Generation of Patient Specific Reagents for the Study of Multiple Myeloma Tumour Progenitor Cells

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Chapter 2 : Materials, Reagents and Solutions

List of Abbreviations

3'	three prime
5'	five prime
APS	ammonium persulphate
Az	sodium azide
bp	base pair
BSA	bovine serum albumin
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ECL	enhanced chemi-luminescence
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
Fab	fragment antigen binding
FBS	fetal bovine serum
g	centrifugal force
g3p	gene III coat protein of filamentous bacteriophage
HRP	horse-raddish peroxidase
i.p.	intraperitoneal
i.v.	intravenous
IgG	immunoglobulin, γ isotype
IPTG	isopropylthio-β-D-galactopyranoside
kDa	kilodalton
mAb	monoclonal antibody
MW	molecular weight
nm	nanometre
NTA	nitrilotriacetic acid
OD	optical density
OPD	o-phenylene diamine
PAGE	polyacrylamide gel electrophoresis
PBS(-T)	phosphate buffered saline (-Tween)
PEG	polyethylene glycol
pfu	plaque forming units
rpm	revolusions per minute
RU	resonance units
SA	streptavidin
scFv	single chain variable fragment
SDS	sodium dodecyl sulphate
TBS(-T)	tris buffered saline (-Tween)
tu	transducing units
UV	ultraviolet
V	volts
V_H	heavy chain variable region
V_L	light chain variable region
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galoctoside

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Abstract

Hybridoma production was investigated as a means to produce patient specific reagent for the characterisation of putative tumour precursors in multiple myeloma. Purified Fab fragments were used as immunogen so as to avoid an immune response towards antigenic regions within the Fc. However, all of the paraprotein-reactive murine antibodies isolated also demonstrated reactivity against pooled normal human immunoglobulin. Induction of tolerance towards human immunoglobulin was investigated as a means focus the immune response towards idiotypic determinants. Although successful in ablating the response towards unrelated immunoglobulins, serum antibody response against immunising paraprotein was also absent.

Three linear libraries were screened against a total of six IgG patient paraproteins, either as immobilised targets or in solution. Three different peptides were recovered (P3-20, P6-15 and P4-7), each of which recognised a different patient paraprotein. All three peptides exhibited binding in ELISA to the original target paraprotein and an absence of binding to normal human immunoglobulin or unrelated paraprotein when expressed by phage. P3-20 retained its binding characteristics in synthetic form, as determined by surface plasmon resonance. However, P6-15 and P4-7 failed to bind to their respective targets as synthetic peptides. The loss of function for P6-15 (VLLFHEPAGLPVYFW) may be attributed to the highly hydrophobic nature of this motif.

It was hypothesised that the presence of structural elements within the minor coat protein were required for P6-15 to adopt the appropriate conformation for binding to target paraprotein. Recombinant proteins were engineered containing the peptide sequence fused to the first N-terminal domain of g3p. Expressed protein did not exhibit the desired binding to paraprotein and neither did a second recombinant protein containing the first and second

domains together. P3-20 expressed as recombinant g3p fusion proteins also failed to bind to its target.

In conclusion, this work has demonstrated the value of phage-displayed peptide libraries for the generation of reagents specific towards myeloma tumour paraproteins. It is reasonable to predict that the extension of this work with additional patient subjects would enable exciting research into the true nature of the malignant precursor involved in multiple myeloma.

Declaration

This work contains no material which 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.

Felicity Louise Holbrook

B. Biotech. (Hons.)

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Chapter 1

Introduction

1.1 MULTIPLE MYELOMA

1.1.1 A Brief History

The first documented case of multiple myeloma was in 1845, which reported the features of extreme bone pain as well as the presence of a unique protein in the urine, known as Bence Jones protein. A detailed report of the symptoms of myeloma was not published until 1889. By the early 1900's, the involvement of plasma cells as the recognisable tumour population was reported. The relationship between Bence Jones protein and the immunoglobulin within the patient's serum was revealed in 1956 (Kyle, 1994). Since the late 1950s, multiple myeloma has been considered a model neoplastic disease both in experimental animals and man. Techniques for determining patient tumour burden in relation to the serum concentration of monoclonal IgG were derived from murine myeloma studies in 1970. Today the ability to monitor treatment success and overall prognosis depends on this ability to measure tumour burden. Durie and Salmon published the first formal staging system for multiple myeloma in 1975, which is still in use today (Durie, 1986). Significant advances in the understanding of myeloma biology have been made over the last two decades with the development of improved molecular, cellular and cytogenetic techniques. Despite these advances, much controversy exists to this day in regard to the involvement and identity of a tumour progenitor cell subpopulation responsible for disease establishment and progression.

1.1.2 Disease Characteristics and Epidemiology

Multiple myeloma is a B-cell malignancy resulting in the presence of a clonal population of plasma cells that produce monoclonal immunoglobulin. These proteins are unique to the patient and are referred to as either M-Component or paraprotein. Malignant plasma cells reside predominantly within the bone marrow and disseminate into the peripheral blood during disease progression. Chemical signaling by myeloma cells within the bone marrow

microenvironment results in the recruitment of osteoclasts, which in turn are responsible for skeletal involvement.

Multiple myeloma is reported to occur in 4 out of every 100,000 individuals (Morgan, 1999), accounting for 10 percent of all haematologic cancers and killing approximately 10,000 Americans each year (Alexanian and Dimopoulos, 1994). Disease incidence is two-fold higher amongst the black population, occurs more frequently in men, with risk increasing with age (Ozaki and Kosaka, 1998) and in individuals with immune defects (Riedel and Pottern, 1992). The Asian populations have the lowest incidence of multiple myeloma, occurring in 2.2 per 100,000 people (Ozaki and Kosaka, 1998).

Exposure to radiation or carcinogens has also been implicated as potential risk factors in the development of myeloma. A pattern of inheritance has been documented in some families for both myeloma and monoclonal gammopathy of undetermined significance (MGUS), suggesting the existence of common genetic factors in both diseases (Ozaki and Kosaka, 1998). Finally, individuals with MGUS have been shown to have a higher risk of developing multiple myeloma and as wells as other haematolgical malignancies. Within a group of 241 patients diagnosed with MGUS, Kyle (1996) found that 16% developed overt disease within 10 years, and 40% of patients were identified with a lymphoproliferative malignancy by 25 years post diagnosis.

1.1.3 Diagnosis

Multiple myeloma is characterized by an increased number of plasma cells within the bone marrow; the presence of monoclonal immunoglobulin within the serum and/or urine, and bone involvement such as lytic bone lesions (Boccadoro and Pilari, 1997) or diffuse osteoporosis which may be associated with bone fractures (Durie, 1986). Originally the most frequent presenting symptom was bone pain, however these symptoms are not always

present at diagnosis. Almost one third of newly diagnosed patients today are identified during routine examinations (Boccadoro and Pilari, 1997). The criteria followed for the diagnosis of multiple myeloma is as follows:

(1) It has been proposed that a minimum of 10 – 15% bone marrow plasma cells be required for diagnosis of MM, however it has been demonstrated that a significant proportion of patients exhibited less than 10% bone marrow plasma cells. Morphology, nuclear abnormality and variations in cell size are also valuable indicators of multiple myeloma. The proliferative activity of malignant plasma cells can be used as a direct indicator of disease progression (Boccadoro and Pilari, 1997).

(2) The minimum level of monoclonal immunoglobulin for diagnosis of myeloma is 30g/L. However, conflicting reports exist of people with higher levels and stable disease as well as lower levels having overt myeloma. Regular measurement of serum M-component is essential for the monitoring of variations of tumour burden (Ozaki and Kosaka, 1998).

(3) Lytic lesions of the bone are caused by the abnormal expansion of the plasma cell compartment within the bone marrow and subsequent osteoclast involvement. Conventional radiography or magnetic resonance imaging are used to assess skeletal involvement (Boccadoro and Pilari, 1997).

1.1.4 Prognosis

Prognostic indicators are important in the appropriate selection of therapy with tumour burden and proliferation rate being the main indicators currently used. Overall tumour burden is assessed by the levels of M-component, haemoglobin, albumin, calcemia, the percent of bone marrow plasma cells, and number and size of lytic bone lesions. These parameters

enable patients to be classified into defined risk groups within staging systems such as Durie and Salmon (Boccadoro and Pilari, 1997). Myeloma is initially chemoresponsive but with current treatment regimes the median survival is only 36 months with all patients eventually progressing to advanced disease and a diminishing quality of life (Morgan, 1999). Patterns of survival are highly heterogenous with life span post diagnosis ranging from between 1 to over 10 years (Boccadoro and Pilari, 1997).

1.1.5 Treatment

The objective of current treatment strategies is to attain a stable disease phase (plateau) during which the patient is relatively free of symptoms despite the presence of residual disease. A variety of chemotherapeutic agents exist, including alkylating agents and steroids (Boccadoro and Pilari, 1997). A variety of different regimens are available depending on patient age and response to treatment. Stem cell transplantation has been available for the last fifteen years and is generally reserved for the patients in the younger age group. Despite the benefit of avoiding transplant rejection, autologous transplants have the potential of reintroducing residual malignant cells.

Complete remission of disease is defined by at least a 75% reduction in the production of serum myeloma protein, a 95% reduction in Bence Jones proteinuria, and less than 5% of plasma cells within the bone marrow. The median duration of remission is ~2 years, and the median survival ~3 years. Less than 10 percent of patients live longer than 10 years, and there is no evidence that even a small subgroup is cured. The refractory nature of this disease, a low frequency of complete remission and inevitable relapse highlights the importance of definitively characterising a putative clonal precursor target for improved treatment (Alexanian and Dimopoulos, 1994).

1.2 MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE (MGUS)

1.2.1 Disease Characteristics

This condition is considered to be the benign form of multiple myeloma. As the name suggests, MGUS is characterised by the presence of abnormally high levels of monoclonal immunoglobulin in the absence of any other clinical features. Large community based surveys have shown that the cause of elevated M-component is unknown in 60-90% of cases (Herrinton, 1996). As with myeloma, incidence of MGUS increases with age and is reported to occur in between 7 to 8% of persons over 65 (Boccadoro and Pilari, 1997). Other aetiological and epidemiological factors are shared with multiple myeloma (Herrinton, 1996).

1.2.2 Diagnosis

A stable level of serum paraprotein at less than 3g/L is required for the diagnosis of MGUS. Other criteria include less than 10% plasma cells in the bone marrow, at most only small levels of Bence Jones protein in the urine, bone free of lytic lesions, and absence of anaemia, hypocalcaemia or renal insufficiency (Kyle, 1996). It has been suggested that the proliferation index of the plasma cells as well as serum levels of β_2-microglobulin and IL-6 are valuable indicators of malignant potential. Regular monitoring of serum paraprotein and other parameters is required to ensure the patient receives treatment if progression to myeloma occurs (Boccadoro and Pilari, 1997).

1.2.3 Prognosis

Individuals with MGUS have a 20-fold higher risk of developing overt myeloma (Herrinton, 1996). However, the majority of patients with MGUS do not progress and experience no adverse health effects (Boccadoro and Pilari, 1997). Other diseases that involve a

monoclonal component include Waldenström's macroglobulinemia, primary amyloidosis, plasmacytoma, lymphoma and chronic lymphocytic leukemia (Kyle, 1996). Thus it is important to assess risk of disease progression so as to provide appropriate intervention as soon as possible if required.

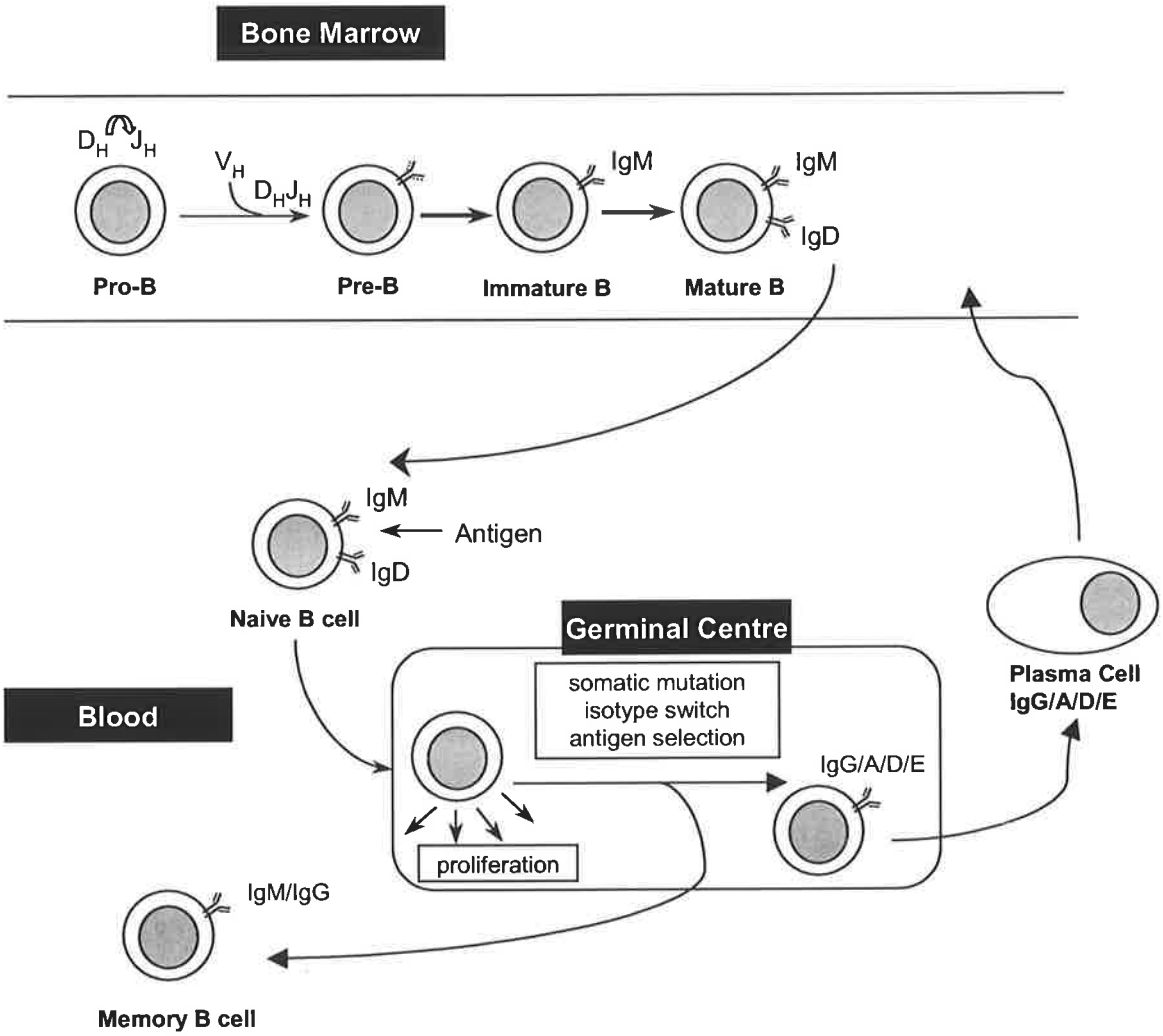
1.2.4 Treatment

Patients with MGUS can be divided into two groups, most requiring only routine follow-up. Those showing evidence of overt disease progression require immediate chemotherapy as detailed for myeloma patients (Boccadoro and Pilari, 1997).

1.3 NORMAL B-CELL DEVELOPMENT

A schematic diagram summarising the developmental stages from pro-B cell through to terminally differentiated plasma cells is provided in Figure 1.1. B-lymphocytes are responsible for the production of antibody constituting the humoral arm of the immune system. Five unique tertiary structures of immunoglobulin exist (M,D,G,A,E) and are referred to as the isotype. The generation of functional antibodies is an involved process and commences at the earliest stages of B-cell commitment. Initially D_H and J_H germline sequences randomly combine (McBlane *et al.*, 1995), followed by the addition of a V_H gene segment (Cook and Tomlinson, 1995). Sequence complexity within the CDR3 region is enhanced by the random addition of nucleotides by terminal deoxynucleotide transferase (TdT) (Kirkham and Schroeder, 1994). Finally, pairing with the surrogate light chain leads to surface expression of the immature immunoglobulin. Cells expressing both IgM and IgD migrate to the peripheral blood to receive antigen challenge and the activated cells either mature into short-lived plasma cells or proceed to the germinal centres for antibody maturation (Stevenson and Sahota, 1999). The final stages of B-cell development are dependent on the generation of high affinity antibodies, with cells expressing low affinity

Figure 1.1 B-cell developmental pathway in relation to immunoglobulin gene sequence (Stevenson and Sahota, 1999)



immunoglobulin being induced to apoptose. Sequence variation is achieved by the process of somatic hypermutation within centroblasts and the final, antigen selected cells undergo isotype-switch reaction leading to the expression of mature immunoglobulin within the centrocytes (Liu *et al.*, 1996). From this point, two potential differentiation pathways are open. Centrocytes can proliferate and develop into memory-B cells residing within the germinal centre, or leave to mature into long-lived plasma cells that migrate to the bone marrow. Analysis on the lifespan of this terminally differentiated population of cells in mice has indicated a survival period of several weeks (Ho *et al.*, 1986) to over a year (Slifka *et al.*, 1998). This process of proliferation, differentiation, and function of lymphohaematopoietic cells is regulated by a complex network of growth factors and cell surface molecules.

1.4 BIOLOGY OF MULTIPLE MYELOMA

1.4.1 Antigen Involvement

The majority of the V_H sequences in multiple myeloma cells are somatically mutated, with a median value of 8.2% base changes apparent in a study involving 48 patients (Vescio *et al.*, 1995). A subsequent finding has also demonstrated the involvement of V_L (Sahota *et al.*, 1997), which further strengthens the argument for antigen selection. The nature of antigen is unknown and likely to vary, although there is the intriguing observation that many myeloma proteins have autoantibody activity. Recently, the possibility of viral involvement supporting development of multiple myeloma has been raised in which the infectious agent produces analogues of human cytokines. However, there is no evidence to date for antibody activity against viral antigens in myeloma proteins. A totally disparate role of antigen, in the form of anti-idiotype antibody, has also been raised. A decrease in anti-idiotype producing B-cells during the progression of disease may reflect an autoregulatory mechanism in MGUS and the early stages of myeloma (Bergenbrant *et al.*, 1991). However, it is also possible that this anti-idiotype promotes disease progression (Abdou and Abdou, 1975). If an immune

regulatory mechanism exists, escape from such control may theoretically require an alteration in paraprotein structure. Amplification of the IgH gene by PCR consistently produced a product of identical length throughout disease progression indicating that no further gene rearrangement had occurred (Ralph *et al.*, 1993).

1.4.2 Adhesion Molecules and Cell Signaling

Multiple myeloma has traditionally been regarded as a tumour consisting predominantly of terminally differentiated plasma cells. However, the malignant cells involved in multiple myeloma have an abnormal biology as evidenced by their aberrant differentiation and survival pathway in the bone marrow. A characteristic of myeloma cells is a marked immaturity resulting in a plasmablastic type cell and they are usually $CD19^-$, $CD56^+$ whereas normal plasma cells are $CD19^+$, $CD56^-$ (Harada *et al.*, 1993). The majority of tumour cells are found in the bone marrow, suggesting the existence of important receptors on the surface of myeloma tumour cells that bind to growth factors and matrix proteins within the bone marrow stromal environment (van Riet, 1999). Significant heterogeneity both within and between patients is observed in the expression of adhesion receptors such as CD54 (ICAM-1), CD56 (N-CAM), CD40, CD44, CD49d (VLA-4), CD49e (VLA-5), and CD138 (syndecan-1) (Ozaki and Kosaka, 1998). These receptors mediate the interaction between myeloma cells and the surrounding bone marrow microenvironment, resulting in the secretion of a variety of haematopoietic growth factors. Of particular importance is the production of interleukin-6 (IL-6), which promotes the differentiation of B-cells into immunoglobulin secreting plasma cells. It is generally believed that this cytokine is produced as a result of a paracrine stimulation of bone marrow stromal cells, osteoclasts and osteoblasts. However, autocrine production induced by CD40 activation on myeloma cells as well as viral IL-6 from Kaposi's sarcoma-associated virus has also been proposed. Whatever the source, IL-6 is a critical factor in the pathogenesis of multiple myeloma, stimulating cell proliferation and preventing cell death in apoptotic conditions (Hallek *et al.*, 1998). Several other cytokines (ciliary neurotropic factor,

oncostatin M, leukemia inhibitory factor, IL-11) also bind to a common β-subunit of the IL-6 receptor and may also be involved in the growth of multiple myeloma. The presence of the soluble IL-6 receptor α subunit is believed to sensitize myeloma cells towards IL-6. A direct correlation with success of treatment and a reduction in IL-6 and IL-6Rα levels in the patient serum has been reported (Hallek *et al.*, 1998).

As with normal B-cell development, granulocyte colony-stimulating factor (G-CSF), IL-10, granulocyte-macrophage-CSF, stem cell factor, tumour necrosis factor-a, hepatocyte growth factor, and insulin-like growth factor-1 and 2 have all been implicated in the support and development of myeloma cells. The observation of G-CSF stimulation should herald caution against the use of this cytokine after high-dose chemotherapy or for stem cell harvests (Hallek *et al.*, 1998).

Finally, the Fas antigen (alternatively termed APO-1 or CD95) has been observed on the majority of myeloma tumours and cell lines. Activation of Fas induces programmed cell death. However in myeloma, IL-6 activation of cells has been associated with antagonism of Fas activity. This antagonism of IL-6 and Fas may serve as a paradigm for the many interactions that regulate myeloma growth in a fine-tuned network of cytokines and growth factors in the bone marrow microenvironment (Hallek *et al.*, 1998).

1.5 Evidence for Tumour Progenitor Cell Involvement

Over 150 years have passed since the first known case of multiple myeloma. However, much controversy exists to this day in regards to the stage in B-cell development at which malignant transformation occurs. Observations of disease dissemination despite localisation of malignant cells within the bone marrow, recurrence of disease despite depletion of the malignant plasma pool to undetectable levels, and difficulty in sustaining malignant plasma cells in culture led to the theory of precursor cell involvement.

The search for these cells began over two decades ago and has since been attempted by a number of groups. Paraprotein produced by the myeloma plasma cells is patient specific and provides a marker for tumour involvement. Unique structures within the antigen binding site are referred to as the idiotype (Wells *et al.*, 1973). Initial studies into myeloma precursor cells involved the production of anti-idiotypic antibodies to screen for the expression of the paraprotein idiotype in conjunction with cell surface markers. Significant evidence has accumulated implicating the involvement of B-cells prior to the plasma cell stage. However, doubts in regards to anti-idiotype specificity, idiotype absorption onto the surface of non-malignant cells and advances in cell-typing support the need for further investigation to clarify the specific phenotype of the malignant precursor population. The information provided below highlights the changing beliefs in this field of research up until the commencement of this work.

1.5.1 Stem cells => pre-B cells

Initial studies made use of unique structural determinants on the paraprotein referred to as the idiotype (Id), which is both tumour and patient specific. A number of research groups have utilised anti-Id antibodies in conjunction with lineage and developmental markers to characterise the earliest idiotype-expressing clonal population. Kubagawa *et al.* published one of the earliest studies in 1979. These studies identified a population of cells of lymphoid morphology that were surface Id negative but weakly positive for cytoplasmic Id expression. Interestingly, this research also revealed the association of isotypes different from that of the paraprotein. It was concluded that the clonal precursor population in multiple myeloma involved cells at the pre-B stage of differentiation prior to isotype commitment (Kubagawa *et al.*, 1979).

Within a few years, reports confirming the presence of B-cells in the early stages of development began to emerge. These studies demonstrated the presence of CD10 (CALLA)

on Id-expressing cells circulating in the peripheral blood of patients (Mellstedt *et al.*, 1974; Ruiz-Argüelles *et al.*, 1984; Pilarski *et al.*, 1985; Grogan *et al.*, 1987). During this time it was believed that CD10 was expressed solely on pre-B cells (Caligaris-Cappio *et al.*, 1985), however it is now known to be re-expressed on pre-plasma cells (Zola, 1987). Evidence of CD10 expression in the presence of cIg and the mature plasma cell marker R1-3 has been reported in ~50% of patients studied by Epstein and coworkers (1988). From these observations, it was proposed that the various combinations of CD10+/cIg+, CD10-/cIg+ and CD10+/cIg- aneuploid cells represent discrete stages in the development of the myeloma clone (Epstein *et al.*, 1988).

The presence of lymphoma-like translocations and the involvement of *c-myc* (Selvanayagam, 1988) and *Bcl1* also support the concept of pre-B cell involvement (Epstein, 1988). The involvement of T-lymphocytes bearing idiotype and antigen-binding receptors was also proposed early on in this argument (Preud'homme *et al.*, 1977). This finding has been supported by a separate study by Lee and co-workers (1987) in which it was proposed that the malignant transformation may occur prior to the commitment to lymphoid lineage differentiation. However, a report stating that clonal rearrangement of the T-cell receptor gene was unable to be detected in myeloma patients has also been published (Berenson *et al.*, 1987). The presence of Fc receptors on T- and B-cells may account for the conflicting findings in regards to surface paraprotein expression.

Cells with typical myeloma-cell features (CD10, PCA-1) expressing erythroid and megakaryocytic antigens (GP11b/11a, CD11b, glycophorin A) have also been observed. Such infidelity in surface antigen expression may indicate that the initial neoplastic lesion occurred in an early haematopoietic stem cell and that the multilineage markers may indicate specific stages at which oncogenic transformation occurred (Epstein *et al.*, 1990). However the counterargument should be considered, in that these anomalies reflect the existence of gene de-regulation within malignant cells.

1.5.2 B-cells => pre-Plasma Cells

Peripheral blood lymphocytes (PBLs) were the first cells to be implicated in multiple myeloma besides the myeloma plasma cell (Mellstedt *et al.*, 1974). Within the next decade, various groups either confirmed (Abdou and Abdou, 1975; Bast *et al.*, 1982; Carmagnola *et al.*, 1983; Cassel *et al.*, 1990) or disputed (King and Wells, 1981; Gobbi *et al.*, 1984; Pilarski *et al.*, 1984) their existence.

More recent studies have identified cells at a stage intermediate between lymphocytes and plasma cells containing idiotypic immunoglobulin localised within a cytoplasmic spot. These cells were shown to be highly proliferative and to express HB4 and HB6 but not antigens associated with preplasmacytoid cells (CD9, 5, 10, 19-22 and 25) or plasma cells (PCA-1, PC1, CD38, HAN-PC2) (Lokhorst *et al.*, 1987). More recent studies have described a clonal component at this stage of development exhibiting the same aneuploidy features as the myeloma plasma cells and are cIg-. These cells were only detected in patients with overt stage III myeloma and hence implicated in disease progression (Shimazaki *et al.*, 1992).

The presence of myeloma precursor cells within the peripheral blood has been demonstrated by culturing circulating mononuclear cells in the presence of various growth factors. Studies with interleukin-3 and interleukin-6 (Bergui *et al.*, 1989) as well as TNF-α and interleukin-4 (Sawamura *et al.*, 1994) demonstrated the ability to generate plasma cells expressing the original monoclonal immunoglobulin, however the possibility of this representing an undetected sub-population of malignant plasma cells should be considered.

It has also been demonstrated that the stimulation of CD10+ cIg- sIg- cells with phorbol ester leads to the generation of plasma cells expressing the same heavy and light chain isotypes as the original cells. These cells were classified as pre-plasmacytic based on their ability to transform despite the absence of TdT and the B-lineage markers CD9, CD19, CD22 and

CD24. Interestingly, only Stage II and III patients exhibited this cell population (Caligaris-Cappio, 1985).

Three-colour immunofluorescence has enabled the characterization of monoclonal B-lymphocytes at a late stage of differentiation based on their expression patterns of CD19, CD20, CD10 and PCA-1. Evidence of heterogeneity in the expression of the RA and RO isoforms of CD45 was interpreted as representing cells within various stages of differentiation (Jensen *et al.*, 1991).

Malignant cells have been found in all fractions of CD20+ and CD34+ cells in the peripheral blood of 11 patients ranging from Stage IA to IIIB. The expression of CD20 in conjunction with CD10, CD21 or CD19 facilitated the characterisation of cells as either a pre-B or activated B-cell, surface Ig+ cell or a pre-plasma cell respectively, whilst CD34 indicated haematopoietic stem cells. Identical CDR3 sequences were found in cells from each of these subsets that had been isolated from peripheral blood. The lack of recombinase activity despite the presence of CD34+ and CD20+ CD10+ cells led to the hypothesis that the myeloma precursor cell emerges during the later stages of B-cell differentiation, with the re-expression of CD34 enabling the resumption of self-renewal (Takishita *et al.*, 1994).

B-cells from a patient with a high proportion of CD19 B-lymphocytes in their circulation were sorted and assessed for expression of CD34 and patient IgH mRNA. Only CD19+, CD34-, IgH+ cells could be detected which also expressed CD45 and CD38. These cells were identified as either late-stage B cells or pre-plasma cells and were susceptible to treatment, leading to the hypothesis that a B-cell population at an earlier stage development existed which was resistant to therapy. The involvement of stem cells in the malignant clone is also jeopardised by the lack of $CD34^{+}$ IgH^{+} cells (Rasmussen *et al.*, 1999).

One group has reported the ability to delineate several stages in pre-B cell development based on the intensity of CD24 in conjunction with a variety of early B-cell markers. Investigation of the pre-B cell ($CD24^+$) compartment of multiple myeloma patients revealed a reduction of these cells compared to normal bone marrow samples. Duperray and coworkers (1991) proposed that this indirectly supports the concept of the major malignant clone is likely to be either at the late or (pre)-plasma cell stage of B-cell development. However, it was acknowledged that the initiating oncogenic event could have occurred at this early stage. Furthermore, the stability of V_H-DJ_H joining sequences within multiple myeloma indicates a lack of recombinase activity in myeloma cells and their precursors. As V_H replacements are generated by these enzymes during pre-B and virgin-B cell stages, this observation does not support the hypothesis of early B-cell involvement in the multiple myeloma clone (Takashita *et al.*, 1994).

Perhaps the strongest and most reliable determinants of clonal origin lie within the monoclonal immunoglobulin gene. DNA sequencing of the CDR2 and CDR3 regions of the monoclonal immunglobulin gene have revealed evidence of somatic hypermutation, that the sequence is conserved amongst various populations of monoclonal B-cells, and the involvement of other isotypes besides the main isotype. Based on these findings it was proposed that the clonogenic precursor arises post initiation of affinity maturation and prior to isotype-switching events (Bakkus *et al.*, 1994). These cells are most likely to be either memory B-cells or plasmablasts that have passed through the germinal centre (Bakkus *et al.*, 1999).

1.6 MM *vs.* MGUS

Unlike immunoglobulin genes in multiple myeloma, MGUS sequences frequently demonstrate a high degree of intraclonal variation. This observation implies that the clonal precursors in MGUS are still exposed to the hypermutation process within the germinal

center and thus differ from the clonal population involved in multiple myeloma (Sahota *et al.*, 1996). A continuation of this concept is that a second transformation event is required for the progression of MGUS to myeloma (Bakkus, 1999).

1.7 ONCOGENIC EVENTS IMPLICATED IN MONOCLONAL GAMMOPATHIES

Malignancies occur as a result of the loss of regulation over cell proliferation and programmed cell death. Such regulatory changes can arise as a result base modifications, chromosomal translocations, or from the loss or gain of chromosomes. The observation that chromosomal aberrations increase with disease progression, and the concurrent gain in independence from the bone marrow microenvironment, has led to the proposal of a multistep transformation process (Hallek *et al.*, 1998).

Karyotypic abnormalities have been detected at a frequency of 30 to 50% in large studies of myeloma tumours using conventional analyses (Hallek *et al.*, 1998), and recent reports using interphase fluorescent *in situ* hybridization have detected involvement in essentially all tumours (Avet-Loiseau *et al.*, 1999). Considering the high degree of rearrangement that occurs during the production of functional antibodies, it may not be surprising that the most frequent translocations involve the IgH locus at position 14q32.3. Such events regularly lead to the relocation of oncogenes and either transcriptional enhancer or promoter regions resulting in the over expression of various oncoproteins. Karyotypically detectable translocations involving 14q32 have been reported to occur in 10 to 40% of myeloma tumours (Chesi *et al.*, 1996). The gain of chromosomes (hyperploidy) may also contribute to overexpression of oncoproteins. On the other hand, monosomies and methylation of various genes may lead in the loss of various regulatory proteins. The involvement of various oncogenes, the process of their activation and their putative role in the development of multiple myeloma is discussed.

1.7.1 Numerical abnormalities

Aneuploidy is a common event in multiple myeloma with the characteristic abnormalities being monosomy 13, and trisomies of chromosomes 3, 5, 7, 9, 11, 15, and 19 (Ozaki and Kosaka, 1998) and (Hallek *et al.*, 1998). As previously mentioned, such abnormalities could be expected to have far reaching consequences in terms of major loss or gain of important genes.

1.7.2 Translocations

c-myc

c-myc performs an essential role in regulating commencement of the DNA replication cell cycle (Greil *et al.*, 1991). However, in myeloma its involvement may be more concerned with tumour mass rather than proliferative activity. Translocation of t(8;14) in the IgH locus, point mutations within the *c-myc* regulatory units, and amplification of the gene have been implicated in the transcriptional deregulation of this oncogene (Greil *et al.*, 1991). Contradictory reports concerning the involvement of *c-myc* expression in multiple myeloma exist. Sensitive immunfluorescence techniques have revealed a significant heterogeneity of *myc* mRNA and protein expression in plasma cells. These studies have also suggested that, in multiple myeloma, the role of *c-MYC* is not cell cycle transversion (Greil *et al.*, 1991).

c-myc translocations t(8;14) or 8q24 into the switch regions of IgH(L) loci or of 8q24 into other regions, DNA rearrangement of regulatory sequences downstream of *c-myc* or within the gene itself and DNA amplification of *c-myc* indicates that this oncogene may be involved in 10-20% of tumours. Despite the apparently low incidence of genetic involvement, elevated levels of c-Myc protein may occur frequently in myeloma. This disparity may be due to either preferential expression of one allele or as a result of mutations in the 5' untranslated region of the c-myc gene leading to the deregulation of translational control (Hallek *et al.* 1998).

bcl-1, bcl-2

The *bcl-1* locus at 11q13 is involved in recurrent translocations to 14q32 in myeloma. Cloning of the breakpoints from multiple myeloma samples has shown that the major reciprocal sites are located in the switch regions of the IgH locus (Bergsagel *et al.*, 1996).

Bcl-2 is a membrane protein known to regulate apoptosis and is found in the outer membrane of mitochondria and nuclei as well as with the endoplasmic reticulum (Korsmeyer, 1992). Overexpression of *bcl-2* in cancer cells can result in chemoresistance and blocks apoptosis. Recent work has shown the existence of several Bcl-2-related proteins that can inhibit (Bcl-X_L, Mcl-1, NR-13, A1, Bcl-W) or enhance (Bax, Bcl-X_S, Bak, Bad) apoptosis. The various actions of Bcl-2 are facilitated by the formation of inactivating or activating heterodimers with other proteins encoded by these genes of the *bcl-2* superfamily (Hallek, 1998).

Expression of *bcl-2* has only been observed in up to 15 % of multiple myeloma patients. Despite this low frequency, overexpression of Bcl-2 is seen in the majority of multiple myeloma cells and cell lines and is though to assist in the prevention of apoptosis induced by IL-6 deprivation and to confer drug resistance (Hallek *et al.*, 1998). High levels of Bcl-2 protein are likely to mediate the resistance of myeloma cells to apoptosis induced by dexamethasone, IL-6 deprivation, staurosporine, or other drugs.

c-maf

The oncogenic potential of *c-maf* (16q23) was first demonstrated in myeloma by Chesi and co-workers (1998a). A study of 21 cell-lines revealed that 5 exhibited translocations within the IgH locus. Translocations and corresponding expression of c-maf mRNA was detected at a similar frequency in primary myeloma tumours. c-maf is a member of a large family of transcription factors that play a role in cellular proliferation, differentiation and responsiveness to IL-6.

Cyclin D1

Cyclin D1 is frequently overexpressed in immature myeloma (Hallek *et al.*, 1998). The 11q13 locus is reportedly associated with approximately 30% of myelomas exhibiting translocations within the IgH locus (14q32.3) (Gabrea *et al.*, 1999). Two out of three human myeloma cell lines that overexpress cyclin D1 are reported to display the 11;14 translocation within a gamma switch region, indicating that an error in switch recombination has occurred. Precise mapping of one of these translocations revealed the breakpoint to reside 100kb centromeric to cyclin D1, close to the MTC region often involved in 11;14 translocations in mantle-cell lymphoma (Chesi *et al.*, 1996). Recently an alternative scenario to the process of reciprocal translocation has been observed in cells from the U266 myeloma cell line. In normal cells, intervening sequences arising during switch recombination are released and lost from the cell. However, in U266 it has been proposed that overexpression of cyclin D1 has resulted from the insertion of the 3' α-1 enhancer, present on an excised intervening sequence, adjacent to the cyclin D1 oncogene on chromosome 11q13. Furthermore, this event most likely occurred during a productive IgHμ–ε switch recombination (Gabrea *et al.*, 1999).

In 21 myeloma cell lines, 6 of which have t(11;14) (bcl-1 locus), there is a direct correlation between the presence of the translocation and over-expression of cyclin D1 protein (Hallek *et al.*, 1998). The presence of the t(11;14)(q13;q32) translocation in myeloma is associated with a lymphoplasmocytic cell morphology as well as with more aggressive disease and a poorer prognosis.

Cyclin D1, together with CDK4, inactivates pRB by phosphorylation resulting in progression through the G1 phase of the cell cycle. Unlike other genes identified in this region of 11q13, there is a close association between t(11;14) and enhanced expression of cyclin D1. Furthermore, a variant translocation (ie, involving a light chain gene) just telomeric to cyclin D1 and a translocation breakpoint within the 3' untranslated region of cyclin D1 (resulting in a 3' truncated mRNA) serve to effectively define cyclin D1 as the gene targeted by the t(11;14),

although given the distance of the breakpoints from cyclin D1, it is possible that other genes may be involved (Hallek *et al.*, 1998).

FGFR3

Dysregulation of the fibroblast growth receptor 3 gene by t(4;14)(p16;q32) translocations has been observed in both myeloma cell lines and primary tumours (Chesi *et al.*, 1997). It has been suggested that this event may be unique to myeloma, however it has yet to be extensively studied in other tumours (Hallek *et al.*, 1998) A year later, Chesi and co-workers discovered that this translocation caused the simultaneous dysregulation of a novel gene named MMSET which also has oncogenic potential (Chesi *et al,*, 1998b).

Hallek and coworkers (1998) propose that myeloma cells with dysregulated expression of FGFR3 as a result of t(4;14) receive an FGFR3-mediated signal from FGF produced by stromal cells in the bone marrow micro-environment. Presumably the continuous signal interferes with the cells' terminal differentiation and apoptosis, and may also stimulate growth. Evidence exists suggesting that the activating mutations occur after the translocation event, enabling tumour survival and/or growth in the absence of FGF (Hallek *et al.*, 1998).

IRF4

A novel locus *MUM1* (multiple myeloma oncogene 1) located on chromosome 6p25 has been implicated in the t(6;14) translocation located near the interferon regulatory factor 4 (*IRF4*) gene in a myeloma cell line (SK-MM1). The resultant sequence is transcribed to yield a functional *MUM1/IRF4* protein that promotes B-cell differentiation in response to mitogenic signals and may act as an effector of other cytokines known to regulate B-cell development. It has also been proposed that the aberrant phenotype of myeloma cells may be as a direct consequence of this hybrid protein (Iida *et al.*, 1997).

1.7.3 Base Modifications

N-ras & K-ras

The *ras* family of oncogenes are activated by single point mutations at codons 12, 13, and 6 which result in amino acid substitution. N-& K-*ras* genes have been implicated in approximately one-third of patients and occur predominantly in advanced disease (Corradini *et al.*, 1993). Recent studies have shown that activation of the N-*ras* gene promoted independent growth of a previously IL-6 dependent cell line, and prevented apoptosis of cells in the absence of IL-6 (Corradini *et al.*, 1993). It has been proposed that mutations resulting in the constitutive activation of *ras* contribute to the IL-6 independent growth often observed at the later stages of disease (Liu *et al.*, 1996). Interestingly, whilst N-*ras* mutations failed to confer any obvious effect to disease progression compared with patients expressing wildtype *ras,* the presence of activated K-*ras* was associated with more aggressive disease and shorter survival (Liu *et al.*, 1996). Based on these observations it has been proposed that the *ras* oncogenes are responsible for tumour progression rather than the initial oncogenic transformation event (Corradini *et al.*, 1993).

p53

p53 is a tumour suppressor gene which has many effects on cell growth and differentiation and is often viewed as the regulator for entry into the cell cycle. It has been shown that the binding of p53 to response elements on *bcl-2* and *bax* genes leads to their downregulation or upregulation respectively (Mazars *et al.*, 1993). Despite their high incidence in cell lines p53 mutations are rarely observed in patient samples, and are normally associated with the advanced stages. It has been proposed that p53 mutations may prevent apoptosis and differentiation if plasmablasts (Hallek, 1998).

1.7.4 Other Mechanisms Supporting Myeloma Cell Growth

Inhibitors of cyclin dependent kinases

Inactivation of $p16^{INK4A}$ (p16) by hypermerthylation occurs in myeloma, especially in advanced disease states, and may be associated with decreased growth control. Homozygous deletions of genes encoding for $p15^{INK4B}$, $p16^{INK4A}$, and $p18^{INK4C}$ may also occur in some multiple myeloma patients (Hallek *et al.*, 1998).

Deactivation of pRb

The retinoblastoma (Rb) gene is another tumour suppressor gene and is frequently mutated in MM patients (70%) and MM-derived cell lines (80%) (Hallek *et al.*, 1998). The phosphorylation of pRb prevents its ability to arrest cell-cycle transition and myeloma cells have been observed with strong expression of pRb in its phosphorylated form (Urashima *et al.*, 1996).

p21

Also known as $p21^{WAF1}$, this protein is another regulator of cellular proliferation. In particular, p21 protects against apoptosis induced by p53 by arresting cell-cycle and initiating DNA repair. Unlike normal cells, myeloma cells appear to constitutively express p21 and the induction of cell cycle transition by IL-6 is associated with the downregulation of its expression in these cells (Urashima *et al.*, 1997). These observations indicate conflicting roles of this tumour suppressor. Whilst overexpression in multiple myeloma cells may facilitate resistance of these cells to therapy, it also may result in the hampering of tumour development and hence slow clinical progression (Hallek *et al.*, 1998)

Drug Resistance

The multi-drug resistance gene encodes a 170 kDa p-glycoprotein which is responsible for the resistance of tumour cells to a variety of antineoplastic drugs. The amplification of MDR

gene expression is correlated with a resistance to doxorubicin and vicristine (Epstein *et al.*, 1989).

MDM2

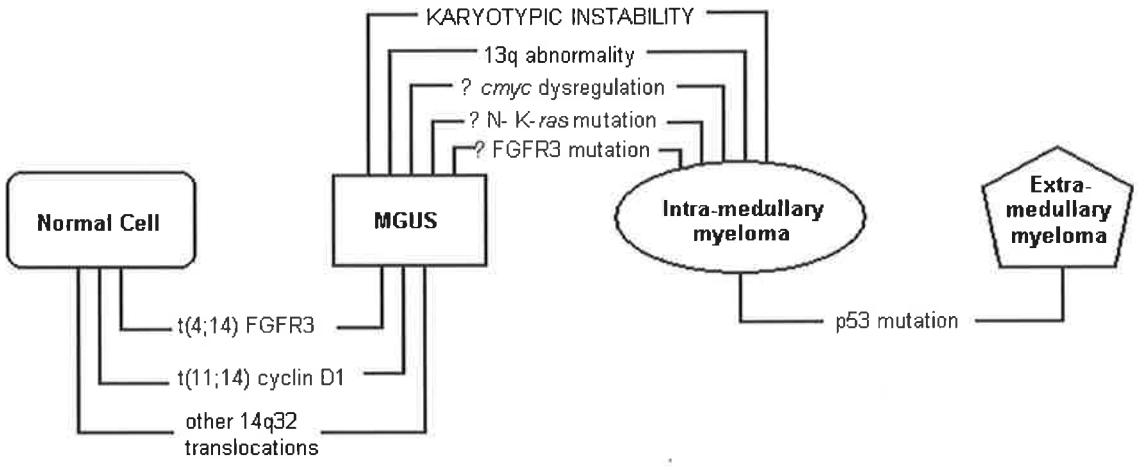
The murine double minute 2 (MDM2) gene product binds directly to DNA, enhancing the transcription of proliferation elements, thus facilitating the transition from G1–S phase. Evidence of it abrogating tumour suppressive functions of wild-type p53 has also been reported. The overexpression of MDM2 protein has been observed in myeloma cell lines and has been proposed to enhance cell-cycle progression in multiple myeloma (Teoh, *et al.*, 1997).

1.7.5 Summary of Genetic Events Implicated in Multiple Myeloma

As highlighted above, the initial oncogenic event leading to the development of either MGUS or multiple myeloma remains elusive. Considering that characteristic translocations exist for other B-cell neoplasms [ie t(8;14)(q24;q32), Burkitt's lymphoma; t(14;18)(q32;21), follicular lymphoma (Rabbitts, 1994)], it is not unreasonable to expect the presence of a common genetic event in monoclonal gammopathies. As mentioned earlier only small numbers of cells within the myeloma clone are in the metaphase stage of DNA replication, which is a requirement of conventional karyotyping. The advent of interphase FISH and single-cell PCR should help to overcome this problem. Secondly, some translocations may be karyotypically silent. For these reasons, it is not necessarily surprising that the primary oncogenic event in multiple myeloma and MGUS is yet to be discovered. Translocations involving 14q32.3 have been reported in 62% of patients with abnormal karyotypes. Furthermore, clinical and cytogenetic observations apparently indicate that the primary transformation of MGUS to myeloma is as a result of such translocations (Nishida *et al.*, 1997). However, more recent studies have reported that monosomy 13 is implicated in this transition and occurs after translocations involving the IgH locus (Avet-Loiseau *et al.*, 1999).

Potential additional events commence with activation of p21ras in the early stages of disease development as a result of IL-6 secretion in the surrounding bone marrow microenvironment, conferring IL-6 independent growth. At later disease stages, activating mutations of N- or K-*ras* replace this function, facilitating expansion and dissemination of the tumour outside the bone marrow. This hypothesis awaits its verification by a careful analysis of the $p21^{ras}$ function at different stages of myeloma (Hallek *et al.*, 1998). A multistep process showing the gradual malignant transformation leading to myeloma has been proposed based on disease evolution (Hallek *et al.*, 1998) and can be seen in Figure 1.2.

Figure 1.2 Flow diagram of the possible mutational evolution of multiple myeloma



1.8 Objectives

As discussed earlier, it is believed that the myeloma tumour plasma cell population is maintained by malignant cell precursors. Furthermore, these cells appear to be resistant to treatment and have been implicated in disease relapse. Despite a large amount of work in this field over several decades, the exact identity of the myeloma precursor cell remains elusive. This knowledge may lead to the development of new treatment strategies that could result in the ability to cure this disease. The first aim of this work was the to produce reagents that recognise myeloma patient paraproteins with a high degree of specificity using hybridoma technology. Monoclonal antibodies that bind to the immunizing paraprotein but not to unrelated isotype and subclass-matched human immunoglobulin or unrelated paraprotein would be classified as idiotype-specific. The second aim of this study was to use these patient-specific reagents to investigate the presence of idiotype-expressing B-cell subpopulations within patient bone marrow aspirates. Stages of B-cell differentiation would be determined by the expression of developmental markers in conjunction with idiotype determinants using multi-parameter flow cytomtery. Furthermore, the presence of karyotypic abnormalities within putative tumour precursor cells would be investigated by fluorescent *in situ* hybridization using sorted cells.

Chapter 2

Materials, Reagents and Solutions

2.1 OVERVIEW

This chapter contains detailed information on the preparation of reagents and solutions used throughout this thesis and are grouped according to particular methods. Chemicals were either analytical, molecular biology or electrophoresis grade. All components used for the preparation of bacterial growth media were purchased from Oxoid, antibiotics from Sigma.

2.2 PATIENT MATERIAL

Six IgG multiple myeloma paraproteins were used in this study (1 Stage I, 1 Stage II and 4 Stage III). Patient serum samples were kindly donated by Professor Bik Toh and Dr.Andrew Zannettino from the Division of Haematology, Institute of Medical and Veterinary Science, Adelaide, South Australia. Informed consent was obtained from all patients involved in this research, according to institutional requirements.

Serum was stored in 2ml cryovials at -80°C until required.

2.3 BACTERIAL STRAINS

ER2738 (Supplied with the Ph.D-7 library, New England Biolabs)

F′ *proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15 zzf::Tn10(Tet*^{R}*)/fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5*

JM109 (Promega)

F′ *traD36 proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15/Δ(lac-proAB) glnV44 e14*$^{-}$ *gyrA96 recA1 relA1 endA1 thi hsdR17*

K91 (Kindly donated by Dr. Joanne Casey, La Trobe University, Victoria, Australia)

Hfr-Cavalli with chromosomal genotype *thi λ*$^{-}$

2.4 MAMMALIAN CELL-LINES

p3653

A non-secretory murine myeloma cell line used as a fusion partner for hybridoma production. p3653 cells were maintained in RF10 medium in a 37°C humidified incubator with 5% CO_2.

2.5 IMMUNOCHEMISTRY REAGENTS

2.5.1 Commercial Antibodies and Conjugates

Conjugates were purchased from Silenus-Amrad Biotech unless otherwise stated. Anti-FLAG mAb kindly donated by Dr. S. Nuttall, CSIRO Division of Health Sciences and Nutrition, Victoria.

anti poly-HISTIDINE mAb, ascites fluid (Sigma)

HRP sheep anti-mouse immunoglobulin

HRP sheep anti-human immunoglobulin

Streptavidin-HRP

Biotinylated horse anti-mouse (H+L) (Vector Laboratories)

anti-M13 mAb (Pharmacia)

2.5.2 Hybridoma Supernatants

HB61 - anti human kappa light chain

HB60 - anti human IgG Fc common

HP6001 – anti human IgG_1 Fc

HP6002 – anti human IgG_2 Fc

HP6003 - anti human IgG_3 Fc

HP6014 – anti human IgG_2 Fab

HP6020 – anti human IgG_4 Fab

HP6025 – anti human IgG_4 Fc

X63 - Non-secretory hybridoma

2.6 BUFFERS

2.6.1 Agarose Gel Loading Buffer

10mg Bromophenol Blue

2ml Glycerol

10ml dH_2O

Loading buffer was boiled for 30 minutes and stored at room temperature.

2.6.2 Blocking Buffer (7-mer Peptide Library)

Per 100ml dH_20:

0.1M $NaHCO_3$ (840mg), pH 8.6

500mg BSA

0.03% NaN_3

Filter sterilized (0.22µm) and stored at 4°C.

2.6.3 Electrophoresis Sample Buffer (ESB)

Per 100ml dH_20:

1.54g Tris Base

2g SDS

20ml Glycerol

5mg Bromophenol Blue

Made to volume and stored at room temperature. Reducing buffer contained 1.54g DTT per 100ml and was stored at -20°C.

2.6.4 Periplasmic Extraction Buffer (Borate Buffer)

Per Litre dH_20:

200mM $Na_2B_4O_7$ (76.3g)

100mM NaCl (5.8g)

100mM EDTA (37.2g)

Titrated to pH 8.0 and made to volume.

2.6.5 Phosphate Buffered Saline (20x PBS), PBS-Azide and PBS-Tween

Per Litre dH_20:

160g NaCl

4g KCl

23g NaH_2PO_4

4g KH_2HPO_4

Made up to volume with dH_2O and stored at room temperature. This solution was diluted 1 in 20 as required with dH_2O and stored at room temperature:

0.01% PBS Azide (PBS-Az) was prepared by adding 0.5ml 20% sodium azide to 1L PBS.

0.05% PBS-Tween20 (PBS-T) was prepared by adding 1ml Tween-20 to 2L PBS.

2.6.6 0.05M Phosphate-Citrate Buffer, pH 5.0

A 5mg Phosphate-Citrate Buffer tablet (Sigma) was dissolved in 100ml H_2O, filter sterilized (0.22μm) and 10ml aliquots stored at room temperature.

2.6.7 20mM Phosphate / 10mM EDTA Buffer, pH 7.0

A stock solution of 200mM phosphate buffer was prepared according to the method described in Gomori *et al.* (1917). 930.6mg EDTA was dissolved in 250ml of 20mM phosphate buffer, titrated to pH 7.0 with 5M NaOH.and stored at 4°C.

2.6.8 Tris Borate EDTA Buffer (10x TBE)

Per Litre milliQ H_20:

108g Tris Base

55g Borate

9.3g EDTA

2.6.9 Tris-Buffered Saline (TBS)

Per Litre dH_20:

50mM TrisHCl (7.88g)

150mM NaCl (8.776g)

Titrated to pH 7.5 with NaOH, made to volume, autoclaved and stored at room temperature

TBS-Az and TBS-T were prepared as for PBS-Az and PBS-T respectively.

2.7 REAGENTS & SOLUTIONS

2.7.1 Anti-Phage Solution (1% SDS, 0.1M NaOH)

Used as a cleaning solution for all glassware, plastics, pipetted and surfaces that come into contact with filamentous bacteriophage.

Per Litre dH_20:

10g SDS

4g NaOH

2.7.2 4-chloro-1-napthol

A stock solution of 4-chloro-1-napthol (Sigma) was prepared by dissolving a 30mg tablet in 1ml methanol and stored at -20C. Immediately prior to use, a 10ml working solution was prepared by diluting the stock solution 1 in 60 with 50mM Tris / 150mM NaCl (pH 7.4) and adding 5µl 30% v/v H_2O_2.

2.7.3 Phage Precipitation Solutions

Per 400ml dH_20:

80g PEG 8000 (20%w/v)

58.44g NaCl (2.5M)

Autoclaved and stored at room temperature.

2.7.4 *o*-Phenylenediamine (OPD)

A 5mg OPD.2HCl tablet (Sigma) was dissolved in 10ml 0.05M phosphate-citrate buffer and 5µl 30% v/v H_2O_2 added immediately prior to use.

2.7.5 Red Cell Lysing Solution (10x)

Per 100ml dH_2O:

8.26g NH_4Cl

1.0g $KHCO_3$ or $NaHCO_3$

37mg EDTA

Made to volume and filter sterilized using a 0.22µm filter.

2.7.6 SDS-Polyacrylamide Gels

The recipes in Table 2.1 provide sufficient acryalmide for a single minigel using a Biorad minigel apparatus.

Table 2.1 SDS-polyacrylamide gel recipes

	4% Stacking Gel	6% Resolving Gel
40% Acrylamide/ Bisacrylamide (BioRad)	0.5ml	1.5ml
milliQ H_2O	3.2ml	5.8ml
1.5M Tris, pH 8.8	-	2.7ml
0.5M Tris, pH 6.7	1.25ml	-
10% SDS	100µl	100µl
40% Ammonium Persulphate	15µl	15µl
TEMED (Merck)	15µl	15µl

2.8 GROWTH MEDIA

2.8.1 Bacterial

2.8.1.1 Antibiotics

Tetracycline: - 50mg/ml Tetracycline HCl in ethanol, filtered (0.22µm) and stored at -20°C protected from light.

Chloramphenicol – 25mg/ml in ethanol, filtered and stored at 4°C.

2.8.1.2 1M IPTG

Diaoxane-free IPTG was purchased from Promega. 2.38g IPTG was dissolved in 10ml sterile water, filtered using a 0.22µm syringe filter and stored at 4°C protected from light.

2.8.1.3 2YT

Per Litre dH_2O:

16g Tryptone

10g Yeast Extract

5g NaCl

Made to volume, autoclaved and stored at room temperature.

2.8.1.4 LB

Per Litre dH_2O:

10g Bacto-tryptone

5g Yeast Extract

5g NaCl

Made to volume, autoclaved and stored at room temperature.

2.8.1.5 Nutrient Agar

1.6g Bacterial Agar was dissolved per 100ml LB or 2YT media, autoclaved and stored at room temperature. Antibiotics were added to cooled, melted agar immediately prior to pouring and stored at 4°C.

2.8.1.6 Agarose Top

Per 500ml dH_2O:

5g Bacto-tryptone

2.5g Yeast Extract

2.5g NaCl

0.5g $MgCl_2.6H_2O$

3.5g Agarose

Made to volume, autoclaved and stored at room temperature.

2.8.1.7 IPTG-XGal Plates

Per 500ml LB Agar:

105µl 1M IPTG

400µl 50mg/ml X-Gal (Promega)

Plates stored at 4°C protected from light.

2.8.2 Mammalian Cell Culture

2.8.2.1 Heat-Inactivated Fetal Bovine Serum (HI-FBS)

Fetal Bovine Serum (CSL) was heat-inactivated for 30 minutes in a 55°C water bath and aliquots of 50ml stored at -20°C.

2.8.2.2 Penicillin Streptomycin Glutamine (100x PSG)

10,000 U/ml penicillin, 10mg/ml streptomycin, 200mM L-glutamine

The following was dissolved into 500ml milliQ H_2O:

3.125g	Benzyl Penicillin (~1600U/mg)
5g	Streptomycin Sulphate
14.615g	L-Glutamine

After filter-sterilizing using a 0.22µm screw top filter into a sterile 500ml bottle, aliquots of 5ml were stored at -20°C.

2.8.2.3 RF10

50ml HI-FBS and 5ml 100x PSG was added to 500ml RPMI 1640 (Cell Image) and stored at 4°C.

2.8.2.4 Freezing Media

50% HI-FBS and 30% DMSO were prepared separately in RF10 and equal parts combined immediately prior to use. These solutions were stored at 4°C with the DMSO solution protected from light.

2.8.2.5 HAT media

HAT Media Supplement (50x) Hybri-Max® (Sigma)

Per 10ml: $5x10^{-3}$M hypoxanthine

$2x10^{-5}$M aminopterin

$8x10^{-4}$M thymidine

One vial of 50x HAT supplement was reconstitute in 10ml sterile water, added to 500ml RF10 and stored at 4°C.

2.8.2.6 HT media

HT Media Supplement (50x) Hybri-Max® (Sigma)

Per 10ml: $5x10^{-3}$M hypoxanthine

$8x10^{-4}$M thymidine

One vial of 50x HT supplement was reconstituted in 10ml sterile water, added to 500ml RF10 and stored at 4°C.

2.9 Supplier Information

BDH Chemicals	Poole, Dorset, UK
CC-Biotech	Poway, CA, USA
Cell Image	Adelaide, South Australia
Commonwealth Serum Laboratories (CSL)	Parkville, Victoria, Australia
Dynal Biotech	Oslo, Norway
Gene Works	Adelaide, South Australia
Invitrogen (Novex)	Mt.Waverly, Victoria, Australia
Merck	Poole, Dorset, UK
New England Bioland (NEB)	Beverly, MA, USA
Oxoid Ltd.	Hampshire, England
Pharmacia	Peapack, NJ, USA
Pierce	Rockford, IL, USA
Promega	Madison, WI, USA
Qiagen	Basel, Switzerland
Roche Applied Science	Mannheim, Germany
Sigma-Aldrich	St Louis, MI, USA
Silenus	Boronia, Victoria, Australia
Vector Laboratories	Burlinghame, CA, USA

Chapter 3

Evaluation of Traditional Hybridoma Techniques

for the Generation of Anti-Idiotype

Monoclonal Antibodies

3.1 OVERVIEW

Identification of putative tumour progenitor cells is dependant on the development of highly specific reagents. Unique structures within the antigen-binding site of myeloma paraproteins, otherwise referred to as the idiotype, provide a valuable marker for disease involvement. This chapter details the use of mice immunised with purified Fab fragments of patient paraprotein for the production of anti-idiotype secreting murine hybridomas. All of the supernatants that tested positive for binders against the immunising paraprotein were also reactive against normal human immunoglobulin. A tolerisation strategy to render mice unresponsive to normal human immunoglobulin was subsequently investigated. It was thought that this approach would increase the proportion of B-lymphocytes reactive against the paraprotein idiotype and hence increase the probability of recovering anti-idiotype specific clones. Screening of serum antibodies revealed that tolerised animals completely failed to respond to the immunising paraprotein.

3.2 INTRODUCTION

Multiple myeloma is characterised by the over-production of monoclonal immunoglobulin, known as the paraprotein. Unique structural determinants within the paraprotein are referred to as the idiotype and are patient specific. Both phenotypical and genotypical methods have been employed for the assessment of idiotype expression by putative tumour precursors. Investigation of clonal involvement using the former approach was selected for this research for the following reasons. Firstly, idiotypic cells can be simultaneously characterised for the expression of idiotype and B-cell related cell surface markers using fluorescent activated cell sorting. In addition, flow cytometry offers the advantages of high-throughput and accuracy, enabling small subpopulations to be identified within a large pool of cells. Finally, analysis of clonal involvement by the detection of tumour immunoglobulin sequences requires a prediction to be made regarding the expected phenotype.

Production of antibody-based reagents against the idiotype of myeloma paraproteins has been well documented (Lindström *et al.*, 1973; Abdou and Abdou, 1975; Kubagawa *et al.*, 1979; Bast *et al.*, 1982). In each of these cases, antiserum was recovered from animals immunised with paraprotein and non-specific antibodies removed by multiple rounds of absorption against normal human immunoglobulin. Patient specificity was determined using immunodiffusion techniques and defined by a lack of binding to either normal human immunoglobulin or isotype matched paraproteins. Several years later, Kiyotaki and co-workers generated anti-idiotypic monoclonal antibodies from mice immunised with whole myeloma paraprotein (Kiyotaki *et al.*, 1987). A major advantage of hybridoma technology over antisera production is that monoclonal antibody specificity and reactivity is consistent between preparations. However, the fact that there is not a wealth of studies using this approach suggests that successful production of specific reagents is not a frequent event. In fact some studies have reported the identification of anti-idiotype reagents that have later been reclassified as anti-isotype specific (Kampe *et al.*, 1994). In addition, some putative anti-idiotypes that have failed to demonstrate binding to normal human serum by ELISA have subsequently shown to be inhibited by normal human serum in immunofluorescence cell studies (Stevenson *et al.*, 1986).

It was hypothesized that the use of antigen-binding fragments (Fab) instead of whole paraprotein would enhance the recovery of antibodies reactive against the variable regions. Paraproteins from Patient 1 and Patient 2 were purified from serum using ProteinG affinity chromatography and digested with immobilized papain. ProteinG affinity chromatography was used to remove contaminating Fc and the flow-through collected. Balb/C mice were immunised using a rapid immunisation schedule that has previously been reported for the production of anti-idiotype monoclonal antibodies against B-cell lymphoma paraproteins (Thielemans *et al.*, 1984). All of the monoclonal antibodies recovered recognized both the paraprotein and normal human immunoglobulin. Two hybridoma clones were analysed by

Western blot, one of which clearly bound to the heavy chain of reduced normal human immunoglobulin.

Tolerisation of mice towards normal human immunoglobulin was investigated as a means to focus the immune response towards the idiotype determinants. Lebrón and co-workers (1999) reported the tolerisation of adult mice to immunodominant epitopes within heterodimeric proteins according to the phenomenon of "high-zone" tolerance. These mice were then used to produce antibodies against rare and weakly immunogenic determinants present on the partner protein. A small study was conducted to assess if this approach could induce immunological unresponsiveness to normal human immunoglobulin in adult Balb/C mice. Unfortunately, analysis of serum antibodies showed that these mice still recognized normal human Ig.

Tracing further back into the literature revealed that Balb/C mice are particularly sensitive to human gamma globulin (Golub and Weigle, 1969). This tendency to become sensitized may be due to enhanced macrophage function in this particular strain of mice. It is therefore vital that the immunoglobulin preparation used to induce tolerance is highly free of aggregated material. It has been shown that unresponsiveness towards human immunoglobulin can be achieved provided aggregates are rigorously removed using sodium sulphate fractionation (Golub and Weigle, 1969). Normal human immunoglobulin was deaggregated according to the methods of Christian (1958) and mice tolerised as before. Although tolerance to normal human immunoglobulin was successfully induced, no serum antibody response was detected following immunisation with patient paraprotein Fab.

3.3 METHODS

3.3.1 Preparation of Myeloma Paraprotein Fab

3.3.1.1 Purification of Patient Paraprotein

IgG paraprotein was purified from patient serum using ProteinG sepharose affinity chromatography (GammaBind® G Sepharose®, Pharmacia Biotech). Prior to loading, patient serum was diluted 1 in 4 with PBS and centrifuged at 13,200 rpm (17,500g) for 1min in a benchtop centrifuge to remove solid material. The column was equilibrated with PBS and 10mg of IgG paraprotein was loaded per ml of ProteinG sepharose at a flow rate of 1ml/min. Bound immunoglobulin was eluted with 0.5M acetate (pH 3) and titrated immediately with 2M TrisHCl, pH 11.2.

Following affinity chromatography, pooled paraprotein fractions were dialysed against PBS-Az overnight at 4°C with stirring. Paraprotein solution was either stored at 4°C or concentrated to ~5ml using polyethylene glycol (PEG8000) and buffer exchanged with 20mM Phosphate / 10mM EDTA Buffer, pH 7.0 for subsequent digestion with papain.

3.3.1.2 Protein Determination

Protein concentrations were determined by visible region spectrophotometry using the BioRad Protein Assay: Bradford Reagent. In summary, samples were diluted in PBS and 200µl added to 800µl Biorad Bradford Reagent. Reactions were vortexed immediately and incubated for 5 minutes at room temperature. Samples were then transferred to a disposable microcuvette (BioRad) and the absorbance read at 595nm. A standard curve was constructed using bovine immunoglobulin in the range of 0.5 to 20µg/ml for each assay.

3.3.1.3 Papain Digest

Purified patient paraprotein was digested using an Immunopure® Fab Preparation Kit (Pierce) according to the manufacturer's instructions. Papain Digestion Buffer was freshly prepared before each digestion by dissolving 42mg cysteine.HCl in 12ml Phosphate Buffer (pH 10, supplied by manufacturer). Briefly, 0.5ml of a 10mg/ml paraprotein solution in 20mM Phosphate / 10mM EDTA (pH 7.0) and 0.5ml Papain Digestion Buffer was added to 0.5ml 50% slurry of immobilized papain in a glass test tube. Immunoglobulin was digested overnight at 37°C with gentle mixing. ProteinG affinity chromatography (3.3.1.1) was used to remove Fc fragments and the flow through containing the Fab fragments was collected. Pooled Fab was dialysed against dH_2O overnight at 4°C and concentrated using PEG8000 to ~5ml. The purity and concentration of resultant Fab fragments were determined as for the purified paraprotein. 100µg aliquots were freeze dried and stored at -20°C.

3.3.1.4 SDS-Polyacrylamide Gel Electrophoresis

Protein purity and yield was monitored by visualization on a 6% SDS polyacrylamide gel using the BioRad Mini-Gel Apparatus according to Laemmli (1970).

Samples were diluted in ESB with or without DTT prior to loading and acrylamide gels were electrophoresed at 200V in a BioRad minigel electrophoresis tank containing 1x Running Buffer.

Unless otherwise stated acrylamide gels were stained with Coomassie Blue as follows. After fixing for 30 minutes in Destain at 37°C, Coomassie Blue was added at a concentration of ~0.5% and allowed to gently mix for 2-3 hours. After removal of stain, fresh Destain was added and allowed to mix gently overnight at room temperature.

Silver staining was performed using a commercial Sigma kit (Cat. N° AG-25) according to manufacturer's instructions. Briefly, gels were fixed for 1 hour or greater in 30% ethanol / 10% acetic acid. Following a 30 minute wash in milliQ H_2O gels were incubated in silver solution for a further 30 minutes, rinsed with milliQ H_2O and developed for approximately 15 minutes. After sufficient banding was visible, the developer was removed and the reaction stopped. The gel was then washed for 30 minutes as before and reduced for ~30 seconds or until background diminished. Finally, gels were washed for 1 minute under running water. Gels were restained when a higher degree of sensitivity was desired.

3.3.1.5 Western Blotting

Proteins were transferred from an acrylamide gel onto PROTRAN® nitrocellulose membrane (Schleicher & Schuell) using a semi-dry western blotting apparatus (Pharmacia LKD Novablot Multiphor II, Pharmacia) at 75mA for 90 minutes.

Membranes were blocked in 3% skim milk / PBS-T for 30 minutes at 37°C or overnight at 4°C. Blots were probed for 1 hour with appropriate murine hybridoma supernatant (neat), washed 3 times for 5 minutes with PBS-T and probed with HRP-conjugated anti-mouse Ig diluted 1:1000 with 3% skim milk / PBS-T. Blots were detected with Enhanced Chemi-Luminescence Western Blotting Reagent (ESL, Pharmacia Biotech) and visualized by exposing X-ray film for 1 to 5 minutes. Alternatively, nitrocellulose membranes were developed with 0.5mg/ml 4-chloro-1-napthol (Sigma).

3.3.2 Monoclonal Antibody Production

Approval from the institutional animal ethics committee was obtained for all of the work detailed below and animal handling was performed according to committee regulations.

3.3.2.1 Immunisation

A rapid immunisation protocol previously used to produce anti-idiotype antibodies against human B-cell lymphomas was selected for this study (Thielemans *et al.,* 1984). Briefly, 10-week old Balb/C mice were immunised with 100µg human paraprotein Fab reconstituted in 100µl GERBU adjuvant (C-C Biotech) by intraperitoneal injection. Mice were boosted intravenously on Day7 with 100µg Fab in PBS and antibody response was determined on Day10 by ELISA. Spleens were removed 3-5 days post booster injection for hybridoma fusion.

3.3.2.2 Tolerisation – 1

5-week old male Balb/C mice were tolerised against pooled normal human immunoglobulin (CSL) according to Lebrón *et al.* (1999). In brief, 250µg immunoglobulin in PBS was administered intraperitoneally on Day 1 and Day 5. Mice were challenged on Day 9 and boosted according to the immunisation procedure outlined in 3.3.2.1.

3.3.2.3 Tolerisation – 2

Due to their lower solubility, aggregated material within normal human immunoglobulin (hIg) can be removed from solution using sodium sulphate precipitation (Christian, 1958). Golub and Weigle (1969) demonstrated the ability to render Balb/Cj mice unresponsive to hIg using particular fractions of immunoglobulin obtained by this method.

Briefly, a 1ml volume of a 20% solution of hIg in 0.15M NaCl (pH 8.0; 8.766g NaCl in 1L) was brought to a final concentration of 0.36M Na_2SO_4 by the addition of 200µl of 2.18M Na_2SO_4 (pH 8.0; 15.478g in 50ml). After centrifugation at 13,200 rpm (17,500g) in a benchtop centrifuge the solution was carefully transferred to a fresh 1.5ml Eppendorf tube using a

pasteur pipette. This process of incrementally increasing Na_2SO_4 was repeated to obtain final concentrations of 0.62M, 0.81M and 0.96M. The final pellet obtained was resuspended in 0.15M NaCl, pH 8.0 and stored at 4°C.

5-week old Balb/C mice were tolerised with 1mg deaggregated hIg in 100µl intraperitoneally s previously described (Golub and Weigle, 1969). Mice were challenged on Day 7 and boosted according to the immunisation procedure in 3.3.2.1.

3.3.2.4 Anti-Idioype ELISA

Wells were coated with 100µl of a 5µg/ml solution of either patient paraprotein Fab or hIg in PBS, and incubated overnight at 4°C in a humidified container. After washing three times with PBS, wells were blocked for two hours at room temperature in 3% skim milk / PBS-T. Wells were washed a further three times with PBS prior to the addition of mouse serum or hybridoma culture supernatant. X63 supernatant was used as the negative control and HB61 supernatant (anti-kappa) as the positive. After incubating for 1 hour at room temperature, wells were washed three times with PBS-T and three times with PBS. 100µl of a 1:1000 dilution of HRP α–mouse Ig antibody in 3% skim milk / PBS-T was added per well and incubated for a further hour at room temperature. Wells were washed as before prior to detection with 100µl OPD reagent. After 30-60min at room temperature, the optical density of the wells at 450nm was measured on a DYNATECH MR7000 plate reader using BioLinx 2.0 software. Optical densities two-fold greater than background were defined as positive.

3.3.2.5 Spleen Single Cell Suspension

Mouse spleens were placed in a sterile petri dish containing 10ml RF10 and mechanically disrupted to release splenocytes. After centrifugation in a 30ml V-bottom tube for 5min at (1,500 rpm) 200g (HERMLE Z 383 K centrifuge), the cell pellet was resuspended in 5ml 1x

Red Cell Lysing Solution to remove red blood cells. After incubating for 5 minutes at room temperature the tube was filled with sterile PBS and centrifuged as before. Cells were washed again, resuspended in a final volume of 1ml PBS and counted using Trypan Blue exclusion to determine viability.

3.3.2.6 Polyethylene Glycol Fusion

Splenocytes and p3653 cells were combined in a ratio of between 10:1 and 1:1, mixed and centrifuged at 200g for 5 minutes. Cells were fused by resuspending the pellet in 1ml PEG 1500 (Boehringer Mannheim) and aspirating vigorously for 1 minute. The fusion reaction was then gradually diluted with 10ml pre-warmed RF10 over 20 minutes. Finally, the cells were centrifuged as before and resuspended into 20ml of pre-warmed RF10. The cell suspension was transferred to a pre-gassed sterile petri dish and placed in a CO_2 incubator with 5.0% CO_2 at 37°C for 1-3 hours.

3.3.2.7 Plating

Following the recovery period, the cell suspension was centrifuged as before and resuspended in 100ml HAT media. The cells were aliquoted evenly between two 24-well, pre-gassed plates and incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2. Wells were fed on Day 7 by replacing 1ml of supernatant with fresh HT media and monitored daily for colony growth. Supernatants were screened by ELISA as soon as media yellowed and either fed as before or cloned.

3.3.2.8 Cloning

Positive primary fusion wells were diluted to a concentration of $1x10^4$ cells/ ml in 10ml RF10. Serial dilutions were performed using RF10 to obtain 10ml of 500 and 50 cells/ml.

Subsequent dilutions to 25, 15 and 5 cells per ml were prepared in HT media containing 1/100 HFCS (Hybridoma Fusion and Cloning Supplement; Roche Biochemicals). Lastly, diluted cells were plated in 200µl aliquots into a pre-gassed 96-well plate resulting in an average of 5, 3 and 1 cells per well being plated. Plates were incubated at 37°C in a humidified CO_2 incubator.

3.3.2.9 Expansion, Storage and Thawing

Cloning wells were sequentially expanded into 24- and 6-well plates prior to a 10ml flask using HT media containing 1/200 HFCS.

Confluent 10ml cultures were centrifuged at 1,500rpm (200g) for 5 minutes at room temperature in a HERMLE Z 383 K centrifuge. Supernatants were decanted and stored at -20°C. Pellets were gently resuspended in 2ml freezing media and aliquoted into two 1.5ml cryovials. Cells were placed directly into a -70°C freezer for at least 12 hours before transferring to liquid nitrogen.

Frozen cells were placed in a 37°C water bath and decanted into a 10ml sterile screw-top V-bottom tube. 10ml pre-warmed RF10 media was added drop-wise over 10 minutes. Cells were centrifuged for 5 minutes at 1,500 rpm (200g) and resuspended in 10ml HT media.

3.4 RESULTS

3.4.1 Preparation of Patient Paraprotein Fab

A total of six IgG patient paraproteins were purified from serum using ProteinG affinity chromatography. The first two paraproteins were used in the hybridoma studies outlined in this chapter. Recovered paraprotein was analysed by Serum Gel Electrophoresis by the

Department of Clinical Immunology, Flinders Medical Centre. As can be seen in Figure 3.1, a distinct band was observed for each of the six paraproteins following purification.

Determining the binding specificity of the reagents generated against patient paraproteins is critical. For this reason, an ELISA was performed to classify each of the paraproteins by subclass so that appropriate controls could be selected at a later stage. Wells were coated in duplicate with 10µg undigested patient paraprotein and a variety of hybridoma supernatants used that recognize specific determinants on each of the four human IgG subclasses (Reimer *et al.*, 1984). Based on the data presented in Table 3.1 each of the patients were classified as having the following predominant subclasses: Patient 1, 4, 5 and 6 – IgG1; Patient 3 – IgG2; Patient 2 – IgG4. Several groups have addressed the question of IgG subclass distribution within multiple myeloma patients. Frequency ranges of 70-80% have been observed for IgG1, 10-17% for IgG2, 6-11% for IgG3 and 2-6% for IgG4 (Kyle and Gleich, 1982).

Figure 3.1 Serum gel electrophoresis of purified patient paraproteins. Lanes 1–6, patient paraproteins 1–6; Lane 7, normal human serum.

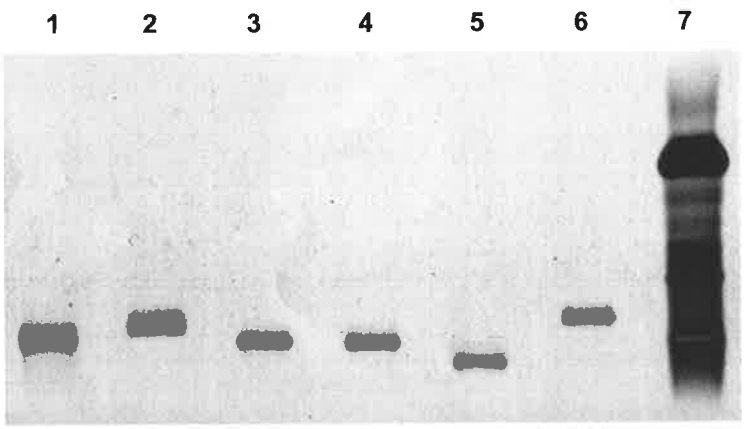
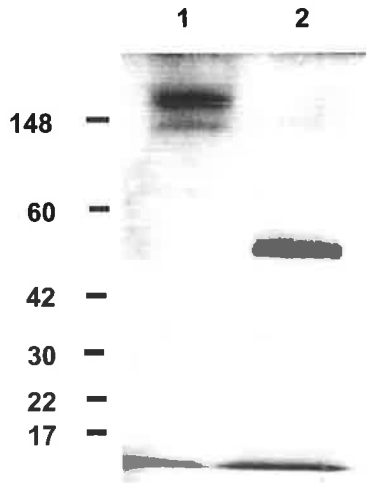


Table 3.1 Paraprotein subclass determination

mAb	Specificity	P1	P2	P3	P4	P5	P6
HP6001	**IgG_1 Fc**	**0.444**	0.175	0.252	**1.616**	**1.492**	**1.477**
HP6002	**IgG_2 Fc**	0.068	0.319	**1.355**	0.651	0.496	0.306
HP6014	**IgG_2 Fab**	0.094	0.394	**1.432**	0.938	0.705	0.453
HP6003	**IgG_3 Fc**	0.020	0.012	0.074	0.109	0.099	0.091
HP6025	**IgG_4 Fc**	0.009	**1.061**	0.069	0.066	0.178	0.129
HP6020	**IgG_4 Fab**	0.007	**0.779**	0.045	0.100	0.243	0.167
X63	-	0.012	0.018	0.066	0.072	0.067	0.084
HB60	**IgG - common**	**1.061**	**0.840**	**1.212**	**1.204**	**1.281**	**1.204**

Protein purity of paraprotein Fab fragments was analysed using silver-stained polyacrylamide gels prior to immunisation of mice. An example of the purity obtained for Patient 1 proteins can be seen in Figure 3.2 and is consistent with Patient 2 paraprotein preparations as well.

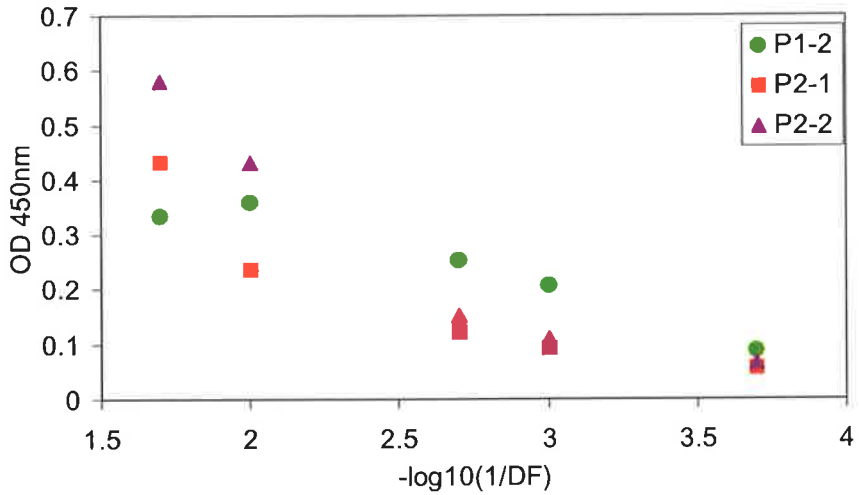
Figure 3.2 Silver-stained SDS-PAGE analysis of whole and papain-digested paraprotein. Patient 1 paraprotein. Lane 1, ~6µg purified paraprotein, Lane 2, ~6µg purified paraprotein Fab. 5µl of MultiMark™ Multi-Colored Standard (Novex) was used as a size reference. Molecular weight is given in kDa.



3.4.2 Hybridoma Production – Standard Immunisation

In the first immunisation trial mice were administered 100µg of either Patient 1 (animals P1-1 & P1-2) or Patient 2 (animals P2-1 and P2-2) purified Fab fragments reconstituted in 100µl GERBU adjuvant. Blood was sampled *via* the retro-orbital plexus three days after boosting and analysed for serum anti-paraprotein antibodies. A summary of the data demonstrating anti-paraprotein antibodies to both patient Fab fragments can be seen in Figure 3.3. Only one mouse immunised against Patient 1 paraprotein Fab was available at the time of serum antibody testing as P1-1 died from anaesthesia during boosting. Mice were anaesthetized by inhalation of methoxyflurane at the time of boosting as they were too restless to perform the intravenous injection.

Figure 3.3 Serum anti-paraprotein response three days post boosting with patient Fab fragments. The negative log10 of the serum dilution factor (DF) has been plotted against absorbance to enable a clear representation of the data.



P1-2 and P2-2 were killed and their spleen cells fused with p3653, a non-secretory myeloma, using standard hybridoma fusion techniques and HAT selection. Supernatant from wells containing viable colonies were screened by ELISA (Table 3.2) to assess binding against

paraprotein Fab as well as whole normal human immunoglobulin. Wells that failed to produce detectable levels of antibody reactive towards the immunising paraprotein have been omitted.

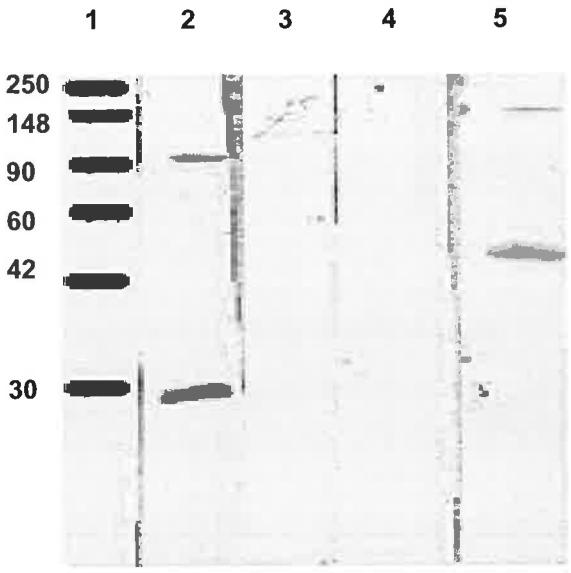
Table 3.2 Binding analysis of supernatant from antibody-producing primary fusion wells. Background and positive control values (- / +) are provided for each ELISA, wells from each fusion are listed sequentially. Highlighted data indicate wells that were subsequently cloned.

Fusion / Well No.	Patient Fab (- / +)	Human Ig (- / +)
P1-2		
1D	0.373 (0.089 / 0.398)	1.616 (0.155/1.911)
2B	0.168	1.765
2E	0.165	1.962
2F	0.150	2.057
3F	0.162	2.172
3F	0.135	1.939
3N	0.136	1.84
3U	0.139	1.537
3V	0.197	0.589
4A	0.141 (0.044/0.347)	0.329 (0.165/2.052)
4I	0.301	2.020
5A	0.197	0.199
P2-1		
1B	0.201 (0.069/0.745)	2.075 (0.155/1.911)
2B	0.113	0.275
2G	0.152	1.916
2Q	0.147	0.282
3R	0.45	1.681
3Q	0.208	1.838
3S	0.195	0.278
P2-2		
1E	0.367 (0.013/0.642)	1.354 (0.176/1.326)
3A	0.05	1.195
3G	0.014	0.848
3H	0.223	1.313
3I	0.014	0.771
3K	0.269	1.361
4B	0.032 (0.109/ND)	1.087 (0.458/1.579)
4M	0.06	1.364
4DD	0.093	1.088
4JJ	0.121	1.412
4WW	0.115	1.071
4ZZ	0.178	1.342

Highlighted data indicates wells that were selected for subsequent cloning based on a higher reactivity to paraprotein Fab and a lower reactivity towards normal human immunoglobulin as compared to other fusion wells. P2-2 was boosted a second time (*i.p.*) on Day 21, sacrificed on Day 26 and the fusion reaction seeded into two 48-well plates in an effort to reduce heterogeneity of cells within each well.

A number of cloned fusion wells secreted antibody that exhibited similar binding profiles to that observed for the primary fusion wells. Two of these, 1E5B and 1D11E, were further characterised by Western blotting (Figure 3.4) to determine their reactivity to normal human immunglobulin. 1E5B bound to a 50kDa band that corresponds to the heavy chain of reduced IgG. No binding was observed for 1D11E or for one of the hybridoma supernatants selected as positive controls (HB60 – IgG). A band corresponding to the light chain was visible with HB61 supernatant (anti-kappa).

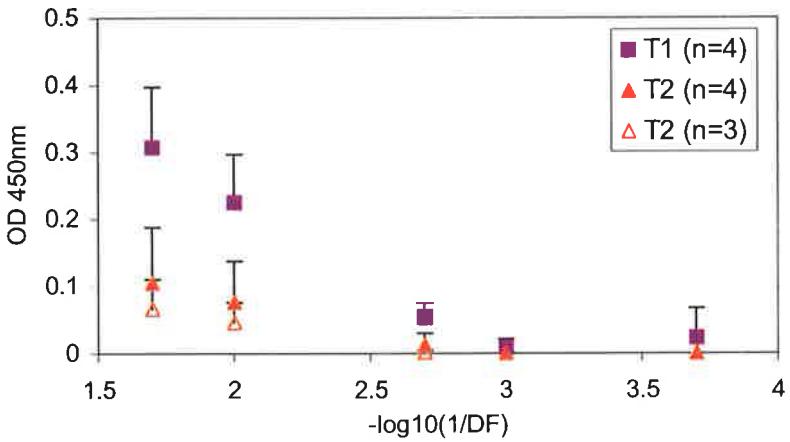
Figure 3.4 Western blot analysis of binding for 1D11E and 1E5B against 4µg reduced normal human immunoglobulin. Bands were visualised with 0.5mg/ml 4-chloro-1-napthol.Lane 1, Low Molecular Weight Marker (Promega) and MultiMark™ Multi-Colored Standard (Novex), Lane 2, HB61 (anti-kappa light chain); Lane 3, HB60 (anti-IgG); Lane 5, 1D11E; Lane 6, 1E5B. Molecular weight is given in kDa.



3.4.3 Tolerisation of Balb/C Towards Normal Human Immunoglobulin

It was hypothesized that mice rendered unresponsive towards normal human immunoglobulin may reveal a response against unique structures present within the idiotype of patient paraproteins. Mice were administered normal human immunoglobulin twice prior to immunisation with paraprotein Fab. Serum antibody analysis showed that there was still a high degree of binding against normal human immunoglobulin. In addition, no increase in the binding to the immunising paraprotein over normal human immunoglobulin was observed. A second tolerisation strategy was investigated in which aggregated material was removed using sodium sulphate fractionation prior to administration. Analysis of serum anti-Ig antibody levels revealed that the mice within this second tolerisation group had been rendered unresponsive towards normal human immunoglobulin. However, subsequent challenge and boosting with patient paraprotein Fab failed to invoke an immune response. The relative effectiveness of these two tolerisation strategies can be seen in Figure 3.5. One of the animals within the second tolerisation group appeared to have been resistant to tolerance induction. A plot of the average values minus the data for this animal is included.

Figure 3.5 **Comparison of serum anti-Ig antibodies following tolerisation as determined by ELISA (T1 – Tolerisation 1, T2 – Tolerisation 2)**



3.5 DISCUSSION

Antibody reagents generated against human paraproteins have a long, but somewhat clouded, history of application in the study of clonal involvement for this disease. Advances in the understanding of developmental markers, the techniques used to produce anti-idiotype reagents and perform phenotype analysis as well as the issue of shared idiotypes means that there is a lot of work remaining. It was the aim of this research to produce anti-idiotypic reagents that could withstand rigorous testing for cross-reactivity with unrelated immunoglobulin. Hybridoma technology was selected as a means to generate stable cell-lines that produce specific anti-idiotype monoclonal antibodies. If successful, these reagents would enable subsequent study of patient bone marrow and peripheral blood for the presence of putative tumour progenitors.

Rigorous testing of putative anti-idiotype reagents is crucial for the ability to confidently use them in subsequent cell-based studies. The first criterion applied in this work was the ability of hybridoma supernatant to bind the immunising paraprotein but not normal human immunoglobin by ELISA. These mAbs would then be tested for binding to unrelated paraproteins as well as subclass matched controls to confirm that binding was not occurring to framework epitopes. As subclass information was not provided with the diagnostic information an ELISA was performed to classify the six paraproteins involved in this study. Multiple subclasses were evident in five of the six IgG paraprotein preparations. For these patients the paraprotein was classified according to the strongest binding signal. The presence of multiple isotypes within patient serum may reflect different stages of disease. However, no correlation was observed between patients at an earlier stage displaying multiple isotypes as might be expected.

Due to the high degree of colony formation, fusion wells were first screened for reactivity against patient Fab and positive clones retested in conjunction with normal human Ig (Table

3.2). Wells were selected for cloning based on the signal being higher than 2-fold background against paraprotein Fab and lower than 2-fold background against normal human immunoglobulin. The fusion wells selected for cloning are highlighted in Table 3.2. P1 fusion wells 1D and 3U were selected despite their high reactivity against normal human Ig in the hope that they represented a heterogeneous population for which cloning would successfully isolate a specific clone. Intensive screening of cloning wells failed to detect any specific hybridomas, however non-specific clones from P1 1D and 3U exhibiting reactivity equipotent to HB63 (anti-IgG) supernatant were observed. A second mouse (P2-2) had been primed and boosted with P2 Fab at the same times as the first two mice. This animal was boosted a second time on Day 21 intraperitoneally with 100μg P2 Fab in PBS and sacrificed five days later. Hybridoma fusion was performed as before except that plating was performed in four rather than two 24-well plates to reduce the risk of heterogeneous cell populations. A total of 94 out of 96 wells (~98%) produced supernatants for screening. From these wells, 3 recognised both P2 and human Ig (3.2%) and 9 reacted against normal human Ig alone (9.6%). Hybridoma clones 1D11E (P1) and 1E5B (P2) were selected as they demonstrated the strongest binding by ELISA. Figure 3.4 shows the binding of 1E5B to the heavy chain of reduced normal human immunoglobulin (~50kDa). Very faint banding could be seen for 1D11E at this position however it was not discernable in the scanned image. However, strong banding was seen on non-reduced whole Ig by Western blot (data not shown). HB60 and was included as a positive control for IgG heavy chain and had previously demonstrated strong reactivity in ELISA and Western blot of non-reduced Ig (data not shown). The observed reactivity of 1E5B against the heavy chain could represent binding to the CH1 domain present in the Fab fragment, alternatively it could reflect contamination of the Fab preparation with Fc. 1E5B and 1D11E monoclonal antibodies were characterised as IgG κ and IgM κ respectively using the Isostrip™ murine monoclonal antibody isotyping kit (Boehringer Mannheim). Although there is no use for such reagents under the objectives of this project, strong anti-human Ig monoclonal antibodies may be beneficial in a range of routine diagnostic situations.

Production of only hIg reactive or co-reactive antibodies after cloning of the three P2 fusion wells highlighted in Table 3.2 is not necessarily surprising. Firstly, all primary fusion wells demonstrated only low reactivity against patient Fab compared with HB61 (~4-7 fold reduced) and also bound normal human immunoglobulin. These wells were selected in the hope that they represented a heterogeneous population of cells, some of which produced idiotype specific antibodies. However subsequent retesting of 3U revealed that the reduced level of reactivity against normal human immunoglobulin initially observed may have been misleading and hence the expectation of recovery any idiotypic-specific clones was optimistic.

From the results of the initial hybridoma fusion study it became apparent that the purified Fab preparations may be contaminated with a small degree of Fc / whole paraprotein at levels undetectable by silver-stained SDS-PAGE (Figure 3.2). This possibility was confirmed by evidence of low reactivity with HB60 (anti human IgG) in a standard ELISA (P2 = 0.146; P1 = 0.247; hIg = 1.598). Subsequent literature searches revealed that the Fc region of human immunoglobulin contains immunodominant epitopes (Novotný *et al.*, 1986) and their presence would be anticipated to dominate an immune response in mice. Based on the impact on the presence of small amount of IgG Fc, several alternatives were considered to focus the immune response against the unique, idiotypic structures within patient paraprotein Fab. One such approach is the suppression of immune response by the passive administration of antibody (Hutchinson and Zola, 1977). This is achieved by either previously reacting or simultaneously administering antigen with murine monoclonal antibodies or antisera against the immunodominant regions. Due to the number of monoclonal antibodies required to block all immunodominant regions and the time required to produce antisera, this approach was not selected. Instead, the induction of tolerance to normal human immunoglobulin prior to challenge with paraprotein Fab was pursued. There are three main options for tolerance induction: neonatal tolerance, adult high zone tolerance and immunosuppression. The use of neonates increases the technical difficulty as well as the

time between inducing tolerance and priming. Whilst immunosuppression is performed on adult mice, the use of cytotoxic drugs to remove lymphocytes stimulated by the tolerogen may adversely affect the entire lymphocyte population (O'Doherty *et al.*, 1993). After consideration, it was decided that the administration of normal human immunoglobulin under conditions to induce tolerance in adult animals was the most easily attainable method with the greatest likelihood of success. The tolerisation method described here was based on the protocol successfully employed by Lebrón *et al.* (1999) for the rapid generation of "high zone" tolerance in adult mice towards immunodominant epitopes in β-2 microglobulin and CD94. This approach failed to induce tolerance towards normal human immunoglobulin in the animals used in this study (Figure 3.5 – T1). After reading further back into the literature it was revealed that the induction of tolerance is not a simple issue. Firstly, the ability to induce a state of immunologic tolerance is highly influenced by the type of tolerogen. A further complicating factor of particular importance to this study is the high degree of variability between strains of mice in their response towards human Ig. Balb/C mice are highly responsive towards immunoglobulin and even the supernatant from an ultracentrifuged solution will elicit a strong immune response. Golub and Weigle (1969) demonstrated that this particular strain of mouse could be made tolerant to human immunoglobulin providing additional measures were taken in the removal of aggregated material and a second approach to tolerisation (T2) was developed based on these methods. As can be seen in Figure 3.5, a marked reduction in reactivity towards normal human immunoglobulin was achieved. Unfortunately these animals failed to exhibit a response against paraprotein Fab following immunisation and boosting.

There are a number of variables that could have been modified in an effort to improve the recovery of anti-idiotype specific mAbs. These include: i) increasing the number of rounds of boosting; ii) increasing the number of animals in each group; iii) increasing the number of primary fusion wells seeded per fusion; iv) altering the strain of mouse. A recent study investigating the effect of lyophilisation on a biopharmaceutical anti-idiotype antibody

revealed that immunogenicity was lost due to structural alterations of the Fab moieties (Taschner *et al.*, 2001). Whole purified paraprotein or Fab fragments stored at 4°C in solution may be a more reliable form of immunogen. However the time involved in modifying the existing ethics applications, combined with the possible increase in immunisation duration of one to two months was prohibitive. Screening of phage display libraries that express either antibody fragments or peptides has enabled the discovery of binders to a wide variety of targets, including antibodies (eg Mennuni *et al.*, 1997; Willems *et al.,* 1999). This technology was selected as a means to recover highly specific reagents in a significantly shorter time (1-2 weeks per target) as compared to the hybridoma approach.

Chapter 4

Discovery of Paraprotein Mimotopes

From Linear Phage Display Peptide Libraries

4.1 OVERVIEW

Six IgG myeloma paraproteins were screened against three linear random peptide phage-displayed libraries ranging in size from 7 to 20 amino acids. Biopanning was performed against both immobilized and free targets in solution. Three unique peptide sequences were identified against three separate paraproteins. Only one of these three peptides retained biological activity as a synthetic biotinylated compound, as determined by surface plasmon resonance using BIAcore analysis. This chapter discusses the development and use of random peptide phage-displayed libraries in the past, with an emphasis on antibody mimotopes. Details of the libraries selected along with the biopanning strategies used are provided as well as suggestions for improvement in the recovery of binders.

4.2 INTRODUCTION

George Smith proposed the engineering of phage for the display of novel proteins in 1985 and subsequently published the first paper on the construction of random peptide libraries five years later (Scott and Smith, 1990). At the same time two other groups published the construction of similar peptide libraries (Cwirla *et al.*, 1990; Devlin *et al.*, 1990). Since then the minor coat protein (g3p) of filamentous bacteriophage has also proved immensely valuable as a scaffold for the display of diverse libraries based on Fab fragments (Hoogenboom *et al.*, 1991) and single chain variable fragments (Marks *et al.*, 1991; Nissim *et al.*, 1994). A major feature of these libraries is the ability to isolate the genetic sequence of recovered binders for subsequent recombinant expression. Other advantages include the high degree of diversity, fast throughput and independence from animal experimentation.

There have been two recent reports regarding the use of phage-display technology for the recovery of specific binders against myeloma paraproteins. Willems *et al.* (1998) isolated a number of scFv fragments after panning with myeloma paraprotein Fab fragments in the

presence of pooled normal human immunoglobulin as a competitor. Antibodies, and hence scFvs, which bind another antibody within its antigen binding-site are referred to as anti-idiotypes. The idiotypic network is a natural feature of the immune system and may serve a regulatory function (Richter, 1975).

Szecsi *et al.* (1999) reported the use of two linear random peptide libraries for the recovery of binders specific for six out of a panel of nine paraproteins. As for Willems *et al.*, specificity was confirmed by absence of binding to pooled normal immunoglobulin or unrelated paraprotein. Besides Szecsi's group, numerous others have reported the use of phage-displayed peptides for the recovery of sequences that bind specifically within an antigen-binding site (Zwick *et el.*, 1998, review). Such compounds are referred to as mimotopes as they mimic the original epitope recognized by the antibody. Mimotopes can be structurally identical to the antigen epitope (Adda *et al.*, 1999) and some have demonstrated the ability to elicit the production of antibodies that cross-react with the original antigen (Mennuni *et al.*, 1997). Peptides have also been discovered that bind to anti-DNA (Sibille *et al.*, 1997) and anti-carbohydrate antibodies (Harris *et al.*, 1997). In most of the reports concerning mimotope discovery the original antigen is already known, enabling confirmation of binding within the antigen-site by competition studies.

Diversity is introduced by using a degenerate coding sequence containing the nucleotide formula $(NNK)_x$, where N represents equimolar G, A, T and C, K is equimolar G and T and x is peptide length. This core repeat allows for all 20 amino acids to be encoded whilst minimizing the probability of in-frame stop codons. Phage bearing peptides within the minor coat protein are typically selected for by tetracycline, with a single infection event conferring resistance. Clones containing inserts possessing affinity for a given target are isolated from the diverse number of non-specific phage by a process known as biopanning. This method provides a selective pressure by increasing washing stringency over a total of three to four repeat exposures of phage with the target protein. Phage particles are re-amplified between

each round so that, by the final pan, the majority of phage recovered should bind specifically to the target. Individual clones are selected and those exhibiting the desired binding specificity sequenced. Synthetic analogues of the peptide sequences of interest can then be purchased and typically incorporate a biotin group at the N-terminus, enabling their use in a range of applications including ELISA and analysis of binding by surface plasmon resonance.

4.3 METHODS

4.3.1 Random Peptide Phage-Displayed Libraries

Three libraries were used in this investigation, ranging in size from 7 to 20 amino acids in length. The 7-mer library was purchased from New England Biolabs. The 15-mer library was originally constructed by Dr. George Smith (Adda *et al.*, 1999) and the 20-mer library was constructed by Dr. Joanne Casey using the same method. Both the 15- and 20-mer libraries were kindly donated by Dr. Joanne Casey, Department of Biochemistry, La Trobe University, a collaborative partner in the CRC for Diagnostics.

4.3.2 Panning Against Immobilised Target Paraproteins

An 8 well polysorp ELISA strip (Greiner) was coated overnight at 4°C with 100µl 10µg/ml whole patient paraprotein (or 5µg/ml 5G8 positive control mAb) per well. Coating solution was tipped off and excess removed by tapping onto paper towel. Wells were washed twice for 15 seconds each time with PBS and blocked for 2 hours at room temperature with 5% skim milk in PBS. After washing with PBS as before, 100µl 10^{11} tu/ml primary phage previously blocked with 1% skim milk in PBS for 15 minutes at room temperature was added per well and incubated for 1 hour at room temperature. Wells were again washed with PBS and phage eluted with 100µl 100mM glycine HCl (pH 2.2) for 10 minutes. Phage were neutralised immediately with 7µl 2M Tris added directly to each well and pooled. For each

subsequent round of panning, reamplified phage were diluted 1 in 2 with 1% skim milk and blocked for 15 minutes prior to panning. Washing stringency after phage incubation was increased in each subsequent round as follows: R1: 2 x PBS, R2: 2 x PBS-T + 2 x PBS, R3: 4 x PBS-T + 2 x PBS, and R4: 8 x PBS-T + 2 x PBS.

4.3.3 Phage Amplification

2ml 2YT was inoculated with K91 *E. coli* from a -80°C glycerol stock and grown overnight at 37°C with shaking. On the following day, 10ml 2YT was inoculated with 200µl overnight K91 culture and grown to log-phase (~2 hours) at 37°C with shaking. K91 cells were then left at room temperature for 15 minutes to allow regeneration of F pili. Eluted phage (~800 µl) were added to 8ml of recovered K91, mixed briefly and incubated at room temperature for 30 minutes. Following infection, K91 cells were transferred to a sterile 1L flask containing 100ml 2YT and 50µg/ml tetracycline (2YT Tet50). Cultures were grown overnight at 37°C with vigorous shaking. The remaining 2ml of recovered K91 was used for determining phage recovery if required.

4.3.4 Phage Precipitation

Phage particles were purified from overnight cultures by precipitation as follows. Cultures were clarified by centrifugation at 8,000 rpm (10,000 g) for 20 minutes at 4°C in a Beckman JA-10 rotor with maximum brake. Supernatant was decanted into a second sterile 500ml centrifuge bottle containing 0.2 volumes chilled 20% PEG-8000/2.5M NaCl, mixed briefly and incubated for two or more hours on ice. Glycerol stocks were prepared by resuspending the cell pellet in ~2ml 2YT. Aliquots of 1ml were placed into two 1.5ml Eppendorf tubes containing 400µl sterile 50% glycerol, vortexed and stored at -80°C.

Precipitated phage was recovered by centrifugation at 10,000 rpm (15,700 g) for 50 minutes at 4°C in a Beckman JA-10 rotor with maximum brake. The supernatant was carefully decanted and residual solution aspirated prior to incubation of the pellet with 1ml PBS-Az on ice for 10 minutes. Phage were resuspended by vigorous aspiration, transferred to a sterile 1.5 ml Eppendorf tube and clarified by centrifugation at 13,200 rpm (17,500 g) for 3 minutes in a benchtop centrifuge. Aliquots were stored at -80°C for subsequent analysis and use.

4.3.5 Phage Count Determination

10µl re-amplified phage was added to 90µl 2YT and serially diluted from 10^{-1} to 10^{-12} in a sterile 96-well plate. 110µl recovered log-phase K91 was added to the 90µl of diluted phage and incubated at room temperature for 30 minutes. Following reinfection, 20µl of each dilution was plated drop-wise onto a 2YT agar plate containing 40µg/ml tetracycline and allowed to dry. Plates were incubated overnight at 37°C and the number of tetracycline resistant colonies counted. Phage concentration [ϕ], expressed as transducing units per ml (tu/ml), was determined using the following equation:

$$[\phi] = (x / 90) \times 200 \times (10^{y} / 20) \times 1000$$

where x = number of colonies and y = dilution factor.

4.3.6 Phage ELISA

ELISA wells were coated and blocked as described for Phage Panning (4.3.2). Phage were diluted to ~$1x10^{11}$ tu/ml in PBS and 100µl added per well. Plates were incubated for 1 hour at room temperature with shaking and washed three times for 15 seconds each with 0.05% PBS-T. 100µl 1:1000 biotinylated anti-fd bacteriophage antibody diluted in PBS was added per well and incubated as before. Wells were washed once with 0.05% PBS-T and once with

PBS alone prior to incubation with 100µl 1:1000 streptavidin-horseradish peroxidase for 30 minutes. After washing once in PBS with and without 0.05% Tween as before, the assay was developed with OPD and the optical density at 450nm measured as described in Section 3.3.2.4.

4.3.7 Phage Cloning and Sequencing

110µl of log-phase K91 were infected with 90µl of phage diluted so as to obtain ~100 colonies on a large plate. After incubating for 30 minutes at room temperature, the infection mix was spread onto a 2YT agar plate containing 40µg/ml tetracycline and grown overnight at 37°C. Large, discrete colonies were picked and grown in 2ml 2YT Tet50 overnight and stored as glycerol stocks at -80°C. Specificity was confirmed by ELISA and phage exhibiting binding to the target paraprotein and no binding to pooled normal human immunoglobulin were selected for sequencing. DNA Taq Polymerase (5U/µl) was purchased from Promega.

Two primers, fdf 5' for (5' GTA TTC TTT CGC CTC TTT C 3') and gIII 3' rev (5' TGT AGC ATT CCA CAG ACA G 3'), were used to amplify and sequence peptides.

100µl PCR reaction :

fdf 5' for (1µg/µl)	0.8µl
gIII 3' rev (1µg/µl)	0.8µl
Taq DNA Polymerase	0.5µl
25mM $MgCl_2$	10µl
10x Magnesium-Free Buffer	10µl
5mM dNTPs	4µl
Sterile water	23µl

1µl of clone glycerol stock was added as template.

A Perkin Elmer or Corbett thermocycler was used according to the following conditions:

94°C 10 minutes

30 cycles of:

94°C 30 seconds
50°C 30 seconds
72°C 30 seconds

72°C 10 minutes

4°C soak

PCR products were cleaned using a QIAquick PCR Purification Kit according to the manufacturer's instructions. Recovery of amplified DNA was confirmed by visualising on a 1% agarose gel containing 5µl 5mg/ml ethidium bromide per 200ml using a UV transilluminator.

Cleaned PCR fragments were sequenced using the gIII 3' rev primer by the Sequencing Facility, Department of Haematology, Flinders Medical Centre.

4.3.8 Panning in Solution

The 7-mer library (PhD-7) was panned based on an alternative strategy provided by the manufacturer (New England Biolabs). $2x10^{11}$ pfu of naïve phage was incubated with 300ng paraprotein in 200µl 0.1% TBS-T (Round 1) or 0.5% (Round 2-4) for 20 minutes at room temperature. Phage/paraprotein complexes were isolated by magentic separation using Dynabeads® (Dynal Biotech) previously blocked for 1 hour at 4°C with 5mg/ml BSA in 0.1M $NaHCO_3$, 0.02% NaN_3. ProteinA and ProteinG magnetic beads were used in alternate rounds to avoid recovery of ProteinA or ProteinG reactive phage. A selective pressure was applied by increasing washing stringency as follows: Round 1 – 10 x 0.1% TBS-T; Round 2-4 – 10 x 0.5% TBS-T. Bound phage were eluted in 1ml 0.2M glycine, pH 2.2 as described in 4.3.2 and used to infect 20ml of log-phase ER2738 *E. coli*. Cultures were grown for 4.5 hours at

37°C with vigorous shaking and the cells pelleted by centrifugation at 10,000rpm (~10,000g) for 10 minutes in an IEC Centra MP4 centrifuge. Culture supernatant was respun and 16ml transferred to a fresh 50ml tube containing 2.7ml 20% PEG8000 / 2.5M NaCl. Phage were incubated at 4°C overnight and pelleted by centrifugation in a chilled rotor for 15 minutes as before. Pelleted phage were resuspended in 1ml TBS, precipitated with 167µl 20% PEG8000 / 2.5M NaCl (1 hour on ice) and resuspended in a final volume of 200µl 0.02% TBS-Az. Amplified phage stocks were stored at for up to 7 days at 4°C or at -20°C in glycerol for long term storage.

4.3.9 Plaque-Assay

The phage vector used to construct the 7-mer library contains the *lacZα* gene enabling blue-white selection of plaques. 10µl phage serially diluted in LB was added to 200µl log-phase ER2738, vortexed and incubated at room temperature for 5 minutes. The infection mix was then transferred to 3ml pre-warmed Agarose Top (45°C), mixed by inversion and poured onto IPTG-Xgal plates. After incubating overnight at 37°C, blue plaques were counted and the number of pfu per 10µl was determined.

4.3.10 BIAcore Analysis of Biotinylated Synthetic Peptides

Characterisation of the binding of synthetic peptides was performed using a BIAcore® X system. Biotinylated peptides were reconstituted to 2mg/ml and diluted to 10µg/ml in commercial HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20). 20µl of diluted peptide was applied to the second flow cell of a streptavidin biosensor chip at a flow rate of 10µl/min. Following coating, 100µg/ml paraprotein or normal human immunoglobulin was injected at a flow rate of 10µl/min through both flow cells. Biosensor chips were regenerated with a 1 minute pulse of 1M NaCl between samples.

4.4 RESULTS

4.4.1 Panning Against Immobilised Paraprotein

Amplified phage recovered after each round of panning was assessed for the presence of binders by ELISA. 5G8, an anti-malarial monoclonal antibody, has previously recovered specific binding motifs from the 20-mer library and was used as a positive control. As can be seen in Figure 4.1, a significant increase in absorbance was observed for both 5G8 and P3 paraprotein following four rounds of panning. Subsequent panning of the 15-mer library under the same conditions resulted in the recovery of specific phage against P6 (Figure 4.2).

Figure 4.1 Summary of ELISA data following panning of the 20-mer peptide library. 10^{10} phage particles from either the naïve library or following amplification after each round of panning were assessed in duplicate for binding to 1μg of the target paraprotein as indicated.

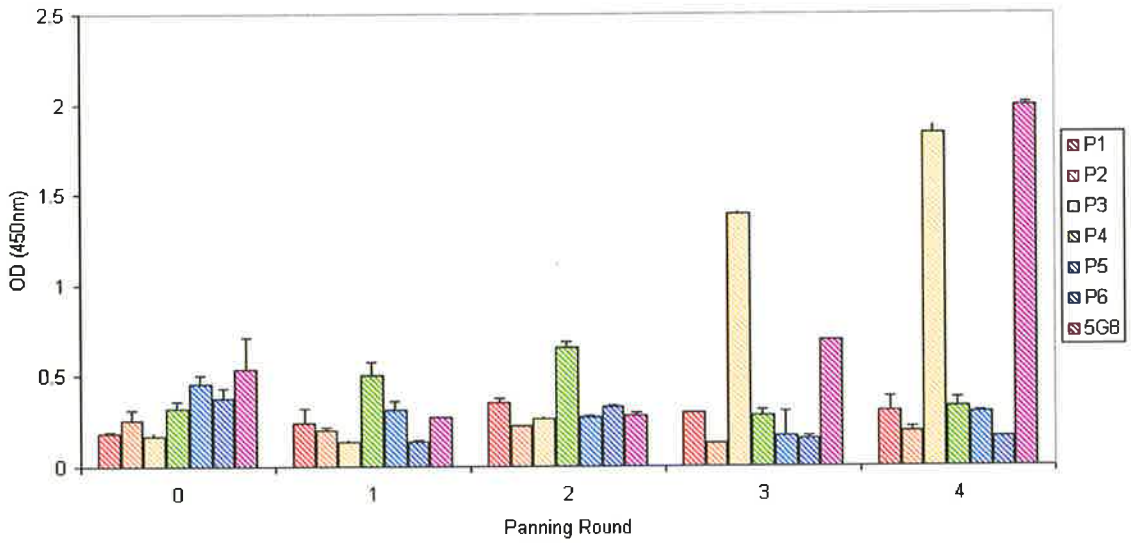
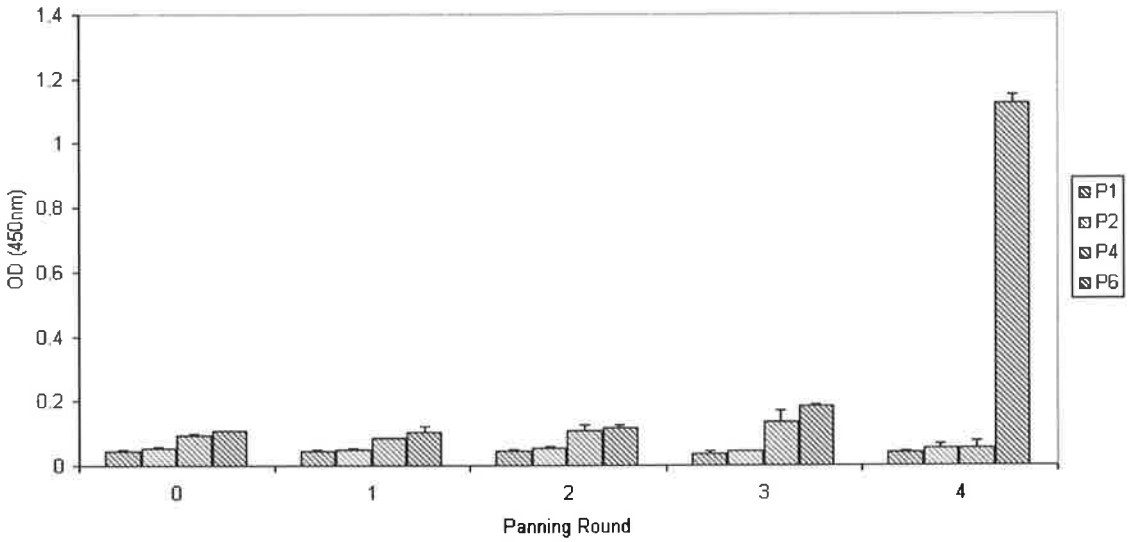


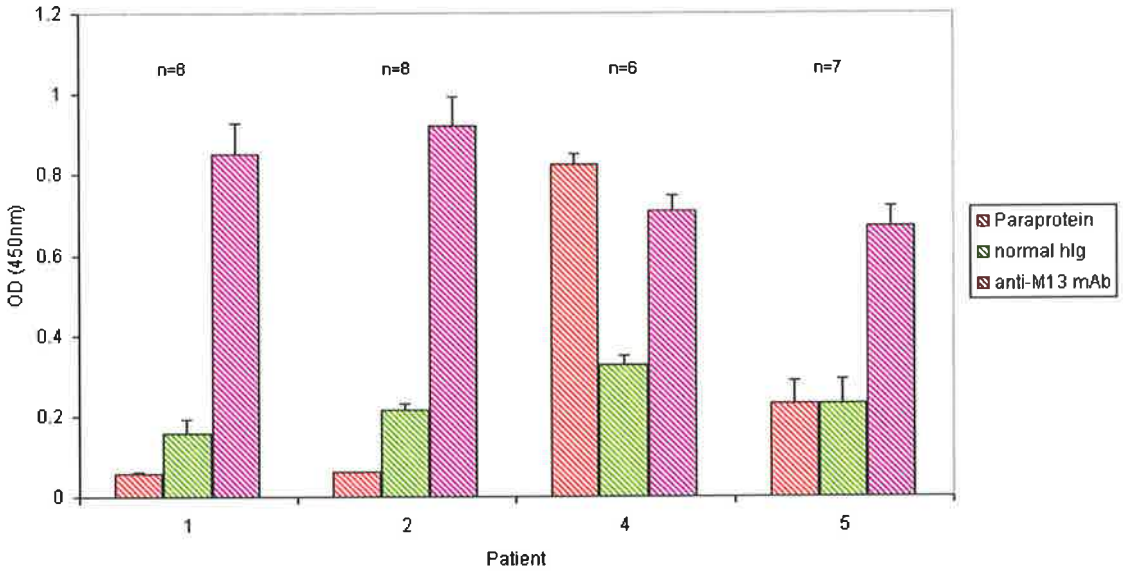
Figure 4.2 Summary of ELISA data following panning of the 15-mer peptide library. 10^{10} phage particles from either the naïve library or following amplification after each round of panning was assessed in duplicate for binding to 1μg of the target paraprotein as indicated.



4.4.2 Panning in Solution

A commercial 7-mer peptide library was panned against paraprotein in solution. The presence of binders was indicated by an increase of phage titre following elution over subsequent rounds of panning. Phage clones isolated and amplified after the third round of panning were tested for the presence of binders to the target paraprotein by ELISA. In addition, wells were coated with either monoclonal anti-M13 antibody or normal human immunoglobulin to confirm phage production and specificity. Phage were recovered which bound to P4 paraprotein and not to normal human (Figure 4.3).

Figure 4.3 Summary of ELISA data following panning of the 7-mer peptide library against paraproteins from patients 1, 2, 4 & 5. Average of ELISA signal against 1μg target paraprotein, 1μg pooled normal human immunoglobulin or 1:1000 anti-M13 mAb for each of the phage clones isolated after Round 3. Total number of clones tested against each target paraprotein is indicated.



4.4.3 Identification of Isolated Peptide Motifs

Clones were randomly selected for sequencing following the fourth round of panning. Phage were also isolated and sequenced following the third round of panning for P4 against the 7-mer library. In all cases a single peptide motif was observed that recognized the target paraprotein. Amino acid sequence details and the number of clones sequenced for each are provided in Table 4.1.

Table 4.1 Paraprotein, corresponding peptide and number of clones sequenced.

Patient	Sequence	# Clones
3	RDRLKYTSVHDLFMHRFIVE	5
6	VLLFHEPAGLPVYFW	4
4	SLDPKVK(GGGS)	9

4.4.4 BIAcore Analysis of Synthetic Peptides

Biotinylated peptides corresponding to the peptide sequences identified above were analysed for their ability to bind paraproteins and normal human immunoglobulin using BIAcore analysis. Data has been presented such that t=0 seconds represents that commencement of injection. As can be seen in Figure 4.4, the 20-mer peptide isolated against P3 paraprotein (P3-20) retained its functional conformation in synthetic form as demonstrated by its ability to bind to P3 paraprotein. In addition this peptide failed to bind to unrelated paraprotein or normal human immunoglobulin. Neither P6-15 (Figure 4.5), the 15-mer peptide recovered against P6 paraprotein, or P4-7 (the 7-mer peptide against P4 paraprotein) were recognised in synthetic form by their respective target paraproteins. Both synthetic peptides showed a high degree of retention on a streptavidin biosensor chip, indicating that the biotin group retained its functional conformation under the conditions used for reconstitution.

4.4.5 Analysis of the Binding Specificity of P3-20, P4-7 and P6-15 Expressing Phage

As P4-7 and P6-15 synthetic peptides failed to bind their respective target paraproteins, binding specificity of the original phage clones was investigated by ELISA. Individual clones were amplified overnight (P3-20 and P6-15) or for 4.5 hours (P4-7) and the culture supernatant used directly without purification. Production of phage was confirmed using wells coated with monoclonal anti-M13 mAb. Each of the phage clones demonstrated specific binding to their respective target panning protein and significantly reduced reactivity against each of the unrelated paraproteins (Table 4.2). Binding of P3-20 expressing phage to normal IgG2 was also examined to test for binding against subclass-specific epitopes (Table 4.3). Strong binding to the subclass control immunoglobulin was observed that was equipotent to the signal against the same amount of P3 paraprotein. However, naïve 20-mer library phage also demonstrated binding to IgG2 but not against P3 paraprotein.

Figure 4.4 Demonstration of the binding of P3 paraprotein (a) but not unrelated immunoglobulin (b-g) to P3-20 peptide by BIAcore analysis. 10µl 100µg/ml immunoglobulin was injected at a flow rate of 10µl/min onto a streptavidin biosensor chip coated with 794 RU biotinylated P3-20.

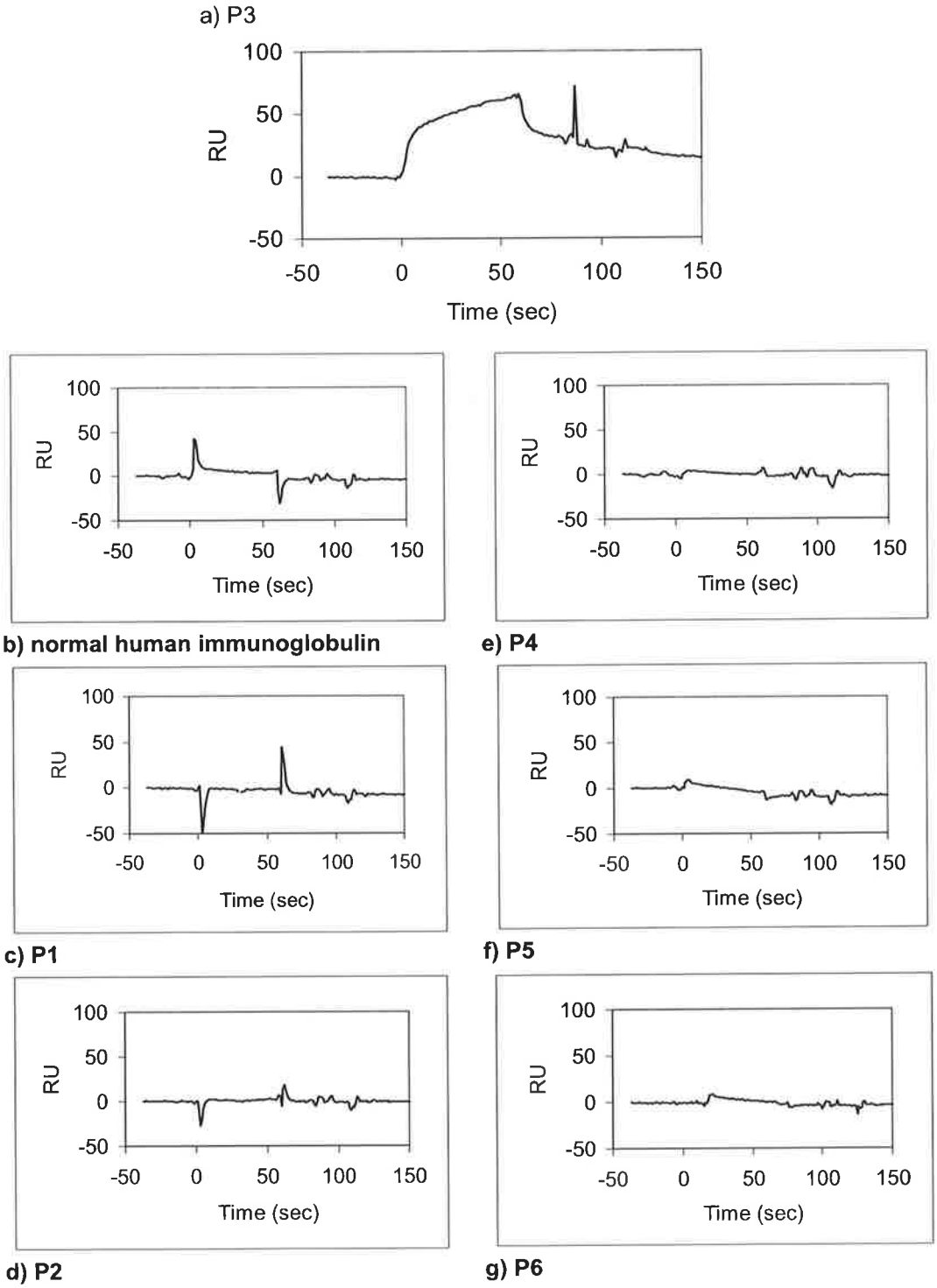
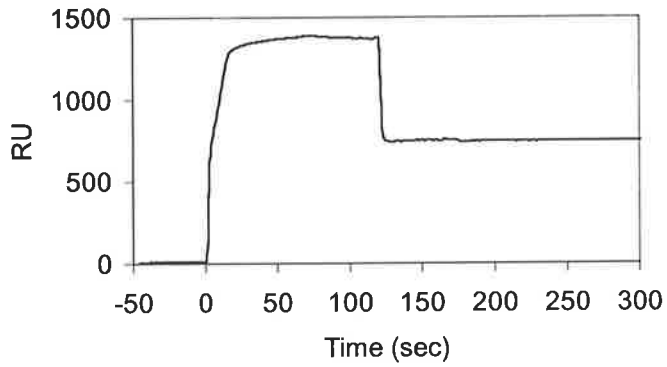


Figure 4.5 BIAcore analysis of the synthetic P6-15 peptide.

a) Coating of FC-2 on a streptavidin sensor chip with 20µl 10µg/ml biotinylated P6-15 at a flow rate of 20µl/min.



b) 20µl injection of 100µg/ml P6 paraprotein at a flow rate of 10µl/min

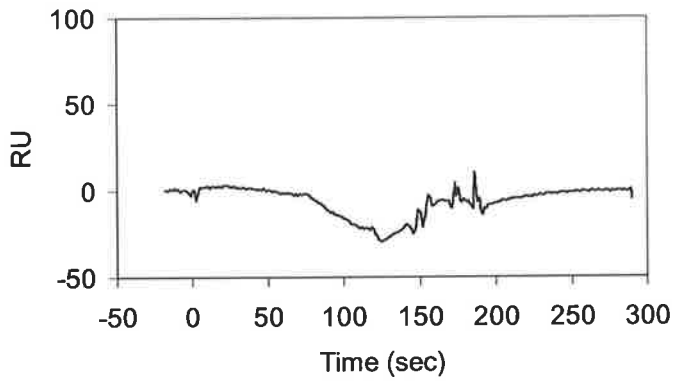


Table 4.2 ELISA reactivity of phage clones expressing P3-20, P4-7 and P6-15 against 1μg target or unrelated paraprotein. Paraproteins from patients 1- 6 denoted P1 - P6.

	P3-20 Phage	P4-7 Phage	P6-15 Phage
P1	0.0815	0.114	0.177
P2	0.117	0.108	0.239
P3	**0.560**	0.092	0.181
P4	0.132	**0.486**	0.098
P5	0.219	0.105	0.245
P6	0.100	0.063	**0.546**
PBS	0.098	0.072	0.171
Anti-M13	**0.618**	**0.663**	**0.697**

Table 4.3 ELISA reactivity of phage expressing P3-20 and naïve 20-mer library phage against 1μg P3 paraprotein or normal human IgG_2 control antibody.

	P3-20 Phage	Naïve 20-mer phage
P3	0.529	0.223
IgG_2	0.453	0.474
PBS	0.221	0.073
Anti-M13	0.516	0.465

4.5 DISCUSSION

A primary stock of naïve 20-mer library was first amplified and stored in individual ampules at -80°C so as to provide sufficient phage for subsequent biopanning experiments. Prior to screening this library against patient paraproteins an anti-malarial monoclonal antibody (5G8), against which binders have previously been recovered from this library, was used to establish the biopanning protocol and confirm that library diversity was maintained. Recovery of specific binders was indicated by an increase in ELISA signal over successive rounds of biopanning. Six whole patient paraproteins, all from IgG expressing myeloma, were then screened as immobilized targets under the same conditions used for 5G8. Phage ELISAs were performed in duplicate to determine the degree of binding within 10^{10} phage particles to wells coated with 1μg of target antibody. A summary of the ELISA data, including standard deviation error bars, for all targets screened against the 20-mer library is provided in Figure 4.1. It can be seen that the selective pressure applied to the 20-mer library resulted in recovery of specific binders for both the positive control (5G8) and the P3 paraprotein, binding was not observed for the remaining five patient paraproteins. Five clones were selected from Round 4 amplified phage against the P3 paraprotein and tested by ELISA for binding to both the original target and to pooled normal human immunglobulin. All cloned phage exhibited specific binding with an average OD(450nm) of 0.88 ± 0.076 with P3 paraprotein compared with 0.096 ± 0.008 against the same amount of pooled normal human immunoglobulin (data not shown). The g3p domain was amplified by PCR and sequenced using ABI Dye Prism Terminator automated sequencing. All five clones contained an identical amino acid insert, RDRLKYTSVHDLFMHRFIVE, referred to as P3-20. BIAcore analysis was performed using a streptavidin sensor chip coated with synthetic N-terminal biotinylated P3-20. Binding to the original P3 paraprotein was retained and no binding was observed with equal amounts of either normal human immunoglobulin or the other five paraproteins, Figure 4.4. As P3 is an IgG_2 paraprotein, phage expressing P3-20 were also screened against a subclass-matched control. Binding equipotent to that observed against

the P3 paraprotein was observed by ELISA indicating that this peptide may bind to subclass specific epitopes. However, it was also observed that phage from the naïve 20-mer library bound and exhibited a signal comparable to that observed for the P3-20 phage (Table 4.3). As a result, the peptide against P3 cannot be classified as either non-specific or specific at this time as the IgG_2 control appears to bind non-specifically to fd bacteriophage.

A 15-mer library of identical construction to the 20-mer library was screened in an attempt to recover more specific binders and hence increase the number of patient cell studies which could be conducted. Biopanning was performed as for the 20-mer library against four of the remaining paraproteins. P4 was omitted due to the observation that it exhibited higher background binding to the phage compared with the other five paraproteins in previous experiments. A summary of the ELISA results for the four paraproteins screened against the 15-mer library is shown in Figure 4.2. As for the Round 4 amplified phage selected against P3, five clones were isolated from P6 amplified Round 4 phage and their specificity assessed. Four out of the five clones exhibited strong signal with P6 paraprotein with an average OD(450nm) of 1.378 ± 0.113 and background binding to pooled normal human immunoglobulin, 0.154 ± 0.013. As P6 is an IgG_1, the most prevalent subclass in normal human serum, a lack of binding to normal human Ig is a good indicator that the binding is paraprotein-specific. Sequencing of these four isolated phage clones revealed that, as for P3-20, a single amino acid motif was recovered (P6-15; VLLFHEPAGLPVYFW). P6-15 is highly hydrophobic, containing only two hydrophilic residues (His-5, Glu-6). Calculating the average hydrophobicity ranking of the peptide gives a value of 0.732 compared with 0.481 for P3-20. Synthetic P6-15 required a solvent environment of 25% acetonitrile : 50% acetic acid for reconstitution which is not appropriate for subsequent immunochemistry on live cells. As can be seen in Figure 4.5, the N-terminal biotin remained functional and bound readily to the streptavidin sensor chip providing an overall increase in surface plasmon resonance of ~755 RU. However subsequent injections of P6 paraprotein, even at twice the amount used to demonstrate binding between P3 and its peptide P3-20, failed to show even a weak

binding interaction. There are a number of potential explanations for this loss of function. Firstly, peptide folding may have been disrupted due to the fact that a number of attempts were performed prior to finding the correct environment, each of which were separated by a freeze-drying step. Alternatively the peptide may require additional structural elements provided by the minor coat protein to form the required structural conformation.

As there was still only one functional peptide recovered out of the two libraries, and as chance would have it no patient material was available for P3, a third library was selected. An alternative biopanning strategy was employed in which the phage interact with target paraprotein in solution and are then retrieved by magnetic bead separation. The beads are then washed with PBS and selective pressure applied by increasing Tween20 concentration and number of washes in subsequent rounds. Phage were pH-eluted as before, titres determined using a plaque assay and the remainder of phage reamplified for subsequent rounds. Eight phage plaques were isolated and amplified after Round 3 to assess binding to equal amounts of original target paraprotein and pooled normal human immunoglobulin. Phage production was confirmed by ELISA using wells coated with 100µl 1:1000 αM13 mAb as counting would have been too labor intensive. A summary of the results for the 7-mer library is provided in Figure 4.3 (data was omitted in cases where phage production was low). It is clearly demonstrated that binders were recovered after three rounds of biopanning against P4, whilst no binding was observed for P1, P2 or P5. Five clones that bound to P4 were sequenced from Round 3 and Round 4, nine of which contained the amino acid motif SLDPKVK(GGGS). The Gly_3-Ser linker is included as it is not present in the 15-mer and 20-mer libraries. One clone did not produce a good signal for sequence analysis and was not included in the final results. Unfortunately the synthetic peptide containing an N-terminal biotin group failed to bind to paraprotein as determined by BIAcore analysis. It was hypothesized that the biotin may interfere with paraprotein binding due to the shortness of the peptide. Relocation of the biotin to the C-terminus would better reflect the presentation of the peptide by the phage and may aid in subsequent binding of the P4 paraprotein. A variant

of P4-7, in which Ser11 is replaced with Cys, was purchased with the intention of chemically coupling a biotin group as a thiol ester to cysteine. However, due to a combination of time limitations and technical complications, C-terminal biotinylated P4-7 was not tested for functional binding to P4 by BIAcore analysis.

There are two striking observations regarding the three peptides isolated in this study. Firstly no diversity was observed amongst the phage clones sequenced, even in Round 3 clones against P4 from the 7-mer library. Many groups have reported a diverse array of sequences that bind to a particular target, some may contain a common motif whilst others show no apparent relationship (Adda *et al.*, 1999; Szecsi *et al.*, 1999). It is possible that a selection of a larger number of clones would have revealed the presence of alternative sequence motifs that bind to the three paraproteins discussed. However sequence analysis of five clones selected after the fourth round of biopanning against 5G8 revealed three unique sequences all containing an (A/S)YP motif which has been routinely observed for 5G8 using the same library (J.Casey, Pers. Comm, 2001). This indicates that diversity would most likely have been detected if present after Round 4 for the peptides against P3, P4 and P6. In addition, there have been previous reports that some targets within a panel have recovered a single peptide sequence (Szecsi *et al,.* 1999). The second observation is that, unlike Szecsi *et al.* (1999), no cross-reactivity was observed with pooled normal human immunoglobulin for any of the three recovered peptides. One difference between the two biopanning protocols is the use of 0.5% PBS-T as a blocking agent as opposed to 5% skim milk used to block microtitre wells in this study. In addition, phage were also blocked in 1% skim milk for 15 minutes prior to panning and were incubated in paraprotein coated wells for 1 hour compared to 2 hours used by Szecsi *et al.*(1999). Blocking with a complex protein mixture will aid in the adsorption of phage which bind non-specifically whilst the shorter incubation time of phage with target paraprotein promotes the recovery of higher affinity binders.

Specific binders were isolated for 3 out of a panel of 6 patient paraproteins after screening three different libraries. In comparison, Szecsi *et al.* (1999) reported that peptides were identified for 6 out of 9 paraproteins using two different peptide libraries. Binders were recovered for each of these six paraproteins from a single 15-mer library, two paraproteins recovered binders from both. In sharp contrast, the three libraries discussed here each recovered a single binder to one of the paraproteins screened. There are a number of reasons for the low recovery rate of binders observed in this current work. Firstly, the sequence motif may simply not be present within the libraries that have been screened. For this reason, it is advisable to screen a number of different libraries to increase the probability of the sequence of interest being recovered. Libraries containing shorter peptides offer the advantage of being able to represent the majority, if not all, of the potential peptide sequences of that length. As peptide size increases, the proportion of possible sequences represented decreases significantly. However this is somewhat balanced by the fact that, for liner epitopes, a multiple reading frames will be present within a given peptide. In addition, longer peptides may able to form secondary structures that could enable recovery of mimotopes for antigens that are non-linear or discontinuous. Another form of library, which could have been screened if time had permitted, contains constrained peptides that form a loop structure due to the presence of conserved cysteine residues. In addition there have been libraries developed in which peptides sequences are expressed on the N-terminus of the major coat protein g8p, of which there are ~100 copies per virion. Such high valency enables the recovery of mimotopes that are of lower affinity compared to the high affinity mimotopes typically recovered from libraries expressed on g3p.

Two different panning strategies have been discussed in this chapter, however there are many more parameters that could be modified. Improvement in the recovery of binders exhibiting high specificity has been reported for antibody immobilized directly onto polystyrene beads as opposed to streptavidin-capture of biotinylated antibody onto polystyrene plates (D'Mello and Howard, 2001). However the report did not include antibody

immobilized directly to polystyrene plates and did mention that, besides increasing the recovery of non-specific binders, streptavidin may disrupt antibody structure. Removal of non-specific binders can be achieved by extensive washing (Barrett *et al.*, 1992) or a stepwise reduction in elution pH from 5.0 to 2.0 during the final round of biopanning (D'Mello and Howard, 2001). Increasing competition for binding, and hence improvement in the recovery of high affinity clones can be facilitated by reducing the concentration of target antibody in the final round (Scott and Smith, 1990). However it is important not to apply conditions that are overly stringent in the early stages of panning as the binding clones are present in low copy number and will be lost. All of these options for were attractive to further investigate the remaining three paraproteins for which binding motifs were not identified. However it was decided that it would be more prudent to use the remaining time to take the peptides already discovered through to the final stage of patient studies.

Chapter 5

Design of g3p Fusion Proteins

in an Attempt to Recover Peptide Function

5.1 OVERVIEW

Two recombinant g3p fusion proteins have been designed to investigate their ability to mimic the presentation of peptides by phage. If successful, these constructs would facilitate future patient cell-phenotype studies using patient-specific peptides which are not functional as synthetic analogues. However, BIAcore analysis of recombinant g3p D1 and D1-D2 proteins containing the N-terminal P3-20 sequence failed to demonstrate binding to P3 paraprotein.

5.2 INTRODUCTION

The minor coat protein of filamentous bacteriophage (g3p) is comprised of three separate domains (D1, D2 and D3) each joined by a glycine-rich linker (Stengele *et al.*, 1990). Uptake of phage into *E. coli* involves the binding of D2 to the tip of the F' pilus followed by the interaction of D1 with its co-receptor, Tole (Marvin, 1998). Structural studies of g3p-D1 and g3p-D1D2 have been performed by NMR spectroscopy (Holliger and Riechmann, 1997) and X-ray crystallography (Holliger *et al.*, 1999) respectively using recombinant proteins. This latter study revealed that D1 and D2 form a "horse-shoe" structure with the N-terminal domain of D1 resting near the C-terminus of D2 in solution.

Two out of the three sequences isolated from phage-displayed peptide libraries against myeloma patient paraproteins failed to bind as synthetic analogues to their respective targets. Due to the highly sticky nature of bacteriophage it is not feasible to use isolated phage clones as reagents in flow cytometry for the detection of idiotypic paraprotein expression on patient cells. It was hypothesised that, at least for the hydrophobic peptide P6-15, structural elements provided by g3p may be essential for the peptide to adopt the conformation required for binding. Two constructs were designed for each peptide motif that contained the first N-terminal domain with or without the second domain in conjunction with the novel N-terminal peptide sequence. A C-terminal 6-His tag was introduced to enable

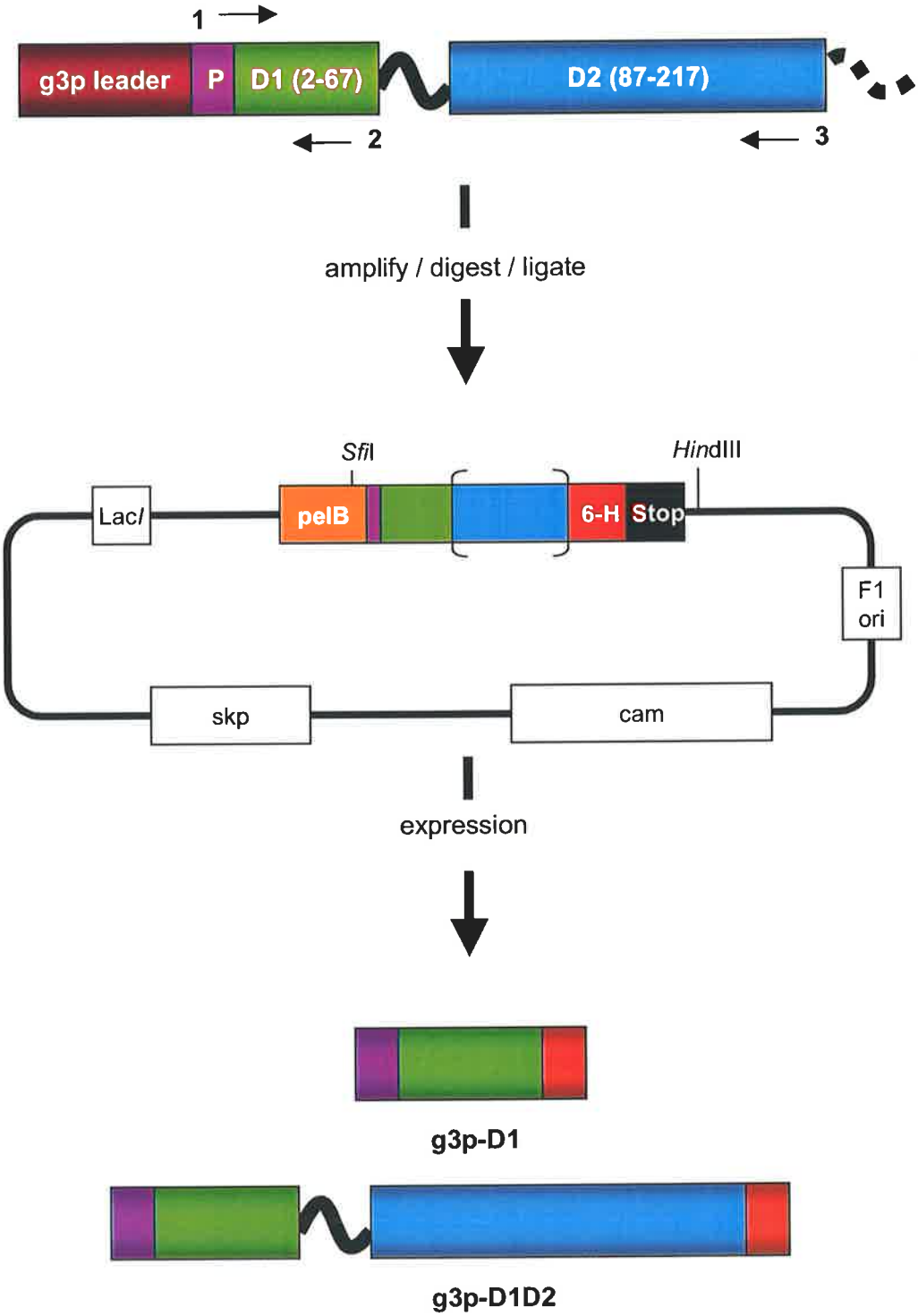
subsequent characterisation. Protein expression was performed by the induction of log-phase cultures with IPTG for three hours prior to borate extraction and analysed by slot blot. Recombinant proteins were assessed for binding to original target paraprotein by BIAcore analysis using a nickel activated NTA biosensor chip to capture the 6-His tag *via* metal chelation. Recombinant P3-20 D1 and D1D2 was produced to establish 'proof of principle' as it bound to P3 paraprotein as a synthetic molecule. However, neither of the recombinant constructs for P3-20 exhibited binding.

5.3 METHODS

5.3.1 Design of Recombinant g3p Fusion Proteins

The g3p leader sequence of the filamentous bacteriophage genome was replaced with the pelB leader sequence to facilitate periplasmic expression in *E. coli*. Novel 5' primers that introduced an *Sfi*I restriction site and the C-terminal region of pelB were designed for each peptide sequence. Two 3' primers that annealed to the C-terminal region of g3p-D1 and g3p-D2 respectively were also constructed. Both of these encoded for a 6-His tag, stop codon and a *Hind*III restriction site as well. An overview of the construction of g3p-D1 and g3p-D1D2 can be seen in Figure 5.1. The DNA sequence of wild-type fd bacteriophage g3p is provided in Appendix I.

Figure 5.1 Schematic representation of the construction of g3p-D1 and g3p-D1D2 recombinant proteins (P, fusion peptide; 6-H, 6-His tag).



5.3.2 Primers

Oligonucleotide primers (GeneWorks, Australia) were reconstituted to 0.5nmol/µl in sterile water and stored at -20°C. Sequence details are provided in Table 5.1 and their regions of annealment to g3p phage clones P3-20, P4-7(11) and P6-15 are shown in Figure 5.2 a) - e).

Table. 5.1 Primers used for the amplification of patient specific g3p-D1 and g3p-D1D2

Name	Sequence (5' - 3')
pelB –P3for	ttactcgcggcccagccggccatggcgcgtgacagg
pelB-P4for	ttactcgcggcccagccggccatggcgtcgttgg
pelB-P6for	ttactcgcggcccagccggccatggcggtgctgctttttcatgag
g3pD1back	agctaagcttactaatgatggtgatgatggtgattttcagggatagcaag
g3pD2back	agctaagcttactaatgatggtgatgatggtgagcattgacaggagg

5.3.3 Polymerase Chain Reaction: g3p-D1 and g3p-D1D2

g3p-D1 and g3p-D1D2 were amplified from phage expressing either P3-20 or P6-15 as follows.

Per 100µl reaction:

4µl 10pmol/µl forward primer (pelB-3for, pelB-4for, pelB-6for)

4µl 10pmol/µl reverse primer (g3pD1back, g3pD2back)

0.5µl Taq Polymerase (Promega)

10µl 25mM $MgCl_2$

10µl 10x Magnesium-free Buffer (Promega)

4µl 5mM dNTPs

67µl sterile H_2O

Figure 5.2 **Locations of primer annealment to g3p-peptide fusion genes.**

a) pelB-P3for

```
                                        |P3-20
                                        cgt gac agg ctt aag tac acc agc
                                        gca ctg tcc gaa ttc atg tgg tcg
tta ctc gcg gcc cag ccg gcc atg gcg cgt gac agg

                                                    ||g3pD1
gtc cac gac ctt ttt atg cat agg ttt att gtt gaa gct *** *** *** ***
cag gtg ctg gaa aaa tac gta tcc aaa taa caa ctt cga *** *** *** ***
```

b) pelB -P4for

```
                                        |P4-7
                                        tcg ttg gat cct aag gtt aag ggt
                                        agc aac cta gga ttc caa ttc cca
tta ctc gcg gcc cag ccg gcc atg gcg tcg ttg g

            ||g3pD1
gga ggt tcg gcc gaa act gtt gaa agt tgt tta gca aaa cct *** *** ***
cct cca agc cgg ctt tga caa ctt tca aca aat cgt ttt gga *** *** ***
```

c) pelB-P6for

```
                                        |P6-15
                                        gtg ctg ctt ttt cat gag cct gct
                                        cac gac gaa aaa gta ctc gga cga
tta ctc gcg gcc cag ccg gcc atg gcg gtg ctg ctt ttt cat gag

                                  ||g3pD1
ggt ttg cct gtg tat ttt tgg gct gaa act gtt gaa agt tgt *** *** ***
cca aac gga cac ata aaa acc cga ctt tga caa ctt tca aca *** *** ***
```

d) g3pD1 back

```
*** *** *** ggt gac gaa act cag tgt tac ggt aca tgg gtt cct att ggg
*** *** *** cca ctg ctt tga gtc aca atg cca tgt acc caa gga taa ccc

ctt gct atc cct gaa aat
gaa cga tag gga ctt tta
gaa cga tag gga ctt tta gtg gta gta gtg gta gta atc att cga atc ga
               End D1 ||          6-His          |Stop |HindIII|
```

e) g3pD2back

```
*** *** *** ttc gtt tgt gaa tat caa ggc caa tcg tct gac ctg cct caa

*** *** *** aag caa aca ctt ata gtt ccg gtt agc aga ctg gac gga gtt

cct cct gtc aat gct
gga gga cag tta cga
gga gga cag tta cga gtg gta gta gtg gta gta atc att cga atc ga
            End D2||       6-His            |Stop||HindIII|
```

1µl of clone glycerol stock was added as template and the reactions incubated under the following thermocycling conditions:

95°C 5 minutes

30 cycles of:

94°C 1 minute
55°C 1 minute
72°C 2 minutes

72°C 10 minutes

4°C soak

5.3.4 Restriction Enzyme Digestion

PCR reactions were cleaned using a Qiagen PCRClean Kit according to the manufacturer's instructions and eluted in 30µl sterile H_2O. Restriction enzymes were purchased from New England Biolabs and were used in conjunction with manufacturer supplied reaction buffer. 5µl of *Hin*dIII (20U/µl) and 6µl NEB Buffer 2 was added to 25µl cleaned PCR product and the reaction volume made up to 50µl with water. Reactions were overlayed with oil and incubated overnight at 37°C. *Hin*dIII digested inserts were cleaned as before performing a second digest with *Sfi*I as follows. 3µl *Sfi*I (20U/µl) and 5µl NEB Buffer 2 was added to 25µl cleaned *Hin*dIII digested inserts and made up to 50µl with sterile water. Reactions were incubated at 50°C for 3 hours.

5.3.5 Ligation Reactions

pHB400 is based on the pAK series of vectors routinely used for the expression of scFv proteins (Krebber*et al.*, 1997). This vector was chosen for the expression of recombinant g3p as It contains the Skp chaperone protein whch improves the folding of recombinant proteins expressed in the periplasm of *E. coli* (Mavrangelos *et al.*, 2001).

Digested PCR inserts and pHB400 vector were combined in a ratio of approximately 1:1 as determined by visualization on a 1% agarose gel (4.3.7). Reactions were performed in 10µl using 1µl T4 DNA ligase and 1µl 10x buffer as supplied by the manufacturer (Promega) and incubated overnight at 16°C.

5.3.6 Heat-Shock Transformation

Ligation reaction were transformed into heat-competent JM109 (Promega) as follows. JM109 cells were thawed on wet ice and 2ml of ligation mix added to 50ml of cells. Cells were incubated for 30 minutes on ice prior to being heat-shocked for 90 seconds at 42°C. Following a 10 minute recovery on ice, transformation reactions were diluted with 1ml 2YT and incubated at 37°C for 1 hour with shaking. 100µl of transformed JM109 cells were spread onto 2YT agar plates containing 25µg/ml chloramphenicol and incubated overnight at 37°C.

5.3.7 Selection of g3p-D1 and g3p-D1D2 Clones

Chloramphenicol resistant colonies were picked and grown in 2ml 2YT containing 25µg/ml chloramphenicol (2YT Chlor25). Cultures were incubated for several hours at 37°C with shaking until turgid and 1µl used as template for PCR as described in 5.3.3. Clones containing the desired inserts were diluted with 2YT Chlor25 and grown overnight as before. On the following day, 200µl of sterile 50% glycerol was added to 1ml of overnight culture, mixed thoroughly and stored at -80°C.

5.3.8 Expression and Recovery of Recombinant Proteins

Overnight cultures were diluted 1 in 100 with 2YT Chlor25 with or without 0.4M sucrose supplementation (~6.84g per 50ml) and incubated at 37°C for 2-3 hours. Log-phase cultures

were induced with 50µl 1M IPTG and grown for a further 3 hours at room temperature with gentle shaking. Following induction, cultures were centrifuged in a Beckman J-6M/E centrifuge using a swing-out rotor for 30 minutes at 2,500rpm (~1,300g), 4°C. The supernatant was decanted and pelleted cells either stored overnight at -20°C or resuspended immediately in 2ml borate buffer. Reactions were incubated on ice and shaken for at least an hour prior to centrifugation in two 1.5ml Eppendorf tubes (13,200 rpm (17,500g), 5 minutes). The resulting supernatant was carefully removed by aspiration, filtered through a 0.22µm syringe filter and stored at 4°C.

5.3.9 Slot Blot Analysis

Levels of protein expression were determined by detecting for 6-His in 100µl samples of extracts applied to nitrocellulose using a BioRad slot-blot apparatus. After loading, nitrocellulose was blocked with 5% skim milk in PBS-T for 30 minutes at room temperature. Membranes were rinsed briefly with PBS and incubated for 1 hour with 1:1000 anti poly-HISTIDINE (Sigma) in blocking solution. Following three 5 minute washes with PBS-T, membranes were incubated for 1 hour in HRP conjugated anti-mouse Ig diluted 1:1000 with blocking solution. Blots were washed as before and bound HRP detected with ECL western blotting reagent according to manufacturer's instructions (Pharmacia Biotech). X-ray film was typically exposed for 1 to 5 minutes to obtain clear banding patterns.

5.3.10 BIAcore Analysis – NiNTA Biosensor Chip

The 6-His tag present in recombinant g3p-fusion proteins chelates with nickel, enabling subsequent analysis of binding to the original target paraproteins.

NTA biosensor chips were equilibrated in NTA running buffer (0.01M HEPES, 0.15M NaCl, 50µM EDTA, 0.005% Surfactant P-20; pH 7.4) at a flow rate of 10µl/minute. The second flow

cell (FC-2) was activated with 10µl 500µM $NiSO_4$ in NTA running buffer prior to the loading of both flow cells with 20µl of periplasmic extracts diluted 1:5 with running buffer. 20µl of 100µg/ml paraprotein was injected after the signal had stabilized. The biosensor chip was regenerated between each protein analysed by injecting 20µl of NTA regeneration buffer (0.01M HEPES, 0.15M NaCl, 0.35M EDTA, 0.005% surfactant P-20; pH 8.3) at 20µl/min.

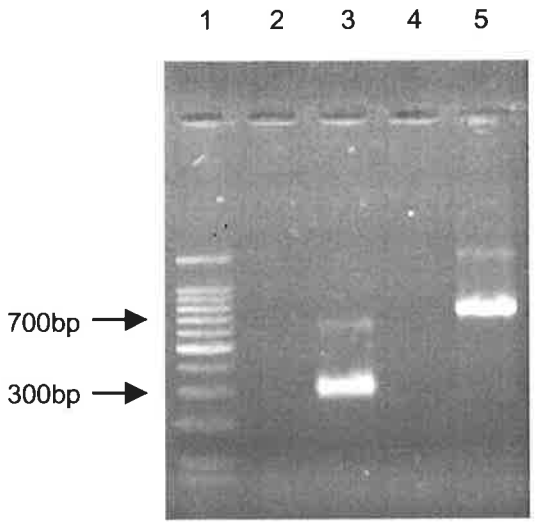
5.4 RESULTS

5.4.1 Cloning of g3pD1 and g3p-D1D2

Phage DNA encoding P3-20, P4-7(11) and P6-15 were amplified in conjunction with the g3p-D1 and g3p-D1D2 using primers specific to the peptide of interest. Inserts were digested with *Sfi*I / *Hin*dIII and ligated into pHB400. Following transformation, colonies were selected and screened by PCR using the primers originally used to produce the inserts. As primers are unique to each insert, detection of a PCR product also confirms that the peptide sequence is correct.

Expected insert sizes are as follows: P3-20 g3pD1 – 320 bp; P3-20 g3p-D1D2 – 770 bp; P4-7(11) g3p-D1 – 293 bp; P4-7(11) g3p-D1D2 – 743 bp; P6-15 g3p-D1 – 305 bp; P6-15 g3p-D1D2 – 755 bp. An example of the inserts obtained for P3-20 g3p-D1 and g3p-D1D2 can be seen in Figure 5.3. Inserts corresponding to the correct size for P6-15 g3p-D1 and g3p-D1D2 were also observed (data not shown) however P4-7(11) failed to be amplified using the primers described here. In addition, negative controls consistently failed to produce product in the absence of template.

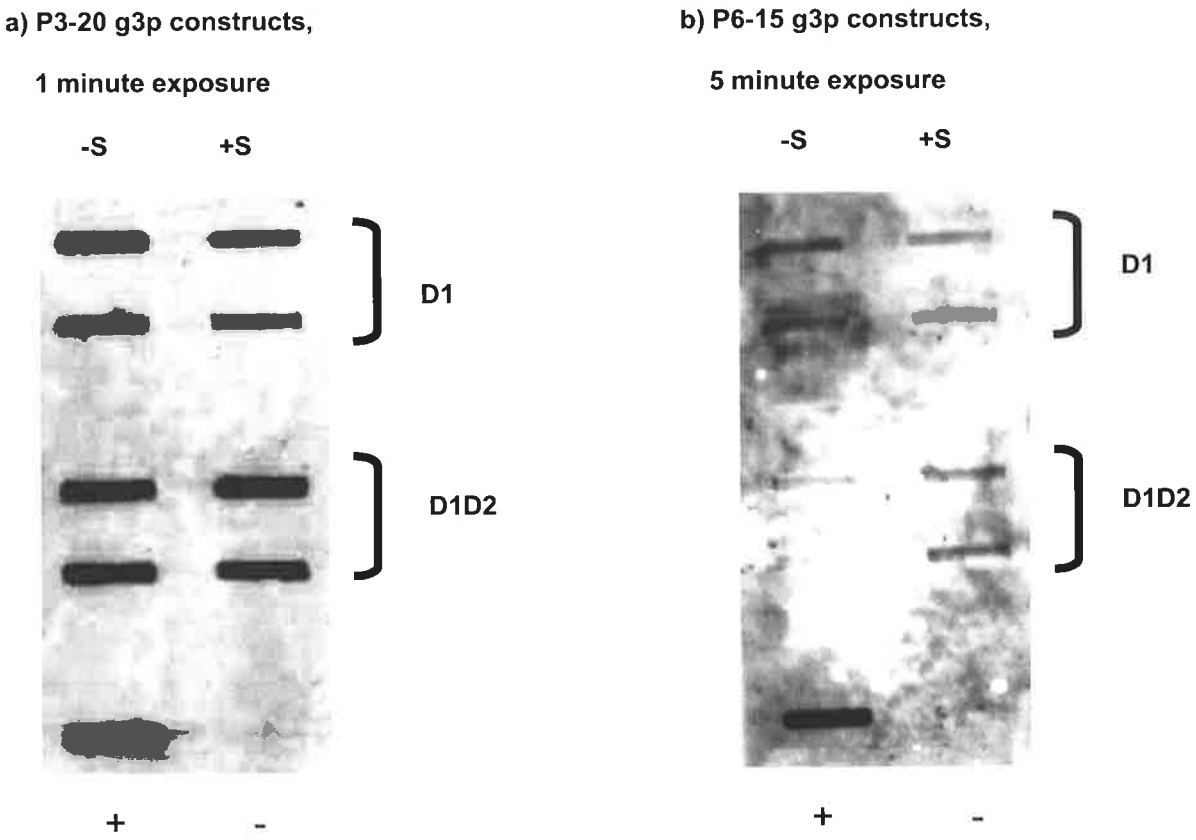
Figure 5.3 Amplification of P3-20 g3p-D1 and g3p-D1D2 inserts from *E. coli* infected with P3-20 expressing phage. Lane 1, 5µl 100bp marker (Promega); Lane 2, 5µl g3p-D1 negative control; Lane 3, 5µl g3p-D1; Lane 4, 5µl P3-20 g3p-D1D2 negative control; Lane 5, 5µl P3-20 g3p-D1D2.



5.4.2 Expression of Recombinant g3p Fusion Proteins

g3p-D1 and g3p-D1D2 were expressed for P3-20 and P6-15 containing recombinant proteins. A short induction of three hours was performed due to the potential toxicity of g3p (Dr. M. Foley, Pers. Comm., 2001). Supplementation with sucrose at 0.4M was also investigated based on reports of improved expression of recombinant proteins (Sawyer *et al.*, 1994). The expression profiles of P3-20 and P6-15 fusion proteins can be seen in Figure 5.4. Both g3p-D1 and g3p-D1D2 fusion proteins containing P3-20 expressed well and no difference was observed for cultures supplemented with sucrose. In comparison, P6-15 containing fusion proteins were expressed at markedly lower levels by *E. coli*. Furthermore, g3p-D1D2 demonstrated negligible expression in the absence of sucrose. 100ng of purified CD20 scFv was used as a positive control for 6-His, 100µl of borate buffer was loaded as a negative control.

Figure 5.4 Slot blot analysis of g3p-D1 and g3p-D1D2 fusion protein expression. Two clones were tested for each construct and 100µl of periplasmic extract was loaded per well. 100µl 1µg/ml CD20-scFv and borate buffer were included as positive (+) and negative (-) controls respectively, sucrose supplementation (0.4M) is indicated as –S (no sucrose) or +S (with sucrose). Levels of expressed protein determined by the detection of 6-His

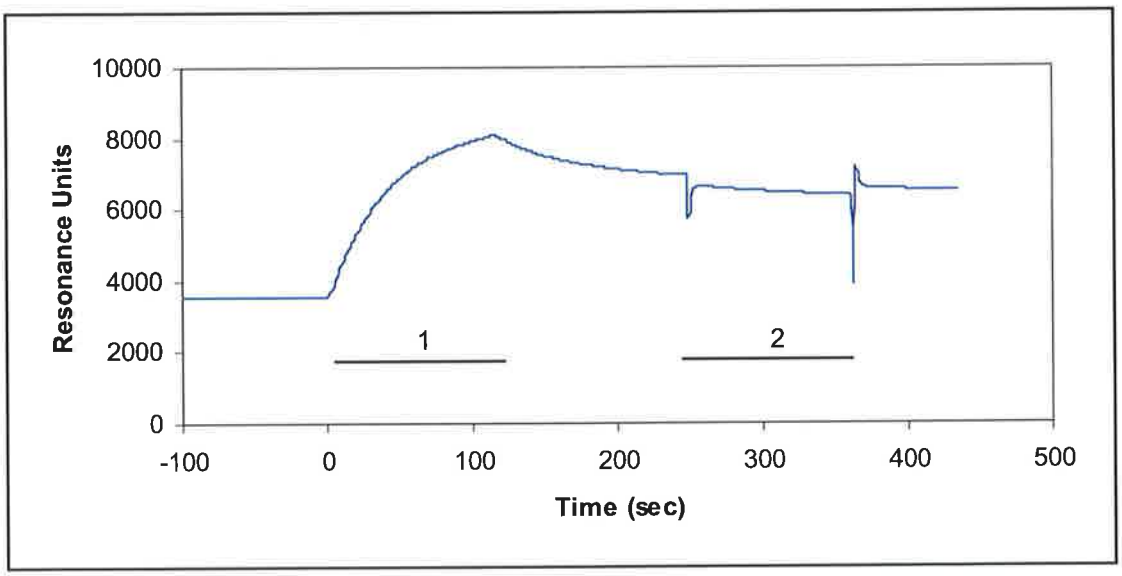


5.4.3 BIAcore Analysis of Recombinant g3p

Recombinant proteins for both g3pD1 and g3pD1D2 successfully bound through 6-His to Ni activated NTA biosensor chips. However, no binding could be observed between the recombinant g3p and the paraprotein against which the expressed peptide was originally recovered. The suitability of the method for analyzing binding was assessed using a closely

analogous protein, CD20 scFv (Figure 5.5). A single chain was selected as it contains a 6-His tag and a FLAG tag, which could be considered similar in function as compared to the unique peptide motifs present in the recombinant g3p proteins. The scFv was applied to a NTA biosensor chip and the ability to detect binding of anti-FLAG mAb (WEHI) was assessed. No detectable binding was observed when 20µl of 100µg/ml anti-FLAG was injected. The reactivity of this particular antibody with the CD20 scFv used to coat the biosensor chip had previously been demonstrated by slot-blot (B. Sergi, Pers. Comm., 2002). It was concluded that the BIAcore system selected to analyse the binding of target paraproteins to g3p recombinant proteins was not appropriate.

Figure 5.5 Assessment of the suitability of BIAcore analysis for the detection of binding between recombinant proteins immobilised to Ni-NTA *via* 6-His and a monoclonal antibody directed against a unique peptide motif. 20µl CD20scFv extract diluted 1/5 with NTA-running buffer (1) followed by 20µl 100µg/ml anti-FLAG mAb (2) at a constant flow rate of 20µl/min.



5.5 DISCUSSION

The aim of the work presented in this Chapter was to investigate whether peptides isolated from phage display libraries would retain their binding properties when expressed as recombinant proteins of g3p. Initially, peptides were expressed in conjunction with the first N-terminal domain alone. The second domain was introduced based on the reports of g3p D1D2 forming a horse-shoe like structure. It was thought that this interaction between domains may be important for the appropriate presentation of the N-terminal peptide. g3p-D1 and –D1D2 constructs were successfully cloned from both P3-20 and P6-15 expressing phage. As can be seen in Figure 5.6, reasonable levels of expression were obtained for P3-20 fusion proteins. In comparison, significantly lower levels of P6-15 g3p-D1 and –D1D2 were expressed. This observation indicates that the hydrophobic P6-15 sequence is highly detrimental to the production of g3p *in vivo*. Supplementation of cultures with 0.4M sucrose was investigated based on the reports of improved expression of recombinant proteins (Sawyer *et al.*, 1994). Although no benefit was observed for the expression of P3-20 containing g3p proteins, P6-15 g3p-D1D2 showed a marked improvement in expression compared to cultures grown in the absence of sucrose. P4-7 failed to be amplified using the peptide-specific primer described here. Redesign of the primer to include the majority of the unique peptide sequence combined with a higher annealing temperature may have enabled the recovery of P4-7 g3pD1 and D1D2. However this option was not investigated as the synthetic analogues had yet to be tested for binding to P4. Furthermore, the main aim of this work was to recover a reagent which bound to the P6 paraprotein which failed to bind synthetic P6-15.

BIAcore analysis was selected for the assessment of the ability of g3p fusion proteins to be bound by the original target paraproteins (P3 and P6). Although all constructs were retained *via* 6-His to a nickel activated NTA biosensor chip, no binding was observed with their respective target paraproteins. For this reason, the ability to detect binding against a small

peptide region on a recombinant protein immobilised by 6-His chelation to nickel was investigated. Single chain variable fragments (scFv) are routinely prepared within the laboratory where this research was performed. A recently expressed scFv against CD20 containing C-terminal 6-His and N-terminal FLAG sequence (DYKD) was selected. This construct provided the closest model available to the recombinant g3p constructs. Furthermore, anti-FLAG mAb provides a model for the interaction of paraproteins binding to their specific peptide sequences in g3p. As can be seen in Figure 5.5, no binding was detectable for 20µl 100µg/ml anti-FLAG mAb injected onto a chip coated with CD20 scFv. As this antibody had demonstrated significant binding by slot blot at 350ng/ml, it was concluded that the BIAcore system was unable to detect binding of antibodies to small peptide inserts within recombinant proteins bound to NTA biosensor chips. P3-20 recombinant g3p proteins were subsequently analysed by slot-blot based on the ability to detect binding of anti-FLAG mAb to scFv20 using this technique. However, no binding was observed to either of the P3-20 constructs when probed with P3 paraprotein at the same concentration as was previously used for anti-FLAG detection.

There are a number of possibilities to explain the inability to detect binding of peptide motifs within recombinant proteins by antibodies. Firstly, the running buffer recommended for use with the Ni-NTA biosensor chip may disrupt the conformation of the recombinant protein. Secondly, and perhaps more likely, the orientation of recombinant protein on the chip surface may hinder subsequent binding of antibody to the peptide sequence. Recombinant g3p used for three-dimensional structure determination was expressed periplasmically using the pelB leader (Holliger *et al.*, 1997). It therefore seems unlikely that the problem is due to post-translational processing occurring in the periplasm rather than within the membrane of *E. coli,* the site where the g3p-leader sequence is cleaved during phage assembly.

Chapter 6

Discussion

6.1 SUMMARY AND CONCLUSIONS

Two different strategies were investigated as a means to generate patient-specific reagents for the study of myeloma cell precursors. Both of these approaches aimed to exploit the uniqueness of the antigen-binding site (idiotype) of individual myeloma patient paraproteins. Production of anti-idiotype specific murine monoclonal antibodies was first investigated based on the long history of anti-idiotype antibodies in the investigation of haematological malignancies (Kubagawa *et al.*, 1979; Carmagnola *et al.*, 1983; Thielemans *et al.*, 1984). A common element of previous research is the use of whole patient paraprotein as immunogen. Several groups have reported the identification of anti-idiotypes that exhibit cross-reactivity with other paraproteins (Stevenson *et al.*, 1986; Kiyotaki *et al.*, 1987; Miller *et al.*, 1989; Berenson *et al.*, 1990; Kampe *et al.*, 1994). However, no common antigenic stimulation event has been linked to the establishment of multiple myeloma. This observation of cross-reactivity between paraproteins may simply reflect a common epitope shared by these antibodies, which may not necessarily be directed against a common antigen. Another possibility that, to the best of the author's knowledge, hasn't been explored is that these anti-idiotype antibodies recognise allotypic determinants.

Balb/C mice were immunised with purified paraprotein Fab fragments in an effort to minimise an immune response against strongly immunogenic epitopes within the constant regions of human immunoglobulin. Following a rapid immunisation procedure (Thielemans *et al.*, 1984), hybridomas were produced and screened for the production of monoclonal antibodies exhibiting anti-idiotype specificity. Despite extensive screening, all of the hybridomas recovered bound to normal human immunoglobulin as well as the immunising paraprotein. Induction of immunological unresponsiveness towards pooled normal human immunoglobulin was attempted using the phenomenon of "high-zone" tolerance. It was hypothesised that a response towards the unique structures of the antigen-binding site would be revealed in animals tolerised against the constant regions present in the Fc region. These animals

demonstrated a significant decrease in serum anti-human immunoglobulin antibodies. However, no response was observed against patient paraprotein following immunisation. This failure to respond to patient paraprotein Fab may be due to the immunisation schedule selected. Repeated boosting over a longer period of time or varying the dose or route of administration may have resulted in an anti-idiotype response being evoked. Alternatively, epitopes within the antigen-binding site may have been denatured by freeze-drying and subsequent storage at -20°C. In this case, the use of paraprotein stored in solution at 4°C may be more appropriate for use as antigen. Although the attempts described here for hybridoma production were unsuccessful, the principle of producing anti-idiotype monoclonal antibodies is still valid. Nevertheless due to the time required to modify the ethics application and a potential increase in the duration of the immunisation schedule of one to two months, the decision was made to investigate other approaches.

Phage-displayed peptide libraries were chosen as an alternative approach to discover highly patient-specific reagents. Three different libraries containing linear peptides of varying length fused to the N-terminal of g3p were screened with either immobilised or free whole patient paraprotein. Three unique peptides were identified (P3-20, P4-7 and P6-15), each against a different paraprotein. Synthetic analogues containing an N-terminal biotin were assessed for their ability to bind target but not unrelated immunoglobulin using surface plasmon resonance. P3-20 retained its ability to bind to P3 paraprotein and no binding was observed against normal human immunoglobulin or unrelated paraprotein. However, both P4-7 and P6-15 failed to exhibit binding to their respective targets. It is proposed that, for P4-7, the positioning of the biotin tag at the N-terminus may interfere with the binding within the 7-mer peptide sequence. If this is so, relocating the biotin group to the C-terminus of the glycine rich sequence which links the 7-mer to the first domain of g3p may restore binding to P4 paraprotein. The second peptide, P6-15, has a particularly hydrophobic sequence and difficulties were experienced during the reconstitution of the synthetic peptide. Although the N-terminal biotin retained its ability to bind to streptavidin, no binding was observed with P6

paraprotein. As assessment of the binding specificity of the synthetic constructs was not possible for P4-7 and P6-15, cloned phage that expressed each of the three peptides were tested for binding to unrelated paraprotein by ELISA. Each of the three peptides demonstrated specific binding to their target paraproteins, indicating that the peptide may represent a mimotope of the original antigen recognised by the patient paraprotein. P3-20 phage were also tested for binding against normal IgG_2 as P3 is the only IgG_2 paraprotein within the panel used in this study. Strong binding was observed against the subclass control and was equipotent to that observed against P3 paraprotein. However, naïve 20-mer library phage also demonstrated strong binding against the IgG2 and not P3. Consequently, P3-20 cannot be confidently classified as specific or non-specific at this stage.

The work detailed herein has demonstrated the ability to produce specific peptide reagents against paraproteins. Interestingly, only a single sequence motif was recovered for each of the three patients. It is common to observe a number of different motifs against a single target (Adda *et al.*, 1999; Szecsi *et al.*, 1999). Within this collection of sequences, some may show a common motif within a diverse range of sequences whilst others may share no sequence homology at all. Such variability enables the production of a number of synthetic peptides in the event that binding is lost in one of the motifs. If time and resources had permitted, additional functional synthetic peptides may have been recovered through the screening of other libraries or with the inclusion of more patient paraproteins. Re-screening of the current libraries against the existing paraprotein panel using alternative panning strategies may also lead to additional motifs being discovered.

It was hypothesised that, due to its hydrophobicity, P6-15 required structural elements within the minor coat protein (g3p) to adopt the conformation required for binding to P6 paraprotein. Recombinant g3p constructs were designed that contained the N-terminal fusion peptide with either the first domain alone or in combination with the first and second domains. As synthetic P3-20 bound to P3, recombinant g3p proteins containing this peptide sequence

were also produced to assess the feasibility of this approach. P6-15 containing constructs expressed extremely poorly in comparison to P3-20 g3p recombinant proteins. Assessment of the binding of P3 to P3-20 fusion proteins failed to demonstrate binding by surface plasmon resonance. The ability to detect binding of antibody against a peptide sequence within a recombinant protein was assessed using a scFv and anti-FLAG monoclonal antibody, both of which had previously demonstrated high levels of binding by slot-blot. However, no binding was observed between anti-FLAG mAb and the CD20 scFv by BIAcore analysis. Binding of P3 to P3-20 fusion proteins was subsequently assessed by slot-blot. Unfortunately, no binding was observed indicating that the P3-20 sequence is not detectable under the conditions selected. During the assembly of filamentous phage, g3p passes through the bacterial membrane where the N-terminal leader sequence is cleaved. In the strategy presented here, the g3p leader sequence was replaced with pelB to enable periplasmic expression in or recombinant g3p in *E. coli.* One possible explanation for the failure to express functional peptides as recombinant fusion proteins with g3p may be due to the different processing pathway. However the structural studies using recombinant g3p have used the same approach for periplasmic expression.

It was the intention of this research to use the reagents described above for the identification of clonally related B cells within patient bone marrow aspirates. Multi-parameter immunofluorescence studies were to be conducted to investigate the phenotype of cells expressing either surface or cytoplasmic paraprotein. A new monoclonal antibody, VAC69, was recently produced which recognises a 78-120 kDa glycoprotein on multiple myeloma plasma cells (Krueger *et al.*, 2001). This antibody failed to bind to a number of other malignant and normal cell types. Weak binding was observed against some unrelated cell types. It would be interesting to see if this antigen is expressed by putative clonal precursors as well on mature myeloma plasma cells. In addition, it was hoped that idiotype-positive cells could be further implicated as part of the malignant clone by the use of single cell fluorescent *in situ* hybridisation studies for the detection of genetic abnormalities. Nickenig and co-

workers (2001) recently investigated whether CD34+ cells within patient bone marrow smears contained the same genetic aberrations as those observed in the myeloma cells. It was concluded that this cell population does not contribute to the malignant clone based on the inability to detect any transformation event within these cells. However, these results do not rule out the possibility of finding a malignant precursor population at a later stage of B-cell development.

6.2 REASSESSMENT OF THE "PRIME TARGET"

Despite continued research, the identity of the putative myeloma precursor population remains unclear. The low proliferative rate of myeloma plasma cells, difficulties in culturing them *in vitro* and relapse in patients despite the eradication of the tumour population led to the original hypothesis of a circulating pool of precursor cells which fed the recognisable tumour clone. Towards the end of the first year of the research presented here, Yaccoby and Epstein (1999) elegantly demonstrated the proliferative potential of myeloma plasma cells. CD38++CD45- myeloma patient plasma cells purified from bone marrow aspirates by fluorescent cell sorting and plasma cell-containing peripheral blood was injected into human bone engrafted into severe combined immunodeficient mice (SCID-hu). Both of these sources of myeloma cells successfully populated non-myelomatous human bone and disease characteristics common to myeloma, such as bone decalcification, were observed. In contrast, peripheral blood depleted of plasma cells ($CD38^{++}CD45^{-}$ and $CD38^{++}CD45^{+}$) by flow cytometry did not induce disease in this model. It is possible that the small percentage of non-myeloma plasma cells present in the $CD38^{++}CD45^{-}$ fraction may contain a precursor population that led to disease establishment. The inability of plasma cell depleted blood to induce disease raises questions about the presence of drug resistant precursors within the peripheral blood. However, the possibility that the environment required by the precursor cells to establish disease is not provided by this model cannot be excluded at this time. The ability of peripheral cells in G-CSF mobilised blood to produce myeloma in NOD-SCID mice

has also recently been demonstrated (Pilarski *et al.*, 2000). Furthermore, migration and homing of clonotypic B and plasma cells was observed. Considerable evidence has amassed demonstrating the existence of B-cells at developmental stages prior to the terminally differentiated plasma cell population within the tumour clone. Sorting of these distinct populations and investigating their ability to produce disease within SCID-hu or NOD-SCID mice may help to clarify their role in malignancy. Furthermore, a recently published method using dual epitope staining has been shown to be able to detect specific cells at frequencies lower than one in one million (Townsend *et al.*, 2001). This technique may reveal new populations of putative tumour precursor cells that exist at levels below the detection limit of previous studies.

People diagnosed with MGUS rarely require treatment and are free from disease symptoms. The majority of problems associated with myeloma are related to complications arising from bone degradation. Osteoclasts, which are responsible for bone resorption, become activated as a result of cytokine signalling by myeloma tumour cells within the bone marrow microenvironment (Caligaris-Cappio *et al.*, 1991). It has recently been shown that myeloma, but not monoclonal gammopathy of undetermined significance, elevates the expression of tumour necrosis factor-related activation-induced cytokine (TRANCE) whilst down-regulating the expression of its decoy receptor (osteoprotegerin) on stromal cells. The resulting imbalance in TRANCE levels leads to bone destruction by osteoclasts and myeloma survival (Pearse *et al.*, 2001). Myeloma cell proliferation was inhibited in SCID-hu mice treated with either TRANCE inhibitor (RANK-Fc) (Pearse *et al.,* 2001) or anti-osteoclast agents (bisphosphonate) (Yaccoby *et al.*, 2002). Myeloma cells isolated from patients with extramedullary disease, and hence independent of the bone marrow for survival, were not affected by osteoclast inhibition (Yaccoby *et al.*, 2002). Targeting of both osteoclasts and tumour plasma cells, perhaps in combination with osteoporosis therapy, may greatly improve the duration of survival for myeloma patients whilst we wait for a definitive answer regarding tumour precursor involvement.

Appendix

Appendix I

DNA sequence of the minor coat protein (g3p) of fd bacteriophage, SWISS-PROT accession number INFDXX. Domain 1 and Domain are highlighted green and blue respectively.

```
2051  ********** TCGGTTATGC GTGGGCGATG GTTGTTGTCA TTGTCGGCGC
2101  AACTATCGGT ATCAAGCTGT TTAAGAAATT CACCTCGAAA GCAAGCTGAT
2151  AAACCGATAC AATTAAAGGC TCCTTTTGGA GCCTTTTTTT TTGGAGATTT
2201  TCAACGTGAA AAAATTATTA TTCGCAATTC CTTTAGTTGT TCCTTTCTAT
2251  TCTCACTCCG CTGAAACTGT TGAAAGTTGT TTAGCAAAAC CTCATACAGA
2301  AAATTCATTT ACTAACGTCT GGAAAGACGA CAAAACTTTA GATCGTTACG
2351  CTAACTATGA GGGCTGTCTG TGGAATGCTA CAGGCGTTGT GGTTTGTACT
2401  GGTGACGAAA CTCAGTGTTA CGGTACATGG GTTCCTATTG GGCTTGCTAT
2451  CCCTGAAAAT GAGGGTGGTG GCTCTGAGGG TGGCGGTTCT GAGGGTGGCG
2501  GTTCTGAGGG TGGCGGTACT AAACCTCCTG AGTACGGTGA TACACCTATT
2551  CCGGGCTATA CTTATATCAA CCCTCTCGAC GGCACTTATC CGCCTGGTAC
2601  TGAGCAAAAC CCCGCTAATC CTAATCCTTC TCTTGAGGAG TCTCAGCCTC
2651  TTAATACTTT CATGTTTCAG AATAATAGGT TCCGAAATAG GCAGGGTGCA
2701  TTAACTGTTT ATACGGGCAC TGTTACTCAA GGCACTGACC CCGTTAAAAC
2751  TTATTACCAG TACACTCCTG TATCATCAAA AGCCATGTAT GACGCTTACT
2801  GGAACGGTAA ATTCAGAGAC TGCGCTTTCC ATTCTGGCTT TAATGAGGAT
2851  CCATTCGTTT GTGAATATCA AGGCCAATCG TCTGACCTGC CTCAACCTCC
2901  TGTCAATGCT GGCGGCGGCT CTGGTGGTGG TTCTGGTGGC GGCTCTGAGG
2951  GTGGCGGCTC TGAGGGTGGC GGTTCTGAGG GTGGCGGCTC TGAGGGTGGC
3001  GGTTCCGGTG GCGGCTCCGG TTCCGGTGAT TTTGATTATG AAAAAATGGC
3051  AAACGCTAAT AAGGGGGCTA TGACCGAAAA TGCCGATGAA AACGCGCTAC
3101  AGTCTGACGC TAAAGGCAAA CTTGATTCTG TCGCTACTGA TTACGGTGCT
3151  GCTATCGATG GTTTCATTGG TGACGTTTCC GGCCTTGCTA ATGGTAATGG
3201  TGCTACTGGT GATTTTGCTG GCTCTAATTC CCAAATGGCT CAAGTCGGTG
3251  ACGGTGATAA TTCACCTTTA ATGAATAATT TCCGTCAATA TTTACCTTCT
3301  TTGCCTCAGT CGGTTGAATG TCGCCCTTAT GTCTTTGGCG CTGGTAAACC
3351  ATATGAATTT TCTATTGATT GTGACAAAAT AAACTTATTC CGTGGTGTCT
3401  TTGCGTTTCT TTTATATGTT GCCACCTTTA TGTATGTATT TTCGACGTTT
3451  GCTAACATAC TGCGTAATAA GGAGTCTTAA TCATGCCAGT TCTTTTGGGT
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