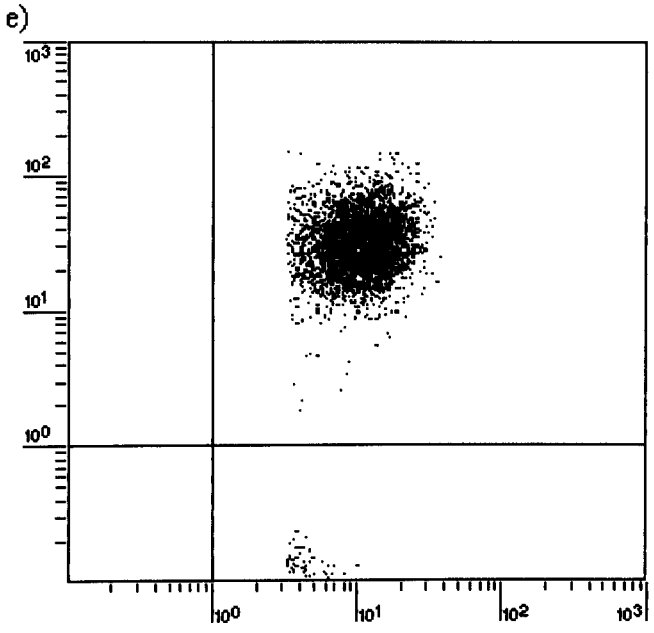


Figure 7.4.9e: Staining of the Gated Peripheral Blood Lymphocytes with CD20 scFv and CD19 CyChrome. CD20 is a B cell surface antigen. The gates were A from figure 7.4.9c and J from figure 7.4.9d.

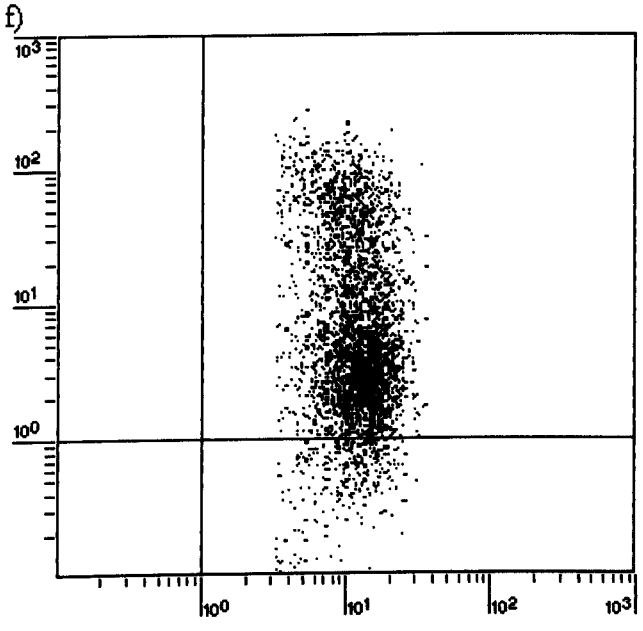


Figure 7.4.9f: Staining of Gated Peripheral Blood Lymphocytes with CD20 Monoclonal Antibody (HB13d) and CD19 CyChrome.

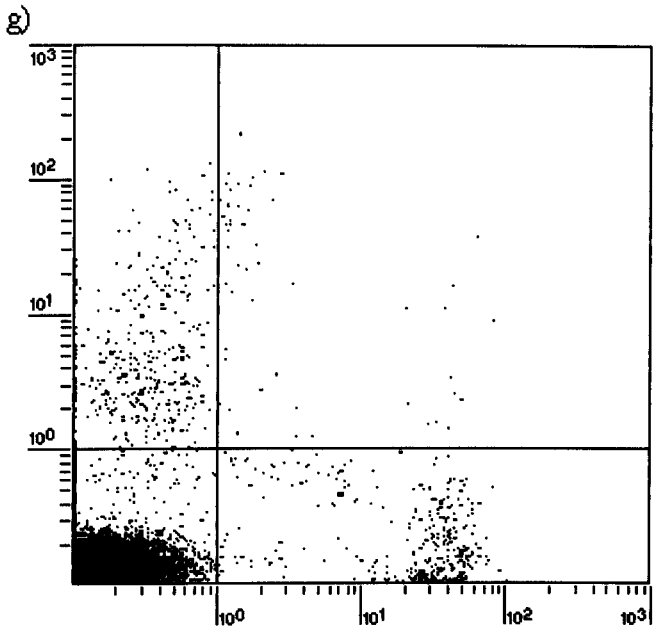


Figure 7.4.9g: Peripheral Blood Lymphocytes Stained with CD20 Monoclonal Antibody and CD3 FITC.

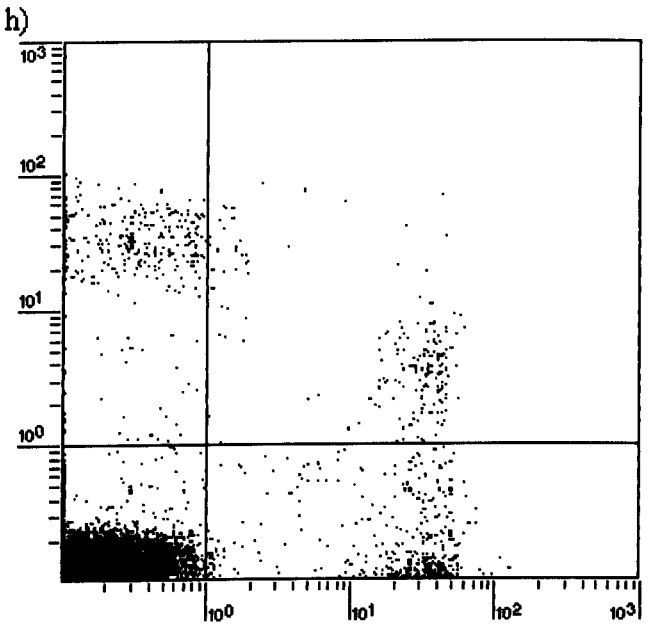


Figure 7.4.9h: Peripheral Blood Lymphocytes Stained with CD20 ScFv Antibody and CD3 FITC.

dual staining of cells with CD3 FITC and both CD20 antibodies, but the majority of T cells do not stain with the CD20 reagents.

Cell Line	Mean Fluorescence Intensity		
	X63	B-Cl	CD20 scFv
HRIK	1.6	49.2	56.7
REH	0.3	8.2	35.4
Namalwa	0.3	1.8	4.5
Jurkat	0.3	0.3	0.2

Table 7.4.1: CD20 scFv Binding to Cell Lines. Mean Fluorescence Intensity (MFI) values for the CD20 scFv and positive and negative controls on 4 cell lines, HRIK, REH, Namalwa and Jurkat. The secondary antibody was 50µl 1/100 monoclonal anti poly-histidine clone HIS-1. The tertiary antibody was 50µl 1/50 biotinylated horse anti mouse. The final antibody was 50µl 1/50 SAPE.

7.4.5 *Methods for Improving scFv Production*

The CD20 scFv was transferred from pAK100 into both the pAK400 and pHB400 vectors. The HB13d-7 scFv was cloned into the pHB400 vector, excising the scFv from the pAK100 vector with SfiI and inserting it into the pHB400 vector. 1µg HB13d-4 and 1µg HB13d-11 (both in pAK100) were digested with 20U SfiI, 10X buffer 2 and water in a volume of 30µl and incubated at 50°C overnight. Digested inserts were gel purified and then inserted into pHB400 in a molar ratio of 1:1. 160ng of each HB13d-4 and HB13d-11 were ligated into 20ng pHB400 vector with 2U T4 DNA ligase in 10X ligase buffer (Promega) and water in a volume of 20µl and incubated at 16°C overnight. 10% of these ligations were transformed into HB2151 in a volume of 42µl. 200µl was plated on 2YT/25µg/ml chloramphenicol/0.5% glucose plates. Colonies were screened for production of protein using a slot blot and for functional protein using flow cytometry on HRIK cells. Figure 7.4.10 shows the production of HB13d-7 in pAK400 and pHB400 in an ELISA. The positive control, B-Cl, and the CD20 scFv produced in the pHB400 vector both bound to the CD20 peptide. The CD20 scFv

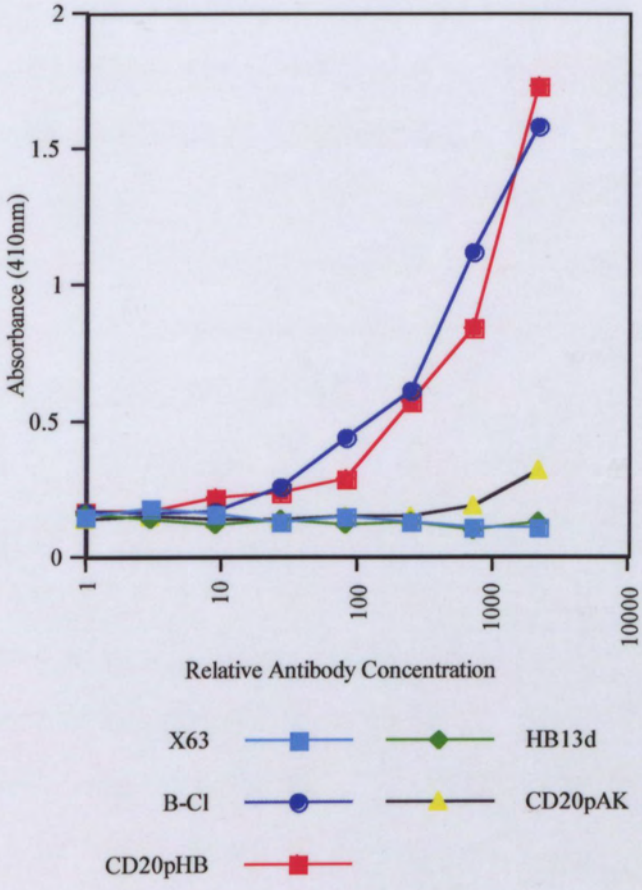


Figure 7.4.10: Comparison of CD20 scFv Expression in pAK400 and pHB400.

produced in the pAK400 vector also bound the CD20 peptide but not as well as the CD20 scFv produced in the pHB400 vector. This could be due to the chaperone protein expressed in the pHB400 construct. Neither FMC63, an anti-CD19 antibody, or supernatant from the HB13d hybridoma bound the CD20 peptide.

7.4.6 *Determination of Optimal Glucose Concentration for Production of scFv*

Experiments were set up to determine the best conditions for production of the CD20 scFv antibody. Flasks were set up with 270ml TB, 30ml TB supplement, 25µg/ml chloramphenicol and various concentrations of glucose, from 0%, 0.25%, 0.5%, 0.75% and 1%. One flask was set up with 300ml 2YT and 25µg/ml chloramphenicol and another the same except for the addition of 0.5% glucose. The broths were inoculated with CD20/pAK400/HB2151 to a final OD_{600nm} of 0.2 and incubated at 37°C with shaking until the OD_{600nm} reached 1.0 when they were induced with 0.1mM IPTG. These were incubated at room temperature overnight with shaking. The cells were centrifuged at 6000rpm (6200g) for 25min at 4°C. Some supernatant was retained and filtered through a 0.2µM filter. The pellets were resuspended in 10ml periplasmic borate extraction buffer (Chapter 2) and were vigorously shaken on ice for 1hr. These fractions were centrifuged at 19500rpm (49000g) for 30min at 4°C. The supernatant was collected and filtered through a 0.2µM filter. The pellet was resuspended in 10ml HEPES buffer (Chapter 2) and the French press fraction collected. The French press fraction was centrifuged at 19500rpm (49000g) for 30min at 4°C and the supernatant filtered a 0.2µM filter and all fractions stored at 4°C. Cell supernatants, periplasmic fractions and French press fractions were analysed by slot blot and flow cytometry at concentrations of neat and 1/3 for the supernatant, 1/10 and 1/30 for the periplasmic fraction and 1/100 and 1/300 for the French pressed fraction. The slot blot showed the greatest amount of protein in the French press fractions (not shown) but this does not correlate to the activity in the fractions. The scFv fractions were assayed for activity using flow cytometry. Figure 7.4.11 shows the results of the flow cytometry. These results show that the best conditions for the production of the CD20 scFv is growth in TB media with no glucose.

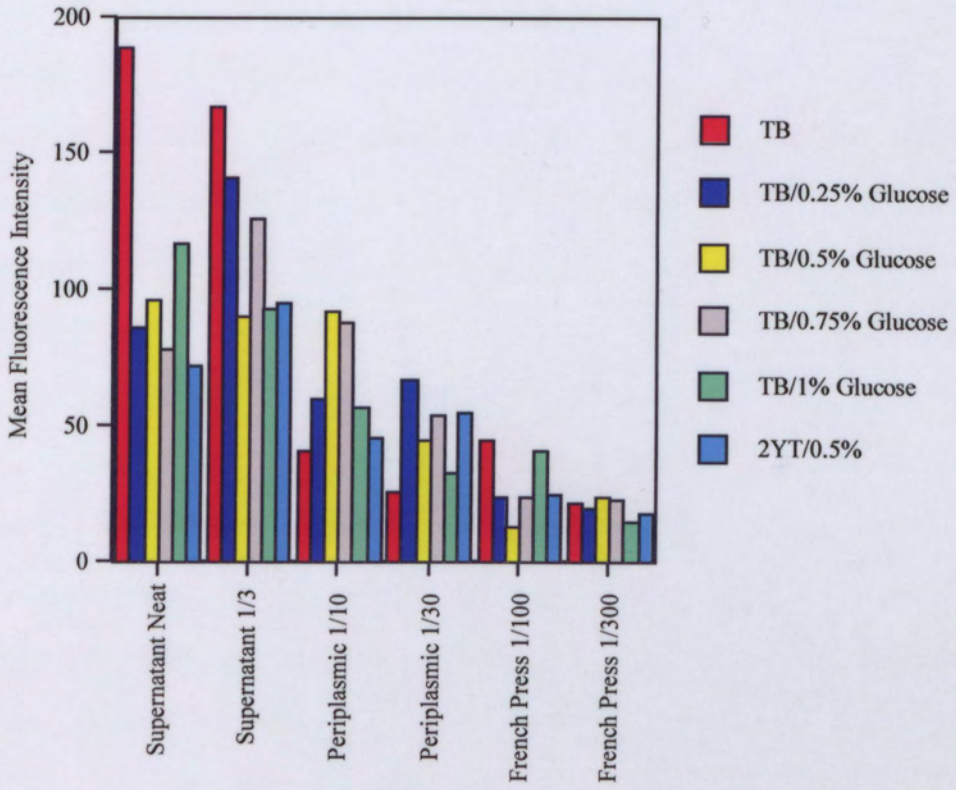


Figure 7.4.11: Growth of CD20 scFv in Media with Different Glucose Concentrations.

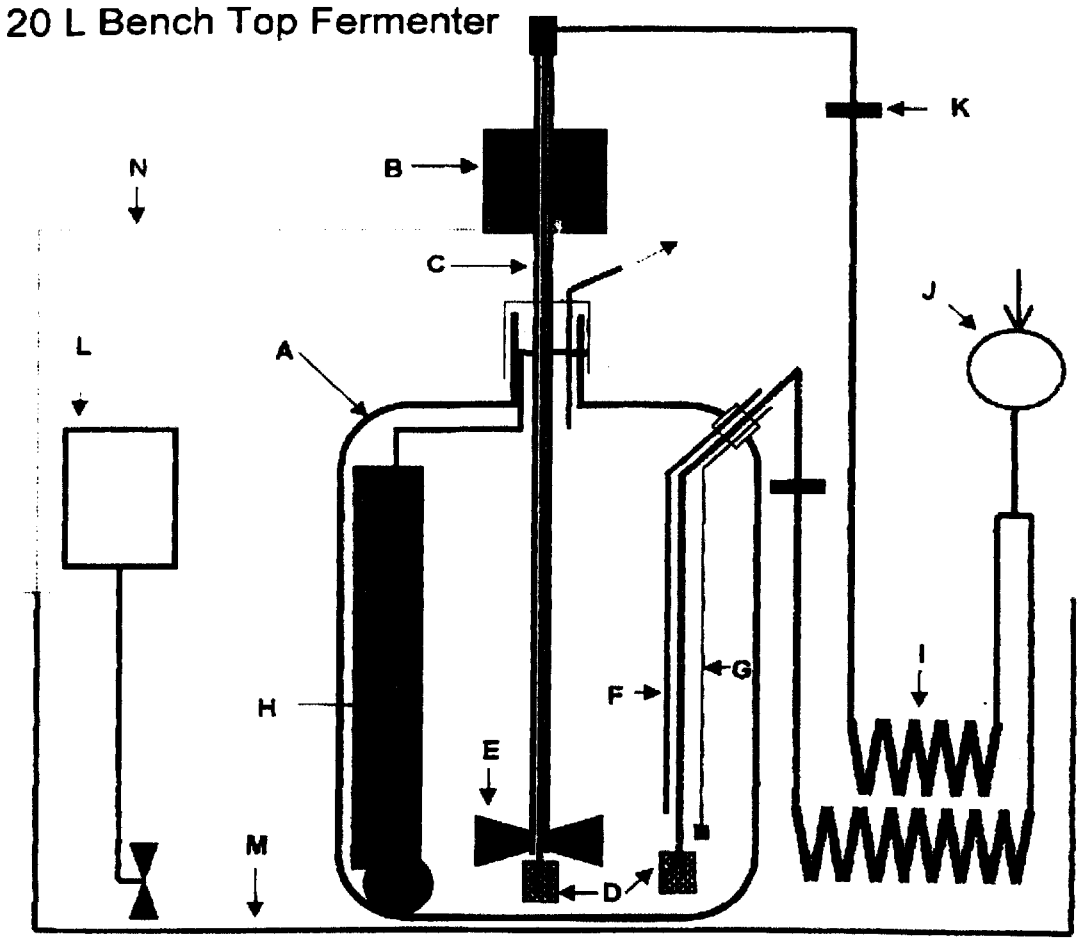
7.4.7 *Scale up of Protein Production Using Fermentation*

The 20L fermenter designed by Michael Thiel and provided by Flinders Medical Centre was used for large scale production of the scFv (figure 7.4.12). On the day before fermentation 2 x 1L broth cultures of TB, TB supplement, 5mM $MgSO_4$, 1% Glucose and 25µg/ml chloramphenicol were inoculated with 400µl of an overnight culture of CD20 scFv in pHB400. The 2 x 1L cultures were incubated at 30°C overnight with shaking. Early the next day the fermenter was set up with 2L 4.5X TB, 1L 9X TB supplement with 0.5% glucose, 6L RO water, 10mM $MgSO_4$, 25µg/ml chloramphenicol and the 2 x 1L precultures of CD20 scFv. The fermenter was incubated at 37°C for 3.5hr and then cooled over 1hr to 25°C. The culture was grown to a final OD_{600nm} of 7.25. The bacterial growth curve can be seen in figure 7.4.13. The fermentation culture was induced with 0.5mM IPTG when the optical density showed that the culture was reaching the end of the log phase. It was incubated at temperatures between 19 - 25°C for 2.5hr during induction and the cells were harvested by centrifuging the culture, 6L at 4000rpm (4650g) for 30min at 4°C and 5L at 8000rpm (11000g) for 10min at 4°C.

The pellet was collected and weighed. 2ml of periplasmic borate extraction buffer was added for every gram of pellet. The pellets were incubated for 2hr on ice with vigorous shaking. This was centrifuged at 8000rpm (11000g) for 20min at 4°C. The supernatant was collected and 55µg DNase I (Boehringer Mannheim) added to it. The pellet was resuspended in the same volume of borate extraction buffer as was added initially and the extraction repeated. A fraction of this was retained for analysis. The periplasmic fraction was dialysed using an Amicon diafiltration system exchanging the borate extraction buffer with 300mM NaCl, 50mM NaH_2PO_4, pH 8.0. A fraction of the permeate was collected for analysis. The dialysed scFv solution was filtered through a large volume Satobran 300 filter (Sartorius) with a pore size of 0.45 - 0.2µM.

7.4.8 *Purification of scFv*

The periplasmic extraction from the fermenter was purified using an immobilised metal affinity chromatography (IMAC) column. 4ml of regenerated Ni NTA beads (QIAGEN) was added to a column and washed with 30ml of 6HIS wash buffer (Chapter 2) at a flow rate of 3ml/min.



A	20 L Nalgene polycarbonate bottle
B	mixer with through shaft design (Cole Parmer P50002-35 + P-50002-07)
C	hollow shaft and fixed, non-rotating air pipe
D	porous stainless steel air sparger
E	high shear radial flow impeller
F	stainless steel tube to remove samples
G	thermometer probe
H	removable stainless steel baffle with stabilisation weight and spring-fixation to the bottle neck
I	copper coils for air-temperature equilibration
J	flow meter and pressure valve for air-flow control
K	air filter (0.2μm)
L	water bath heater
M	water bath
N	stand for mixer engine

Fragile parts that require careful handling:
- **Mixer shaft**: This long and hollow shaft must not be bent. Keep always in a vertical position
- **Peripheral sparger, sampling unit**: do not remove or insert this unit into the fermenter when mixer is running

Figure 7.4.12: 20L Bench Top Fermenter. This figure and the fermenter were designed by Michael Thiel (Flinders University & Flinders Medical Centre, SA, Australia).

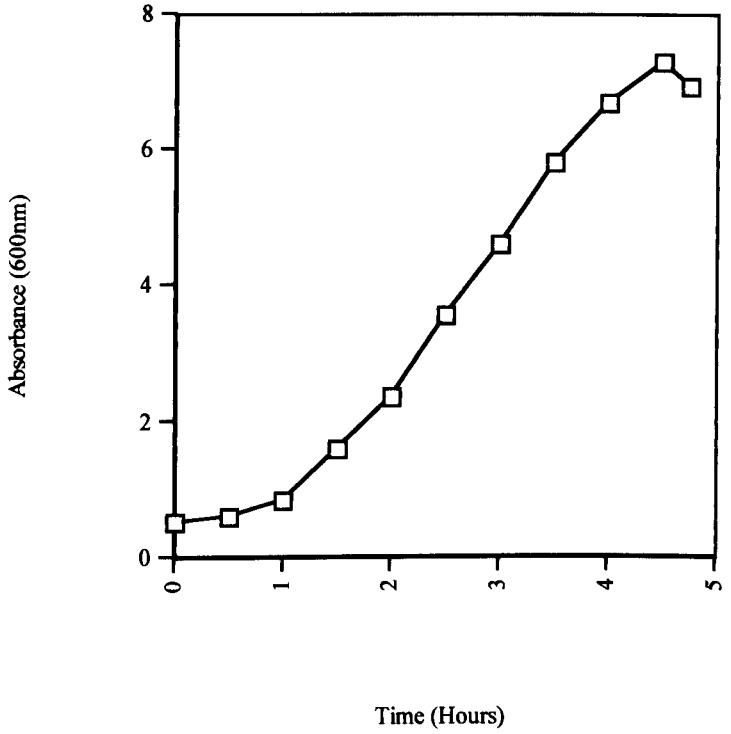


Figure 7.4.13: Bacterial Growth Curve for the CD20 scFv Fermentation.

20mM imidazole was added to the scFv periplasmic fraction and this was loaded onto the column at a flow rate of 1ml/min. Any unbound protein was washed from the column with 6HIS wash buffer. The scFv was eluted with 50, 100 and 250mM imidazole, washing with 6HIS wash buffer between each elution and finally the column was stored in 6HIS wash buffer at 4°C. The purified fractions, flow through and the first wash were analysed by SDS-polyacrylamide gel electrophoresis. Figure 7.4.14 shows that protein of the correct size, 28 - 30kD was eluted in all three fractions, 50, 100 and 250mM imidazole. A band of the correct size was also observed in the wash fraction.

Densitometry measurements of the bands on the gel was carried out on the wash fraction and the three eluted fractions using VDS Imagemaster software (table 7.4.2).

Fractions	Relative Mass
Wash	244.6
50mM Imidazole	973.9
100mM Imidazole	1448.7
250mM Imidazole	1623.6

Table 7.4.2: Densitometry Measurements from the SDS-PAGE.

All the fractions were tested for activity of the scFv. Flow cytometry results can be seen in table 7.4.3.

These results show that there are significant levels of functional CD20 scFv in all samples collected from the column purification. The sample from the fermenter, pre column, and the 3 elution fractions with imidazole have mean fluorescence intensities in a similar range to the positive control.

Comparison of the results of the flow cytometry to the density of the bands on the SDS-PAGE show that the most dense band, 250mM imidazole, shows the least activity of the eluted fractions. The 50mM and 100mM eluted fractions had lower densities but greater biological activity. Mean fluorescence intensity can be used to measure binding activity if the antibody is not in excess.

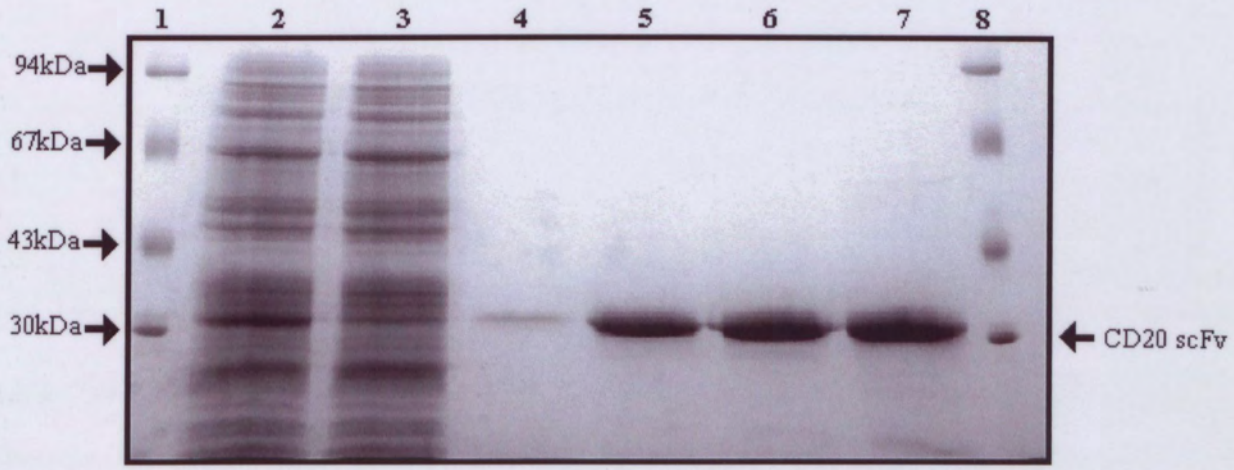


Figure 7.4.14: SDS-PAGE Analysis of CD20 scFv Purification using IMAC. Lanes 1 & 8 are Low Molecular Weight Markers (Promega); Lane 2 is crude periplasmic extract; Lane 3 is flow through from the IMAC column; Lane 4 is the wash before elution; Lane 5, 6 & 7 are 50mM, 100mM and 250mM Imidazole elutions of CD20 scFv from the column.

Conditions	MFI
PBS	3.36
CD20 scFv Control	65.5
CD20 pre column	41.3
CD20 flow through	14.7
Column Wash	21.2
50mM Imidazole	52.2
100mM Imidazole	53.1
250mM Imidazole	31.4

Table 7.4.3: Flow Cytometry for Purified CD20 scFv Fractions. Mean fluorescence intensity by flow cytometry for the fractions purified using an IMAC column.

7.4.9 *Determination of Protein Concentration*

The amino acid sequence of the scFv was derived from the nucleotide sequence of the CD20 scFv. Using a site on the internet (Rodgers, 1998) the extinction coefficient of the CD20 scFv could be determined from the amino acid composition of the protein. The optical density of the protein at 280nm was measured and the concentration of the scFv determined. $1OD_{280nm}$ = 0.45mg/ml CD20 scFv.

7.4.10 *Blocking and Competition Assays Using Flow Cytometry*

Flow cytometry was used to compare binding of the CD20 scFv to a commercial CD20 monoclonal antibody. The antibodies used were FITC conjugated mouse anti 6-histidine (BAbCO) and PE conjugated mouse anti human CD20 (Leu-16; Becton Dickinson). The concentration of the scFv was determined using the method described above and was 210µg/ml. The scFv was diluted 1/210 to the same concentration as the working concentration of CD20 PE used, 1µg/ml. Phycoerythrin is 240kDa (Oi *et al.*, 1982) and its conjugation to the monoclonal antibody was taken into consideration and the scFv was tested at a 1/525 dilution or 0.4µg/ml. Due to the size difference between a whole antibody molecule and the scFv (about

5-fold smaller), the scFv was diluted 1/2625 (0.08μg/ml) to the same molar ratio as the whole antibody. The other dilution 1/1315 (0.16μg/ml) was to account for the bivalency of the monoclonal antibody in comparison to the monovalency of the scFv. 1/50 CD20PE was added to the cells already incubated with the scFv dilutions and the scFv dilutions were added to the cells already treated with 1/50 CD20PE. The competition experiment was carried out at the same time as the blocking experiments and instead of incubating the antibodies on the cells one after the other the dilutions of the antibodies were mixed together in a 1:1 ratio before the addition of the scFv/CD20PE to the cells. The results of the competition and blocking flow cytometry can be seen in table 7.4.4.

The dilutions of the scFv were applied to the cells by themselves, before, directly following or simultaneously with the CD20PE. As can be seen in table 7.4.4 all dilutions of the CD20 scFv blocked the binding of the CD20 monoclonal antibody and likewise the CD20 monoclonal antibody blocked the action of the CD20 scFv. The competition assays showed that the CD20 scFv and the CD20PE compete equally against each other.

7.4.11 *Enzyme Treatment*

Experiments were carried out to determine whether reason the CD20 scFv competes so effectively with the monoclonal antibody is that it is small and can more easily access the CD20 molecule which only has a small extracellular region (figure 1.3). HRIK cells were treated with neuraminidase, an agent which cleaves the sugar residues from the non-reducing end of the carbohydrate side chain. Neuraminidase (Sigma) was resuspended at 0.5U/ml in serum free RPMI. HRIK cells were resuspended at 5 x 10^6 cells/ml in serum free RPMI. To 1ml of the cell suspension was added 1ml enzyme mix. This was incubated at 37°C for 1hr. A control of 5 x 10^6 cell/ml was treated with 1ml serum free RPMI instead of the enzyme. The cells were resuspended in 8ml cold RF10 medium and centrifuged at 1500rpm (430g) for 5min at 4°C. The cells were washed with 10ml PBS/0.01% azide and centrifuged as above. The cells were resuspended in 0.5ml PBS/0.01% azide and then used in the competition and blocking flow cytometry. Results of flow cytometry using the blocking and competition assays described above on neuraminidase treated HRIK cells can be seen in table 7.4.5.

Conditions	MFI FITC	MFI PE
PBS - αHis FITC	0.33	
2.1μg/ml CD20 scFv - αHis FITC	0.81	
1μg/ml CD20 scFv - αHis FITC	0.84	
0.4μg/ml CD20 scFv - αHis FITC	0.79	
0.16μg/ml CD20 scFv - αHis FITC	0.54	
0.08μg/ml CD20 scFv - αHis FITC	0.44	
1/50 CD20 FITC	2.9	
PBS - SAPE		0.35
CD20PE 1/50		5.25
1μg/ml CD20 scFv - CD20PE - αHis FITC	0.78	0.85
0.4μg/ml CD20 scFv - CD20PE - αHis FITC	0.63	0.86
0.16μg/ml CD20 scFv - CD20PE - αHis FITC	0.60	0.93
0.08μg/ml CD20 scFv - CD20PE - αHis FITC	0.41	1.31
CD20PE - 1μg/ml CD20 scFv - αHis FITC	0.37	3.57
CD20PE - 0.4μg/ml CD20 scFv - αHis FITC	0.38	3.50
CD20PE - 0.16μg/ml CD20 scFv - αHis FITC	0.37	3.58
CD20PE - 0.08μg/ml CD20 scFv - αHis FITC	0.30	3.83
1μg/ml CD20scFv + CD20PE - αHis FITC	0.69	1.66
0.4μg/ml CD20scFv + CD20PE - αHis FITC	0.64	2.32
0.16μg/ml CD20scFv + CD20PE - αHis FITC	0.48	2.21
0.08μg/ml CD20scFv + CD20PE - αHis FITC	0.34	2.69

Table 7.4.4: Blocking and Comptition Experiments with CD20 scFv and a Commercial Monoclonal Anti-CD20 Antibody. Table of the mean fluorescence intensity values for the blocking and competition experiment. CD20PE and CD20FITC are 1μg/ml. - means followed by and + is added at the same time.

Conditions	HIRK Cells Without Treatment		HRIK Cells Without Neuraminidase		HRIK Cells With Neuraminidase	
	FITC	PE	FITC	PE	FITC	PE
PBS - FITC	0.363		0.234		0.260	
scFv 0.4µg/ml - α HIS FITC	0.546		0.410		0.512	
scFv 0.16µg/ml - α HIS FITC	0.388		0.289		0.333	
PBS - SAPE		0.313		0.286		0.399
CD20PE		2.76		2.73		4.46
scFv 0.4µg/ml - CD20PE - αHISFITC	0.490	0.831	0.391	0.978	0.520	1.39
scFv 0.16µg/ml - CD20PE - αHISFITC	0.366	1.09	0.277	0.890	0.350	1.59
CD20PE - scFv 0.4µg/ml - αHISFITC	0.370	1.70	0.291	1.53	0.340	2.95
CD20PE - scFv 0.16µg/ml - αHISFITC	0.318	1.89	0.247	2.03	0.284	2.63
scFv 0.4µg/ml + CD20PE - αHISFITC	0.396	1.21	0.347	1.46	0.406	2.12
scFv 0.16µg/ml + CD20PE - αHISFITC	0.342	1.30	0.310	1.82	0.302	2.01

Table 7.4.5: CD20 scFv Binding to Neuraminidase Treated Cells. Table of mean fluorescence intensities for cells treated with neuraminidase and control cells incubated with CD20 scFv in blocking and competition assays. PE is phycoerythrin, FITC is fluorescein isothiocyanate. CD20PE is 1µg/ml. - means followed by and + means at the same time.

7.4.12 *BIAcore Studies*

The biotinylated CD20 peptide was coupled to a BIAcore streptavidin sensor chip and samples of the purified HB13d-7 scFv was passed across its surface using a BIAcore biosensor (Pharmacia Biotech). The streptavidin sensor chip was neutralised with BIAneutralising solution and conditioned 3 times with 1M NaCl/50mM NaOH. Flow cells 2, 3 and 4 were coated with the biotinylated CD20 peptide (SynPep Corporation) at different concentrations. Flow cell 2 of the chip was coated with 6µl 10µg/ml CD20 to 488.9RU. Flow cell 3 was coated with 25µl 10µg/ml CD20 peptide to 1429.8RU. Flow cell 4 was coated with 30µl of 10µg/ml CD20 peptide to 846.7RU. Flow cell 1 was not coated with the CD20 peptide and was used as a reference cell. 20µl 2.5mM HCl was used to regenerate the surface as this was determined to be adequate for the removal of antibody from antigen bound to a streptavidin chip. Samples were added in volumes of 50µl. Dilutions of the samples were made in the running buffer, 1X HBS (Chapter 2). The binding curve of the scFv was determined on the BIAcore (figure 7.4.15). Time constraints did not allow further kinetic analysis of this binding.

7.4.13 *scFv Computer Modelling*

The amino acid sequence of the CD20 scFv was used to predict a 3 dimensional structure for the scFv. Dr. Terry Mulhern from the University of Adelaide, SA, Australia carried out the modelling of the scFv. BLAST searches were carried out for identification of proteins with known 3-dimensional structure that had similar sequence to the known sequence of the CD20 scFv. Once some close matches had been identified and those closest to the real sequence chosen they were transported into the Insight II program. This program was used to predict the structure of the scFv. The results of this work can be seen in figure 7.4.16. The orange region is the light chain, the yellow is the heavy chain, blue is the light chain CDRs, green is the heavy chain CDRs, red is the linker and the pink indicates the regions which mutations between the 3 clones occur. The mutation in the linker of HB13d-7 would make no difference to the binding of the antibody to its target. The mutations that are most likely to have an effect on antigen binding are those in CDRH1 and CDRH2 which are in the region of antibody-antigen

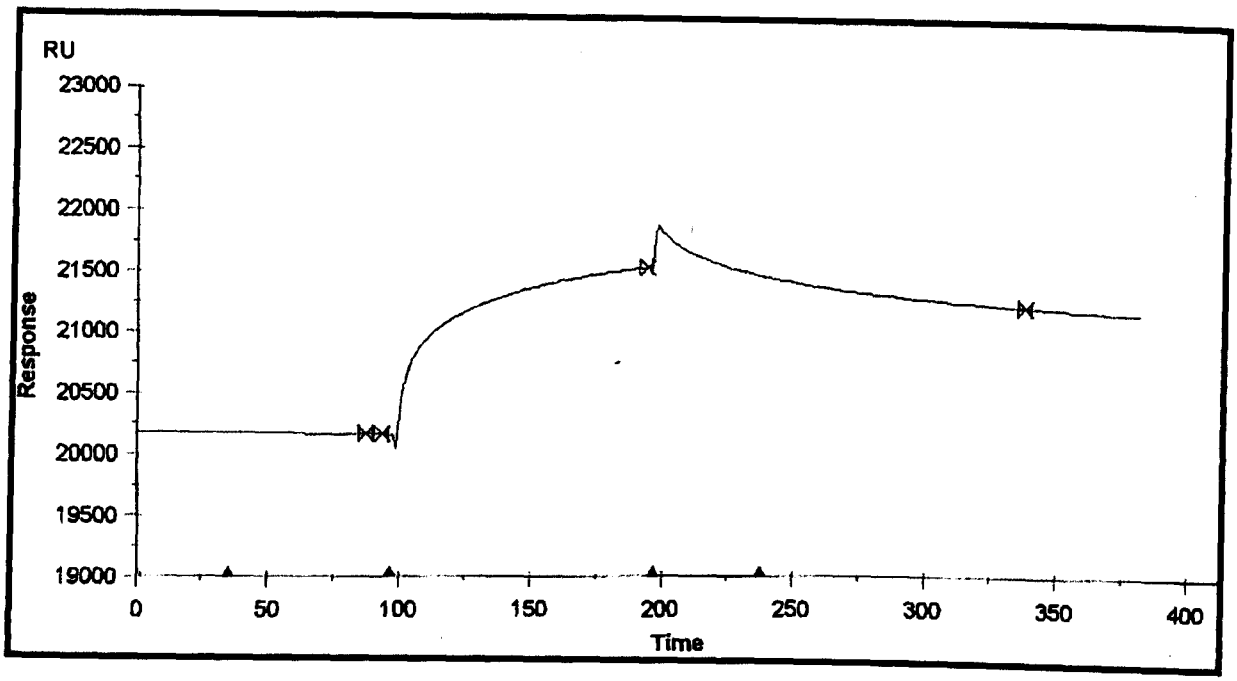
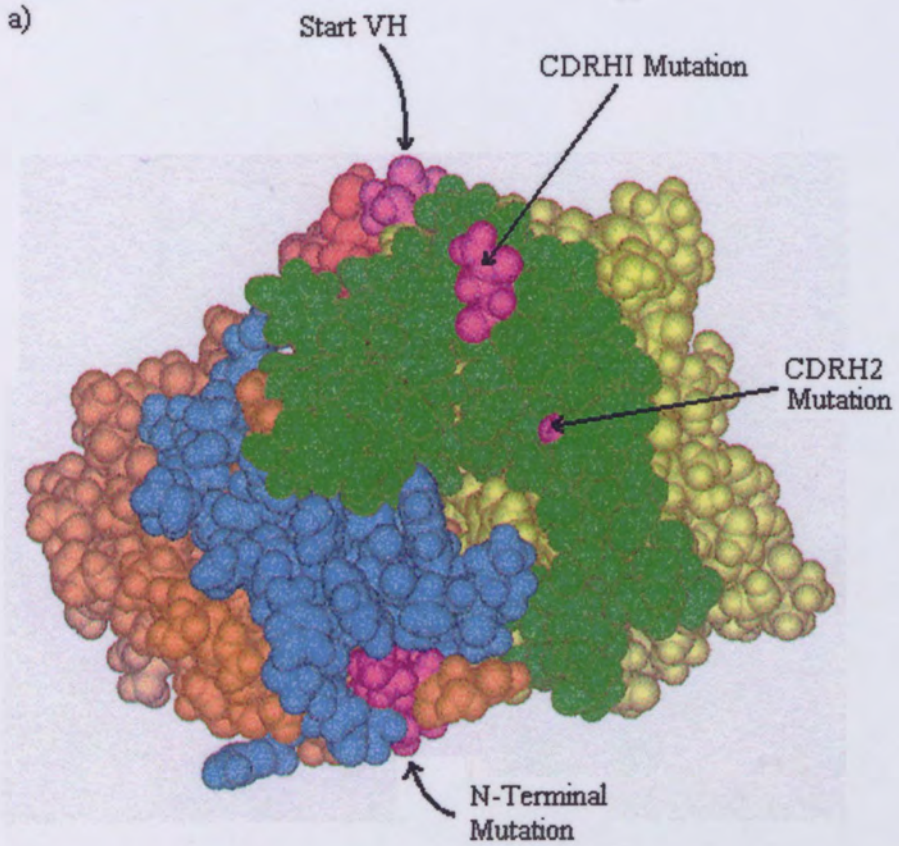
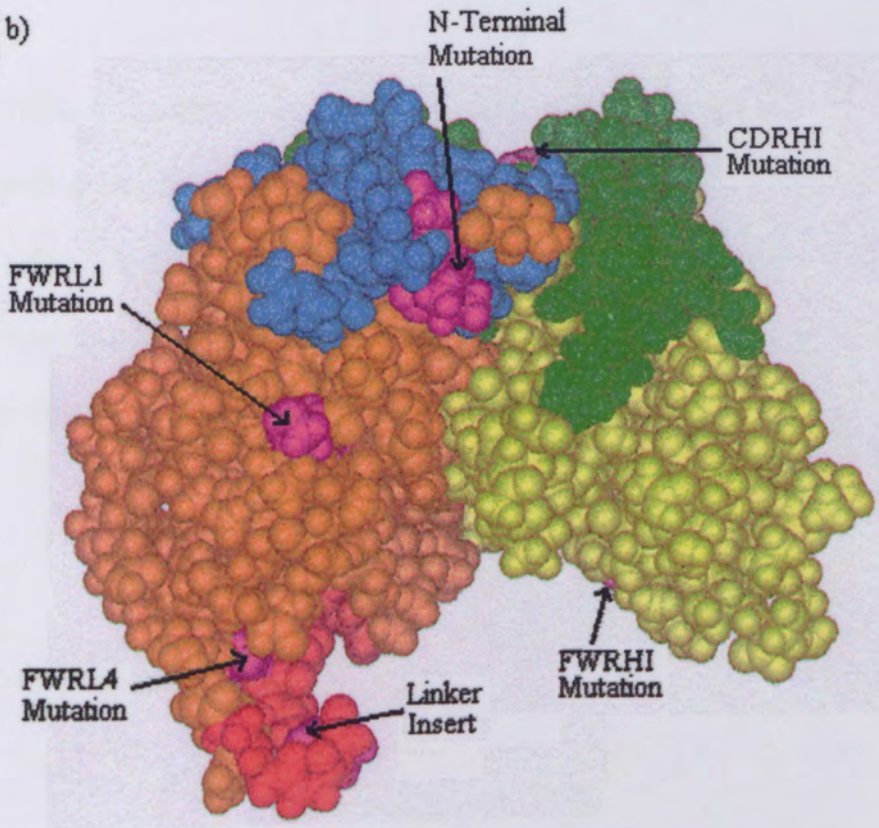


Figure 7.4.15: BIAcore Analysis of CD20 scFv.


a)
Start VH
CDRHI Mutation
CDRH2
Mutation
N-Terminal
Mutation


b)
N-Terminal
Mutation
CDRHI
Mutation
FWRL1
Mutation
FWRL4
Mutation
Linker
Insert
FWRHI
Mutation

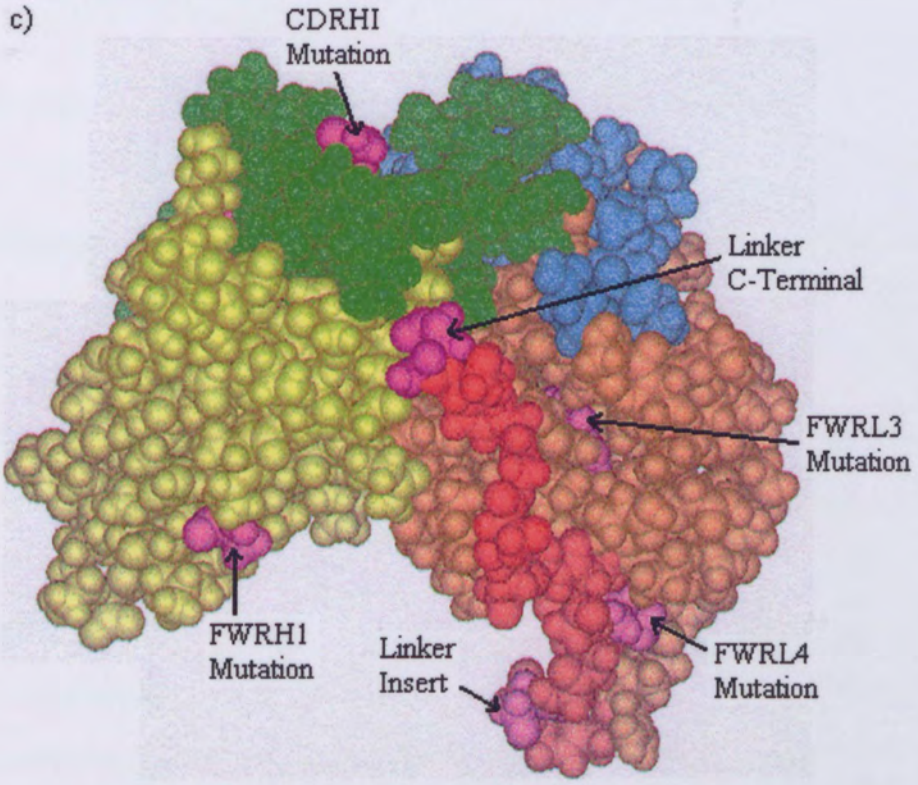


Figure 7.4.16: Computer Modelling of CD20 scFv. Figure a) is a view of the scFv from above looking into the site of antigen-antibody interaction. Figures b) and c) are both side views of the scFv from opposite angles. The light chain is orange; Light chain CDRs blue; Heavy chain is yellow; Heavy chain CDRs green; Linker is red; Mutations between the 3 single chains (figure 7.4.7) are pink.

interaction. In HB13d-7 the aspartate in CDRH1 is mutated to asparagine in HB13d-4 and -11. In the CDRH2 the hydrophobic isoleucine is mutated in HB13d-11 to threonine. Some framework mutations, although they are not directly involved in the binding, are near enough to the CDR regions that they may affect the antibody binding region.

7.4.14 *Comparison of the Modelling to Affinity Measurements*

An ELISA plate coated with CD20 peptide was used to make an estimated comparison of the binding affinities of the 3 scFvs, HB13d-7, HB13d-4 and HB13d-11. Concentrations of the 3 scFvs were determined and equal concentration of these plated in an ELISA plate coated with CD20 peptide. 1/1000 monoclonal anti poly-histidine clone HIS-1 was the secondary antibody and 1/1000 alkaline phosphatase conjugated anti-mouse Ig was the tertiary antibody. Alkaline phosphatase substrate, p-nitrophenyl phosphate in diethanolamine buffer, was used to detect binding. Figure 7.4.17 shows the results of the ELISA. The HB13d-4 mutations completely abrogated the ability to bind CD20 and the mutations in HB13d-11 almost did. The residues mutated in the HB13d-4 and HB13d-11 clones play an important part in the binding of the scFvs.

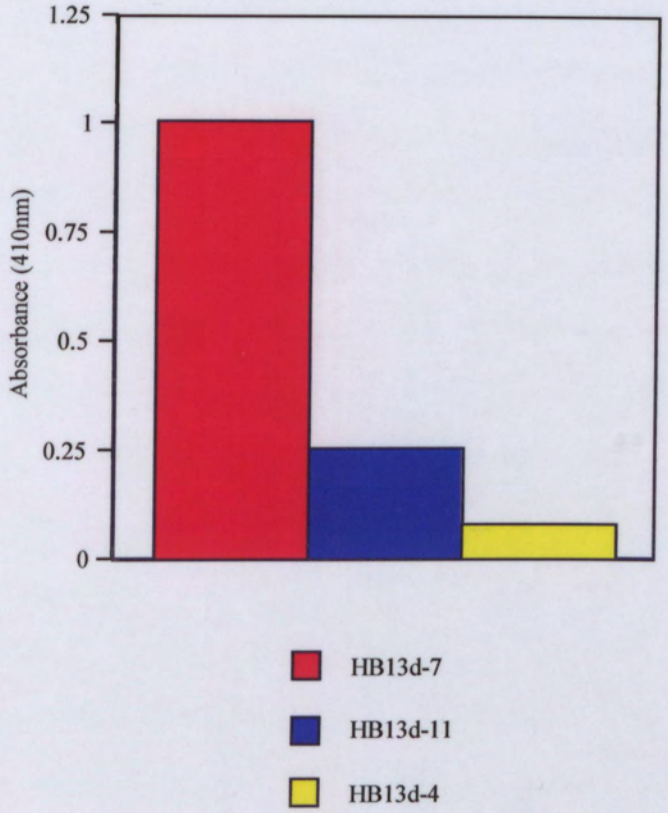


Figure 7.4.17: Comparison of HB13d-7 Binding with HB13d-4 and HB13d-11 Mutants in an ELISA Against CD20 Peptide.

7.5 *Discussion*

Described here is a scFv fragment which has been produced from the HB13d (anti-CD20) hybridoma. The scFv bound to CD20 expressing cell lines and CD20 peptide in other binding assays.

Improved production of the scFv was achieved firstly by cloning the fragment from the pAK100 vector to pAK400. Cloning into the pHB400 vector increased production further. Optimal expression conditions were determined by growing bacteria containing the scFv/pAK400 vector in medium with different glucose concentrations. Optimising conditions for the production of scFvs is important. Glucose in the growth medium represses the production of any background recombinant protein by the bacterial cells (Krebber *et al.*, 1997). Aggregation of recombinantly expressed protein in bacterial cells reduces the biological activity of the protein if it can not be refolded. The addition of sugars to the growth medium can significantly reduce this aggregation (Bowden and Georgiou, 1988). This allows much improved activity of the recombinant protein. Growth in medium with no added glucose is the best expression conditions for the CD20 scFv. This suggests that it is a protein which folds well. The type of medium used is also important with an approximate 3-fold drop in production of the CD20 scFv with the use of 2YT instead of TB medium. More information about optimising scFv production is described by Thiel *et al.* (in preparation).

Purified CD20 scFv was used to determine the characteristics of the binding of the scFv to the CD20 antigen. In ELISA the CD20 scFv was shown to bind to the 45aa CD20 peptide and as mentioned above it binds CD20 expressing cell lines and B lymphocytes in peripheral blood. Binding to the peripheral blood lymphocytes showed CD19 and CD20 binding to the same cell population although some of the CD19 positive cells were not stained with CD20. This could be due to the earlier expression of CD19 on B lymphocytes than CD20. Incubation of the PBLs with CD20 scFv/CD3 or CD20 monoclonal antibody/CD3 showed a small number of cells stained with both antibodies. CD3 staining of these cells indicates that they are T cells, however it is widely accepted that CD20 expression is restricted to B cells. This staining could indicate CD20 expression on a wider range of cells than previously detected, or alternatively could result from non-specific uptake of one of the detection reagents. The majority of T cells

did not stain with the CD20 antibody or scFv. Taken together, the results indicate that the scFv has essentially the same specificity as the parent antibody.

In flow cytometry the CD20 scFv blocked the action of a monoclonal CD20 antibody, Leu16, and conversely the monoclonal antibody was shown to block the CD20 scFv. Using both the monoclonal antibody and the CD20 scFv in competition experiments showed that the CD20 scFv effectively competes for binding to the CD20 antigen. Treatment of the cells with neuraminidase, a glycosidase which is involved in the cleavage of sugar residues from the end of the carbohydrate side chain, appeared to improve the binding of the monoclonal antibody to the CD20 antigen. The use of this enzyme on cells can effect the binding of some antibodies to their epitope by either directly affecting the binding site or by making the binding site more accessible to the antibody. FMC1 antibody binding is abolished by treatment of the cells with neuraminidase (Zola *et al.*, 1984a) whereas FMC7 binding is improved with treatment with the glycosidase (Zola *et al.*, 1984a; Zola *et al.*, 1984b). The use of these enzymes to treat cells gives some insight into the properties of the antibody and the antigen it binds. The CD20 antigen on B cell lines becomes more accessible with neuraminidase treatment. Antibody fragments against CD20 would be more useful for treatment of B cell diseases than whole antibodies because their small size allows the fragment easier access to the target antigen.

The 3 dimensional structure of the CD20 scFv was predicted using Insight II and the known 3-D structure of antibodies with similar amino acid sequence to the CD20 scFv. The structural comparison of the HB13d-7 scFv to the mutant scFvs, HB13d-4 and HB13d-11, showed that the mutations were in or close to the area which was identified as the site of antigen-antibody interaction. This may explain the reduction in the affinities of the two mutant scFvs in comparison to the HB13d-7 clone.

There are many proteins that have had their 3 dimensional structure predicted using modelling protocols. Some have later gone on to have their crystal structure determined and have shown remarkable similarity to their predicted structure (Perisic *et al.*, 1994). Modelling is widely accepted as reliable for predicting antibody structures.

Since the HB13d-7 scFv was constructed, and up until August 1999, there have been 4 variations of anti-CD20 scFv antibodies described (Haisma *et al.*, 1998; Jensen *et al.*, 1998; Schmidt *et al.*, 1999; Shan *et al.*, 1999). CD20 scFvs were constructed from the mouse monoclonal antibodies IH4 (Haisma *et al.*, 1998) and IF5 (Shan *et al.*, 1999) and selected from

a phage display library (Schmidt *et al.*, 1999) panned against fixed cells. Haisma *et al.* (1998) described a scFv against CD20 fused to ß-glucuronidase. The enzyme catalyses the activation of the nontoxic drug N-[4-doxorubicin-N-carbonyl(-oxymethyl) phenyl]O-glucuronyl carbamate to doxorubicin at the site of the tumour due to the scFv targeting the enzyme to the malignant cells. Shan *et al.* (1999) constructed a series of CD20 scFv fragments with different linker lengths. These single chains were then linked to a portion of human IgG1, hinge with CH2 and CH3 domains, for purification purposes. These constructs successfully bound in ELISA and flow cytometry but the scFv with the 5aa linker was far superior in its binding to the others. Scatchard analysis of this scFv showed a binding avidity of 1.35 x 10^8 M^{-1} compared to 7.56 x 10^8 M^{-1} for the monoclonal antibody. A functional anti-CD20 scFv was isolated from a phage display library of antibody genes cloned from a hybridoma which was selected against CD20 by panning the library against CD20 positive cells fixed to ELISA plates (Schmidt *et al.*, 1999). Jensen *et al.* (1998) cloned a CD20 scFv fragment and fused it to human IgG1 hinge, CH2 and CH3 and the human CD4 transmembrane and intracellular signalling domain of the human CD3 complex's zeta chain. Transfection of this construct into Jurkat cells produced a transfected cell line which targeted CD20 expressing cells and caused the production of IL-2 and the destruction of the targeted cells.

Described here is a CD20 scFv antibody which binds a variety of cell lines and the B cell population of peripheral blood lymphocytes. Due to the specificity of the scFv for the CD20 antigen and its ability to compete effectively for antigen this scFv shows great potential for a future in the therapy or diagnosis of B lymphocyte leukemias and lymphomas.

Chapter 8

Construction of a Fab Antibody Against CD20

Chapter 8

Construction of a Fab Antibody Against CD20

8.1 *Summary*

This chapter describes the construction of a Fab antibody fragment directed against CD20. Primers designed from the sequence of the CD20 scFv, together with constant region primers, were used to amplify the variable and constant regions of the light chain and the variable region and the first constant domain of the heavy chain from cDNA extracted from the HB13d hybridoma. The light and heavy chains were inserted into pComb3H. For production of soluble Fab, clones containing both light and heavy chains were digested with SpeI and NheI to remove gene III from the vector. Clones were screened for Fab expression and one which was producing Fab was tested for activity against CD20 expressing cells. The Fab antibody produced by this clone was functional.

8.2 *Introduction*

Antibody fragments, such as scFv and Fab, have been developed as an alternative to whole antibodies for therapeutic purposes (Chapter 1). The Fab fragment has been of particular interest to this project as human Fab antibodies against CD19 and CD20 were to be isolated from phage display libraries constructed in Chapter 6. No anti-CD20 Fabs were isolated during library screening. To determine the efficiency of library panning the library could be spiked with an anti-CD20 Fab antibody at a known frequency. Isolation of the Fab antibody would confirm the lack of anti-CD20 antibodies in the original library, and the Fab could be used to optimise panning conditions. This chapter describes the construction of an anti-CD20 Fab antibody to be used for this purpose.

The HB13d hybridoma, used for the production of the Fab fragment, has already been described in the production of a scFv fragment which binds CD20 (Chapter 7). Fab antibodies have been constructed from mRNA of established hybridomas against human molecules such as the LDL receptor binding domain of apolipoprotein E (Raffai *et al.*, 1999) and pathogens like dengue virus (Thullier *et al.*, 1999).

Fab fragments consist of the whole light chain and the variable and the first constant domain of the heavy chain (CH1) (figure 1.1). The Fab light and heavy chains are transported to the periplasm separately and associate through a disulphide bond (figure 8.2.1). The constant regions and the presence of the disulphide bond which connects the heavy and light chains provide stability for the molecule. The Fab protein can be recovered by extracting the periplasmic fraction of the bacterial cell pellet or from collection of cell supernatant.

The size of the Fab molecule gives certain advantages over scFvs and whole antibodies in therapy. Smaller fragments show improved tumour penetration (Yokota *et al.*, 1992; Lane *et al.*, 1994) and lack the more immunogenic Fc region (LoBuglio *et al.*, 1989; Brüggemann *et al.*, 1989) of the whole antibody. The Fab fragment is larger than a scFv and is retained in the circulation longer (Colcher *et al.*, 1990; Milenic *et al.*, 1991). This molecule is better suited for use in therapy, where longer retention times can be an advantage, than diagnostic work to which scFvs are more suitable.

The vector used for the expression of the Fab fragment was pComb3H, a modification of the pComb3 described by Barbas *et al.* (1991). The pComb3H vector differs from the original

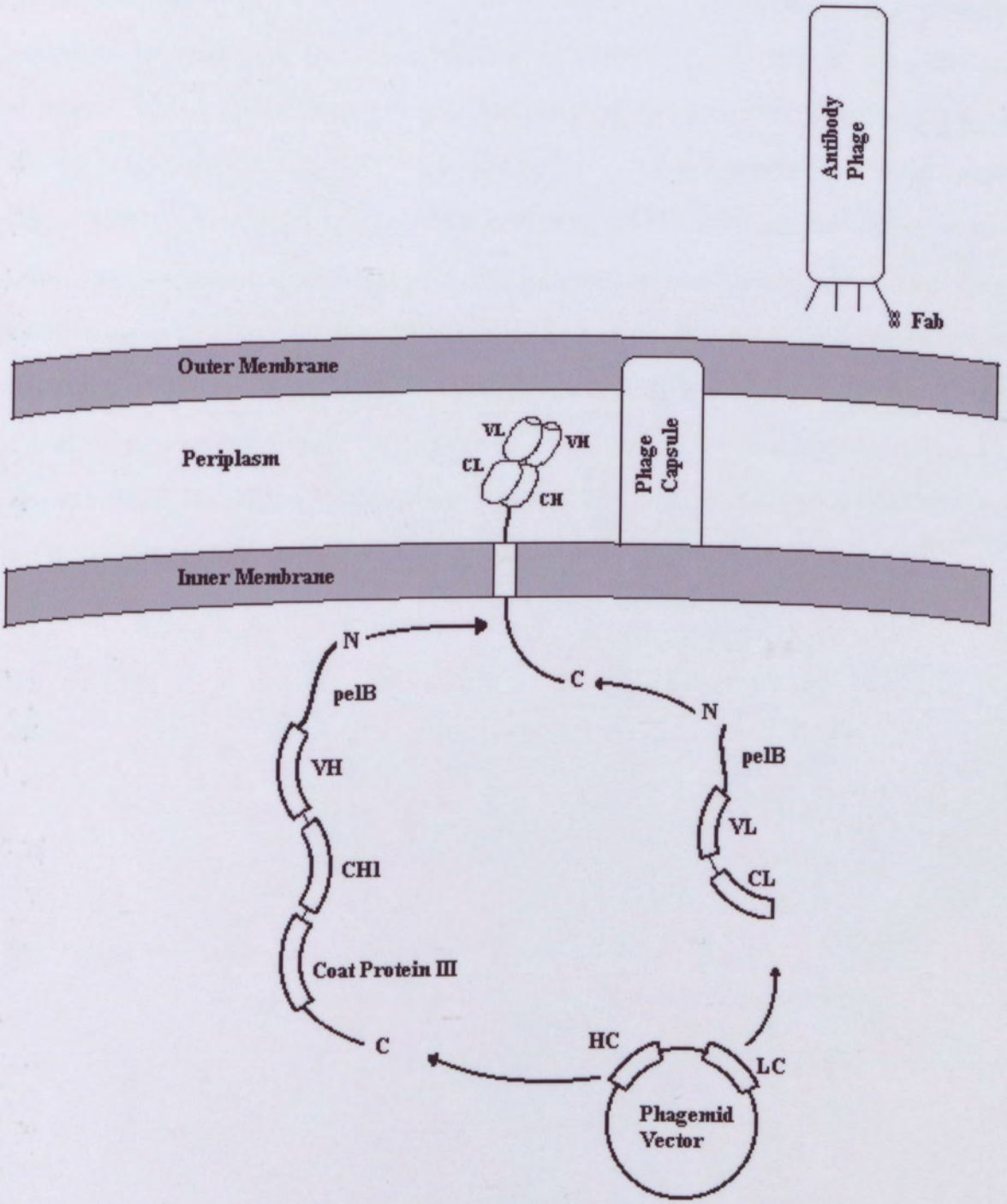


Figure 8.2.1: Transport of Light and Heavy Chains to the Periplasm and Association into a Fab Molecule.

pComb as it has been designed to provide a human consensus sequence to the amino terminus of the heavy chain (Barbas and Burton, 1993). pComb3H has been used in the construction of libraries for production of antibodies to tetanus toxoid (Ward *et al.*, 1996), proteins that interact with PAI-1 (Lang *et al.*, 1996) and characterisation of IgE antibodies against a grass pollen antigen (Steinberger *et al.*, 1996). This phage display vector was selected as the Fab produced was to be used to spike a library. Unlike the vectors pHEN-1 (Hoogenboom *et al.*, 1991) or some from the pAK series (Krebber *et al.*, 1997) the pComb vectors and others based on them have no amber stop codon so gene III needed to be removed for soluble production of protein.

CD20 has proven to be a very good target for therapy of B cell disease with at least one antibody against it, Rituximab (Reff *et al.*, 1994; Coiffier *et al.*, 1998), already in clinical use. Described here is a CD20 Fab fragment produced from the HB13d hybridoma and assays carried out to determine the extent of its activity.

8.3 *Materials*

8.3.1 *pComb3H Vector*

The pComb3H vector was kindly provided by The Scripps Institute (Barbas and Burton, 1993). Figure 8.3.1 shows the pComb3H vector. It is a modified version of the original pComb3 vector (Barbas *et al.*, 1991). There are stuffer fragments in the light and heavy chain insert sites of 1200bp and 300bp, respectively and stop codons directly following each insertion site which ensures independent expression of each chain. The chains are transported separately to the periplasm by the leader sequences ompA, for the light chain, and pelB, for the heavy chain.

8.3.2 *Fab Heavy and Light Chain Primers*

Primers for amplification of the heavy and light chains of the Fab fragment were designed based on the results of the sequencing of the CD20 scFv. These results indicated that the 5' primers for the light and the heavy chains of the single chain were LB12 and HB6 respectively from the Pluckthun series (figure 7.4.2; Krebber *et al.*, 1997). The primers designed for the Fab were modified versions of these. New restriction sites, SacI and XhoI respectively, were incorporated so the chain could be inserted into the Fab vector, pComb3H. The 5' primers were CD20.VL.SENSE for the light chain and CD20.VH.SENSE for the heavy chain (table 8.3.1). 3' primers for the constant regions of the light and heavy chains were based on the HB13d hybridoma having a kappa light chain and being an IgG1 antibody. The primer for the constant region of the heavy chain was designed in the hinge region between CH1 and CH2 and the primer designed for the light chain was on the 3' end of the light chain. The 3' primers were CD20.CL.ANTI for the light chain and CD20.CH.ANTI for the heavy chain (table 8.3.1).

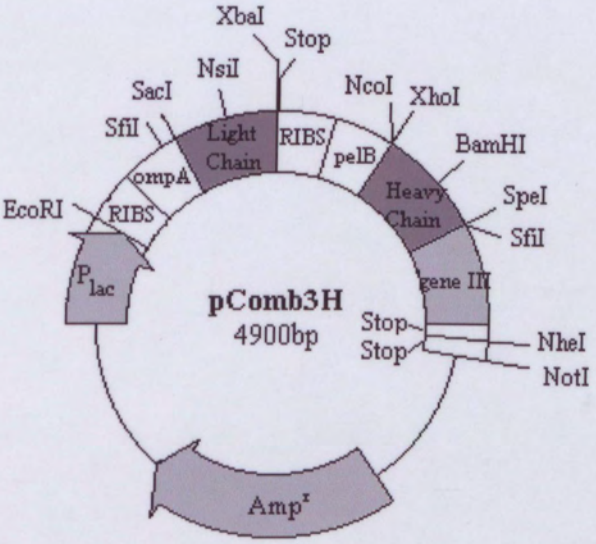


Figure 8.3.1: pComb3H.

Primer Name	Primer Sequence 5' - 3'	Restriction Site
CD20.VL.SENSE	tttagagctcgttgtgatgacccaaac	SacI
CD20.CL.ANTI	cgtctagaacactcattcctgttgaagct	XbaI
CD20.VH.SENSE	ttatctcgagcaggttcaactgcagcagtc	XhoI
CD20.CH.ANTI	gcactagtacaaccacaatccctggg	SpeI

Table 8.3.1: CD20 Fab Primers. Primers designed for amplification of the light and heavy chains of the Fab fragment.

8.4 *Methods and Results*

8.4.1 *Construction of the CD20 Fab*

Light and heavy chains were amplified from the HB13d cDNA with the CD20.VL.SENSE - CD20.CL.SENSE and CD20.VH.SENSE - CD20.CH.SENSE primers. Second round amplification was carried out on the initial PCR product to produce enough light or heavy chain to use in the digestions. The bands of approximately 670 - 700bp were cut from an agarose gel and purified.

The Fab light chains and the pComb3H vector were digested with SacI and XbaI. 1µg PCR product and 1µg pComb3H were digested with SacI (40U for the insert and 20U for the vector) and XbaI (70U for the insert and 20U for the vector) in a reaction containing 10X buffer 4 (New England Biolabs), 100X BSA and water in a volume of 40µl for the light chains and 20µl for the vector. Digestions were incubated at 37°C overnight. The pComb3H digestion was treated with 3U Calf Intestinal Alkaline Phosphatase, 10X alkaline phosphatase buffer and water in a volume of 30µl at 37°C for 30min. To stop this reaction 5mM EDTA was added and incubated at 65°C for 10min. Digestion of the pComb3H vector with the SacI and XbaI enzymes resulted in 2 bands on an agarose gel. The larger band was the linearised pComb3H and the smaller band was a stuffer fragment of 1200bp (figure 8.4.1). The digested pComb3H vector was gel purified.

100ng light chains were ligated into 50ng pComb3H in a molar ratio of 6:1. The reaction consisted of insert, vector, 3U T4 DNA ligase, 10X ligase buffer (Promega) and water in 20µl. The ligation was incubated at 16°C overnight. The control ligation consisted of the above reaction without the insert.

The light chain/pComb3H construct was transformed into HB2151 *E. coli* . 10% of the ligations were mixed with 50µl electrocompetent HB2151 cells in an eppendorf tube and then transferred to a 0.2cm gap electroporation cuvette. Directly after the electroporation the cuvette was flushed with 1ml 2YT and incubated at 37°C with vigorous shaking for 1hr. 100 - 200µl of the electroporation mix was plated on LB/200µg/ml ampicillin and incubated at 37°C overnight.

After transformation, colonies were screened for light chains. Colonies from the

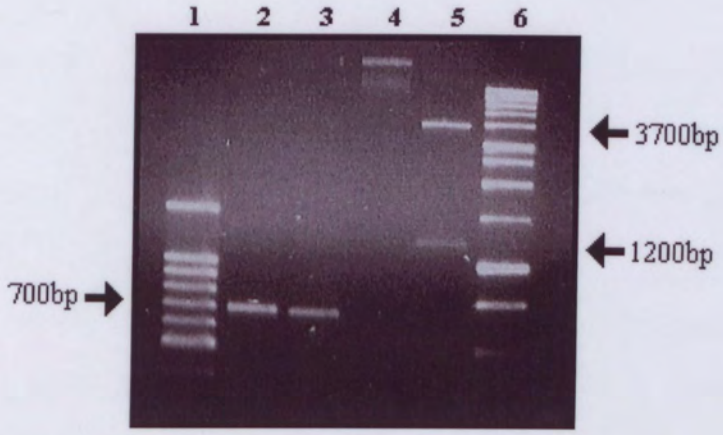


Figure 8.4.1: Digestion of pComb3H and Fab Light Chains. Lane 1 is 100bp DNA ladder; Lane 2 is undigested light chains; Lane 3 is SacI/XbaI digested light chains; Lane 4 is undigested pComb3H; Lane 5 is SacI/XbaI digested pComb3H, 1200bp is the light chain stuffer fragment; Lane 6 is 1kb DNA ladder.

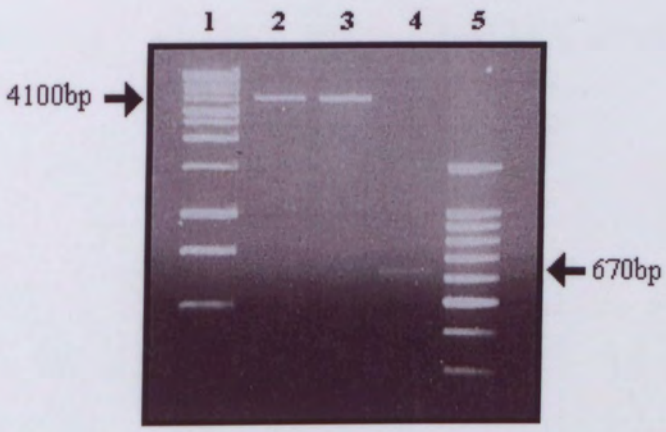


Figure 8.4.2: Digestion of pComb3H/Light Chain and Fab Heavy Chains. Lane 1 is 1kb DNA ladder; Lane 2 & 3 is SpeI/XhoI digested pComb3H/Light Chain; Lane 4 is SpeI/XhoI digested heavy chains; Lane 5 is 100bp DNA ladder.

electroporation were grown in 3ml 2YT/100μg/ml ampicillin/1% glucose for 4hr at 37°C with shaking and screened by PCR using CD20.VL.SENSE and CD20.CL.ANTI primers. Positive clones were grown in 100ml 2YT/100μg/ml ampicillin/1% glucose at 37°C with shaking overnight. Plasmid was recovered from the bacteria and resuspended in 300μl or 50μl water depending on the kit used. From 10 colonies screened by PCR, 3 had bands of the correct size indicating the presence of light chains. One of these clones was grown up and the pComb3H/light chain plasmid recovered.

The heavy chains and the light chain vector were digested. 1μg heavy chains and 1μg pComb3H/light chain plasmid were digested with XhoI (70U for the insert and 20U for the vector) and SpeI (20U for the insert and 10U for the vector), 10X buffer 2 (New England Biolabs), 100X BSA and water in a volume of 40μl for the heavy chain and 20μl for the plasmid. Digestions were incubated at 37°C overnight. Digestion of the pComb3H vector with the XhoI and SpeI enzymes resulted in 2 bands on an agarose gel. The larger band was the digested plasmid and the other a stuffer fragment of 300bp (figure 8.4.2). The plasmid was gel purified.

The heavy chains were ligated into the pComb3H light chain vector in a molar ratio of 8:1. 100ng of heavy chain and 50ng of light chain vector were ligated in a reaction containing 3U T4 DNA ligase, 10X ligase buffer (Promega) and water in a volume of 40μl. The ligation was incubated at 16°C overnight. 10% of the ligation mix and controls were used to transform HB2151 *E. coli*.

Colonies from the electroporation were grown up in 0.5ml 2YT/100μg/ml ampicillin/1% glucose for 4hr at 37°C with shaking and screened for the presence heavy chains by PCR using CD20.VH.SENSE and CD20.CH.ANTI primers. From 50 colonies screened, 4 clones were identified by PCR as having heavy chains. The four colonies were screened to determine whether both light and heavy chains were present. PCR amplification using the primers CD20.VL.SENSE and CD20.CL.SENSE showed that all four colonies contained both the light and heavy chains (figure 8.4.3). These colonies were grown in 10ml 2YT/100μg/ml ampicillin/1% glucose at 37°C with shaking overnight. The pComb3H/light and heavy chain plasmid was recovered from these colonies.

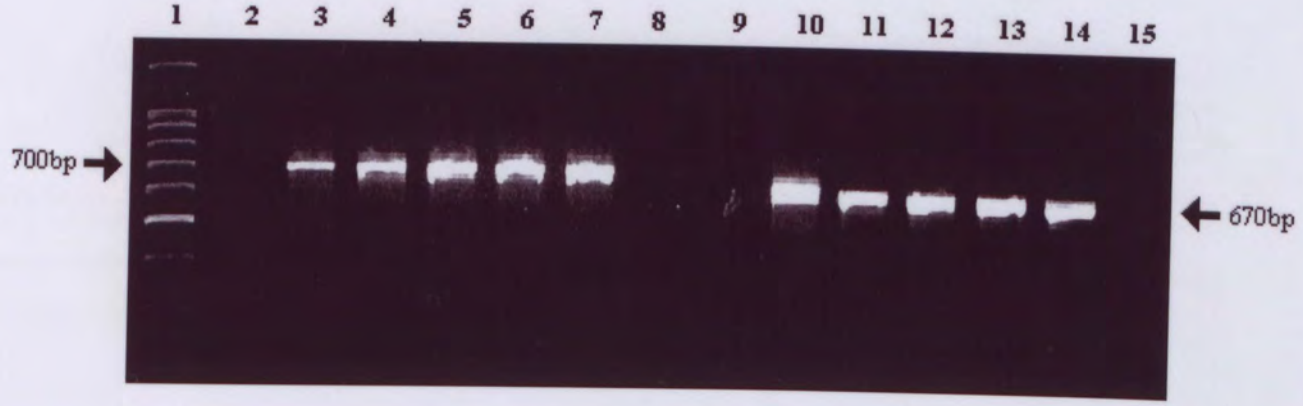


Figure 8.4.3: PCR Screen for Light and Heavy Chains in the Fab Colonies. Lane 1 is the 100bp DNA ladder; Lanes 2, 8, 9 & 15 are water controls; Lane 3 is the light chain PCR control; Lane 10 is the heavy chain PCR control; Lane 4 & 11 is colony 26; Lane 5 & 12 is colony 33; Lane 6 & 13 is colony 35; Lane 7 & 14 is colony 36. Lanes 2 – 8 were amplified with CD20.VL and CD20.CL, Lanes 9 – 15 were amplified with CD20.VH and CD20.CH.

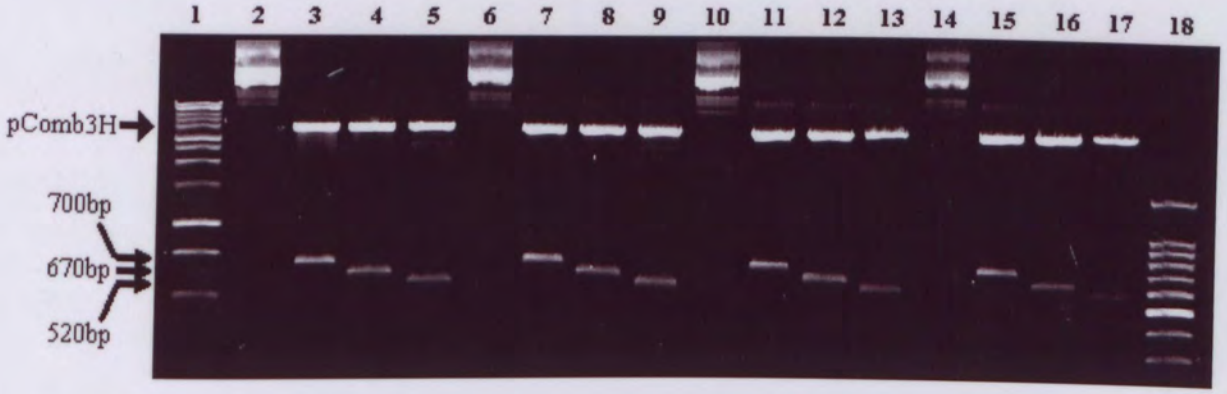


Figure 8.4.4: Digestion of Light and Heavy Chains and Gene III. Lane 1 is 1kb DNA ladder; Lane 18 is 100bp DNA ladder; Lanes 2 – 5 are colony 26; Lanes 6 – 9 are colony 33; Lanes 10 – 13 are colony 35; Lanes 14 – 17 are colony 36. Lanes 2, 6, 10 & 14 are undigested Fab plasmid; Lanes 3 ,7, 11 & 15 were digested with SacI/XbaI (LC; 700bp); Lanes 4, 8, 12 & 16 were digested with XhoI/SpeI (HC; 670bp); Lanes 5, 9, 13 & 17 were digested with SpeI/NheI (gene III; 520bp).

8.4.2 *Production and Screening of Soluble Fab*

The gene III from each of the plasmids was excised for production of soluble protein (figure 8.4.4) and the plasmid digested to remove both the heavy and light chains to show the presence of both chains in each plasmid (figure 8.4.4). Plasmid was digested with SpeI and NheI in reactions containing 1µg plasmid, 10U SpeI, 10U NheI, 10X buffer 2 (New England Biolabs), 100X BSA and water in a volume of 20µl were incubated at 37°C overnight. Digestions were gel purified. The plasmid was ligated back together in a reaction containing 100ng DNA, 3U T4 DNA ligase, 10X ligase buffer (Promega) and water in a volume of 20µl and incubated at 16°C overnight. 10% ligation mix was electroporated into HB2151 *E. coli* and colonies from the plate grown in 0.5ml 2YT/100µg/ml ampicillin at 37°C overnight with shaking. These cultures were then used to inoculate 3ml 2YT/100µg/ml ampicillin which were grown at 37°C for 5hr or until they reached log phase and then induced with 0.1M IPTG and incubated at room temperature overnight with shaking. Cultures were centrifuged at 13200rpm (17500g) in a microfuge for 2min. The supernatants were collected and the pellets were resuspended in 100µl PBS/0.01% azide and freeze/thawed three times. The pellets were centrifuged at 13200rpm (17500g) for 2min and the supernatant collected and screened by slot blot (figure 8.4.5). The controls were C215 Fab (Antibody specific for human colon carcinoma cells; Dohlsten *et al.*, 1994), X63 or HB13d supernatants. The nitrocellulose was incubated with one of 3 antibody combinantions. Antibody 1 was 1/2500 HRP conjugated anti-mouse Fab (Sigma); Antibody 2 was 1/1000 HRP conjugated anti-mouse Ig; Antibody 3 was 1/5000 biotinylated anti-mouse Fab (Sigma) with 1/5000 HRP conjugated avidin (Sigma). Tables 8.4.1 - 8.4.4 show the number of colonies screened for each clone, the antibodies used for screening and the number of positive colonies.

8.4.3 *Functional Testing of Fab Using Flow Cytometry*

Colonies which were producing Fab protein were tested for Fab specificity for CD20 using flow cytometry. The negative control was PBS and the positive control was the HB13d hybridoma supernatant. After incubation with the primary antibodies the cells were incubated with 1/50 biotinylated anti-mouse Fab antibody (Sigma) and 1/50 SAPE. One clone, called

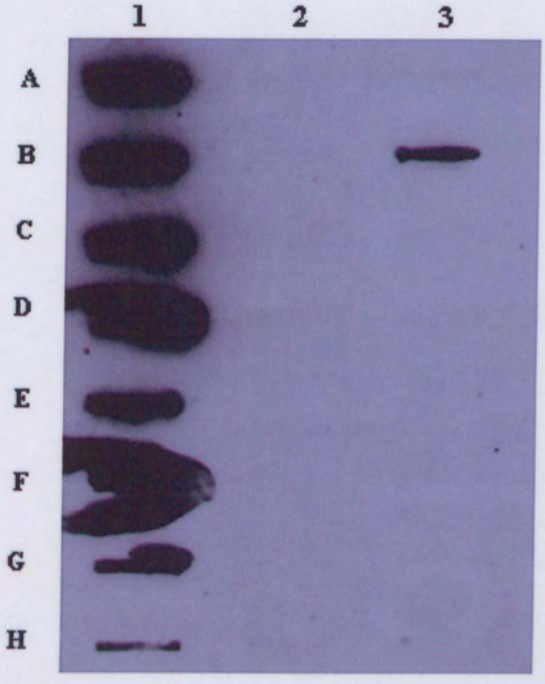


Figure 8.4.5: Slot Blot Screening of Colonies for Fab Protein Production. Slot A1 – H1 are dilutions of C215 Fab; Slot B3 is a colony producing Fab fragment. The other slots are colony supernatants. Nitrocellulose filter was incubated with 1/2500 HRP conjugated anti-mouse Fab.

Supernatant or Cell Extract Screened	Number of Colonies Screened	Antibodies Used #	Number of Positives
Supernatant	24	1 & 2	_
Supernatant and Extract	24	1	_
Supernatant and Extract	10	3	Sup - 1 Ext - 1

Table 8.4.1: Slot Blot Screen of Clone 26 Colonies. Antibody 1 is HRP conjugated anti-mouse Fab; Antibody 2 is HRP conjugated anti-mouse Ig; Antibody 3 is biotinylated anti-mouse Fab with HRP conjugated avidin. Sup is supernatant, Ext is extract.

Supernatant or Cell Extract Screened	Number of Colonies Screened	Antibodies Used #	Number of Positives
Supernatant	24	1 & 2	_
Supernatant and Extract	24	1	_
Supernatant and Extract	10	3	Sup - 2 Ext - 1

Table 8.4.2: Slot Blot Screen of Clone 33 Colonies. Antibodies are the same as those described above.

Supernatant or Cell Extract Screened	Number of Colonies Screened	Antibodies Used #	Number of Positives
Supernatant and Extract	24	1	Ext - 1 *
Extract	24	1	–
Supernatant and Extract	10	3	Ext - 2

Table 8.4.3: Slot Blot Screen of Clone 35 Colonies. Antibodies are the same as those described above. * is the colony continued for other applications.

Supernatant or Cell Extract Screened	Number of Colonies Screened	Antibodies Used #	Number of Positives
Supernatant and Extract	24	1	–
Supernatant and Extract	10	3	–

Table 8.4.4: Slot Blot Screen of Clone 36 colonies. Antibodies are the same as those described above.

35.2, bound HRIK cells in flow cytometry (figure 8.4.6). The Fab bound to the HRIK cells in this test but due to unreliable expression of the Fab fragment this result could not be repeated.

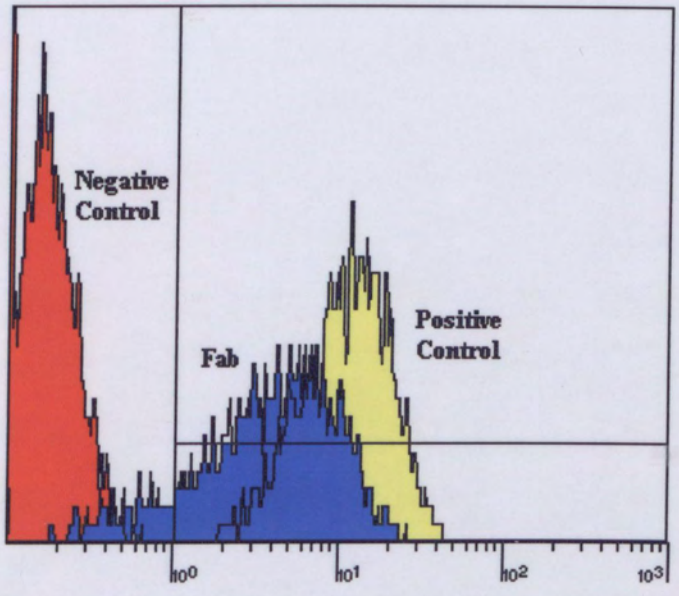


Figure 8.4.6: Flow Cytometry Screening of Supernatant From the Fab Producing Colony.

8.5 Discussion

A Fab fragment against CD20 was engineered and expressed. One clone, 35.2, was shown to be producing Fab protein by slot blot and bound HRIK, a CD20 expressing cell line, in flow cytometry. This result was not reproducible due to unreliable expression of Fab protein by the bacteria.

The periplasm is the site in *E. coli* where antibody fragments are transported for final folding and in the case of Fab antibodies, dilsulphide bond formation. Proteases have been identified in the periplasm of bacteria by an number of different groups. Some include *sohB* (Baird *et al.*, 1991), *degP* (Strauch *et al.*, 1989) and protease III (ptr) (Cheng and Zipser, 1979; Baneyx and Georgiou, 1991). The presence of these enzymes, and others, cause degradation of proteins and may be responsible for the lack of CD20 Fab expression. It has been suggested though, that the degradation of protein in the periplasm usually occurs as a consequence of production of poorly folded protein, which would likely result in poor binding if any, and does not usually cause poor expression (Pluckthun *et al.*, 1996). There have been some multiple deletion strains of *E. coli* described which could be used as an alternative to the strains currently used to produce Fab fragments (Meerman and Georgiou, 1994).

Long term storage of Fab antibody can also expose protein to attack by proteases which may be coexpressed with the antibody fragment. Some of the proteases described above, including *degP* and protease III, are secreted and others, such as OmpT (Grodberg *et al.*, 1988) and OmpP (Kaufmann *et al.*, 1994), are known to be active when the cell ruptures. If proteases are present in the collected protein solution they have the potential to dramatically reduce the activity of the Fab fragment during storage.

As well as being useful for many applications the Fab fragment produced here could be used in a model system of phage display. Unfortunately the unreliable expression of the Fab fragment restricted its use in this application. Due to time constraints this route of investigation was not pursued further, but if it had been, the Fab molecule would have been reconstructed and used in comparative binding studies on an affinity biosensor with the scFv and the parent monoclonal antibody, comparisons of circulation retention times and tumour penetration and testing for effective tumour targeting *in Vivo*.

Chapter 9

General Discussion

Chapter 9

General Discussion

For many years researchers have been developing new therapies for diseases which have poor prognosis, such as cancer. The conventional therapies of surgery, chemotherapy and radiotherapy are effective in the treatment of some cancers but in many cases patients are prone to relapse and alternative treatments which are more efficient and less destructive to non tumour tissue need to be pursued.

The search for new therapies for these diseases has so far yielded many options. One of the most promising has been the introduction of monoclonal antibodies. With the development of hybridoma technology (Kohler and Milstein, 1975) came opportunities for extensive research into different immunological systems and also the possibility for the production of reagents which have potential for use in human disease therapy. There are, however, limitations to the use of whole murine monoclonal antibodies in humans. The major problem is immunogenicity of the antibodies and the production of the HAMA response (Grossbard *et al.*, 1992) which has initiated research into the production of less immunogenic antibodies.

This thesis describes some of the techniques used for the production of therapeutic monoclonal antibodies and the antigen targets of these antibodies. The main aim for the project was to produce antibody fragments which could be used in the treatment of B cell leukemias and lymphomas. This required the production of the CD19 and CD20 cell surface antigens for antibody screening, generation of a phage Fab antibody display library and then the construction of scFv and Fab antibody fragments.

The cell surface antigens CD19 and CD20 were engineered. The CD20 peptide, provided by Intracel, was synthetically constructed based on the amino acid sequence of the extracellular loop of the antigen. This 45aa peptide is the site in the molecule where most anti-CD20 antibodies are expected to bind. Antibodies bound to the CD20 peptide using ELISA and the epitopes bound by antibody were not affected by the shortened peptide structure or the changed physical environment of the peptide.

The extracellular region of CD19 is very large, 275 amino acids. This prevented the synthesis of a CD19 peptide in the same manner as the CD20. Attempts were made to recombinantly

express the CD19 protein in a soluble form using a mammalian expression system. The extracellular CD19 constructs transfected into CHO-K1 cells, for stable expression, and COS-1 cells, for transient expression, did not produce transfectants which expressed any detectable amounts of CD19 protein. Either there was no correctly folded recombinant protein produced or the assay was not sensitive enough to detect the small amounts of protein being produced. Poor folding of the soluble CD19 protein would prevent binding of the detection antibody, and the extracellular domain of CD19 may be insoluble or not efficiently transported to the cell membrane. The solubility problem was addressed by producing a new CD19 construct which linked the CD19 extracellular region to a murine immunoglobulin CH2-CH3 tail. Western blotting of the fusion protein showed a product of ~50kDa, the expected size of the CD19MuIgG fusion protein. This construct showed some promise with the detection of both mouse (Ig) and human (CD19) protein although not enough protein was isolated for use.

As an alternative to the production of soluble human CD19, the molecule was expressed as a membrane bound protein in CHO-K1 cells. These transfected cells were used for studies with CD19 antibodies and may also find application in the search for the ligand for CD19.

The future for the production of purified soluble human CD19 could include the use of different expression systems such as bacterial or baculoviral expression systems although these systems do not glycosylate proteins to the same extent as mammalian systems. Another approach could involve the use of phosphatidylinositol linkage. In 1997 Okada *et al.* described the replacement of the TCR transmembrane domain with sequences encoding phosphatidylinositol-linked TCR expression on the surface of the cells. The recombinant protein could be cleaved from the surface of the cell for the production of soluble receptor which was then purified. This technology could be applied to the production of soluble human CD19 which could be purified for ELISA or for use in BIAcore testing of the CD19 scFv (Nicholson *et al.*, 1997) and the mutants which have been produced from it (Hohmann *et al.*, 1999).

The display of proteins on the surface of bacteriophage is just one of the many methods which has been used in an effort to produce fragments of both murine and human antibodies. This technique, initially established by Smith (1985) who displayed small peptides on the surface of bacteriophage, has expanded to the display of other proteins and antibodies (Barbas *et al.*, 1991). Most commonly the antibodies displayed are scFv or Fab fragments. Libraries have been made from the lymphocytes of immunised mice and humans, unimmunised sources and

sequences modified *in vitro* for the construction of synthetic libraries. Some of these libraries have been useful in the isolation of human antibodies against diseases which are potentially lethal in humans like HIV (Pilkington *et al.*, 1996) and cancer (Ridgway *et al.*, 1999).

The phage display library was initially produced from the tonsil lymphocytes of a young child who had undergone a routine tonsillectomy. The eventual aim was to produce antibody libraries from the bone marrow of patients in remission with a B cell lymphoma or leukemia. The hypothesis was that patients with this type of disease may produce autoantibodies against antigens on the surface of their malignant cells. Chapter 5 described the screening of serum and plasma from patients with cancer or autoimmune disease and control adults. The conclusions from this work was that all individuals produce different levels of autoantibody to CD19 and CD20. The few people that produced high levels were not restricted to the patient groups. This indicates that to construct a phage display antibody library which is biased toward a particular antigen, many individuals should be screened for the production of antibody to that antigen. This will ensure that a better proportion of the antibodies in the library will be specific for the target antigen.

The phage Fab antibody display library produced was 2.6×10^5. The percentage of heavy and light chains present in the library reduced the size of the library to 7.9×10^4. After the analysis of the library to determine the percentage of clones with both heavy and light chains, the library was to be transformed into *Escherichia coli*. Due to the small size of the library, screening was not persevered with.

A characteristic which is deemed important in the construction of phage display libraries is the size of the library. To date there have been few really large libraries produced (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996; de Haard *et al.*, 1999). Simple techniques such as the quality of PCR product, ligation reactions and electrocompetent cells can all improve library size but to construct really large libraries with $\sim 10^{10}$ clones other methods must be implemented. The library described by Vaughan *et al.* (1996) was produced by ligating large amounts of the antibody chains into the vector and performing several hundred transformations. Another method involved the infection of bacteria containing a repertoire of heavy chain antibodies with phage encoding a repertoire of light chains. The heavy chain genes were translocated to the phage plasmid by recombination within the bacterium resulting in a library size of 6.5×10^{10} (Griffiths *et al.*, 1994). de Haard *et al.* (1999) introduced another technique for production of

large libraries which involved cloning κ and λ light chain PCR products into 2 different libraries in the pCES1 vector. The heavy chain PCR products were cloned into a variation of this vector and all libraries transformed into bacteria. The VH fragments were digested from the vector and cloned into the light chain libraries which made it possible to generate a repertoire of 3.7×10^{10}.

More important than the size of the library is the diversity of the library. It is better to screen a small library with a wide range of antibodies than a really large library with a very narrow diversity. Efforts to increase library diversity has seen the development of synthetic repertoires which allow the isolation of unique antibodies to a wide range of antigen (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996).

The most common method of screening libraries is to immobilise pure antigen on a solid surface, such as an ELISA plate. With recent developments in library panning (Pasqualini and Ruoslahti, 1996; Watters *et al.*, 1997; Tordsson *et al.*, 1997; Schmidt *et al.*, 1999) the transfected cell line which expresses CD19 could be used for panning. The libraries that were screened, the Einstein kappa and lambda libraries, yielded no anti CD20 antibodies when panned against the synthetic CD20 peptide. A method of determining the efficiency of library panning is to spike a library with an antibody at a known frequency in the library. If the antibody can be recovered from the spiked library, some idea of the frequency of antibody necessary for isolation could be gained. An anti-CD20 Fab antibody was produced which bound to CD20 expressing cells. After the initial expression of the fragment, which showed biological activity, further expression was unreliable. This fragment would have been ideal to use to spike a phage display antibody library to determine the efficiency of panning libraries. Phage display libraries are reported to be readily constructed and panned in many laboratories. Unfortunately in this instance the manipulation of phage display libraries proved difficult.

This led to the engineering of antibody fragments from the anti-CD20 hybridoma, HB13d. An anti-CD20 scFv fragment was constructed which bound the synthetic CD20 peptide in ELISA and BIAcore and also bound CD20 expressing cell lines. Extensive characterisation of the scFv was carried out. This fragment effectively blocked and competed for binding with a whole monoclonal antibody. The predicted 3-dimensional structure of the scFv was determined using computer modelling. This allowed the identification of sites in the HB13d-7 scFv and the mutants, HB13d-4 and HB13d-11, in which binding could be affected. The CD20 scFv

appears to have a high affinity for antigen. This is only speculative however, as affinity of the fragment has never been accurately measured. Although the affinity could not be quantitated the competition experiments show that it displays a high activity in comparison to other anti-CD20 monoclonal antibodies. This is remarkable, bearing in mind the lower avidity of monovalent fragments. Treatment of the cells with neuraminidase improved the binding of the CD20 monoclonal antibody, making the antigen more accessible, but did not effect the competition between the two fragments. The CD20 scFv would be ideal to develop into a multimeric fragment, bispecific antibody fragment or as a suitable partner in the immunotherapy of B cell malignancies.

Many different methods have been developed in the search for better therapeutic antibodies (Chapter 1). Initial over-optimism by researchers led to monoclonal antibodies being labelled as magic bullets for targeting therapeutic reagents to cancer cells. The problems associated with the application of these antibodies in a clinical setting were not anticipated and resulted in apathy towards research in this field which was just beginning to show its potential. Due to ethical constraints many of the antibodies constructed were trialed in patients in the late stages of their disease after conventional therapies had failed. However some antibodies showed promise despite this. There are now a small number of antibodies which have been approved by the FDA for use in disease therapy, including the chimeric anti-CD20 molecule Rituximab (Rituxan; IDEC Pharmaceuticals Inc., San Diego, CA), and many more still in clinical trials. The graduation of therapeutic antibodies to the level of approval has taken many years. These antibodies have been constructed using what are now considered older antibody engineering methods such as the formation of chimeras between mouse and human antibodies or the humanisation of mouse antibodies. Antibody engineering technology is developing so quickly that when the antibodies just entering clinical trial now are being approved for use the focus of antibody engineering research will have turned else where.

Some of the most recent highlights in this field have arisen from developments in basic immunology. Interest has increased in the production of the smallest fragment of an antibody that will retain its binding properties with the discovery of a class of antibodies found in camelids and sharks which consist of heavy chains only. The result has been the development of single domain antibody fragments.

Fragments of antibodies are problematic in that they are monovalent and do not provide the same avidity as a whole antibody. There is much greater effort being made toward the construction of multivalent fragments which are smaller or remain equal in size, but with better valency, to whole monoclonal antibodies. The multivalent fragments retain or improve the avidity of whole antibodies and are good agents for conjugation to therapeutic reagents. It has been widely recognised that antibodies and antibody fragments are more effective in tumour therapy if used together with an immunoconjugate. This field is quickly expanding and new and safer methods of delivery of these substances is being developed. The conjugation of immunoliposomes (Ahmad *et al.*, 1993) for less toxic drug delivery and the further development of targeted enzyme treatment (Bagshawe *et al.*, 1999) have both worked to reduce the effects of nonspecific targeting of cytotoxic agents by releasing their drugs at the tumour site.

Multispecific antibodies have emerged as an effective way of recruiting effector cells to target cells and are a good alternative to the Fc regions of a whole antibody which can trigger the HAMA response. The bispecificity can also be used to increase the avidity of the antibody by binding two different epitopes of the same antigen. Conjugation of viruses for use in gene therapy (Martin *et al.*, 1999) and cytokines for recruitment of cytotoxic lymphocytes to tumour sites (Peng *et al.*, 1999) can replace the second antibody of the bispecific antibody to produce a bifunctional antibody. The production of antibodies with antigen specificity and effector function combined is an approach which is becoming more popular.

Although this thesis describes mostly difficulties with phage display libraries, much work has gone into improving library techniques in terms of panning but more particularly library size and the quality of antibodies which are isolated from them. Variations of antibody libraries which could be used as alternatives to phage display, namely bacterial cell display and ribosomal display, have emerged. There are problems inherent with phage display. Bacteriophage are relatively unstable, package the phagemid DNA poorly and are inclined to lose expression of the antibody from their surface. The methods used for phage display panning involve selection against antigen bound on a surface. This relies upon the antigen binding in an orientation which allows interaction of the antibody phage with the binding epitopes (Francisco and Georgiou, 1994). Also as part of the panning process the phage must reinfect bacteria. This step is prone to contamination with opportunistic wild type phage which

will be preferentially selected over the recombinant phage (Francisco and Georgiou, 1994). The final step of the panning process involves acid elution which can be detrimental to phage viability (Francisco and Georgiou, 1994). The other display methods have advantages over phage display, described in detail in chapter 6, which indicate that these systems may have more potential for easy isolation of antibody fragments.

One of the latest developments has been the use of mice for the production of human antibodies. Human PBL repopulated SCID mice have been constructed which have the ability to produce human antibodies in response to challenge with antigen. Another approach involves the integration of human antibody genes into mice (Jakobovits, 1995; Mendez *et al.*, 1997). With the generation of these mice has come a renewal of interest in the traditional hybridoma technology for the production of human monoclonal antibodies. The major problem with producing human antibodies in the first instance was the lack of a truly effective human fusion partner. This is still the case today. The production of human antibodies in mice also presents a new and interesting problem. Borrebaeck *et al.* (1993) first voiced a concern about this problem which involves differences in glycosylation patterns between human and mouse antibodies. Although the antibodies produced in these mice are essentially human their glycosylation differs from human antibodies. More significantly the glycosylation contains the Galα1-3Gal residue which is known to produce an immune response in humans (Gallili, 1993). These problems will need to be overcome before any useful therapeutic reagents can be produced from these antibodies.

The future of antibody engineering lies partly in greater development of the techniques described above and the progression of the products derived from these into a clinical setting but also in the discovery of new fields of research which will be developed into a new generation of immunoreagents. Until this time the focus will remain on the improvement of the reagents already in use. Development of less immunogenic antibodies and better delivery systems, combined with the use of antibody reagents earlier in the treatment regimen will ensure more effective treatment of disease. A synergistic approach of combining treatments would also certainly improve the prognosis of some diseases. There is a great future for the products of antibody engineering. The methods described above are those which show promise but there will be other new revolutionary discoveries which will supersede these techniques and products.

Appendix

Appendix 1

Patient and Control Plasma and Serum Samples

A1.1 Patient Plasma Samples

Sample Identification	Sample Specification	Sample Type and Origin
PS1	62001	CLL, FMC
PS2	91911	CLL, FMC
PS3	93607	CLL, FMC
PS4	93673	CLL, FMC
PS5	94185	CLL, FMC
PS6	95674	CLL, FMC
PS7	95682	CLL, FMC
PS8	95739	CLL, FMC
PS9	95933	CLL, FMC
PS10	Plasma 1	CLL, IMVS
PS11	Plasma 2	CLL, IMVS
PS12	Plasma 3	CLL, IMVS
PS13	2479486	CLL, IMVS
PS14	2692252	CLL, IMVS
PS15	2759743	CLL, IMVS
PS16	2766733	CLL, IMVS
PS17	2809653	CLL, IMVS
PS18	2825048	CLL, IMVS
PS19	2831404	CLL, IMVS

Table A1.1: Patient Plasma Samples. Shows the code used for the ELISA and flow cytometry assays. CLL is Chronic Lymphocytic Leukemia, FMC is Flinders Medical Centre, Bedford Park, South Australia, IMVS is Institute of Medical and Vetinary Science, Adelaide, South Australia.

A1.2 Control Plasma Samples

Sample Identification	Sample Specification	Sample Origin
PN1	-	CHRI
PN2	-	CHRI
PN3	-	CHRI
PN4	-	CHRI
PN5	-	CHRI
PN6	-	CHRI
PN7	-	CHRI
PN8	-	CHRI
PN9	-	CHRI
PN10	-	CHRI
PN11	-	CHRI

Table A1.2: Control Plasma Samples. Shows the control samples used in the ELISA and flow cytometry. CHRI is Child Health Research Institute, North Adelaide, South Australia.

A1.3 Patient Serum Samples

Sample Identification	Sample Specification	Sample Origin
SS1	-	Sjogren's Syndrome, QEH
SS2	-	Sjogren's Syndrome, QEH
SS3	-	Sjogren's Syndrome, QEH
SS4	-	Sjogren's Syndrome, QEH
SS5	-	Sjogren's Syndrome, QEH
SS6	-	Sjogren's Syndrome, QEH
SS7	-	Sjogren's Syndrome, QEH
SS8	-	Sjogren's Syndrome, QEH
SS9	-	Sjogren's Syndrome, QEH
SS10	-	Sjogren's Syndrome, QEH
SS11	-	Sjogren's Syndrome, QEH
SS12	22254	Myeloma or NHL, FMC
SS13	30470	Myeloma or NHL, FMC
SS14	32873	Myeloma or NHL, FMC
SS15	382807	Myeloma or NHL, FMC
SS16	41748	Myeloma or NHL, FMC
SS17	47982	Myeloma or NHL, FMC
SS18	52482	Myeloma or NHL, FMC
SS19	52486	Myeloma or NHL, FMC
SS20	53103	Myeloma or NHL, FMC
SS21	6403	Myeloma or NHL, FMC

Table A1.3: Patient Serum Samples. Shows the code used for the ELISA and flow cytometry assays. QEH is Queen Elizabeth Hospital, Woodville, South Australia and NHL is Non Hodgkin's Lymphoma.

A1.4 Control Serum Samples

Sample Identification	Sample Specification	Sample Origin
SN1	Normal 1	FMC
SN2	Normal 2	FMC
SN3	Normal 3	FMC
SN4	Normal 4	FMC
SN5	Normal 5	FMC
SN6	Normal 6	FMC
SN7	Normal 7	FMC
SN8	Normal 8	FMC
SN9	Normal 9	FMC
SN10	Normal 10	FMC
SN11	Normal 11	FMC
SN12	Normal 12	FMC

Table A1.4: Control Serum Samples. Shows the control samples used for the ELISA and flow cytometry assays.

Appendix 2

Statistical Equations

All equations described were obtained from Weiss (1999).

A2.1 *Quartiles*

Arrange the data in increasing order.

The first quartile is at position $(n + 1)/4$

The second quartile is at position $(n + 1)/2$

The third quartile is at position $3(n + 1)/4$

n is the number of samples

A2.2 *The Interquartile Range (IQR)*

IQR = Q3 - Q1

Q3 is the third quartile, Q1 is the first quartile

A2.3 *Determining Outliers Using the Upper Limit*

Upper limit = Q3 + (1.5 x IQR)

Outliers lie above the upper limit

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