STUDIES ON THE CLONOGENIC CELL IN MULTIPLE MYELOMA

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PREFACE

The work described in this thesis was initiated in order to characterise the clonogenic cell in multiple myeloma and to determine whether any of the previously identified abnormal circulating lymphocyte populations in myeloma patients belong to the malignant clone. All experiments were performed between February 1988 and October 1991 in the Department of Haematology at the Royal Prince Alfred Hospital, a teaching hospital of the University of Sydney.

This work is original and has not been presented for the purpose of obtaining any other degree. Some investigations required to characterise a series of newly derived cell lines described in Section 2 were kindly performed by colleagues in other departments and institutions. These investigations and the colleagues who performed them are identified in the Materials and Methods of Section 2. Similarly, investigations performed as part of the routine monitoring of patients with multiple myeloma were used to assist in the interpretation of the experimental results discussed in Sections 3 and 4. These investigations are identified in the Materials and Methods of these sections.

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ABSTRACT

There is debate about the maturation status of precursor cells in multiple myeloma. Some investigators have hypothesised that there are haemopoietic stem cells or pre-B cells belonging to the malignant clone but others believe that precursor cells arise at a later stage in B cell ontogeny. The studies described in this thesis were based on the hypothesis that the earliest point in B cell ontogeny at which oncogenic transformation can give rise to a malignant clone manifest as multiple myeloma is a germinal centre B cell.

The first approach to the problem involved cell culture studies on a new germinal centre B cell line derived from the malignant cells of a patient with follicular non-Hodgkin's lymphoma. Culture of these cells with some non-physiological reagents resulted in changes in the surface antigen expression and the proliferation of the cells. Culture with physiological stimuli produced no detectable changes, so this cell line was not a useful model for studying the maturation of germinal centre B cells to plasma cells.

The second approach was to undertake immunophenotypic studies of peripheral blood lymphocytes expressing the CD10 or CD38 antigens. Both of these antigens are expressed on germinal centre B cells. Increased numbers of peripheral blood lymphocytes expressing CD38 in myeloma patients are associated with a poor prognosis. Recent studies have suggested that CD38 is expressed on increased numbers of T cells and NK cells rather than precursor cells. These findings were confirmed but the lymphocyte subsets responsible for the expansion of CD38 positive cells were shown to be different in untreated versus treated patients. In untreated patients, CD38 was detected on an

increased number of helper T cells and was associated with the maintenance of relatively normal humoral immunity. In treated patients, there were increased numbers of activated suppressor/cytotoxic T cells and NK cells expressing this antigen.

The CD10 antigen has been consistently reported as being expressed by precursor cells in myeloma. The peripheral blood lymphocytes expressing CD10 were shown to be CD5 positive B cells that also express CD38. There was no definitive proof that these cells were related to the malignant clone, although this possibility was not excluded. The immunophenotype of these cells was similar but not identical to germinal centre B cells. Alternatively, these cells may be related to the immunosuppressive CD5 positive B cells previously described in myeloma patients.

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LIST OF ABBREVIATIONS

ALL	acute lymphoblastic leukaemia
β2M	β2 microglobulin
BJP	Bence-Jones protein
BSA	bovine serum albumin
CD	cluster of differentiation
cId	cytoplasmic idiotypic immunoglobulin
cIg	cytoplasmic immunoglobulin
CLL	chronic lymphocytic leukaemia
cμ	cytoplasmic μ immunoglobulin heavy chain
EDTA	ethylenediaminetetraacetic acid
EBV	Epstein Barr virus
FCS	foetal calf serum
FITC	fluorescein isothiocyanate isomer 1
GM-CSF	granulocyte-macrophage colony stimulating factor
HLA-DR	human leucocyte antigen DR molecules
Id	idiotypic immunoglobulin
IFN-α	interferon-α
IgA	immunoglobulin A
IgD	immunoglobulin D
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
LCIS	light chain isotype suppression
LI	labelling index
LPS	lipopolysaccharide
NK cell	natural killer cell

MHC	major histocompatibilty complex
MGUS	monoclonal gammopathy of uncertain significance
NHL	non-Hodgkin's lymphoma
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PBS	phosphate-buffered saline
PE	R-phycoerythrin
PWM	pokeweed mitogen
sβ2M	serum β2 microglobulin
SD	standard deviation
sId	surface idiotypic immunoglobulin
sIg	surface immunoglobulin
SMM	smouldering multiple myeloma
STK	serum thymidine kinase
TCR	T cell receptor
TdT	terminal deoxytidyl transferase
TNF	tumour necrosis factor
TPA	12-O-tetradecanoyl-phorbol-13 acetate
TRITC	tetramethylrhodamine isothiocyanate
U	units
+	positive
-	negative
<	less than
>	greater than

SECTION 1: INTRODUCTION

Multiple myeloma is a B cell malignancy characterised by the accumulation in the bone marrow of neoplastic plasma cells which usually secrete a monoclonal immunoglobulin. Manifestations of the disease include osteolytic bone lesions which are frequently associated with pathological fractures, hypercalcaemia, renal failure which is multifactorial in origin, recurrent bacterial infections, hyperviscosity and bone marrow failure (Kyle and Greipp, 1988).

1.1: MONOCLONAL GAMMOPATHIES

Multiple myeloma is one of the diseases associated with the presence of a monoclonal immunoglobulin (paraprotein) in the serum or urine. Patients with a paraprotein are said to have a monoclonal gammopathy. Other diseases associated with monoclonal gammopathy include Waldenstrom's macroglobulinaemia, NHL, amyloidosis, heavy chain disease and light chain disease. There are patients who have a monoclonal gammopathy but no evidence of any of these diseases. Such patients usually have <30g/l paraprotein and <5% plasma cells in the bone marrow. They are classified as having MGUS. In an unselected population, only 0.5% of paraproteins detected by current routine laboratory techniques are associated with a malignant disorder (Lightart and Hijmans, 1989). If patients with MGUS are monitored over a long period of time, a proportion will develop one of the diseases associated with monoclonal gammopathy (Axelsson, 1986; Kyle and Greipp, 1988). The incidence of this varies between series, probably reflecting differences in the population being studied. Kyle and Greipp (1988) studied a referred group of patients, whereas the study of Axelsson (1986) was a population survey.

Studies in both humans and a murine model are attempting to clarify the relationship between MGUS and multiple myeloma. Radl (1985) has divided monoclonal gammopathies into 4 categories based on pathogenesis: (1) B cell malignancies; (2) B cell benign neoplasia (BMG); (3) immunodeficiency characterised by greater T cell than B cell dysfunction; (4) antigen driven. Immunodeficiency causing monoclonal gammopathy may be either primary or secondary, the latter including ageing, immunosuppressive treatment and the period of immune reconstitution following bone marrow transplantation. The mouse strain C57BL/KaLwRij has proved a good model for the spectrum of monoclonal gammopathies in humans and has enabled a study of these disorders in the context of the above classification (Van Den Akker, 1989). In categories 1 and 2, the clone producing the monoclonal immunoglobulin exhibits autonomous proliferation but, only in category 1, is the clone immortal. In category 3, there is no autonomous proliferation of the clone and, in both the murine and human situation, the monoclonal gammopathy is transitory. These findings have provided support for a previously proposed model for the development of BMG (Radl, 1979). The presence of T cell dysfunction and relatively normal B cell function leads to transient mono- or oligoclonal B cell expansions. In those with a susceptible genetic background, these expansions, if recurrent, lead to a higher probability of genetic mutations, some of which may result in the development of a BMG. However, studies in the murine model offer no support for the concept that BMG is a pre-myeloma (Radl, 1991). Myeloma in the murine model does not develop from a clone causing BMG. This suggests that patients with MGUS who develop myeloma have a separate disorder compared to that in those who do not progress. The former patients actually have early myeloma, albeit asymptomatic and stable, at the time of diagnosis. The laboratory tests enabling the determination of this distinction

are not adaptable to the clinical situation, necessitating follow-up as the only way of differentiating these conditions in patients with a provisional diagnosis of MGUS.

1.2: DISEASE ACTIVITY IN MULTIPLE MYELOMA

1.2.1: Smouldering or Indolent Myeloma

The term "smouldering multiple myeloma" was first used by Kyle and Greipp (1980) to describe a small group of patients who fulfilled the criteria for the diagnosis of myeloma but who did not require therapy or progress over a period of 5 years. These patients had no lytic bone lesions, anaemia, renal failure or hypercalcaemia. As this term was applied retrospectively, there were patients who appeared to have this entity at diagnosis but who progressed within months to years. At about the same time, Alexanian (1980) used the term "indolent myeloma" to describe those patients who were asymptomatic at diagnosis, did not have renal impairment, were not anaemic or hypercalcaemic and did not have more than 3 lytic lesions. The median time before progressive disease necessitated the institution of treatment was 28 months. Among such patients, the presence of a paraprotein concentration >30g/l, osteolytic bone lesions or ≥20% plasma cells in the bone marrow is associated with a shorter interval before the occurrence of progressive disease (Alexanian et al, 1988; Wisløff et al, 1991). The survival of these patients from the time of commencing treatment is no different to those requiring treatment at diagnosis (Alexanian et al, 1988; Wisløff et al, 1991). As delaying therapy is not detrimental to survival and myeloma is not cured by chemotherapy, it is now common practice to withhold therapy and monitor these patients until progression occurs. The term "smouldering myeloma" is now frequently used to mean the same as "indolent myeloma", namely asymptomatic disease with no indications for

therapy at the time of diagnosis. As MGUS, SMM and active myeloma represent a continuous spectrum of clinical disease, it is sometimes difficult to correctly assign individual patients to one of these categories. Some of the newer prognostic factors (section 1.3) can assist in such decisions.

1.2.2: Plateau Phase

In a review of the patterns of response to initial induction chemotherapy, almost half the patients had a response followed by stable disease, despite persistence of the paraprotein and the plasma cell infiltrate in the bone marrow (Durie et al, 1980a). This period of disease stability is termed plateau phase and, during this time, patients have no more than minimal symptoms attributable to active disease, no transfusion requirement and exhibit serological stability. Treatment with either melphalan and prednisone or combination chemotherapy is not curative therapy for multiple myeloma and, for such standard treatment, attainment of plateau is now recognised as being more important than the degree of serological response. Several studies have demonstrated that survival does not correlate with achieving a response as defined by either the South West Oncology Group or the Chronic Leukemia-Myeloma Task Force criteria (Palmer et al, 1987; Baldini et al, 1991a). Nor does the actual percentage fall in paraprotein concentration correlate with the duration of plateau or survival (Palmer et al, 1989; Joshua et al, 1991a). Consequently, patients cannot be considered to have progressive disease requiring therapy with an alternative chemotherapy regimen solely on the basis of failure to achieve a specified decrement in paraprotein level.

Patients may be in plateau phase at diagnosis and this group includes those patients initially assessed as having SMM who do exhibit a period of stability on

follow-up. Such patients do not have a survival different to those who have an obvious serological response followed by plateau phase (Joshua et al, 1991a).

High dose therapy, frequently incorporating autologous or allogeneic bone marrow transplantation, is currently being investigated as curative treatment in appropriately selected patients with multiple myeloma. Even with such therapy, not all patients achieve a complete remission, which is defined as <5% plasma cells in the bone marrow and the absence of a paraprotein in serum or urine using the technique of immunofixation. The majority of patients, including those who do attain complete remission, do subsequently develop progressive disease, sometimes after prolonged periods of stability (Fermand et al, 1992; Jagannath and Barlogie, 1992; Tura and Cavo, 1992). The follow-up on these patients is still too short to know whether any of the patients who achieve complete remission have been cured. Thus, the outcome in the majority of patients treated with high dose therapy may be considered to be the attainment of a plateau phase, albeit frequently with a lower tumour burden than in those patients treated with standard therapy.

The recognition of plateau phase as an entity raised the possibility that chemotherapy could be ceased after reaching this state. There have been several trials of maintenance with chemotherapy versus no maintenance but only two used attainment of plateau phase as a criteria for randomisation (Belch et al, 1988; Medical Research Council Working Party on Leukaemia in Adults, 1985), although the study by Belch et al (1988) also required a defined serological response. The criteria for randomisation in the other trials were a defined serological response and/or a defined period of chemotherapy (South West Oncology Group, 1975; Finnish Leukaemia Group, 1985; Cohen et al, 1986; Peest

et al, 1988; Kildahl-Anderson et al, 1988). Despite these randomisation differences, no trial demonstrated a survival advantage for continuing chemotherapy. Two of the 6 studies which analysed remission duration found an advantage for maintenance but there was no difference in survival, as these patients responded to the reintroduction of chemotherapy (Belch et al, 1988; Peest et al, 1988). In one of these studies (Peest et al, 1988), it is possible that many patients, although responding, were not stable, as they had received only 6 months of therapy prior to randomisation and the median time to plateau is approximately 9 months (Joshua et al, 1991a; MacLennan et al, 1992a).

1.3: PROGNOSTIC FACTORS IN MULTIPLE MYELOMA

Many clinical and routine laboratory parameters have been shown to provide prognostic information on survival duration. The frequently identified prognostic factors are serum creatinine, haemoglobin, serum calcium (corrected or uncorrected) and age (Durie and Salmon, 1975; Durie et al, 1980b; Gassman et al, 1985; Bataille et al, 1986; Greipp et al, 1988; Simonsson et al, 1988; Cavo et al, 1989; Palmer et al, 1989; San Miguel et al, 1989; Cuzick et al, 1990; San Miguel et al, 1992). Factors which have only been identified in a small number of studies or about which there has been debate include serum albumin, performance status, serum lactic dehydrogenase, ESR, excretion of light chains in the urine, serum uric acid, platelet count, percentage of plasma cells in the bone marrow, plasmablastic morphology, extent of bone lesions, λ paraprotein, infection occurring prior to diagnosis, fever and absolute number of $CD4^+$ PBL (Durie and Salmon, 1975; Durie et al, 1980b; Gassman et al, 1985; Bataille et al, 1986; Greipp et al, 1988; Simonsson et al, 1988; Cavo et al, 1989; San Miguel et al, 1989; Cuzick et al, 1990; San Miguel et al, 1992). Over the last decade there has been continued research into the identification of new prognostic factors

which could improve on the predictive ability of the more traditional ones. Such factors include sβ2M, LI, STK, the presence of LCIS and the number of PBL expressing the CD38 antigen.

1.3.1: Staging Systems

There are several staging systems based on traditional prognostic factors. The most commonly used one was devised by Durie and Salmon (1975), who found that the major determinants of survival were measured myeloma cell mass and the clinical features associated with it. They devised a staging system based on haemoglobin concentration, serum calcium level, extent of radiological bone disease and paraprotein level. It divided the patients into 3 groups: those who had low (stage I), intermediate (stage II) and high (stage III) myeloma cell mass. Serum creatinine level was used to further divide each stage into A and B. The survival of patients with stage IA disease was not significantly different to those with stage II disease. Patients with stage IA or II disease had a significantly longer survival than those with stage IIIB disease and there was a trend in the same direction when patients with stage IA or II disease were compared to those with stage IIIA disease. Patients with stage IIIA disease fared significantly better than those with stage IIIB disease. The aim of this staging system was to provide a means of planning, analysing and comparing clinical trials.

There has been controversy about the adequacy of this system to provide prognostic information about the majority of patients, although it is not disputed that stage IIIB patients are a poor prognostic group. There is the lack of difference in the survival of patients with stage I and stage II disease in the initial and subsequent studies (Durie and Salmon, 1975; Pennec et al, 1983; Bataille et al, 1986). Some studies have failed to find a difference in survival

between stage II and stage III patients, either when only A patients were studied or when the groups were not subdivided into A and B (Pennec et al, 1983; Gassman et al, 1985; Greipp et al, 1988; Cavo et al, 1989; San Miguel et al, 1989). One problem is that when advanced lytic bone lesions are defined as more than 3 lesions, the majority of patients have stage III disease (Gassman et al, 1985; Greipp et al, 1988; Cavo et al, 1989). Furthermore, the assessment of the extent of lytic bone lesions is subjective and this results in a lack of uniformity when assigning patients to a given stage (Bataille et al, 1986).

Alternative staging systems have been developed. The most important are those of the British Medical Research Council (MRC) (Medical Research Council's Working Party on Leukaemia in Adults, 1980) and of Merlini-Waldenström-Jayakar (MWJ) (1980). Both of these systems also include a parameter which could be considered subjective (Bataille et al, 1986). In studies comparing these staging systems, the MRC system was superior in 3 studies (Gassman et al, 1985; San Miguel et al, 1989; Gobbi et al, 1990) and the Durie and Salmon system in one (Bataille et al, 1986).

1.3.2: Serum β2 Microglobulin

β2M is the invariant β polypeptide chain of class I MHC molecules which are present on almost all nucleated cells. Serum levels of β2M are the result of membrane turnover. It is freely filtered by the renal glomeruli and catabolised by the tubules (Berggärd and Bearn, 1968; Peterson et al, 1974). The serum level rises when the glomerular filtration rate decreases and there is a strong correlation with serum creatinine (Wibell et al, 1973; Van Dobbenburgh, 1985; Greipp et al, 1988). Serum β2M levels also correlate with total body myeloma

cell (TBMC) mass (Van Dobbenburgh, 1985) and Durie and Salmon stage (Boccadoro et al, 1988; Greipp et al, 1988).

Serum β2M levels at diagnosis have been repeatedly reported to be a prognostic factor for survival duration (Van Dobbenburgh et al, 1985; Boccadoro et al, 1987; Greipp et al, 1988) and, frequently, as the single most important prognostic factor (Bataille et al, 1986; Simonsson et al, 1988; Cuzick et al, 1990; Gobbi et al, 1991; San Miguel et al, 1992). As sβ2M correlates with serum creatinine and TBMC mass, it has been argued that even though sβ2M, both corrected and uncorrected for serum creatinine, predicts survival, it is no more useful than a combination of age, TBMC mass and serum creatinine (Van Dobbenburgh, 1985). However, sβ2M has prognostic significance even in patients with a normal serum creatinine (Boccadoro et al, 1987; Greipp et al, 1988).

Gobbi et al (1991) showed that sβ2M as a continuous variable was better than either the MRC, Durie and Salmon or MWJ staging systems. Bataille et al (1986) found that serum albumin was the only variable which added significantly to the predictive capacity of sβ2M but this study did not include a parameter reflecting plasma cell proliferation.

One group of investigators concluded that sβ2M was not a useful parameter for monitoring disease activity because fluctuations in sβ2M levels did not reflect changes in paraprotein levels (Boccadoro et al, 1989). Some of the limitations of relying on changes in paraprotein levels to monitor disease activity have already been discussed (section 1.2.2). In contrast, several groups have reported that sβ2M is useful for monitoring disease activity in patients with normal renal function (Bataille et al, 1984; Garewal et al, 1984; Brenning et al, 1985; Cuzick

et al, 1985). A detailed analysis of prognostic factors in 1,014 patients in the MRC IVth and Vth Myelomatosis Trials (Cuzick et al, 1990) showed that sβ2M levels at diagnosis were predictive of survival only for the 12-24 months following diagnosis. However, sβ2M measurements at any given time point during the course of the disease were predictive of survival for the subsequent 12-24 months. Once plateau phase was reached, sβ2M levels at that time were more predictive of survival than those obtained at diagnosis.

1.3.3: Plasma Cell Labelling Index

The LI is a cell kinetic measurement of the percentage of cells in the S-phase of the cell cycle. It can be determined using either tritiated thymidine autoradiography or quicker immunofluorescence methods utilising antibodies against 5-bromo-2-deoxyuridine (Gratzner, 1982; Gonchoroff et al, 1985). The method described by Greipp et al (1985) has the advantage of employing a monoclonal antibody (BU-1) which does not require the prior chemical denaturation of DNA, due to the presence of deoxyribonuclease in the hybridoma cell culture supernatant as a result of Mycoplasma infection of the hybridoma cell line (Gonchoroff et al, 1985; Gonchoroff et al, 1986a). This results in the preservation of tissue morphology and antigen molecules.

There is no correlation between the LI and the sβ2M level at diagnosis or at relapse, although there is a trend towards a positive correlation in the remission phase (Boccadoro et al, 1987). The LI has found several roles in assessing myeloma patients. Firstly, it predicts survival duration at diagnosis (Durie et al, 1980b; Latreille et al, 1982; Montecucco et al, 1986; Greipp et al, 1988). Greipp and colleagues (1988) found that the LI added significantly to the prognostic information provided by sβ2M and age. Secondly, it can be used during plateau

to monitor for the onset of disease progression (Boccadoro et al, 1984; Boccadoro et al, 1987; Greipp et al, 1987). It has been reported to be a better discriminator than sβ2M for detecting progression (Boccadoro et al, 1987). Thirdly, it may be used to distinguish MGUS or SMM from active myeloma requiring therapy (Durie et al, 1980b; Greipp and Kyle, 1983; Boccadoro et al, 1984; Boccadoro et al, 1987; Greipp et al, 1987; Büchi et al, 1990).

1.3.4: Serum Thymidine Kinase

Thymidine kinase (TK) is the key enzyme in the pyrimidine salvage pathway, allowing the reutilisation of endogenous thymidine for DNA synthesis. The isoenzyme TK1 is detected in large amounts in the cytoplasm of dividing cells in the G1 to S phases of the cell cycle (Bello, 1974). In multiple myeloma, STK is a prognostic factor predicting survival at diagnosis and it can be used to monitor the response to therapy and to detect the occurrence of progressive disease (Simonsson et al, 1985; Simonsson et al, 1988; Brown et al, 1989; Joshua et al, 1990; Bartl et al, 1991; Luoni et al, 1992). STK may also be used to detect the presence of increasing disease activity necessitating therapy in patients with SMM (Bartl et al, 1991). In cross-sectional analysis, STK does not correlate with sβ2M at any time during the course of the disease (Simonsson et al, 1985; Brown et al, 1990). Thus, these two variables are independent prognostic factors. In multivariate analysis, STK provides additional information to that provided by sβ2M (Simonsson et al, 1988; Luoni et al, 1992). In multiparameter longitudinal analysis, there are significant correlations between STK and LI, number of $CD38^+$ PBL, sβ2M, BJP excretion, serum creatinine and haemoglobin (Joshua et al, 1990). It has been reported that STK may be more reliable than either sβ2M or LI for the longitudinal monitoring of disease activity (Brown et al, 1989). This group noted patients with obvious progressive disease who had

an elevated STK level but normal LI. The LI reflects proliferation of the bone marrow plasma cells only, whereas STK does not reflect proliferation in a specific compartment. They hypothesised that the STK was reflecting proliferation in a malignant pre-plasma cell population.

1.3.5: Light Chain Isotype Suppression

In the majority of patients with multiple myeloma, the total number of plasma cells in the lamina propria of the rectal mucosa is reduced due to the selective loss of plasma cells secreting antibody with the same light chain isotype as the paraprotein (Leonard et al, 1979). This phenomenon was termed "light chain isotype suppression".

Subsequently, the determination of the ratio of cells expressing surface κ light chain to those expressing surface λ light chain (κ:λ ratio) has been used to demonstrate that LCIS occurs within the circulating B cell population of myeloma patients. It is present in a large proportion of patients during plateau (Wearne et al, 1984; Joshua et al, 1988) and is lost with disease progression (Wearne et al, 1985; Joshua et al, 1988). Among newly diagnosed patients, those with LCIS have a significantly longer survival (Joshua et al, 1987) and frequently have stable disease not requiring immediate therapy (Wearne et al, 1987b; Büchi et al, 1990; Oritani et al, 1990). Patients with MGUS rarely have LCIS (Wearne et al, 1984; Büchi et al, 1990; Oritani et al, 1990). At diagnosis, LCIS is associated with sβ2M levels <6 mg/l (Wearne et al, 1987b). At later stages of the disease, there is no correlation between the presence of LCIS and sβ2M levels (Wearne et al, 1987a; Joshua et al, 1988).

It has been proposed that LCIS results from host immunoregulation of the tumour. This occurs in an isotype specific way, affecting normal B cells as well as the malignant clone (Leonard et al, 1979; Wearne et al, 1987b). The presence of LCIS, although contributory, is not sufficient to explain the polyclonal hypogammaglobulinaemia present in myeloma patients (Leonard et al, 1979).

The existence of either LCIS or clonal excess (discussed in section 1.4.2) has been disputed (King and Radicchi, 1992). This group performed dual labelling studies with anti-CD19 and either anti-κ or anti-λ antibodies in 25 patients at diagnosis or in plateau and did not find an abnormal ratio of $CD19^{+}s\kappa^{+}$:$CD19^{+}s\lambda^{+}$ lymphocytes in any patient. They proposed that the phenomena of LCIS and clonal excess resulted from the presence of cytophilic immunoglobulin (paraprotein in myeloma patients) on non-B cells. Washing the cells does not remove all the cytophilic immunoglobulin (King and Wells, 1981) and single parameter flow cytometry analysis does not allow adequate discrimination of B cells from monocytes falling within the lymphoid gate. Complicating the issue further are reports that in some patients the B cells have bound cytophilic immunoglobulin (King and Wells, 1981; Levy et al, 1991).

1.3.6: Number of Lymphocytes Expressing the CD38 Antigen

The CD38 antigen is an integral membrane glycoprotein (Terhorst et al, 1981). The first monoclonal antibody against the CD38 antigen (OKT10) was produced by immunising mice with human thymocytes (Reinherz et al, 1980). CD38 is present on >95% of thymocytes but was initially reported as being expressed on <5% of normal peripheral blood T cells. Since this original report, the percentage of PBL reported as expressing the CD38 antigen has increased due to improvements in technology. In the IVth Leucocyte Typing Workshop (Pezzutto

et al, 1989), the mean percentages of PBL and peripheral blood T cells bearing CD38 were 36% and 31% respectively. In a recent study, the absolute number of lymphocytes expressing this antigen was $0.47\pm0.18x10^9/l$ (mean±SD) in the control group (Gonzalez et al, 1992). CD38 expression is upregulated on mitogen activated T cells (Terhorst et al, 1981). This occurs on both $CD4^+$ and $CD8^+$ T cells following mitogen and alloantigen stimulation but only on $CD4^+$ lymphocytes following stimulation by soluble antigen (Hercend et al, 1981).

It was apparent from the initial description of the tissue distribution of CD38 that it was not a lineage specific antigen, as it was also expressed on ≤20% of bone marrow cells and about 10% of peripheral blood non-T cells (Reinherz et al, 1980). On B lineage cells, the CD38 antigen is expressed on early B progenitor cells, predominantly pre-B cells (Ling et al, 1987; Dörken et al, 1989), germinal centre cells (Hsu and Jaffe, 1984; Ling et al, 1987; Gadol et al, 1988; Liu et al, 1989) and on over 90% of plasma cells in all anatomic sites (Hercend et al, 1981; Bhan et al, 1981; Tedder et al, 1984). The malignant plasma cells in multiple myeloma (Bhan et al, 1984; Aisenberg and Wilkes, 1983), plasma cell leukaemia (Aisenberg and Wilkes, 1983) and soft tissue plasmacytoma (Foon et al, 1982) also characteristically express this antigen. The intensity of expression of CD38 is so high on plasma cells in comparison to other cells that it has been exploited to study the phenotype of both normal and malignant plasma cells, even when they are present only in low numbers (Terstappen et al, 1990; Harada et al, 1992; Kawano et al, 1992). *In vitro* studies utilising PWM to activate and differentiate B lymphocytes have demonstrated that the initial expression of CD38 antigen coincides with the appearance of $cIgM^+$ cells and the downregulation of CD21 and sIgD (Stashenko et al, 1981). As CD38 expression increased, CD20, CD21 and sIgD became undetectable but sIgG, cIgM and cIgG

were dramatically upregulated. The expression of sIgM was unaltered. Tedder et al (1984) demonstrated that these $CD38^+$ cells arise even when the starting population of B cells is depleted of any $CD38^+$ cells.

The identification of the surface antigens on plasma cells, combined with the hypothesis that there are circulating precursor cells in myeloma, prompted investigation of the expression of "plasma cell associated antigens" on the PBL of myeloma patients. Ruiz-Argüelles et al (1984) studied CD38 expression on lymphocytes in 14 patients with myeloma. In 4 patients who had a high tumour burden and a LI >1%, there was an increased percentage of $CD38^+$ cells. One of these patients had 40% circulating atypical plasma cells but the other patients did not have morphologically detectable plasma cells in the peripheral blood. It was concluded that the observations suggested the presence of peripheral blood involvement by immature cells of the malignant clone in these patients and that this was associated with a poor prognosis.

In a larger series of 37 patients studied serially over a period of 8 months (Joshua et al, 1988), 22 patients had an increased absolute number of $CD38^+$ lymphocytes and this was inversely correlated with the presence of LCIS. The presence of an elevated number of $CD38^+$ cells was not correlated with the presence of $CD10^+$ PBL or the sβ2M level. Again, the increased number of $CD38^+$ PBL was thought to represent peripheral blood involvement by the malignant clone, due either to "spill-over" from the bone marrow or to the presence of malignant circulating precursor cells.

Two studies have investigated the phenotype of $CD38^+$ PBL in patients with multiple myeloma. Boccadoro et al (1988) reported that no patient with MGUS

had an elevated percentage of CD38$^+$ PBL. In contrast, the percentage of CD38$^+$ PBL was more than two standard deviations above the mean value of the control group in 2/8 patients at diagnosis, 4/9 during remission and 7/8 with progressive disease, even though no patient had >2% circulating cIg$^+$ cells. Half the patients with elevated levels in remission subsequently experienced early relapse. In 3 controls and 4 myeloma patients, the CD38$^+$ cells were sorted and further immunophenotypic studies were undertaken. There was a significantly increased percentage of CD4$^+$ and CD8$^+$ cells in the CD38$^+$ population in the patients compared to the controls. There was also an increased proportion of activated (HLA-DR$^+$) cells. The majority of B cells did not express CD38. In a study of NK cells in myeloma, there was a significantly increased proportion of CD38$^+$ PBL in untreated myeloma patients compared to normal controls (Gonzalez et al, 1992). Patients with a high percentage of NK cells had a significant increase in the percentage and absolute number of CD38$^+$ lymphocytes compared to those without elevated NK numbers. From the data presented, it was apparent that there were patients without an expanded NK population who had increased CD38$^+$ cell numbers. It was stated that dual labelling studies had shown that the CD38$^+$ cells were T cells and NK cells but not B cells. Further details of these studies were not provided.

Thus, a significant proportion of the CD38$^+$ PBL in myeloma patients are T cells or NK cells rather than cells belonging to the neoplastic clone. However, only a limited number of patients have been studied and, in the study of Boccadoro et al (1988), there were CD38$^+$ cells whose lineage was not assigned, raising the possibility that there could exist a minor population of CD38$^+$ malignant precursors. In agreement with this concept, up to 29% of small Id$^+$ lymphocytes

have been shown to co-express the CD38 antigen (Bloem et al, 1988) but these Id^+ cells constituted <1% of PBL.

Two studies have addressed the usefulness of the number of $CD38^+$ PBL at diagnosis as a prognostic factor. Omedé et al (1990) serially studied 52 patients with myeloma, including 32 patients at diagnosis. Patients with $\geq 0.45 \times 10^9/l$ $CD38^+$ lymphocytes had a median survival of 14 months compared to a median survival that had not been reached at 32 months in those patients with an absolute number of $CD38^+$ PBL below this cutoff value. It was reported that the results were more significant when expressed as percentages, as the myeloma patients had a significant lymphopenia compared to normal individuals. The Australian Leukaemia Study Group MM1 study enrolled 74 patients at diagnosis (Joshua et al, 1991b). Patients with >20% $CD38^+$ PBL had a median survival of 19 months, whereas those with ≤20% had a median survival of 33 months. There was no correlation between the percentage of $CD38^+$ PBL and either the sβ2M level or the Durie and Salmon stage.

1.4: PRECURSOR POPULATIONS IN MYELOMA

Evidence has accumulated over the years that the malignant clone in myeloma does not consist solely of plasma cells but that there is also a pre-plasma cell compartment. These precursor cells are present in the peripheral blood and are hypothesised to home to the bone marrow where they undergo differentiation to plasma cells. The existence of such a population would explain the fact that the disease is widely disseminated in the axial skeleton in most patients at the time of diagnosis. The site of origin of these precursor cells remains unknown.

1.4.1: Expression of Surface Immunoglobulin with Idiotypic Determinants by Peripheral Blood Lymphocytes

Studies with antibodies detecting idiotypic determinants on individual monoclonal immunoglobulins provided early evidence that cells belonging to the malignant clone circulate in the peripheral blood of some patients with MGUS and multiple myeloma, even in the absence of morphologically identifiable plasma cells (Mellstedt et al, 1974; Abdou and Abdou, 1975; Preud'homme et al, 1977; Kubagawa et al, 1979; Lea et al, 1979; Pettersson et al, 1980; Mellstedt et al, 1982b; Bast et al, 1982; Carmagnola et al, 1982; Bloem et al, 1988). This sId^+ population has been reported to constitute from <1% to 47% of PBL. In individual patients, the size of the sId^+ population fluctuates with disease activity (Pettersson et al, 1980; Mellstedt et al, 1982b; Carmagnola et al, 1983). The detailed results of two studies examining the immunophenotype of sId^+ and cId^+ cells are discussed in section 1.4.6.

A major problem associated with studying sIg^+ cells is that endogenous membrane immunoglobulin cannot be distinguished from exogenous immunoglobulin, including the anti-immunoglobulin reagent itself, bound to Fc receptors. Standard methods employed to overcome this problem are the use of $F(ab')_2$ antibody fragments and extensive washing of the cells at 37°C prior to the staining procedure. In two studies in which >15% sId^+ PBL were detected, these precautions were not taken but the idiotypic structures were demonstrated to be synthesised by the cells (Mellstedt et al, 1974; Abdou and Abdou, 1975). Nevertheless, extensive incubation and washing of the cells at 37°C is not sufficient to remove all the cytophilic immunoglobulin from either B or non-B cells (King and Wells, 1981; Levy et al, 1991). In each of these studies, there was a patient with an IgG paraprotein who had a significant number of

sId$^+$ lymphocytes but the sIg molecules were demonstrated to be extrinsic in both cases. This should not be unexpected as B cells express the FcγII receptor (CD32), a low affinity receptor for IgG1 and IgG3 complexes (Anderson and Looney, 1986).

Dual labelling experiments have demonstrated that sId$^+$ cells predominantly express the immunoglobulin isotype of the paraprotein (Kubagawa et al, 1979; Pettersson et al, 1980; Mellstedt et al, 1982b; Bloem et al, 1988). Small numbers of peripheral blood B cells expressing sIgM with or without sIgD (Kubagawa et al, 1979; Pettersson et al, 1980; Mellstedt et al, 1982b) and bone marrow pre-B cells (Kubagawa et al, 1979) have also been shown to express idiotypic determinants. One interpretation of these results is that oncogenic transformation and expansion of the malignant clone occurs at an early stage of B cell ontogeny (Kubagawa et al, 1979; Mellstedt et al, 1980). An alternative explanation is based on the fact that idiotopes, which are the antigenic determinants of immunoglobulin molecules, range in specificity from public to private. Despite extensive absorption of anti-idiotypic antibodies, it is difficult to ensure their specificity for a particular paraprotein (Kiyotaki et al, 1987). If the anti-idiotypic reagents were not specific for the private idiotopes of the paraprotein, then the sIgM$^+$sId$^+$ B cells and Id$^+$ pre-B cells may not belong to the malignant clone but may share public idiotopes with the malignant clone.

Idiotypic structures have been reported to be present on T cells as well as B cells (Preud'homme et al, 1977; Lea et al, 1979). These idiotypic structures were not immunoglobulin molecules, were actively synthesised and were capable of binding the native antigen. These studies were performed prior to the

description of the TCR but this would be the most likely candidate for the idiotype expressing structure.

1.4.2: Surface Immunoglobulin Light Chain Isotype Expression of Peripheral Blood Lymphocytes

As the use of antibodies directed against idiotypic determinants is not feasible for studying large numbers of patients, the possibility that there are circulating precursor cells in multiple myeloma has also been explored by analysing the sIg light chain isotype expression of PBL. The presence of an abnormal κ:λ ratio due to a relative increase in the number of cells expressing the same light chain isotype as the paraprotein has been considered to indicate the presence of malignant precursor cells in the peripheral blood. This phenomenon, which has been termed isotypic dominance or clonal B cell excess, has been documented in a variable proportion of patients with MGUS or active myeloma (Pettersson et al, 1981; Mellstedt et al, 1982b, Österborg et al, 1987; Bagg et al, 1989; Büchi et al, 1990; Oritani et al, 1990). Its presence is predictive of a short remission duration and survival (Österborg et al, 1987). Three of these studies documented a large preponderance of patients with λ paraproteins among those who exhibited clonal excess (Bagg et al, 1989; Büchi et al, 1990; Oritani et al, 1990) and this may be related to the worse prognosis of patients with λ paraproteins (Durie et al, 1980b).

There is no direct proof that the cells expressing the same light chain isotype as the monoclonal immunoglobulin belong to the malignant clone. An alternative explanation is that there may be loss of the immunoregulatory mechanisms responsible for LCIS with a resultant rebound increase in non-

malignant lymphocytes expressing the same light chain isotype as the malignant clone.

The technical problems associated with the analysis of sIg^+ populations has been discussed in the sections pertaining to LCIS (1.3.5) and sId^+ PBL (1.4.1).

1.4.3: Immunoglobulin Secreting Cells in Peripheral Blood

Immunoglobulin secreting cells can be quantified using a reverse haemolytic plaque assay. A large proportion of myeloma patients have increased numbers of peripheral blood mononuclear cells which secrete immunoglobulin of the same isotype as the paraprotein, even in the absence of contaminating plasma cells on morphological examination (Shimizu et al, 1980; Nagai et al, 1981; Dammacco et al, 1985). Only one of these studies detected such cells in patients with MGUS (Dammacco et al, 1985). Changes in the number of isotype concordant immunoglobulin secreting cells reflect disease activity (Nagai et al, 1981; Dammacco et al, 1985).

1.4.4: Immunoglobulin Gene Rearrangements

The development of molecular techniques presented an alternative method for investigating the existence of circulating clonal lymphocytes in multiple myeloma. The sensitivity of Southern Blot analysis varies but usually allows the detection of 0.5-5% clonal cells within a sample (Baldini et al, 1991b; Berenson and Lichtenstein, 1989a; Chiu et al, 1989; Clofent et al, 1989; Griesser et al, 1989; Korsmeyer et al, 1987; Van Riet et al, 1989; Levy et al, 1991). Immunoglobulin gene rearrangements have not been detected in the peripheral blood mononuclear cells of patients with MGUS (Cassel et al, 1990; Chiu et al, 1989). After excluding those cases with circulating plasma cells on

morphological grounds, studies of peripheral blood mononuclear cells (Berenson et al, 1987; Berenson and Lichtenstein, 1989a; Chiu et al, 1989) or T cell depleted preparations (Cassel et al, 1990) have indicated that up to 78% of myeloma patients have circulating cells with immunoglobulin gene rearrangements identical to those detected in the bone marrow plasma cells. The incidence of immunoglobulin gene rearrangements is higher in active than stable disease and in untreated than treated patients (Cassel et al, 1990; Chiu et al, 1989; Van Riet et al, 1989). In one study which failed to demonstrate rearrangements in peripheral blood mononuclear cells, the detection limit of Southern blot analysis in the laboratory was 5-10% clonal cells (Bagg et al, 1989).

It has been argued that the detection of circulating plasma cells by morphological examination is not sufficiently sensitive (Klein and Bataille, 1989). Proponents of this view have found that immunoglobulin gene rearrangements are detected only in peripheral blood mononuclear or negatively selected B cell-enriched preparations that contain >2% circulating cIg^+ cells (Clofent et al, 1989; Baldini et al, 1991b; Levy et al, 1991). As there is evidence that the malignant cell in the peripheral blood of myeloma patients is an adherent B cell (Jensen et al, 1991; Berenson and Lichtenstein, 1989a), two of these studies (Clofent et al, 1989; Baldini et al, 1991b) have been criticised on the basis that monocytes had been depleted by adherence to plastic (Berenson and Lichtenstein, 1989a). In contrast, a study using positive selection of B cells demonstrated that the rearrangements detected in the myeloma cells were present in 3/7 bone marrow and 2/10 peripheral blood preparations containing <2% cIg^+ cells (Van Riet et al, 1989).

Studies using PCR technology may resolve many of the issues regarding the identity and maturation status of sId^+ and isotype concordant sIg^+ PBL and the cells with clonal immunoglobulin gene rearrangements in peripheral blood. The complementarity determining region 3 (CDR3) sequence of rearranged alleles of the immunoglobulin heavy chain genes is unique for each B cell clone due to recombination of the variable (V), diversity (D) and joining (J) gene sequences, random nucleotide deletions and insertions at the V-D and D-J junctions and the occurrence of somatic mutation in this region. Sequencing of the CDR3 region of a malignant clone enables the synthesis of allele-specific oligonucleotide (ASO) probes that are sensitive enough to detect one neoplastic cell in 10^5 normal cells using PCR assays (Billadeau et al, 1991). This technique has been used to detect malignant cells in the peripheral blood of myeloma patients, although it also cannot distinguish plasma cells from circulating precursor cells (Billadeau et al, 1992). Performing PCR with an ASO primer plus an isotype specific immunoglobulin heavy chain constant region primer has enabled the demonstration of pre-switch cells belonging to the malignant clone in the bone marrow of myeloma patients (Corradini et al, 1991). Similar techniques could be used to detect neoplastic pre-switch B cells in the peripheral blood and to determine the immunophenotype of both pre- and post-switch cells belonging to the malignant clone in both blood and bone marrow by performing the studies on cells sorted on the basis of surface antigen expression. If ASO probes to immunoglobulin DNA or the corresponding mRNA sequences were linked to appropriate detection systems (eg. fluorochromes or streptavidin-biotin), the technique of in-situ hybridisation could be used to study the morphology and surface antigen expression of positive cells. The sensitivity of conventional in-situ hybridisation may be a limiting factor in such studies. This could possibly be overcome by

modifications of in-situ hybridisation such as oligonucleotide primed in-situ labelling or in-situ PCR (Mogensen et al, 1991; Nuovo et al, 1991; Embleton et al, 1992).

1.4.5: Miscellaneous Evidence for Circulating Precursor Cells

Peripheral blood B cells in myeloma have an increased LI compared to those in normals and there is a correlation with disease activity (Boccadoro et al, 1983; Witzig et al, 1988). Circulating aneuploid cells have been detected in both MGUS and myeloma (Barlogie et al, 1982).

A cell line with lymphoid characteristics by standard morphological criteria and electron microscopy examination has been derived from the peripheral blood mononuclear cells of a patient with plasma cell leukaemia. This cell line exhibited the same cytogenetic abnormalities as the patient's malignant plasma cells (MacKenzie and Lewis, 1985).

1.4.6: Precursor Populations Identified by Immunophenotypic Analysis

Attempts to study the phenotype of precursor cells in the peripheral blood and bone marrow of myeloma patients have frequently implicated a cell expressing the CD10 antigen. Wearne et al (1987a) demonstrated small but increased numbers of $CD10^+$ PBL in 40% of treated patients. The absolute number of these cells ranged from $0.026\text{-}0.11 \times 10^9/l$ and they constituted from 2.1-8.7% of PBL. Increased numbers were not detected in patients with MGUS or solitary plasmacytoma. Elevated numbers of $CD10^+$ PBL in relapsed patients have also been reported by other workers (Boccadoro et al, 1988; Omedé et al, 1990). The presence of increased numbers of $CD10^+$ cells correlates with the absence of LCIS but not with sβ2M. There is also no correlation between increased

numbers of $CD10^+$ and $CD38^+$ PBL, suggesting that these antigens are not co-expressed (Joshua et al, 1988). There is an increased proportion of small lymphocytes in the bone marrow of some myeloma patients expressing CD9, CD10, CD38 and PCA-1 (Shimazaki et al, 1990). In none of the above studies were dual labelling experiments to identify lineage performed and there was no proof that these lymphocytes were related to the malignant clone.

One group of investigators has published results that were interpreted as showing the existence of a precursor population with a hybrid phenotype of pre-B cell and plasma cell antigens (Durie and Grogan, 1985; Grogan et al, 1987). In a single patient with $CD10^+$ myeloma cells, there were about 20% small lymphoid cells in the bone marrow which expressed CD10, CD19, CD20, CD22, CD71, PCA-1, PC-1, TdT and cμ without detectable immunoglobulin light chains. The relationship of this population to the malignant clone was not established. From this patient, as well as another two who did not have $CD10^+$ plasma cells, these workers have established 3 cell lines composed of immature lymphoid cells with the phenotype $c\mu^+$, TdT^+, $CD10^+$, PCA-1^+, $CD19^+$, $CD20^+$, $CD22^+$, HLA-DR^+ and $CD71^+$ (Grogan et al, 1987). However, these cell lines have not been tested for the presence of EBV and only one has been demonstrated to be derived from the malignant clone of the patient.

Caligaris-Cappio et al (1985) detected a lymphoid population with the following phenotype in the bone marrow of all patients with $CD10^+$ plasma cells: $CD10^+$, $CD38^+$, $CD9^-$, $CD19^-$, $CD20^-$, $CD21^-$, $CD22^-$, $CD24^-$, HLA-DR^+, TdT^-, sIg^- and cIg^-. More than 50% of these patients also had lymphoid cells of the same phenotype in the peripheral blood. After culturing with the phorbol ester TPA for 72 hours, these $CD10^+$ cells matured into plasma cells which had a low mitotic rate

and synthesised immunoglobulin of the same isotype as the malignant plasma cells. A $CD10^+$ lymphoid population was not detected in bone marrow samples from patients with MGUS. In a follow-up study (Bergui et al, 1989), peripheral blood mononuclear cells from 11 myeloma patients were cultured with a combination of IL-3 and IL-6. In day 3 cultures, there were proliferating large blast cells with the phenotype $CD38^+$, HLA-DR^+, $CD19^+$, sIg^- and cIg^-. In 3 samples, these blasts also expressed CD10. After 6 days, 30-70% of the cells were plasma cells. These cells were $CD10^-$, strongly $CD38^+$, weakly $CD19^+$, sIg^-, cIg^+ and PCA-1^+. The expression of class II MHC molecules persisted in 3 cases. The cIg was the same isotype as that expressed by the myeloma cells. This group has also shown that peripheral blood mononuclear cells develop into monoclonal lymphocytes and plasma cells when cultured with autologous bone marrow stromal cells (Caligaris-Cappio et al, 1991).

DNA aneuploidy of the plasma cells occurs in 70-80% of patients with myeloma (Latreille et al, 1982) and has been used as a marker to identify the malignant clone (Epstein et al, 1988). Aneuploid cells in the bone marrow expressed cIg and the plasma cell antigen R1-3 in almost 90% of cases, CD10 in 55% of cases, and CD19 and CD20 in <25% of cases. As there was independent expression of CD10 and cIg, 3 aneuploid populations were identified: $CD10^+cIg^-$; $CD10^+cIg^+$; $CD10^-cIg^+$. All patients with aneuploid $CD10^+$ cells also had $CD10^+$ diploid cells which did not usually express cIg or R1-3. No patient without aneuploid $CD10^+$ cells had diploid $CD10^+$ cells. It was hypothesised that these various populations represented different maturation stages, with the diploid $CD10^+cIg^-$ cells being the least mature and possibly progenitor cells, whereas the aneuploid $CD10^-cIg^+$ cells were the most mature plasma cells.

Pilarski et al (1985c) described an abnormal population in the blood of 88% of myeloma patients and 44% of patients with MGUS. These cells were sIg^-, $c\mu^+$, $CD24^+$ and $CD38^-$, and expressed either class II MHC molecules or peanut agglutinin (PNA) but not both antigens. These cells were hypothesised to be pre-B cells but their relationship to the malignant clone was not elucidated. They may have represented either a malignant precursor pool or polyclonal pre-B cells present in increased numbers due to the presence of an immunoregulatory block of the maturation pathway.

This group of researchers has subsequently identified and studied a much larger B cell precursor population in the blood of 21 untreated and 21 treated patients with multiple myeloma and 13 patients with MGUS (Jensen et al, 1991; Pilarski and Jensen, 1992). The immunophenotypic analysis was performed by flow cytometry on peripheral blood mononuclear preparations. As the abnormal B cells were enlarged and highly granular compared to normal circulating B cells, gating on forward angle light scatter versus side scatter to identify lymphoid cells was not undertaken. These cells were adherent and they were lost by methods used to deplete monocytes. $CD19^+$ B cells constituted from 14-80% of the mononuclear cells in patients with myeloma and 7-39% in patients with MGUS compared to 6-14% in normal controls. These cells were $CD20^+$, $CD21^-$ and $CD24^+$. In the myeloma patients, over 70% of these B cells co-expressed CD10 plus PCA-1 and a variable proportion expressed CD5 and CD9. There was a lower proportion of B cells with this abnormal phenotype in MGUS. Unlike the malignant bone marrow plasma cells, these circulating B cells were $CD56^-$ and CD38 was present only at a low density on a small subset. The T cell antigens CD4 and CD8 and the haemopoietic progenitor cell antigen CD34 were not detected. In an occasional patient, CD14 was present at a high density on

nearly all of the abnormal B cells. CD11b, CD44, VLA-2 (CDw49b/CD29), VLA-4 (CDw49d/CD29), VLA-5, VLA-6 (CDw49f/CD29), LAM-1 (a selectin) and RHAMM (receptor for hyaluronate adhesion mediated motility) were expressed. These molecules are associated with adhesion, interaction with endothelial cells and components of the extracellular matrix, motility and homing.

On the basis of both PCA-1 and CD45 isoform expression, these abnormal cells were identified as late B cells or pre-plasma cells rather than pre-B cells. In normal individuals $CD19^+$ cells are predominantly $CD45RA^+$, whereas in myeloma patients they are predominantly $CD45RA^+RO^+$ and $CD45RO^+$. This latter pattern is characteristic of B cells stimulated *in vitro* to differentiate to plasma cells (Jensen et al, 1989).

The sIg light chain present on these cells was concordant with that of the malignant plasma cells. Clonality was confirmed by the presence of immunoglobulin J_H rearrangements in 6/15 samples. A substantial reduction of the germline band on the Southern blots was consistent with the large size of the abnormal population. The failure to detect rearrangements in all the samples does not necessarily imply lack of monoclonality, as failure to detect rearrangements in bone marrow samples containing large numbers of plasma cells has been documented previously in patients with myeloma (Zaccaria et al, 1989; Van Riet et al, 1989).

Morphologically, these cells were a heterogeneous population consisting of small lymphocytes, lymphoblasts and plasmacytoid and/or monocytoid cells. Between 4-19% of the $CD19^+$ cells were actively cycling. The investigators concluded that the abnormal B cell population in myeloma patients comprises

late stage B cells that are continually differentiating towards plasma cells, are homing to the bone marrow and may be a major proliferative compartment feeding the terminally differentiated compartment.

Two studies have used the idiotype of the paraprotein as a tumour marker (Bloem et al, 1988; King and Nelson, 1989). Care was taken to avoid the technical difficulties associated with the detection of Id^+ cells (section 1.4.1). In 2 patients, cId^+ cells constituted <1% of PBL (Bloem et al, 1988). The majority of these cells were small lymphocytes which expressed class II MHC molecules and CD21. One case expressed CD20. A proportion of the cells expressed CD38. In the other study, lymphoid sId^+ cells were detected in low numbers in the blood of most patients but this did not result in an abnormal $\kappa:\lambda$ ratio (King and Nelson, 1989). The Id^+ blood and bone marrow cells were pleomorphic, comprising lymphoid, lymphoplasmacytoid and plasmacytoid forms. They displayed a spectrum of antigen expression consistent with the occurrence of differentiation within the myeloma clone. The phenotype ranged from $CD19^+$, $CD20^+$, $PCA\text{-}1^-$, sId^+, cId^- lymphocytes to $CD19^-$, $PCA\text{-}1^+$, cId^+ plasma cells with either absent or weak sId expression. In 1/6 patients, the majority of the sId^+ lymphocytes expressed the CD10 antigen.

1.5: THE RELATIONSHIP OF PRECURSOR CELLS TO NORMAL B CELLS

Until recently, the phenotype of normal plasma cells was considered to be relatively restricted, whereas myeloma cells expressed antigens characteristic not only of B lineage cells but also other cell lineages. Recent studies have revealed that the phenotype of normal plasma cells is also heterogeneous and this is relevant to hypotheses about the maturation stage of postulated precursor populations in myeloma.

1.5.1: Plasma Cell Antigens

Traditionally, both normal and malignant plasma cells have been characterised as immunoglobulin secreting cells which express cIg but not sIg. The CD38 antigen is present on >90% of normal plasma cells (Hercend et al, 1981; Bhan et al, 1981; Hsu and Jaffe, 1984; Tedder et al, 1984), as well as the malignant plasma cells in multiple myeloma (Bhan et al, 1984; Aisenberg and Wilkes, 1983), plasma cell leukaemia (Aisenberg and Wilkes, 1983) and plasmacytomas (Foon et al, 1982). The intensity of CD38 expression is much higher on plasma cells than other cells and this has allowed detailed studies of the antigen expression of both normal and malignant plasma cells, even when they are present only in low numbers (Terstappen et al, 1990; Harada et al, 1992; Kawano et al, 1992). Antibodies directed against rough endoplasmic reticulum (RFD6) or antigens expressed by secretory cells (BU-5, BU-7, BU-9, BU-11) also identify normal and malignant plasma cells (Nathan et al, 1986; Johnson et al, 1987; Jackson et al, 1988). The unclustered antibodies PCA-1 (Anderson et al, 1983), PC-1 (Anderson et al, 1984), R1-3 (Gonchoroff et al, 1986b) and MM4 (Tong et al, 1987) have restricted expression within the B cell lineage, reacting predominantly with normal and malignant plasma cells, but not all of them are lineage specific.

1.5.2: Phenotype of Malignant Plasma Cells

Analysis of plasma cells from patients with myeloma has revealed that the phenotype of the malignant cells is highly variable, both between cases and within any single case. The antigens detected on myeloma cells are not only those considered characteristic of plasma cells but also those detected on B lineage cells at other stages in the maturation pathway and those characteristic of other lineages. The following B lineage antigens have been detected: CD19,

CD20, CD21, CD22, CD23, CD24 (almost exclusively the epitope detected by the antibody HB8), CD37, CD39, CD40, CD75, CD78 and sIg (Ruiz-Argüelles et al, 1984; Caligaris-Cappio et al, 1985; Durie and Grogan, 1985; San Miguel et al, 1986; Epstein et al, 1988; Jackson et al, 1988; Grogan et al, 1989; Epstein et al, 1990; Shimazaki et al, 1990; Van Camp et al, 1990; Drach et al, 1991; Hamilton et al, 1991a; San Miguel et al, 1991; Leo et al, 1992). $CD10^+$ plasma cells were reported in all these studies, sometimes in up to 50% of cases, but not infrequently they constituted only a minor subset of the total malignant population. The prognostic significance of CD10 expression remains uncertain, with some investigators reporting an aggressive disease course (Caligaris-Cappio et al, 1985; Durie and Grogan, 1985), whereas others have not found an association with a poor prognosis (Epstein et al, 1988; San Miguel et al, 1991).

The non-lineage specific antigens CD9, CD25, CDw32, CD45, CD45R, CD71 and class II MHC molecules have been detected (Ruiz-Argüelles et al, 1984; Durie and Grogan, 1985; San Miguel et al, 1986; Jackson et al, 1988; Grogan et al, 1989; Shimazaki et al, 1990; Van Camp et al, 1990; Drach et al, 1991; Hamilton et al, 1991a; San Miguel et al, 1991). The myelomonocytic antigens CD13, CD14, CD15 and CD33 (Grogan et al, 1989; Epstein et al, 1990; Van Camp et al, 1990; Drach et al, 1991; San Miguel et al, 1991; Leo et al, 1992), the platelet antigen CD41 (glycoprotein IIb/IIIa) (Epstein et al, 1990) and the erythroid antigen glycophorin A (Epstein et al, 1990) are present on the plasma cells in some cases of myeloma. Grogan et al (1989) reported that patients with myeloma cells expressing 3 or more myelomonocytic antigens had a shorter median survival than those expressing fewer but other workers have concluded that the expression of myelomonocytic antigens is so common that it is unlikely to be of prognostic import (Epstein et al, 1990).

Two groups have investigated the presence of the progenitor cell antigen CD34 on myeloma cells. Grogan et al (1989) reported that the plasma cells in 2/16 cases of myeloma expressed CD34, whereas Van Camp et al (1990) did not detect this antigen on the myeloma cells of any of the 22 studied patients. Further studies are required to clarify this situation, as the expression of CD34 on myeloma cells or precursor populations has therapeutic implications. Positive selection of $CD34^+$ haemopoietic progenitor cells for use in autologous bone marrow transplantation, which is being trialled in other malignancies (Besinger et al, 1990; Berenson et al, 1991), may be an inappropriate treatment strategy for all patients with myeloma or for individual patients, depending on the proportion of cases in which CD34 is expressed by the malignant clone.

Recent interest has focused on the presence of molecules involved in adhesion and homing. CD11b, CD11c, CD18, CD44, CD54, CD56, CD58, CD29, Cdw49d (VLA-α4 chain), VLA-α5 chain and RHAMM are expressed on malignant plasma cells (Grogan et al, 1989; Epstein et al, 1990; Van Camp et al, 1990; Drach et al, 1991; Hamilton et al, 1991a; Van Riet et al, 1991; Barker et al, 1992a; Kawano et al, 1992; Leo et al, 1992; Pilarski and Jensen, 1992).

The T cell antigens have rarely been reported to be present on myeloma cells. The CD4 antigen has been detected on the cells of 3 patients (Grogan et al, 1989), CD7 weakly on 2 cases (Grogan et al, 1989), CD8 on 2 cases (Van Camp et al, 1990) and CD28 on 1 case (Kozbor et al, 1987). The lectin PNA (Rhodes and Flynn, 1989) and the oncogene bcl-2 are almost invariably expressed by myeloma cells (Hamilton et al, 1991b).

Most of these antigens have also been detected on myeloma cell lines. The antigens characteristic of T cells, myelomonocytic cells, erythroid cells and platelets have been less commonly reported to be present on myeloma cell lines than on fresh myeloma cells (Durie et al, 1985; Goldstein et al, 1985; Gazdar et al, 1986; Jernberg et al, 1987; Kozbor et al, 1987; Lohmeyer et al, 1988; Matsuzaki et al, 1988; Jackson et al, 1989; Ohtsuki et al, 1989; Hamilton et al, 1990; Nacheva et al, 1990; Scibienski et al, 1990; Van Camp et al, 1990; Hamilton et al, 1991a; Hamilton et al, 1991b; Van Riet et al, 1991; Barker et al, 1992a). There are 4 EBV^- myeloma cell lines which express sμ and cμ without immunoglobulin light chains and a proportion of the cells in these lines are $CD10^+$ (Duperrray et al, 1989).

1.5.3: Phenotype of Normal Plasma Cells

Phenotypic analysis of normal plasma cells has been difficult, until recently, due to the inability to isolate viable plasma cells from normal tissues in adequate numbers. Most studies have employed staining of lymphoid tissue sections or cytospin preparations of cells from these tissues. PBL induced to differentiate *in vitro* to antibody secreting cells have also been studied. Recent advances in flow cytometry technology have permitted immunophenotypic analysis of the small number of plasma cells in normal lymphoid tissue. The presence of high density surface CD38 antigen has been used to identify plasma cells (Harada et al, 1992; Kawano et al, 1992). One study combined the quantitative expression of CD38 with the location of cells on the dot plot of forward angle light scatter versus transformed orthogonal light scatter to accurately identify plasma cells in bone marrow (Terstappen et al, 1990).

Many of the antigens which are expressed on myeloma cells have now been detected on normal plasma cells or immunoglobulin secreting cells (Hsu and Jaffe, 1984; Caligaris-Cappio et al, 1985; Jensen et al, 1989; Kozbor et al, 1987; Ling et al, 1987; Rhodes and Flynn, 1989; Tominaga et al, 1989; Terstappen et al, 1990; Hamilton et al, 1991b; Van Camp et al, 1991; Van Riet et al, 1991; Barker et al, 1992a; Harada et al, 1992; Leo et al, 1992). The phenotype of normal plasma cells, *in vitro* stimulated peripheral blood B cells, malignant plasma cells, myeloma cell lines and putative precursor populations are compared in table 1.1. The antigen expression is as heterogeneous in normal individuals as it is in myeloma patients. The phenotype varies with the location of the cells (Tominaga et al, 1989) and there is evidence of continuing maturation of immunoglobulin secreting cells within the bone marrow, with the presence of both lymphoplasmacytoid cells and mature plasma cells which can be distinguished by phenotypic differences, as well as morphological criteria (Terstappen et al, 1990).

Of significance is the demonstration of CD10 expression on plasma cells in lymphoid and haemopoietic tissue by immunohistological techniques and flow cytometry (Caligaris-Cappio et al, 1985; Terstappen et al, 1990) and on immunoglobulin secreting cells using reverse haemolytic plaque assay combined with complement dependent cytolysis (Tominaga et al, 1989). CD10 is present on a mean of 26% of bone marrow plasma cells in normal individuals (Terstappen et al, 1990). Some myelomonocytic antigens have also been detected on normal plasma cells (Terstappen et al, 1990) but there have been no reports regarding the expression of glycophorin A or CD41 (platelet glycoprotein IIb/IIIa). The T cell antigen CD28 is expressed on peripheral blood B cells induced to differentiate *in vitro* to plasma cells (Kozbor et al, 1987).

Table 1.1: Immunophenotype of Various Plasma Cell Populations.

	Normal Plasma Cells	Stimulated PBL	Plasma Cells in Myeloma	Myeloma Cell Lines	Precursor Cells in Myeloma
Plasma Cell Antigens					
CD38	+	+	+	+	+
PCA-1	+	+	+	+	+
B Lineage Antigens					
CD10	+		+	+	+
CD19	+		+	+	+
CD20	+	+	+	+	+
CD21			+	+	+
CD22	+		+	+	
CD23			+	+	
CD24	+*		+*	+	+
CD37			+	+	+
CD39	+		+	+	+
CDw40			+	+	
CD75			+	+	
CD76				+	
CD77				+	
CD78			+	+	
sIg	+		+	+	
cμ				+	+†
Non-Lineage Antigens					
CD9	+‡	+	+	+	+
CD25			+	+	
CD30				+	
CDw32			+	+	
CD35	+				
CD45	+		+	+	
CD45R			+		
CD45RA		+			+
CD45RO		+			+
CD71			+	+	
Class II MHC	+	+	+	+	+

*only the epitope detected by the HB8 antibody; †1 patient only;
‡*in vitro* immunoglobulin secreting cells.

Table 1.1 Continued.

	Normal Plasma Cells	Stimulated PBL	Plasma Cells in Myeloma	Myeloma Cell Lines	Precursor Cells in Myeloma
Adhesion and Homing Molecules					
CD11a	+			+	+
CD11b	+		+	+	+
CD11c	+		+		
CD18	+		+	+	
CD44	+		+	+	+
CD29	+		+	+	
CDw49b (VLA-α2)					+
CDw49d (VLA-α4)	+		+	+	+
VLA-α5	+		+	+	+
CDw49f (VLA-α6)				+	+
CD54	+		+	+	
CD56	- or weak		+	+	
CD58	+		+	+	
LAM-1					+
RHAMM			+		+
Myelomonocytic, Progenitor Cell, Platelet and Erythroid Antigens					
CD13	+		+		
CD14	+		+		+
CD15			+	+	
CD33	+		+		
CD34			+		
CD41 IIb/IIIa			+		
Glycophorin A			+		
T Lineage Antigens					
CD4			+		
CD5				+	+
CD7			+		
CD8			+		
CD28		+	+	+	
Miscellaneous Antigens					
TdT				+	+[†]
bcl-2 oncogene	+		+	+	
PNA	+		+		

[†]1 patient only.

CD56 and CD58 are either not or only weakly expressed by normal plasma cells and this contrasts with the strong expression on myeloma cells (Van Camp et al, 1991; Van Riet et al, 1991; Barker et al, 1992a; Harada et al, 1992; Leo et al, 1992). The expression of these antigens may assist in differentiating malignant plasma cells from a non-malignant expansion of plasma cells.

Changes in CD45 isoform expression on B cells have been studied by *in vitro* stimulation of normal peripheral blood mononuclear cells (Jensen et al, 1989). Freshly isolated B cells strongly express CD45RA and a proportion co-express CD45RO. *In vitro* maturation towards plasma cells is accompanied by increasing proportions of cells expressing either CD45RO only or both CD45RA and CD45RO, with very few cells expressing CD45RA only. The common determinant of the CD45 antigen is present on a proportion of plasma cells in normal bone marrow but the isoforms of CD45 were not studied (Terstappen et al, 1990).

1.5.4: The CD10 Antigen

The CD10 (CALLA) antigen is a 95-100,000 dalton single-chain type II integral membrane glycoprotein containing 20-25% carbohydrate (LeBien and McCormack, 1989). It was originally described as a surface antigen present on the cells of some cases of ALL (Greaves et al, 1975) but has subsequently been shown to have a wide tissue distribution. Normal haemopoietic cells expressing CD10 include 2-6% of the nucleated non-myeloid cells in adult bone marrow (Pesando et al, 1983), a much higher percentage of cells in foetal bone marrow (LeBien and McCormack, 1989), lymphoid progenitor cells in the thymus, especially in the foetus (LeBien and McCormack, 1989), segmented neutrophils (Pesando et al, 1983; McCormack et al, 1986) and occasional peripheral blood

mononuclear cells (Canon et al, 1985). Among normal cells of the B lineage, CD10 is expressed on B cell progenitors, principally pre-B cells (Dörken et al, 1989), germinal centre B cells (Hsu and Jaffe, 1984; Weinberg et al, 1986; Ling et al, 1987; Gadol et al, 1988; Liu et al, 1989) and plasma cells (Caligaris-Cappio et al, 1985; Terstappen et al, 1990). Among the haemopoietic malignancies, it is expressed on the cells of the majority of cases of pre-B cell ALL, the occasional case of T-cell ALL, centrocytic/centroblastic lymphoma (Greaves et al, 1975; Ling et al, 1987; Zola, 1987) and the plasma cells in some cases of multiple myeloma (Ruiz-Argüelles et al, 1984; Caligaris-Cappio et al, 1985; Durie and Grogan, 1985; San Miguel et al, 1986; Epstein et al, 1988; Jackson et al, 1988; Grogan et al, 1989; Epstein et al, 1990; Van Camp et al, 1990; Drach et al, 1991; Hamilton et al, 1991a; San Miguel et al, 1991). The CD10 antigen is also present on cultured normal fibroblasts, cultured bone marrow stroma, glomerular and proximal tubular epithelium, foetal intestine and breast myoepithelium, as well as several human solid tumours, including melanomas, gliomas, retinoblastomas and carcinomas of the breast and colon (Pesando et al, 1983; McCormack et al, 1986, LeBien and McCormack, 1989; Shipp et al, 1991).

The CD10 molecule has been identified as the membrane-associated enzyme neutral endopeptidase, which is also known as metalloendopeptidase and enkephalinase (Jongenaal et al, 1989; Letarte et al, 1989; Shipp et al, 1989). It cleaves the alpha amino groups of hydrophobic amino acids. Substrates for CD10 include regulatory peptides such as angiotensins I and II, bradykinin, enkephalins, neurotensin, atrial natriuretic peptide, oxytocin, substance P and the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (LeBien and McCormack, 1989; Shipp et al, 1989; Soleilhac et al, 1992). As CD10 cleaves multiple substrates, it has been hypothesised that its function may reflect

specific biochemical requirements of a given cell type (LeBien and McCormack, 1989). The function of CD10 on lymphoid cells is unknown but the finding that the bioactivity of IL-1β but not IL-2 is abolished after incubation with CD10 suggests that it may be involved in regulating the levels of some cytokines in certain microenvironments (Pierart et al, 1988).

1.5.5: The Maturation Status of Putative Precursor Cells in Myeloma

When the studies documenting the presence of CD10 and myelomonocytic antigens on malignant plasma cells, myeloma cell lines and putative precursor cell populations were published, some workers concluded that these data were consistent not just with aberrant surface antigen expression by malignant cells, or the presence of the malignant counterpart of a rare normal B cell, but with the existence of a significant proliferating precursor population of pre-B cells or haemopoietic stem cells (Durie and Grogan, 1985; Epstein et al, 1988; Barlogie et al, 1989; Epstein et al, 1990). However, as discussed above, CD10 expression within the B cell lineage is not confined to pre-B cells and both CD10 and some myelomonocytic antigens have now been demonstrated to be present on normal plasma cells (table 1.1). It has become increasingly apparent that although the expression of an antigen may be characteristic of a particular cell lineage or maturation stage within a given lineage, it is frequently not specific and that undue emphasis should not be placed on the detection of an apparently inappropriate antigen on malignant cells.

One study has indicated the presence of Id^+ bone marrow pre-B cells in myeloma (Kubagawa et al, 1979) but the significance of this finding is uncertain due to the technical difficulties associated with such studies. These are discussed in section 1.4.1.

Approximately one third of myeloma patients have rearrangements of the TCR γ gene in the malignant plasma cells (Barlogie et al, 1989). TCR β gene rearrangement has been documented in 1/14 patients but this was not accompanied by transcription of mRNA (Berenson and Lichtenstein, 1989). The occurrence of these TCR gene rearrangements has been cited as evidence that the proliferating precursor cell in myeloma is a stem cell. However, cross-lineage rearrangements, which are not uncommon in lymphoid malignancies, may reflect events that occur in normal cells due to the presence of common recombinases, which are responsible for both immunoglobulin and TCR gene rearrangements, and a chromatin configuration allowing accessibility of the recombinases to both these gene loci at the time of rearrangement (Greaves et al, 1987; Korsmeyer, 1987).

The most suggestive evidence of pre-B cell involvement has come from studies reporting isolated cμ expression in patient material or cell lines but these data, which were presented in sections 1.4.6 and 1.52, are limited.

The alternative view is that the precursor cells in myeloma are at a late stage in B cell ontogeny. Pre-B cells, defined as expressing cμ without immunoglobulin light chain, are virtually absent from the peripheral blood of myeloma patients (Zhang et al, 1988). CD24 antigen expression can be used to discriminate between pre-B cells and B cells in normal individuals, as the former have a higher antigen density (Duperray et al, 1990). This finding has been used to study the B cell lineage in the bone marrow and peripheral blood of myeloma patients (Duperray et al, 1991). Patients with active disease but not those with indolent disease have a low percentage of mature B cells in the bone marrow. Both groups of myeloma patients have a decreased proportion of bone marrow

pre-B cells. Only mature B cells, albeit in decreased numbers, are detected in the peripheral blood. The studies by Pilarski's group on the CD45 isoform expression on normal B cells and precursor cells in myeloma indicate that the precursor populations are late B cells or pre-plasma cells rather than pre-B cells (Jensen et al, 1989; Jensen et al, 1991; Pilarski and Jensen, 1992). Thus, neither of these studies provide supportive evidence for an expanded proliferating population of malignant pre-B cells.

We have argued that the proliferating precursor cell may be a germinal centre B cell or a cell derived from the germinal centre reaction (Warburton et al, 1989) but this does not exclude the possibility that at least some of the oncogenic insults occur in multipotential haemopoietic stem cells or early in B cell ontogeny. The siting of the precursor cell as either a germinal centre or later B cell is based on the requirement of the cell to have stable immunoglobulin gene rearrangements, to have been exposed to antigen, to have undergone somatic mutation and to have undergone immunoglobulin isotype switching.

Proliferating precursor cells in myeloma must have stable immunoglobulin gene rearrangements or the malignant plasma cells from any one case would be expected to display multiple and continually changing gene rearrangements, a situation which has not been documented. This requires the cell to be more mature than a pre-B cell. The precursor cell would also be expected to have encountered antigen. Germ-free Balb/c mice rarely develop plasmacytomas after pristane exposure, whereas antigen exposed animals regularly develop such tumours (Potter, 1973).

The paraproteins in multiple myeloma are almost exclusively of the isotypes occurring after immunoglobulin class switching. Among 2,011 successive patients entered into the MRC myelomatosis trials, only 4 had IgM paraproteins (MacLennan, 1992). Nine patients had 2 paraproteins. In 4 cases, the light chain isotypes of the paraproteins were discordant. In 5 cases, the patients had an IgG or IgA paraprotein plus a minor IgM paraprotein of the same light chain isotype. There have been two reported patients in whom there was evidence that some cells of the myeloma clone were sited at the stage of isotype switching. In one patient with IgGλ and IgAλ paraproteins, the N-terminal amino acid sequences were analysed and both the light chains and heavy chains had identical variable regions (Takahashi et al, 1986). In the other case, the myeloma cells were shown to be synthesising both IgG and IgA (Lucivero et al, 1986). In contrast, there is a recent study which used allele specific oligonucleotide primers in a PCR assay to detect pre-switch cells belonging to the malignant clone in the bone marrow of two myeloma patients (Corradini et al, 1991).

The immunoglobulin V_H genes in myeloma patients have undergone somatic mutation but there is no evidence of ongoing mutation as the disease progresses (Bakkus et al, 1992; Ralph et al, 1993). These data strongly support the hypothesis that the precursor population does not include stem cells or pre-B cells, as the progeny of these cells would not undergo identical somatic mutation of the immunoglobulin genes during the maturation process.

As outlined earlier in section 1.4.4, recent advances in molecular techniques, if combined with studies of morphology and surface antigen expression, may lead to accurate description of precursor cells in myeloma and eventual resolution of the debate about the maturation status of the clonogenic cell.

1.6: GERMINAL CENTRES

1.6.1: Germinal Centre Formation

The changes in follicles in secondary lymphoid tissue during the immune response to T-dependent antigens are complex (reviewed by MacLennan et al, 1990; Liu et al, 1992; MacLennan et al, 1992b). Primary follicles consist of recirculating $sIgM^+sIgD^+$ small lymphocytes interspersed between follicular dendritic cells (FDC). Secondary follicle formation can be divided into 3 stages. In the first stage, B blasts (primary B blasts) appear in the FDC network and rapidly proliferate, displacing the small recirculating lymphocytes which form the follicular mantle. The second stage commences when most of the B blasts disappear from the FDC network and a zone of blasts (centroblasts) localises at one pole of the follicle to form the dark zone. They proliferate rapidly but do not increase in number, as they give rise to non-proliferating centrocytes which fill the FDC network forming the light zone. Centroblasts are large or medium sized cells with a round or oval nucleus, usually containing a few nucleoli located at the nuclear membrane, and sparse, intensely basophilic cytoplasm (Stein et al, 1982). Centrocytes are small to medium sized, irregularly shaped cells with a cleaved nucleus. Most have pale cytoplasm but a small number have basophilic cytoplasm. There is a high death rate among the centrocytes and, to a lesser extent, the centroblasts. The follicle in this second stage is known as the germinal centre. In the third stage, which persists for prolonged periods of time, the light and dark zones are not apparent and only a central core of B blasts (secondary B blasts) remains.

1.6.2: Phenotypic Analysis of Germinal Centre Cells

Centroblasts and centrocytes express CD9, CD10, CD11a, CD19, CD20, CD22, CD35, CD37, CD38, CD40, CDw49d, CD45, CD71, CD72, CD74, CDw75, CD76,

CD77, CDw78, PNA and class II MHC antigens. CD23, CD25 and CD39 have not been detected (Hsu and Jaffe, 1984; Ledbetter and Clark, 1985; Weinberg et al, 1986; Ling et al, 1987; Gadol et al, 1988; Dörken et al, 1989; Liu et al, 1989; Soligo et al, 1989; Koopman et al, 1991). CD24 has been reported to be absent (Hsu and Jaffe, 1984), to be present (Weinberg et al, 1986) or to be expressed weakly by some centrocytes (Ling et al, 1987). CD21 has been detected by flow cytometry (Weinberg et al, 1986; Gadol et al, 1988) but not immunohistology (Hsu and Jaffe, 1984; Ling et al, 1987). CD44 is either absent (Heinen et al, 1988) or weakly expressed (Toyos et al, 1989; Koopman et al, 1991). There is disagreement about the expression of CD54 (Boyd et al, 1989; Koopman et al, 1991). Centroblasts are sIg^- (Stein et al, 1980). Centrocytes do not express sIgD (Ledbetter and Clark, 1985; Weinberg et al, 1986; Gadol et al, 1988; Liu et al, 1989; Heinen et al, 1988). Those in tonsils and lymph nodes are predominantly $sIgG^+$, with a smaller proportion being $sIgM^+$, and those in Peyer's patches are mainly $sIgA^+$ (Heinen et al, 1988). The phenotype of secondary B blasts is not known.

1.6.3: Function of Secondary Follicles

Three functions have been ascribed to the secondary follicle: (1) somatic mutation; (2) immunoglobulin heavy chain isotype switching; (3) selection and differentiation of B cells. Affinity maturation of the antibody response is due to the occurrence of somatic mutation in the immunoglobulin variable region germ-line genes (Griffiths et al, 1984; Berek et al, 1985). This occurs in the germinal centre (Jacob et al, 1991). There is some evidence that somatic mutation is triggered during the phase of exponential growth of primary B blasts (Gray, 1991).

Although there is no direct proof, several studies have suggested that immunoglobulin heavy chain isotype switching occurs in the germinal centre (Jacobsen and Thorbecke, 1968; Butcher et al, 1982; Coico et al, 1983).

Germinal centre cells give rise to memory cells (Klaus et al, 1980; Coico et al, 1983). The bone marrow is the major source of all classes of serum immunoglobulin and bone marrow plasma cells are derived from blast cells which migrate from the spleen and peripheral lymph nodes during the established phase of the secondary immune response (Benner et al, 1981). It has been proposed that cells of the secondary lymphoid follicle give rise to these bone marrow plasma cells (MacLennan and Gray, 1986). The secondary B blasts are the most likely candidate, as they could maintain antibody production throughout the established phase of T-dependent antibody responses (MacLennan et al, 1990).

Isolated germinal centre cells cultured *in vitro* undergo rapid programmed cell death, a process known as apoptosis. The onset of apoptosis can be delayed by anti-CD40, immobilised polyspecific anti-immunoglobulin or anti-IgG antibody. Combining antibodies against immunoglobulin and CD40 delays apoptosis for longer than either agent alone (Liu et al, 1989). Germinal centre cells rescued by anti-CD40 antibodies become smaller and the prominent nuclear cleft is lost. These cells have been hypothesised to be memory cells (Liu et al, 1991a). Recombinant 25-kDa CD23 and IL-1α rescue germinal centre cells and this is accompanied by features of plasmacytoid differentiation (Liu et al, 1991a). Anti-CD40 antibodies plus IL-4 cause proliferation of germinal centre cells and IL-2 rescues a subset of these cells and maintains them in cell cycle (Holder et al, 1991). This latter effect is augmented by anti-immunoglobulin reagents. The

rescue of germinal centre cells by any of these combinations is accompanied by expression of the protein encoded by the bcl-2 oncogene (Liu et al, 1991b). The phenotype of these rescued cell populations has not yet been determined.

Some of these rescue signals can be related to the *in vivo* situation (reviewed by Liu et al, 1992; MacLennan et al, 1992b). FDC have antigen on their surface in the form of immune complexes. Centrocytes may be rescued by interaction of their sIg with this antigen. The cells which do not successfully compete for interaction with the antigen on FDC undergo apoptosis, accounting for the high death rate in germinal centres. IL-2 is produced by T cells in the germinal centre (Emilie et al, 1990) and FDC in the apical light zone express large quantities of CD23 (Johnson et al, 1989).

1.7: THE EFFECT OF CYTOKINES ON THE DIFFERENTIATION AND PROLIFERATION OF MYELOMA CELLS AND PRECURSOR CELLS

Recombinant IL-6 has consistently been implicated as an *in vitro* growth factor for the plasma cells of about half the patients with multiple myeloma (Kawano et al, 1988; Anderson et al, 1989; Klein et al, 1989) but it does not augment the secretion of immunoglobulin (Anderson et al, 1989; Tanabe et al, 1989; Sonneveld et al, 1991). Controversy exists about whether IL-6 acts principally through an autocrine (Kawano et al, 1988) or paracrine mechanism (Klein et al, 1989). There is a positive correlation between the LI and the proliferative response of myeloma cells to exogenous IL-6 (Zhang et al, 1989). Serum levels of IL-6 are higher in Durie and Salmon stage II and III than stage I disease (Nachbaur et al, 1991), are positively correlated with disease activity (Bataille et al, 1989; Nachbaur et al, 1991) and are negatively correlated with survival duration (Ludwig et al, 1990; Reibnegger et al, 1991). IL-6 receptors were

detected on myeloma cells from all 6 cases in one study (Kawano et al, 1988) but on only 1/18 cases in another study (Brown et al, 1993). The reason for these discrepant results remains unknown. It may reflect different methodologies but it also highlights the fact that, although IL-6 appears to be an important biological factor in multiple myeloma, its precise role in the *in vivo* situation requires clarification.

Recombinant IL-1α, IL-1β and TNF-α augment the proliferation of myeloma cells (Kawano et al, 1989; Carter et al, 1990; Nagata et al, 1991). These cytokines increase the production of IL-6 (Kawano et al, 1989; Carter et al, 1990) and IL-1α has a direct effect on proliferation (Kawano et al, 1989). The increased amounts of IL-6 have been reported to be derived from both the myeloma cells (Kawano et al, 1989; Carter et al, 1990) and the bone marrow stromal cells (Carter et al, 1990). Purified myeloma cells induce IL-6 production by bone marrow stromal cell cultures and this has been shown to be due to IL-1α and IL-1β, suggesting that these cytokines are produced by the myeloma cells (Carter et al, 1990). IL-1β and TNF but not IL-1α are produced by cultured bone marrow cells in both myeloma patients and normals but the cells producing these cytokines were not identified (Klein et al, 1989; Lichtenstein et al, 1989; Nagata et al, 1991). One study did not confirm a role for IL-1α or IL-1β in the proliferation of myeloma cells (Anderson et al, 1989).

GM-CSF, which is produced by cells in the bone marrow microenvironment but not by myeloma cells, increases the proliferation of myeloma cells by increasing their sensitivity to IL-6 (Zhang et al, 1990) but this finding was not confirmed in another study (Anderson et al, 1989). Recombinant IL-3 and IL-5 increase the proliferation of myeloma cells in either none or only a minority of cases

(Anderson et al, 1989; Sonneveld et al, 1991). Recombinant IL-4 decreases the proliferation of myeloma cells by decreasing IL-6 production (Taylor et al, 1990; Herrmann et al, 1991). IL-4 augments immunoglobulin synthesis by myeloma cells (Sonneveld et al, 1991) but this result is also disputed (Anderson et al, 1989).

Although there has been much research into the effects of cytokines on the neoplastic plasma cells in myeloma, there has been comparatively little investigation of their effect on precursor cells. Peripheral blood mononuclear cells from myeloma patients cultured with a combination of IL-3 and IL-6 give rise to proliferating large blast cells by day 3 and plasma cells of the appropriate immunoglobulin isotype by day 6 (Bergui et al, 1989). IL-3, IL-6, IL-1 and IL-4 as single agents and other combinations of these interleukins are ineffective. Similar results have been obtained with T cell-depleted non-adherent peripheral blood mononuclear cells (Goto et al, 1992). Peripheral blood mononuclear cells cultured with autologous bone marrow stromal cells develop into monoclonal lymphocytes and plasma cells, suggesting that maturation of the precursors may occur in the specialised microenvironment provided by the bone marrow (Caligaris-Cappio et al, 1991). The established bone marrow stromal cultures produce IL-6 and IL-1β and, following culture with autologous peripheral blood mononuclear cells, IL-3 becomes detectable in the supernatant.

1.8: ABNORMALITIES OF NON-MALIGNANT LYMPHOCYTES IN MYELOMA

Non-neoplastic lymphocyte populations in the peripheral blood of myeloma patients have been extensively studied, as they may be involved in both immunoregulation of the malignant clone and the pathogenesis of the immunodeficiency associated with this disease.

1.8.1: T Cells

T Cell Subsets

The T cells in peripheral blood are divided into 2 major subsets on the basis of surface antigen expression and function (Reinherz and Schlossman, 1980). $CD4^+$ T cells provide help in T-B, T-T and T-macrophage cell interactions. $CD8^+$ T cells provide suppression in T-T and T-B cell interactions and are cytotoxic. The $CD4^+$ and $CD8^+$ subsets can be further subdivided as they exhibit immunophenotypic and functional heterogeneity. Morimoto et al (1985a, 1985b) produced the monoclonal antibodies 4B4 (CD29) and 2H4 (CD45RA) which characterise functional subsets of $CD4^+$ cells. $CD4^+29^+$ lymphocytes proliferate poorly on stimulation with concanavalin A or autologous cell antigens but well on exposure to soluble antigen. They provide help for PWM driven immunoglobulin secretion. These cells were initially termed helper-inducer cells. $CD4^+45RA^+$ lymphocytes proliferate well to concanavalin A and autologous cell antigen stimulation but poorly to soluble antigen stimulation. They provide poor help for PWM induced immunoglobulin secretion and induce $CD8^+$ suppressor cells, leading to the labelling of these cells as suppressor-inducers. Anti-CD45RA and anti-CD29 antibodies each react with 41% of $CD4^+$ PBL. Less than 10% of $CD4^+$ cells react with both antibodies but 15-30% of these cells are not labelled with either antibody.

There are other immunophenotypic differences between these two $CD4^+$ subsets (Sanders et al, 1988a; Sanders et al, 1988b). The suppressor-inducer cells are $CD2^{lo}$, CD11a/$CD18^{lo}$ (LFA-1), $CD44^{lo}$, $CD45RA^+$, $CD45RO^-$ and $CD58^-$ (LFA-3), and actually express low levels of CD29 rather than being $CD29^-$. The helper-inducer cells are $CD2^{hi}$, CD11a/$CD18^{hi}$, $CD44^{hi}$, $CD45RA^-$, $CD45RO^+$, $CD58^+$ and $CD29^{hi}$. The intensity of CD44 expression on these subsets is not sufficiently different

to allow their discrimination using single colour flow cytometry analysis. These $CD4^+$ subsets were initially regarded as separate functionally-defined T cell lineages but further studies led to the concept that the phenotypic differences were the result of a post-thymic differentiation pathway triggered by antigen stimulation (Akbar et al, 1988; Serra et al, 1988a; Sanders et al, 1988a; Sanders et al, 1988b). Consequently, the suppressor-inducer cells are now known as naive cells and the helper-inducer cells as memory cells.

CD25, CD54 and MHC class II molecules are expressed at high levels on both activated naive and memory T cells but they are also present at low levels on a variable proportion of resting memory T cells (Wallace and Beverley, 1990). It has been suggested that the low levels of these activation markers on memory cells indicate that they are not truly resting cells (Beverley, 1990).

A minor subset of $CD4^+$ PBL (<5%) is $CD11b^+57^+28^-$ (Velardi et al, 1985b; Morshita et al, 1986). Like $CD8^+11b^+$ cells, they form conjugates with NK-sensitive tumour cell targets without killing them. In patients with various haemopoietic and non-haemopoietic tumours, they may comprise up to 90% of peripheral $CD4^+$ T cells (Velardi et al, 1985a; Velardi et al, 1985c). These cells do not exhibit the characteristic *in vitro* functions of $CD4^+$ cells. They do not support the proliferation and differentiation of B cells or proliferate in response to mitogens or alloantigens. However, they do express the CD25 antigen on activation (Velardi et al, 1985b; Morshita et al, 1986). They have a moderate proliferative response to stimulation with soluble antigen, suggesting that at least some cells in this population are memory lymphocytes (Morshita et al, 1986).

$CD8^+$ PBL can also be divided into naive and memory cells on the basis of their expression of the antigens used to delineate these subsets within the $CD4^+$ population. Using anti-CD45RA and anti-CD29 antibodies, 54% and 43% of $CD8^+$ T cells are naive and memory cells respectively (Morimoto et al, 1985a; Morimoto et al, 1985b).

$CD8^+11b^+28^-$ T cells are the suppressor subset for PWM induced B cell differentiation, the proliferative response of $CD4^+$ cells to soluble antigen and mitogens, and the proliferative response of $CD8^+11b^-$ cells to mitogens. They exhibit minimal proliferative responses to mitogens (Landay et al, 1983; Clement et al, 1984; Morishita et al, 1986). Greater than 95% of these cells are large granular lymphocytes and they include both dim and bright $CD8^+$ cells (Clement et al, 1984). Although >80% of these cells express CD57, they do not bear the CD16 antigen and are distinct from NK cells. The $CD8^+11b^-28^+$ T cells are precursor and effector cytotoxic lymphocytes which recognise class I MHC alloantigens and proliferate in response to stimulation with mitogens (Clement et al, 1984; Morshita et al, 1986). These cells have high ecto-5'nucleotidase (5'NT) (now known as CD73) activity, whereas the $CD8^+11b^+$ cells have low or absent activity (Dianzani et al, 1986).

Alterations in Lymphocyte Counts and T Cell Subsets in Myeloma

Significantly reduced lymphocyte counts have not been found in patients with MGUS (Mellstedt et al, 1982a; Bergmann et al, 1985; Büchi et al, 1985). Lauria et al (1984) described lymphopenia in untreated myeloma patients with Durie and Salmon stage II or III but not stage I disease. In other studies, lymphocyte counts were normal in untreated patients (Mellstedt et al, 1982a; Bergmann et al, 1985; Büchi et al, 1985; Wahlin et al, 1985) but only one of these studies further

analysed the untreated patients according to disease stage (Wahlin et al, 1985). Lymphopenia has been consistently documented in patients who have received treatment for myeloma. It is present both in those patients currently receiving therapy and those who have ceased therapy up to 65 months previously (Mellstedt et al, 1982a; Bergmann et al, 1985; Wahlin et al, 1985).

There is significant disagreement in the literature regarding the presence and nature of immunophenotypic abnormalities of T cell subsets in patients with monoclonal gammopathies. This may be due to numerous factors, including methodology (eg. fluorescence microscopy versus flow cytometry to enumerate positive cells), the small patient numbers in many studies, the varying definitions of clinical subgroups, the expression of results as percentages as opposed to absolute numbers and the use of different statistical tests of significance. In general, the documented abnormalities have been more marked in treated patients than untreated patients, whereas patients with MGUS have had the lowest frequency of abnormalities.

Total T cell numbers have been reported to be either decreased or normal in patients with MGUS or untreated myeloma (table 1.2). Lauria et al (1984) divided the untreated patients into those with Durie and Salmon stage I disease versus those with stage II or III disease. Although both groups had a normal percentage of $CD3^+$ PBL, those with stage II or III disease had a decreased absolute number. Another study found no difference between those patients with stage I or II versus those with stage III disease (Wahlin et al, 1985). Patients who have already received treatment for myeloma have reduced numbers of T cells. This abnormality persists for at least 28 months after cessation of therapy (Wahlin et al, 1985).

Table 1.2: Total T Lymphocytes in MGUS and Multiple Myeloma.						
	MGUS		Untreated Myeloma		Treated Myeloma	
	%	A	%	A	%	A
Mellstedt et al, 1982a	N	-	N	-	↓	-
Lauria et al, 1984	-	-	N	N* or ↓†	-	-
Bergmann et al, 1985	-	↓	-	↓	-	↓
Büchi et al, 1985	-	N	-	N	-	-
Pilarski et al, 1985a	↓	-	↓	-	↓	-
Wahlin et al, 1985	-	-	N	N	N	↓
Pilarski et al, 1989b	↓	↓	↓	↓	-	-

%-results expressed as percentage; A-results expressed as absolute number; N-normal; ↓-decreased; *Durie and Salmon stage I disease; †Durie and Salmon stage II and III disease.

Studies of $CD4^+$ PBL in patients with MGUS have produced conflicting data, with the number of $CD4^+$ cells reported as being either normal or decreased. The majority of studies have found a reduced number of $CD4^+$ lymphocytes in both treated and untreated myeloma patients (table 1.3).

Table 1.3: $CD4^+$ Lymphocytes in MGUS and Multiple Myeloma.								
	MGUS		Myeloma		Untreated Myeloma		Treated Myeloma	
	%	A	%	A	%	A	%	A
Mellstedt et al, 1982a	↓	↓	-	-	↓	↓	↓	↓
Mills and Cawley, 1983	-	-	↓	↓	-	-	-	-
Lauria et al, 1984	-	-	-	-	↓	↓	-	-
Wearne et al, 1984*	-	-	-	-	-	-	-	↓
Bergmann et al, 1985	-	N	-	-	-	↓	-	↓
Büchi et al, 1985	-	N	-	-	-	N	-	-
Pilarski et al, 1985a	↓	N	-	-	↓	↓	↓	↓
Wahlin et al,1985	-	-	-	-	N	N	N	↓
Massaia et al, 1987	-	-	↓	↓	-	-	-	-
Massaia et al, 1988b	-	-	-	↓	-	-	-	-
Pilarski et al, 1989a	-	-	↓	↓	-	-	-	-
Pilarski et al, 1989b	↓	↓			↓	↓	-	-
Shapira et al, 1989	N	-	N	-	-	-	-	-
San Miguel et al, 1992	-	-	-	-	↓	↓	-	-

%-results expressed as percentage; A-results expressed as absolute number; N=normal; ↓=decreased; *only patients in plateau phase were studied.

In untreated patients, an absolute $CD4^+$ count $<0.7x10^9/l$ has been associated with decreased survival and there is a correlation between decreased $CD4^+$ cell numbers and both a high sβ2M level and anaemia (San Miguel et al, 1992).

Shapira et al (1989) did not document a significant alteration in the percentage of $CD4^+$ lymphocytes in patients with MGUS or myeloma but there was a significant elevation of the percentage of $CD4^+45RA^+$ (naive) lymphocytes in MGUS with a trend in the same direction in multiple myeloma. Two other groups have obtained different results when analysing subsets of $CD4^+$ lymphocytes. The proportion of $CD4^+45RA^+$ cells in patients with myeloma but not MGUS was decreased, with a resultant significant reduction in the ratio of naive:memory cells (Serra et al, 1988b; Massaia et al, 1991). The naive:memory cell ratio was abnormal in 70% of untreated and 90% of treated patients (Serra et al, 1988b).

A significant expansion of both the percentage and absolute number of $CD4^+11b^+57^+$ and $CD4^+$HLA-DR^+ cells occurs in MGUS and myeloma but there is not preferential expression of HLA-DR by either the $CD11b^+$ or $CD11b^-$ subsets (Massaia et al, 1988b; Massaia et al, 1990). The absolute number of $CD4^+11b^+$ cells is significantly elevated in MGUS and myeloma at diagnosis, falls markedly with chemotherapy, is lower than that at diagnosis during plateau phase, but does not rise again with the onset of progressive disease (Dianzani et al, 1988). Although the mean absolute number of $CD4^+$HLA-DR^+ cells was higher in all patient groups compared to the controls in this latter study, the results were not stated to be statistically significant. There is an inverse correlation between the $CD4^+11b^+$ and $CD8^+11b^+$ numbers in myeloma patients (Massaia et al,

1988b). The expression of CD25 by the $CD4^+$ population is normal in myeloma (Massaia et al, 1991).

There is no consensus regarding quantitative alterations of $CD8^+$ PBL in any group of patients with plasma cell dyscrasias (table 1.4). Normal, reduced and elevated numbers have all been reported. In MGUS and untreated myeloma, normal studies predominate, whereas no particular pattern of alteration predominates in treated myeloma.

Table 1.4: $CD8^+$ Lymphocytes.

	MGUS		Myeloma		Untreated Myeloma		Treated Myeloma	
	%	A	%	A	%	A	%	A
Mellstedt et al, 1982a	N	N	-	-	N	N	↑	N
Mills and Cawley, 1983	-	-	-	-	↑	N	↑	N
Lauria et al, 1984	-	-	-	-	↑	↑* or ↓†		
Wearne et al, 1984‡	-	-	-	-	-	-	-	↑
Bergmann et al, 1985	-	↓	-	-	-	N	-	↓
Büchi et al, 1985	-	N	-	-	-	N	-	-
Pilarski et al, 1985a	N	↓	-	-	N	↓	N	↓
Wahlin et al, 1985	-	-	-	-	N	N	N	N
Massaia et al, 1987	-	-	↑	N	-	-	-	-
Massaia et al, 1988b	-	-	-	N	-	-	-	-
Pilarski et al, 1989a	-	-	↑	N	-	-	-	-
Pilarski et al, 1989b	N	↓	-	-	N	↓	-	-
Shapira et al, 1989	N	-	N	-	-	-	-	-
González et al, 1992	-	-	-	-	↑	N	-	-
San Miguel et al, 1992	-	-	-	-	N	N	-	-

%-results expressed as percentage; A-results expressed as absolute number; N-normal; ↑-increased; ↓-decreased; *Durie and Salmon stage I disease; †Durie and Salmon stage II and III disease stage; ‡only patients in plateau phase were studied.

Alterations in $CD8^+$ lymphocyte subsets have been investigated in great detail. The initial finding was a depressed mean 5'NT activity of $CD8^+$ PBL in MGUS and multiple myeloma at diagnosis, although some patients did have normal levels

(Massaia et al, 1985). The 5'NT activity was inversely related to the LI, suggesting that low 5'NT activity may be a poor prognostic factor. Subsequent studies have shown that decreased 5'NT activity was not confined to newly diagnosed patients and was related to a reduced proportion and absolute number of $CD8^+11b^-$ lymphocytes (Massaia et al, 1987).

There is an increased percentage and absolute number of $CD8^+11b^+$ and $CD8^+$HLA-DR^+ cells in monoclonal gammopathies (Massaia et al, 1987; Massaia et al, 1988b; Massaia et al, 1990). A significantly increased proportion of the $CD8^+11b^+$ population in myeloma expresses CD57 and HLA-DR but not CD25 (Massaia et al, 1990; Gonzalez et al, 1992). Proliferative activity of peripheral blood T cells is correlated with $CD8^+$HLA-DR^+ cell numbers (Massaia et al, 1988b). Although there is an enrichment of HLA-DR^+ cells in the $CD8^+11b^+$ population, with 63-94% of $CD8^+$HLA-DR^+ cells belonging to this subset, 6-37% of HLA-DR^+ cells are found in the $CD8^+11b^-$ subset (Massaia et al, 1988b). Dianzani et al (1988) have undertaken a more detailed study of the absolute numbers of $CD8^+11b^+$ and $CD8^+$HLA-DR^+ cells. The $CD8^+11b^+$ lymphocytes are significantly elevated to similar levels in MGUS and myeloma at diagnosis. The numbers fall significantly with chemotherapy, return to normal levels in patients who reach plateau but rise to pretreatment levels with the onset of progressive disease. The number of $CD8^+$HLA-DR^+ cells is elevated in MGUS and myeloma at diagnosis, being significantly higher in the latter compared to the former. The numbers do not fall in patients on therapy but do return to the levels seen in MGUS during plateau phase. The levels in progressive disease are similar to those present at diagnosis.

The 5'NT deficiency and decreased cytotoxic response towards allogeneic cells of peripheral blood T cells from patients with MGUS and myeloma are not solely attributable to reduced numbers of $CD8^+11b^-$ cells (Massaia et al, 1988a; Massaia et al, 1988b). They also positively correlate with the expansion of the $CD8^+$HLA-DR^+ subset This suggests that $CD8^+11b^-$ cells expressing the HLA-DR antigen have decreased 5'NT activity and impaired ability to generate alloreactive cytotoxicity. Impairment of this latter function is most marked in patients with a poor prognosis (Massaia et al, 1988a).

$CD8^+11b^+$ lymphocytes are the major contributors to peripheral blood T cell lymphokine-activated killer (LAK) activity but, despite the expansion of this subset in MGUS and myeloma, their LAK activity is substantially diminished. As $CD3^+57^+$ cells in normals have poor LAK activity, the decreased activity in monoclonal gammopathies has been hypothesised to be related to the increased expression of CD57 by the $CD8^+11b^+$ subset in these disorders (Massaia et al, 1990).

The $CD8^+$HLA-DR^+ cells adhere to plastic dishes coated with autologous but not allogeneic IgG paraprotein, consistent with the presence of idiotype-reactive T cells. These idiotype-reactive cells are present in both the $CD8^+11b^+$ and $CD8^+11b^-$ populations (Dianzani et al, 1988). Other investigators have also found evidence of idiotype-reactive cells in MGUS and myeloma (Österborg et al, 1991; Nelson et al, 1993). Some studies have provided evidence for the existence of clonal T cell populations (Wen et al, 1990; Janson et al, 1991). This finding would not be unexpected given the evidence for the presence of elevated numbers of T cells recognising the idiotypic determinants on the paraprotein.

Peripheral blood T cells from patients with multiple myeloma exhibit hyperreactivity on stimulation with suboptimal quantities of plastic-immobilised anti-CD3 monoclonal antibodies, due, at least partially, to the increased percentage of $CD8^+HLA\text{-}DR^+$ cells (Massaia et al, 1991). This response to cross-linking of CD3 receptors is reminiscent of that of normal memory T cells (Sanders et al, 1988b; Schlossman et al, 1989). Consequently, the relative numbers of naive and memory $CD8^+$ PBL have been investigated. There is a normal percentage of $CD8^+45RA^+$ lymphocytes but an increased percentage of $CD8^+45RO^+$ lymphocytes, resulting in a significantly depressed naive:memory cell ratio within the $CD8^+$ population. Just as the majority of $HLA\text{-}DR^+$ cells in normals are found in the memory population, 70-80% of $HLA\text{-}DR^+$ T cells in myeloma patients are memory cells. As a result of reviewing these numerous studies, it has been proposed that the $CD8^+11b^+$ and $CD8^+11b^-$ cells appear to have depressed functional activity *in vitro* as the incorrect assays have been employed. Rather, these populations contain activated memory cells committed to recognition of idiotypic determinants on the monoclonal immunoglobulin and these cells are most abundant in patients with a poor prognosis (Dianzani et al, 1988; Massaia et al, 1991).

As a result of the numerical changes in both $CD4^+$ and $CD8^+$ populations, most studies of currently and previously treated patients have documented a reduced CD4:CD8 ratio (table 1.5). Opinion is divided as to whether untreated patients have a normal or depressed ratio. The findings in patients with MGUS are varied.

Table 1.5: Ratio of $CD4^+$:$CD8^+$ Lymphocytes.				
	MGUS	Myeloma	Untreated Myeloma	Treated Myeloma
Mellstedt et al, 1982a	N	-	N	↓
Mills and Cawley, 1983	-	-	↓	↓
Lauria et al, 1984	-	-	↓	-
Bergmann et al, 1985	↑	-	N	N
Büchi et al, 1985	N	-	N	-
Pilarski et al, 1985a	↓	-	↓	↓
Wahlin et al, 1985	-		N	↓
Massaia et al, 1987	-	↓	-	-
Dianzani et al, 1988	↓	-	↓	↓
Pilarski et al, 1989a	-	↓	-	-
Pilarski et al, 1989b	↓	-	↓	-
San Miguel, 1992	-	-	↓	-
N-normal; ↑-increased; ↓-decreased.				

1.8.2: NK Cells

Function and Phenotype of NK Cells

NK cells constitute up to 15% of normal PBL and are effector cells which lyse target cells without deliberate prior immunisation and without restriction by major histocompatibility antigens (Ritz et al, 1988; Robertson and Ritz, 1990). NK cells are also capable of antibody-dependent cellular cytotoxicity via the CD16 receptor (Perussia et al, 1983; Anegón et al, 1988; Robertson and Ritz, 1990). NK cells may have a role in regulating immunoglobulin production, as they can inhibit *in vitro* production of immunoglobulin by B cells in several experimental systems (Tilden et al, 1983; Arai et al, 1983; Brieva et al, 1984). The physiological functions proposed for NK cells are numerous. These include the destruction of virus infected cells and autologous tumour cells, the resistance to certain bacterial, fungal and protozoan organisms, the participation in the graft-versus-leukaemia effect following bone marrow transplantation and the

regulation of haemopoiesis (Ritz et al, 1988; Robertson and Ritz, 1990; Oldham, 1990).

There is no cell surface antigen which is specifically expressed by all NK cells. The CD16 and CD56 antigens are the most useful but neither is expressed exclusively on lymphocytes. The CD56 antigen is expressed by virtually all lymphocytes capable of non-MHC restricted cytotoxicity (Griffin et al, 1983). Consequently, it is present on >95% of NK cells but it is also present on a $CD3^+$ population which comprises <5% of PBL (Lanier et al, 1986; Schmidt et al, 1986). Approximately 75% of $CD3^+56^+$ cells are $CD8^+$ (Lanier et al, 1986) and this population has rearranged TCR genes in contrast to the $CD3^-56^+$ population (Ritz et al, 1988; Robertson and Ritz, 1990). Among lymphocytes, anti-CD16 monoclonal antibodies bind to <5% of non-NK cells (Lanier et al, 1983; Perussia et al, 1983; Anegón et al, 1988). Less than 2% of T cells express CD16 (Robertson and Ritz, 1990). The vast majority of NK cells are $CD16^+56^+$ but approximately 10% are $CD16^-56^{+\text{"bright"}}$ (Lanier et al, 1986). This latter population does not express CD8, contains both large granular and agranular cells and has less cytotoxic activity than $CD16^+56^+$ NK cells.

The CD57 antigen has been used to identify NK cells but it is expressed by only 50-60% of NK cells and 50% of lymphoid cells bearing CD57 are T cells (Lanier et al, 1983; Perussia et al, 1983). CD2, CD7 and CD11b are detected on 80-90% of NK cells, CD38 on 60-90% and CD8, at a lower density than on T cells, on 30-40% (Ortaldo et al, 1981; Griffin et al, 1983; Perussia et al, 1983; Lanier et al, 1986; Schmidt et al, 1986; Ritz et al, 1988; Robertson and Ritz, 1990).

Alterations in NK Cell Numbers in Multiple Myeloma

There have been a limited number of studies on NK cells in multiple myeloma and the results are not in agreement. Büchi et al (1985) identified an increased absolute number of $CD57^+$ PBL in patients with either MGUS or myeloma. As the number of $CD8^+$ cells was unaltered in these patients, it was concluded that the increase in the number of $CD57^+$ cells was due to an increase the number of NK cells. As the percentage of $CD8^+57^+$ cells is increased in myeloma (Massaia et al, 1990; Gonzalez et al, 1992), this conclusion is flawed.

In a large series of patients, monoclonal antibodies against CD16, CD56 and CD57 were used to determine the relative and absolute number of NK cells (Österborg et al, 1990). In patients with MGUS and untreated myeloma patients with Durie and Salmon stage I or II disease, there was an increase in NK cell numbers compared to normal controls but those with Stage III disease had decreased numbers. In previously treated patients, those with responding or plateau phase disease had elevated numbers of NK cells, while those with progressive disease had reduced numbers. Similar, but not identical patterns, were present in assays of NK cell activity.

Gonzalez and colleagues (1991) studied only untreated patients. They found an increased percentage but normal absolute number of $CD16^+$ PBL compared to controls. Neither the proportion nor the absolute number of $CD56^+$ cells was significantly different compared to the normals but there was a non-significant elevation of $CD3^-56^+$ cells in myeloma patients. The mean percentage of $CD16^+$ and $CD56^+$ cells within the gated lymphoid population in the controls was 6% and 15% respectively. Thus, the mean percentage of NK cells which were $CD16^-56^+$ was much higher than the 10% reported in the literature (Lanier et al,

1986; Österborg et al, 1991). In the patients, the majority of NK cells did co-express CD16 and CD56, thus accounting for the increase in $CD16^+$ but not $CD56^+$ cells compared to the controls. The median value for the number of $CD16^+$ lymphocytes in Durie and Salmon stage I or II patients was higher than in stage III patients but did not reach the level of significance.

1.8.3: B Cells

Alterations in B Cells in Multiple Myeloma

The technical difficulties associated with enumerating B cells by detecting sIg have been discussed in sections 1.3.5 and 1.4.1. When methods for enumerating B cells have included appropriate precautions for the detection of sIg or the use of monoclonal antibodies directed against B cell specific surface antigens, the number of B lymphocytes has usually been found to be reduced in patients with MGUS and multiple myeloma, irrespective of their treatment status (Dillman et al, 1981; Pilarski et al, 1984; Büchi et al, 1985; Pilarski et al, 1989b). Zhang et al (1988) reported that B cell numbers were normal in patients with stable disease but decreased in those with active disease. Bergmann et al (1985) detected normal numbers of B cells in patients with MGUS.

The specificity repertoire of B lymphocytes is different to that present in normal individuals. There is an increased proportion of B cells which have anti-idiotypic specificity (Pilarski et al, 1985b; Pilarski et al, 1989b; Bergenbrant et al, 1991).

$CD5^+$ B Cells (B-1a Cells)

B lymphocytes which express CD5 are also known as B-1a cells (Kantor, 1991). In adults they constitute 0-3% of peripheral blood mononuclear cells (Freedman et al, 1987a; Hardy et al, 1987) and 15-30% of the B cells (Hardy et al, 1987;

Caligaris-Cappio et al, 1989). Approximately 5% of splenic B cells (Mackenzie et al, 1991) but no bone marrow B cells (Freedman et al, 1987a) express this antigen. A small number of $CD3^{-}5^{+}$ cells have been detected by immunohistology in the germinal centres of tonsils and lymph nodes (Caligaris-Cappio et al, 1982). Recent flow cytometry analysis of suspensions of tonsillar B cells detected CD5 on 30% of cells (Defrance et al, 1992). The majority had the phenotype of follicular mantle cells but about 6% co-expressed CD10, suggesting a possible germinal centre origin. Foetal tissues contain higher percentages of these cells (Freedman et al, 1987a) and they are the major B cell population in cord blood (Hardy et al, 1987). In the recovering lymphoid populations following bone marrow transplantation, they are the predominant B cell (Ault et al, 1985). In certain autoimmune disorders including rheumatoid arthritis, $CD5^{+}$ B cells are detected in increased numbers (Plater-Zyberk et al, 1985; Hardy et al, 1987). They are the major source of rheumatoid factor, other autoantibodies and anti-idiotypic antibodies in normal neonates and adults (Casali et al, 1987; Hardy et al, 1987; Raveche, 1990). CLL is a malignant expansion of $CD5^{+}$ B cells (Caligaris-Cappio et al, 1982; Freedman et al, 1987a). There is *in vitro* evidence that CD5 may be an activation antigen on B cells (Freedman et al, 1987b; Freedman et al, 1989; Werner-Favre et al, 1989; Visser et al, 1990), so it remains unclear whether $CD5^{+}$ B cells in humans constitute a distinct lineage, as they do in mice (Hardy et al, 1987; Lydyard et al, 1987; Herzenberg et al, 1992).

CD5 is the only T cell antigen detected on B-1a cells and its level of expression is weaker than on T cells (Caligaris-Cappio et al, 1992; Ault et al, 1985; Hardy et al, 1987; Plater-Zyberk et al, 1987). Other antigens expressed by $CD5^{+}$ B cells include CD19, CD20, CD21, CD22, CD24, and class II MHC molecules (Caligaris-Cappio et al, 1982; Ault et al, 1985; Bofill et al, 1985; Hardy et al, 1987; Plater-

Zyberk et al, 1985; Freedman et al, 1987a; Caligaris-Cappio et al, 1989). Surface immunoglobulin is either absent or weakly expressed and, when detected, it is almost exclusively IgM and IgD (Caligaris-Cappio et al, 1982; Freedman et al, 1987a; Hardy et al, 1987; Lydyard et al, 1987). The CD38 antigen has not been detected (Caligaris-Cappio et al, 1989). CD25 and CD71 are either absent or expressed on only some $CD5^+$ B cells (Hardy et al, 1987; Caligaris-Cappio et al, 1989).

Abnormalities of $CD5^+$ B Cells in Multiple Myeloma

$CD5^+$ B cells have been implicated in the pathogenesis of the immunodeficiency present in patients with multiple myeloma. Both monocytes and monocyte-depleted mononuclear cells from myeloma patients are able to suppress the *in vitro* immunoglobulin production by normal lymphocytes stimulated with either PWM or specific antigen. The mononuclear cell with the greatest suppressive activity has been identified as one forming rosettes with human O Rh^+ erythrocytes coated with human IgG anti-D antibody (EA cell) (Paglieroni and MacKenzie, 1977; Paglieroni and MacKenzie, 1980). Subsequent immunophenotypic studies (Mackenzie et al, 1987) have identified the EA cell as a $CD5^+$ B cell. $CD5^+$ B cells from normal individuals do not inhibit the immunoglobulin production of normal PBL in response to PWM or specific antigen, suggesting an abnormal activation state of these cells in myeloma (MacKenzie et al, 1991).

The phenotype of the $CD5^+$ B cell in myeloma patients is similar to that in normal individuals but they also express CD14, the CD10 antigen has not been detected and the sIg is IgM only. The lack of isotypic concordance with the

paraprotein has been used as the basis for proposing that these cells do not belong to the malignant clone (MacKenzie et al, 1987; MacKenzie et al, 1991).

An elevated percentage of $CD5^+$ B cells is present in the peripheral blood and spleens of myeloma patients (Mackenzie et al, 1991; Paglieroni et al, 1992b). $CD5^+$ B cells with immunosuppressive properties can be detected prior to the development of overt myeloma (Paglieroni et al, 1992a; Paglieroni et al, 1992b). In patients with MGUS, solitary plasmacytoma of bone or extramedullary plasmacytoma, the $CD5^+$ B cells were studied at the time of diagnosis and the patients were reviewed over a period of at least 5 years. Immunosuppressive $CD5^+$ B cells were detected at initial presentation in those patients who eventually developed active myeloma or had multiple recurrences of plasmacytomas. $CD5^+$ B cells are hypothesised to have specificity for autoantigens and to be involved in the idiotypic immune network (Lydyard et al, 1987). As there is no direct evidence that the $CD5^+$ B cells in myeloma patients belong to the malignant clone, it has been proposed that the increase in these cells in myeloma patients is an immune response to idiotypic determinants on the neoplastic clone but that the response of the $CD5^+$ B cells is defective, thus allowing the development of clinically overt disease (Paglieroni et al, 1992a).

However the research group of Mackenzie and Paglieroni needs to do further work to exclude the possibility that these $CD5^+$ B cells do not belong to the neoplastic clone. They have relied on the lack of isotypic concordance of the surface immunoglobulin of these cells with the paraprotein as proof of the non-malignant nature of these cells and have not used molecular biology techniques to compare the immunoglobulin gene rearrangements of the $CD5^+$ B cells to

those of the myeloma cells. Several lines of evidence suggest that CD5 may be expressed on cells of the neoplastic clone. CD5 is weakly expressed by the myeloma cell lines U266 and RPMI 8226 (Duperray et al, 1989). Another cell line, which has been linked to the myeloma cells from which it was derived on the basis of common cytogenetic abnormalities, also expresses CD5 and exhibits the same immunosuppressive characteristics as $CD5^+$ B cells from myeloma patients (Scibienski et al, 1990). Furthermore, CD5 expression has been documented on 20-100% of the cells of a putative peripheral blood precursor population (Jensen et al, 1991; Pilarski and Jensen, 1992). This population was identified on the basis of a surface antigen phenotype that was distinct from that of normal peripheral blood B cells. Although clonal heavy chain immunoglobulin gene rearrangements of peripheral blood mononuclear cells were documented, no attempt was made to demonstrate such clonality on specifically sorted or identified subpopulations, including the cells expressing CD5.

SECTION 2: CELL CULTURE STUDIES OF PRECURSOR CELLS IN MULTIPLE MYELOMA

2.1: AIMS

It is generally accepted that in multiple myeloma there is a compartment of proliferating precursor cells which "feeds" the predominantly non-proliferative plasma cell compartment. Numerous studies have attempted to confirm the presence of and characterise this precursor population. There is ongoing debate about the stage of B cell development at which the tightly controlled processes of proliferation and differentiation become uncoupled, giving rise to proliferating precursor cells. An *in vitro* culture system for growing myeloma colonies may assist in identifying and characterising clonogenic cells, as well as allowing investigation of the effects of cytokines on the proliferation and differentiation of these cells. Millar's group (1988) described a culture system that has the potential of allowing such studies. Myeloma colonies were grown from 80-90% of bone marrow aspirates from myeloma patients, irrespective of the stage of the disease or the degree of plasma cell infiltration. Colonies were also grown from peripheral blood samples from 7/12 patients, even in the absence of circulating plasma cells. The colonies were composed of either large plasmacytoid cells, small lymphoid cells or both cell types. This suggests that the colonies arose from malignant cells at different differentiation stages, including ones representing pre-plasma cell stages.

An alternative approach is to establish permanent cell lines derived from cells of the malignant clone, based on the assumption that a cell line is representative of the proliferating precursor cells rather than the end-stage plasma cells. However, past experience indicates that myeloma cell lines are usually established only from "unusual" cases, such as those with extensive

extramedullary disease or high proliferative activity in the end-stage plasma cell compartment (Durie et al, 1985; Gazdar et al, 1986; Jernberg et al, 1987; Lohmeyer et al, 1988; Matsuzakai et al, 1988; Jackson et al, 1989; Ohtsuki et al, 1989; Hamilton et al, 1990; Nacheva et al, 1990). Nevertheless, it was decided to attempt to establish cell lines from bone marrow aspirates from myeloma patients, as a constant supply of cells would enable more detailed studies of proliferation and differentiation in a given patient than would be possible with a single specimen studied in the colony forming assay.

We have previously hypothesised that the precursor cell in myeloma is of germinal centre origin, possibly an antigen-rescued cell of the germinal centre reaction (Warburton et al, 1989). The establishment of cell lines from patients with malignancies of germinal centre cells (predominantly follicular NHL) would provide a means of studying the effects of differentiation factors on these cells. Although the results from studies of such cell lines may provide important information about the factors required for the maturation of B cells to plasma cells, they would have to be interpreted with caution as the transforming events in these lymphoid malignancies are likely to be different to those in multiple myeloma. Consequently, the effects of growth and differentiation factors on the neoplastic cells of these malignancies may not be identical.

2.2: METHODS

2.2.1: Patients

The peripheral blood and bone marrow samples were obtained from patients attending the haematology outpatient clinic, either for initial assessment or routine follow-up. The samples were taken only if the patient was undergoing venipuncture or bone marrow aspiration for other indications. For the

clonogenic assay of myeloma colonies, samples were obtained from patients with plasma cell dyscrasias, almost exclusively those with multiple myeloma as defined by the criteria of the Committee of the Chronic Leukemia-Myeloma Task Force (1973). For the establishment of permanent cell lines, samples were predominantly from patients with multiple myeloma but a small number of samples were from patients with certain lymphoproliferative disorders such as centroblastic/centrocytic or lymphoplasmacytoid lymphoma. Further details of the patients will be provided in the results (section 2.3) as appropriate.

2.2.2: Tissue Culture Reagents

The following tissue culture media were used:

- RPMI 1640 Medium (RPMI) with 20 mM HEPES buffer and without sodium bicarbonate and L-glutamine as single strength (1x) liquid (Flow Laboratories, Irvine, Scotland).
- RPMI without L-glutamine and Hepes buffer as 1x liquid (Flow Laboratories, Irvine, Scotland).
- Iscove's Modification of Dulbecco's Medium with 25 mM Hepes buffer and L-glutamine and without bovine serum albumin, pure human transferrin and soybean lecithin (Iscove's) as 1x liquid (Flow Laboratories, Irvine, Scotland).
- Ham's F-12 Medium without L-glutamine (Ham's F12) as 1x liquid (Flow Laboratories, Irvine, Scotland).
- Alpha Modification of Minimum Essential Medium Eagles (α-MEM) without L-glutamine, ribosides and deoxyribosides as 1x liquid (Flow Laboratories, North Ryde, NSW, Australia).
- α-MEM with L-glutamine and without ribosides, deoxyribosides and sodium bicarbonate as powder (Flow Laboratories, McLean, Virginia, USA; catalogue no. 10-311-22).

- Minimum Essential Medium Eagle (MEM) 100x (Modified) Vitamins (Flow Laboratories, McLean, Virginia, USA).

FCS was obtained from several sources during the period of these studies:

- Flow laboratories, North Ryde, NSW, Australia; Batch No. 29101868.
- Cytosystems, Castle Hill, NSW, Australia; Batch No. 71215023.
- Cytosystems, Castle Hill, NSW, Australia; Batch No. 71204022.

All culture media mixtures were supplemented with L-glutamine 0.8 mM (CSL, Parkville, Victoria, Australia) just prior to use. Benzylpenicillin 240 U/ml (CSL, Parkville, Victoria, Australia) and gentamycin 160 µg/ml (David Bull Laboratories, Mulgrave, Victoria, Australia) were added to all bottles of culture media when they were opened.

2.2.3: Isolation of Mononuclear Cells

Bone marrow (1-2 ml) was collected into 1 ml RPMI medium containing 100 U preservative free porcine heparin sodium (Fisons Pharmaceuticals, Thornleigh, NSW, Australia). A single cell suspension was prepared and the mononuclear cell fraction was obtained by separation on Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, New Jersey, USA). After washing 3 times in the appropriate tissue culture medium, the cells were resuspended and a cell count was performed. Peripheral blood was also collected into heparinised tissue culture medium. The specimen was diluted 1:4 with medium prior to centrifugation through Ficoll-Paque. It was then processed in the same manner as a bone marrow sample.

2.2.4: Myeloma Colony Assay

The method for growing myeloma colonies from the peripheral blood and bone marrow of patients with multiple myeloma was based on that described by Millar et al (1988). This method uses a double layer agar/agar or agar/liquid system.

Culture of HL-60 Cells

The HL-60 cells (promyelocytic cell line) (Collins et al, 1977) used in the feeder layers were grown in RPMI with 15% FCS. They were seeded at $1x10^5$ cells/ml in either $25cm^2$ or $75cm^2$ tissue culture flasks (Corning Glass Works, Corning, NY, USA) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. To obtain HL-60 conditioned medium (HL60-CM), the cultures of HL-60 cells were grown to saturation density and the supernatant was then harvested and filtered.

Feeder Layers

For the feeder layers, the HL-60 cells were irradiated to 50 Gy. They were plated at 5 or $10x10^5$ cells/plate in either α-MEM with 20% FCS, 1% BSA (RIA Grade, Fraction V Powder; Sigma Chemical Co, St Louis, MO, USA; catalogue no. A7888) and 0.5% agar (Bacto-Agar, Difco Laboratories, Detroit, Michigan, USA) or α-MEM with 1x MEM vitamins, 20% FCS, human transferrin 50 mg/l (Sigma Chemical Co, St Louis, MO, USA; catalogue no. T1147), ascorbic acid 50 mg/l (BDH Chemicals, Kilsyth, Victoria, Australia), 1% BSA and 0.5% agar. One millilitre of the feeder layer mixture was plated into each 35 mm plastic petri dish (Kayline Plastics, Australia). In some experiments, the BSA was omitted from the feeder layers. In both the feeder layers and the overlays, amphotericin 50 μg/ml (Fungizone

for tissue culture; E. R. Squibb & Sons Pty Ltd, Noble Park, Victoria, Australia) was added to the final culture media mixtures.

Overlays Containing Bone Marrow or Peripheral Blood Mononuclear Cells

The mononuclear cells were plated as an overlay at 10^5-10^6 cells/plate in several alternate media mixtures:

(i) 0.5 ml α-MEM with 20% FCS, 1% BSA and 0.23% agar.

(ii) 0.2 ml of 30% HL60-CM plus 70% α-MEM with 20% FCS, 1% BSA and $2x10^{-5}$ M putrescine (Sigma Chemical Co, St Louis, MO, USA; catalogue no. P6024).

(iii) 0.5 ml α-MEM with 20% FCS and 0.23% agar.

(iv) 0.2 ml of 30% HL60-CM plus 70% α-MEM with 20% FCS and $2x10^{-5}$ M putrescine.

(v) 0.2 ml HL60-CM.

(vi) 0.5 ml HL60-CM.

(vii) 0.5 ml HL60-CM with 0.16% agar.

(viii) 0.25 ml α-MEM with 20% FCS, 1% BSA and 30% HL60-CM.

(ix) 0.5 ml α-MEM with 1x MEM vitamins, 20% FCS, transferrin 50 mg/l, ascorbic acid 50 mg/l, 1% BSA and 0.2% agar.

(x) 0.2 ml α-MEM with 1x MEM vitamins, 20% FCS, transferrin 50 mg/l, ascorbic acid 50 mg/l, 1% BSA and 30% HL60-CM.

Overlays (i) and (viii) were those originally described by Millar's group. The rationale for the use of the others will be detailed in the results. When mononuclear cell numbers were sufficient, a specimen was plated at several cell concentrations using both a liquid and agar overlay.

The cultures were incubated for up to 21 days at 37°C in an atmosphere of 5% CO_2, 10% O_2 and 85% N_2. As it was not possible for an entire incubator to be

filled with this gas mixture, the cultures were placed in a Modular Incubator Chamber (Flow Laboratories, McLean, Virginia, USA) containing this gas mixture and placed in the main incubator. The cultures were examined at 7, 14 and 21 days with an inverted microscope. Cells in the overlay were aspirated with a glass pasteur pipette and gently smeared onto glass slides. The morphology of the cells was examined following May-Grünwald-Giemsa staining.

2.2.5: Establishment and Characterisation of Permanent Cell Lines

Cell Culture Method

Mononuclear cells obtained from peripheral blood or bone marrow specimens were plated at 1-2x10^5 cells/ml in a 1:1 mixture of Iscove's and Ham's F12 media with 50 mg/l transferrin and 20% FCS. Culture was initially in 24-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA), which were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Fresh media was added to the cultures weekly for periods of up to 8 weeks. If no obvious proliferation had occurred at that stage, the cultures were discarded. If proliferation occurred, then the cultures were split as necessary and were eventually transferred to 25cm^2 tissue culture flasks.

Screening for EBV and Mycoplasma Infection

Any resulting cell lines were screened to exclude transformation by EBV by testing for the presence of Epstein-Barr nuclear antigen (EBNA) (Henle and Henle, 1966; Reedman and Klein, 1973). These tests were kindly performed by staff of the Microbiology Department at Royal Prince Alfred Hospital, Sydney, Australia. All cell lines, including HL-60, were regularly screened for the presence of Mycoplasma infection using a culture technique (Barile and

McGarrity, 1983) in the Department of Microbiology at the Institute of Clinical Pathology and Medical Research, Westmead Centre, Sydney, Australia.

Cytogenetic and Gene Rearrangement Studies

Cytogenetics on the WL2 cell line were kindly performed by staff in the Kanematsu Laboratories at the University of Sydney. Immunoglobulin and TCR gene rearrangements on cell lines and patient specimens were performed by Southern Blot analysis following digestion of the DNA with the restriction endonucleases BamHI, HindIII and EcoRI. Immunoglobulin gene rearrangements were investigated using J_H, C_κ and C_λ probes and TCR rearrangements using a β chain probe. These studies were kindly performed by Edna Yuen of the Haematology Department at Royal Prince Alfred Hospital.

2.2.6: Culture of WL2 Cells with Mitogens and Cytokines

Cell Culture Method

The cells were plated at $1x10^6$ cells/ml in either $25cm^2$ tissue culture flasks or 24-well flat-bottomed tissue culture plates. The latter were used in those experiments involving the use of large quantities of recombinant cytokines. Appropriate controls were included in all experiments. Cells and supernatants were usually harvested at 2 and 5 days after the initiation of the culture. In two experiments, based on a study of the effects of IL-3 and IL-6 on peripheral blood mononuclear cells in myeloma (Bergui et al, 1989), the harvests were performed on days 3 and 6. Cell counts were performed at the time of harvest. Cell viability was determined by vital staining with Trypan Blue (CSL Laboratories, Melbourne, Victoria, Australia). Morphology was assessed by May-Grünwald-Giemsa staining of cytospin preparations (Cytospin 2 centrifuge, Shandon Southern Products Ltd., Astmoor, Runcorn, Cheshire, UK). The proportion of

cells in the S phase of the cell cycle and the immunophenotype, including the expression of cIg, were determined. The quantity of immunoglobulin in the supernatants was measured.

Reagents

TPA (Sigma Chemical Co, St. Louis, Mo., USA) was diluted to 1 mg/ml in acetone and stored in aliquots at -20°C. LPS derived from Escherechia Coli Serotype 055:B5 (Catalogue No. L6018) and 4-bromo-calcium ionophore A23187 (A23187) were purchased from Sigma Chemical Co (St. Louis, Mo., USA). Stimulation by cross-linking of sIg was undertaken using micron sized hydrophilic polyacrylamide beads with covalently bound highly purified anti-human IgM (μ chain specific) immunoglobulin (Immunobeads). The Immunobeads were a kind gift from Dr H Zola (Flinders Medical Centre, Adelaide, South Australia), who had purchased them from Biorad Laboratories (Richmond, California, USA) and prepared them for use in cell culture experiments. IFN-α2a was obtained from Roche Products Pty. Ltd. (Dee Why, NSW, Australia). The recombinant human cytokines IL-3, IL-4, IL-6 and GM-CSF (Genzyme Corporation, Boston, Massachusetts, USA) were diluted at no more than 1:5 in media supplemented with 5% FCS and stored at -35°C as per the manufacturer's instructions. IL-3 had a specific activity of 10^8 colony forming units/mg protein, with 1 U of activity defined as the amount of growth factor required to produce a single colony from $7.5x10^4$ human bone marrow cells cultured in soft agar for 14 days. IL-4 had a specific activity of 10^8 proliferation units/mg protein, with 1 U being the amount that caused half-maximal thymidine incorporation in 100 μl cultures of $2x10^3$ human tonsillar B cells stimulated with anti-human IgG. IL-6 had a specific activity of 10^7 U/mg protein, with 1 U defined as the amount required to augment the production of immunoglobulin by the CESS lymphoblastoid cell

line to half-maximal level. One unit of GM-CSF was defined as the amount which produced a single colony from $7.5x10^9$ human bone marrow cells cultured in soft agar for 14 days.

Preparation of Leucocyte Conditioned Medium

Leucocyte conditioned medium (LCM) was prepared by culturing peripheral blood mononuclear cells at $4x10^6$ cells/ml with TPA 10 ng/ml and PHA (phytohaemagglutinin P; CSL, Parkville, Victoria, Australia) at 5 µg/ml. The supernatant was harvested after 5 days, filtered and stored in aliquots at -20°C. To control for residual mitogens in the supernatant, culture medium containing the mitogens but no cells was treated in the same manner (control LCM).

Mitogen and Cytokine Combinations

The combinations of mitogens and interleukins used in the stimulation experiments were as follows: IL-4 100-1000 U/ml; IL-6 2-1000 U/ml; IFN-α2a 100 U/ml; GM-CSF 250 U/ml; IL-3 15-250 U/ml; anti-IgM antibodies 10-30 µg/ml and IL-4 100 U/ml; anti-IgM antibodies 10 µg/ml, IL-4 100 U/ml and IL-6 100 U/ml; anti-IgM antibodies 10 µg/ml, IL-4 100 U/ml and IFN-α2a 100 U/ml; GM-CSF 250 U/ml and IL-6 1000 U/ml; IL-3 15-250 U/ml and IL-6 100-1000 U/ml; IL-3 15 U/ml and IL-6 1000 U/ml with additional IL-6 1000 U/ml on day 3; LPS 25 µg/ml; LPS 25 µg/ml and IL-4 100-1000 U/ml; TPA 2-10 ng/ml with and without A23187 25-750 ng/ml; TPA 10 ng/ml and LPS 25 µg/ml; TPA 10 ng/ml and IFN-α2a 100 U/ml; TPA 10 ng/ml, IL-4 100 U/ml and IFN-α2a 100 U/ml; TPA 10 ng/ml, IL-4 100 U/ml and IL-6 100 U/ml; TPA 10 ng/ml, LPS 25 µg/ml and IL-4 100-1000 U/ml; TPA 10 ng/ml and IL-3 15 U/ml; TPA 10 ng/ml and IL-6 1000 U/ml; TPA 10 ng/ml, IL-3 15-250 U/ml and IL-6 100-1000 U/ml; TPA 10 ng/ml, LPS 25 µg/ml and IL-6 1000 U/ml; TPA 10 ng/ml, LPS 25 µg/ml, IL-3 15 U/ml

and IL-6 1000 U/ml; 20% v/v LCM; 20% v/v LCM, IL-3 15 U/ml and IL-6 1000 U/ml with and without additional IL-6 1000 U/ml on day 3.

2.2.7: Immunophenotypic Analysis of Cell Lines

Reagents

The antibodies used to study the surface antigen expression of cell lines were directed against the following molecules: CD3, CD5, CD9, CD10, CD11a, CD11b, CD13, CD14, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD33, CD36, CD37, CD38, CD39, CD54, CD71, PCA-1, class II MHC molecules and immunoglobulin μ, δ, γ, α, κ and λ chains. Not all antibodies were used in every experiment. Specifications for these antibodies are listed in table 2.1. All antibodies were used at the manufacturer's recommended dilution or had been appropriately titrated for use in the routine haematology laboratory.

Immunophenotypic Labelling Procedure

The cells were washed and resuspended in PBS. For the detection of sIg, the cells were incubated with PBS at 37°C for 20 minutes to remove cytophilic immunoglobulin, centrifuged and resuspended in PBS. Unconjugated antibody was added to $5x10^5$ cells suspended in 50 μl PBS. The specimens were vortexed, incubated at 4°C for 30 minutes and then washed twice in PBS at 4°C. Fifty microlitres of a 1:200 dilution of FITC-conjugated affinity-isolated $F(ab')_2$ sheep anti-mouse immunoglobulin (Silenus Laboratories, Hawthorn, Victoria, Australia) were added to the cells, which were then incubated in the dark at 4°C for 30 minutes. The samples were washed twice in PBS at 4°C and resuspended in 1 ml 1% paraformaldehyde (AJAX Chemicals, Sydney, Australia) in PBS. Specimens were stored in the dark at 4°C until analysis.

Table 2.1: Specifications of the Non-Conjugated Antibodies Used to Study the Phenenotype of the Cell Lines.

Specificity	Commercial Name	Clone	Isotype	Source
CD3	OKT*3	-	IgG2a	Ortho Diagnostic Services, Raritan, New Jersey
CD5	Anti-Leu-1	L17F12	IgG2aκ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD9	-	BU-16	IgG2a	Gift: G.D. Johnson, Department of Immunology, University of Birmingham, U.K.
CD10	CALLA	W8E7	IgG2aκ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD11a	DAKO-CD11a	MHM24	IgG1κ	Dakopatts A/S, Glostrup, Denmark
CD11b	DAKO-CD11b	2LPM19c	IgG1κ	Dakopatts A/S, Glostrup, Denmark
CD13	MY7	366	IgG1κ	Coulter Immunology, Hialeah, Florida
CD14	MY4	322-A1	IgG2bκ	Coulter Immunology, Hialeah, Florida
CD18	DAKO-CD18	MHM23	IgG1κ	Dakopatts A/S, Glostrup, Denmark
CD19	B4	89B	IgG1κ	Coulter Immunology, Hialeah, Florida
CD20	B1	H299	IgG2aκ	Coulter Immunology, Hialeah, Florida
CD21	-	BU-34	IgG1	Gift: G.D. Johnson, Department of Immunology, University of Birmingham, U.K.
CD22	Anti-Leu-14	SHCL-1	IgG2bκ	Becton Dickinson Immunocytometry Systems, San Jose, California
CD23	DAKO-CD23	MHM6	IgG1	Dakopatts A/S, Glostrup, Denmark
CD24	-	ALB9	IgG1	Pel-Freez, Brown Deer, Wisconsin
CD25	Anti-Interleukin-2 Receptor	2A3	IgG1Kκ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD33	MY9	906	IgG2bκ	Coulter Immunology, Hialeah, Florida
CD36	OKM5	-	IgG1	Ortho Diagnostic Services, Raritan, New Jersey

Table 2.1: Continued.

Specificity	Commercial Name	Clone	Isotype	Source
CD37	-	BL14	IgG1	Pel-Freez, Brown Deer, Wisconsin
CD38	OKT10	-	IgG1	Ortho Diagnostic Services, Raritan, New Jersey
CD39	-	AC2	IgG1	Gift: Department of Immunology, University of Birmingham, U.K.
CD54	-	ICAM-1	IgG2a	Gift: A. Boyd, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
CD71	OKT9	-	IgG1	Ortho Diagnostic Services, Raritan, New Jersey
HLA-DR framework antigen	OKIa*1	-	IgG2	Ortho Diagnostic Services, Raritan, New Jersey
Unclustered*	PCA-1	138	IgG2aκ	Coulter Immunology, Hialeah, Florida
κ light chains	DAKO-Kappa	A8B5	IgG1κ	Dakopatts A/S, Glostrup, Denmark
λ light chains	DAKO-Lambda	N10/2	IgG1κ	Dakopatts A/S, Glostrup, Denmark
μ heavy chain	DAKO-IgM	R1/69	IgG1κ	Dakopatts A/S, Glostrup, Denmark
δ heavy chain	IgD	SFCID117	IgG1κ	Coulter Immunology, Hialeah, Florida
γ heavy chain	IgG	SFCIIgG	IgG1κ	Coulter Immunology, Hialeah, Florida
α heavy chain	IgA	NIF 2	IgG1κ	Pel-Freez, Brown Deer, Wisconsin
Isotype Control	-	L7	IgG1	Gift: Clinical Immunology and Research Centre, University of Sydney, Australia†
Isotype Control	MsIgG2a	7T4-1F5	IgG2aκ	Coulter Immunology, Hialeah, Florida
Isotype Control	MsIgG2b	MPC-11	IgG2bκ	Coulter Immunology, Hialeah, Florida

*Anderson et al, 1983; †mouse anti-Mycobacterium Leprae (Britton et al, 1985).

Flow Cytometry Analysis

Immunophenotypic analysis was performed with an Ortho Spectrum III flow cytometer (Ortho Diagnostic Services Inc., Westwood, Massachusetts, USA). This machine is equipped with a water cooled argon-ion laser, which emits at 488 nm with an adjustable power (usual power setting: 25-30 mW). Forward angle light scatter is detected with a photodiode. Right angle light scatter is reflected by a 488 nm dichroic mirror to a photodiode. Green (FITC) fluorescence is split off to the photomultiplier tube by a 600 nm short pass filter and collected through a 530 nm band pass filter. Fluorescence intensity is expressed on a linear scale.

The flow cytometer was calibrated daily for forward angle light scatter, right angle light scatter and green fluorescence using glutaraldehyde-fixed human red blood cells which exhibit autofluorescence in the yellow region.

Viable cells were analysed by selective gating based on forward angle and right angle light scatter properties. The fluorescence data from at least 1,000 cells were collected for each sample. The positive analysis region for FITC fluorescence was set manually so that ≥99% of the cells were negative following labelling with the appropriate isotypic control.

2.2.8: Cell Cycle Analysis of WL2 Cells

The method for determining the percentage of cells in the S phase of the cell cycle was based on a method for calculating the plasma cell LI in multiple myeloma (Greipp et al, 1985). The harvested cells were washed three times in RPMI to remove any traces of the culture media, as Ham's F12 contains thymidine. One million cells were added to 2 ml RPMI containing 10 μM 5-

bromo-2-deoxyuridine (Calbiochem Corporation, San Diego, California, USA) and 1 μM 5-fluorodeoxyuridine (Sigma Chemical Co, St Louis, MO, USA). The sample was incubated at 37°C for 1 hour. The cells were then centrifuged and resuspended in 1 ml RPMI with 10% FCS. Cytospin slides (Cytospin 2 centrifuge, Shandon Southern Products Ltd., Astmoor, Runcorn, Cheshire, UK) were prepared and allowed to air dry. The slides were fixed for 10 minutes in 95% ethanol, washed in PBS and incubated with 20 μg of an anti-bromodeoxyuridine antibody (BU-1; gift of Dr P Greipp, Mayo Clinic) at room temperature for 45 minutes. The slides were washed again in PBS and incubated at room temperature for a further 45 minutes with TRITC-conjugated sheep $F(ab')_2$ anti-mouse IgG (Silenus Laboratories, Hawthorn, Victoria, Australia) and either FITC-conjugated $F(ab')_2$ sheep anti-human kappa light chain or FITC-conjugated $F(ab')_2$ sheep anti-human lambda light chain reagents (Silenus Laboratories, Hawthorn, Victoria, Australia). The staining with anti-immunoglobulin light chain reagents allowed a qualitative assessment of the expression of cIg with an inbuilt negative control. Slides were washed once more with PBS and mounted with Glycergel (Sigma Chemical Co, St Louis, MO, USA). Fluorescence microscopy was performed using a Leitz Laborlux 12 microscope (Ernst Leitz Wetzlar GMBH, Germany) and filter sets suitable for FITC and TRITC fluorescence. Any cIg^+ cells appear a bright apple-green and the nucleus of cells in S-phase appears a bright orange-red. Five hundred cells were counted and the LI was calculated as the percentage of cells in S-phase.

2.2.9: Quantitation of Immunoglobulin in Culture Supernatants of WL2 Cells

Immunoglobulin in the cell culture supernatants was measured using enzyme-linked immunoassays of total kappa and lambda light chains. Free kappa and lambda light chains were also assayed.

The purified free kappa and lambda light chains that were used as antigen standards were prepared from the urine of myeloma patients with BJP excretion by ammonium sulphate precipitation followed by column chromatography. The isolated monomeric light chains from 6 patients with kappa BJP and 3 patients with lambda BJP were pooled and lyophilised. Stock solutions of 1 mg/ml in 0.05 M tris/saline buffer pH 7.6 were stored in small aliquots at -35°C.

All antigen standards, supernatant samples and developing antibodies were diluted in milk/veronal buffer. This buffer was prepared by diluting skim milk (Dairy Farmers, NSW, Australia) 1:10 in 0.05 M veronal buffer pH 8.0, centrifuging at 10,000 g for 30 minutes and then passing the supernatant through a 0.2 µm filter (Minisart NML, Sartorius, Goettingen, Germany). Skim milk, although a non-standard biological product, has been successfully used by other research groups to prevent non-specific protein binding in enzyme-linked immunoassays of immunoglobulin light chains (Axiak et al, 1987; Nelson et al, 1992).

Linbro/Titertek E.I.A. microtitre plates (Flow Laboratories, McLean, Virginia, USA) were coated with rabbit antibodies against either human kappa light chain, free kappa light chain, lambda light chain or free lambda light chain (Dakopatts A/S, Glostrup, Denmark). These antibodies were diluted 1:100 in 0.2 M sodium bicarbonate buffer pH 9.2 and 100 µl were dispensed into each well. The plates were incubated overnight at 4°C and then washed four times in PBS pH 7.7 containing 0.005% Tween 20 using a Titertek Microplate Washer 120 (Flow Laboratories, Irvine, Scotland). The standard curve was set up in duplicate for each of the following dilutions of the antigen standards: 2,000 ng/ml, 1,000 ng/ml, 500 ng/ml, 200 ng/ml, 100 ng/ml, 50 ng/ml, 20 ng/ml, 10 ng/ml, 5

ng/ml, 2 ng/ml, 1 ng/ml and buffer only. The cell culture supernatants were appropriately diluted at 1:5-1:25, as the most accurate part of the standard curve covered the range 10-100 ng/ml. A control comprising culture medium with 20% FCS, diluted as for the cell culture supernatants, was also included. Unknown samples were assayed in triplicate. Aliquots of 100 µl of the standards and unknown samples were dispensed into the wells. The plates were incubated at room temperature for 2 hours and then washed as described above. One hundred microlitres of a 1:5000 dilution of either the IgG fraction of sheep anti-human kappa or lambda chain antibody (Silenus Laboratories, Hawthorn, Victoria, Australia) were dispensed into each well. The plates were incubated at room temperature for 1 hour and washed again. A 1:5000 dilution of horse radish peroxidase conjugated affinity-isolated donkey anti-sheep immunoglobulin (Silenus Laboratories, Hawthorn, Victoria, Australia) was prepared and 100 µl were added to each well. The plates were incubated for 1 hour at room temperature and washed. The assay was developed by the addition of the substrate o-phenylenediamine dihydrochloride (Sigma Chemical Co, St. Louis, Mo, USA) and colour development was halted by the addition of 3 M sulphuric acid. The optical density of the wells was read on a Titertek Multiskan MC (made for Flow Laboratories by Eflab Oy, Helsinki, Finland) The readings were averaged, the standard curves were drawn up and the values for the unknown supernatants calculated.

2.2.10: Statistical Analysis

Results are summarised as mean±SD unless otherwise stated. The paired t-test was used to determine whether the difference between the means of paired observations was statistically significant. Results were considered statistically

significant if $p \leq 0.05$. The analyses were performed using the statistical software package Minitab version 8.2 (Minitab Inc., State College, PA, USA).

2.3: RESULTS

2.3.1: Myeloma Colony Assay

The myeloma colony assay was unable to be established in the laboratory, despite multiple modifications to both the feeder layers and the overlays. The various combinations of feeder layers and overlays are listed in table 2.2 and each combination has been identified with a letter. A total of 62 specimens (56 bone marrow aspirates; 6 peripheral blood samples) from 46 patients were placed into culture. An individual specimen was usually cultured using several different modifications of the colony assay. Each modification was tested on 5-10 specimens. As the published success rate for this technique is 80-90% for bone marrow aspirates (Millar et al, 1988), these numbers should have been sufficient to detect a successful change in the methodology.

Attempts to establish the technique were commenced prior to the publication of the method and were based on one presented at a myeloma workshop held in 1987. At this stage, the media mixtures used for the overlays were 0.5 ml α-MEM with 20% FCS, 1% BSA and 0.23% agar (method A) and 0.2 ml of 30% HL60-CM plus 70% α-MEM with 20% FCS, 1% BSA and 2×10^{-5} M putrescine (method B). Colonies of more than 50 cells were present in 3/9 specimens, with the other specimens containing clusters of 25-50 cells. The cells in these colonies were predominantly granulated cells, either macrophages containing ingested debris or mast cells. Only occasional plasma cells were present.

Table 2.2: Modifications to the Myeloma Colony Assay		
Method No.	Feeder Layer	Overlay Containing Bone Marrow or Peripheral Blood Mononuclear Cells at 10^5-10^6 Cells/Plate
	GROUP 1: HL-60 cells grown in RPMI 1640 with 20 mM HEPES buffer	
A	HL-60 cells* at $5x10^5$ cells/plate in α-MEM with 20% FCS, 1% BSA, 0.5% agar	0.5 ml α-MEM with 20% FCS, 1% BSA, 0.23% agar
B	As for method A	0.2 ml of 30% HL60-CM plus 70% α-MEM with 20% FCS, 1% BSA, $2x10^{-5}$ M putrescine
C	HL-60 cells at $5x10^5$ cells/plate in α-MEM with 20% FCS, 0.5% agar, no BSA	0.5 ml α-MEM with 20% FCS, 0.23% agar, no BSA
D	HL-60 cells at $10x10^5$ cells/plate in α-MEM with 20% FCS, 0.5% agar, no BSA	As for method C
E	As for method C	0.2 ml of 30% HL60-CM plus 70% α-MEM with 20% FCS, $2x10^{-5}$ M putrescine, no BSA
F	As for method D	As for method E
G	As for method C	0.2 ml HL60-CM
H	As for method D	As for method G
I	As for method A	As for method G
J	As for method A	0.5 ml HL60-CM
K	As for method A	0.5 ml HL60-CM with 0.16% agar
L	As for method A	0.25 ml α-MEM with 20% FCS, 1% BSA and 30% HL60-CM
	GROUP 2: HL-60 cells grown in RPMI 1640 without HEPES buffer	
M	HL-60 cells at $5x10^5$ cells/plate in α-MEM with 1x MEM vitamins, 20% FCS, human transferrin 50 mg/l, ascorbic acid 50 mg/l, 1% BSA, 0.5% agar	0.5 ml α-MEM with 1x MEM vitamins, 20% FCS, human transferrin 50 mg/l, ascorbic acid 50 mg/l, 1% BSA, 0.2% agar
N	As for method M	0.2 ml α-MEM with 1x MEM vitamins, 20% FCS, human transferrin 50 mg/l, ascorbic acid 50 mg/l, 1% BSA, 30% HL60-CM
*HL-60 cells were irradiated to 50 Gy prior to use in feeder layers		

The initial modifications were the omission of BSA from both the feeder layers and the overlays and a doubling of the number of HL-60 cells in the feeder layers from $5x10^5$ to $10x10^5$ cells/plate (methods C-F). The BSA was omitted as there are multiple grades of this product and the one being used had not been tested previously in tissue culture systems and was hence potentially inhibitory. The HL-60 cells had been hypothesised to provide growth factors for the myeloma colonies, so an increased concentration was conceivably beneficial. The results were no better.

When the published article became available, the liquid overlay was changed to 0.2 ml HL60-CM only and was trialled both with and without BSA in the underlay (methods G-I). As the plates were becoming dehydrated, the volume of the liquid overlay was increased to 0.5 ml (method J). This overlay gave the most promising results. In 3/6 patients, there were floating "colonies" or "clusters". Some of these contained predominantly plasma cells but a significant number were composed of a mixture of granulocytes, eosinophils and macrophages. The presence of these "colonies" at day 7, as well as days 14 and 21, suggested that they were clumps of cells rather than proliferating colonies. In an attempt to immobilise these colonies so that they could be counted and to prevent the occurrence of confluent sheets of either aggregated or proliferating cells, 0.16% agar was added to the overlay of HL60-CM (method K). There were no cultures in which the presence of groups of plasma cells was the dominant feature. Two further samples were examined at days 7, 14 and 21. The numbers of "colonies" and "clusters" decreased with time and there was no evidence of proliferation of plasma cells or plasma cell precursors on the basis of the number of cells in the "colonies". These findings supported the possibility

that cells of multiple lineages were clumping at the time of plating and then gradually dying.

After corresponding with Millar, it became apparent that my interpretation of the liquid overlay described in the published article had been incorrect. This led to the trialling of an overlay containing α-MEM, FCS, BSA and HL60-CM (method L). A final attempt to perfect the technique was made visiting Millar's laboratory. It was suggested that HEPES buffer, present in the media used to grow the HL-60 cells, was toxic to the cultures. Furthermore, the growth media based on α-MEM was now supplemented with vitamins, transferrin and ascorbic acid. Thus, the media mixtures for the overlays recommended by Millar were 0.5 ml α-MEM with 1x MEM vitamins, 20% FCS, transferrin 50 mg/l, ascorbic acid 50 mg/l, 1% BSA and 0.2% agar (method M) and 0.2 ml α-MEM with 1x MEM vitamins, 20% FCS, transferrin 50 mg/l, ascorbic acid 50 mg/l, 1% BSA and 30% HL60-CM (method N). Despite implementing these changes, myeloma colonies could still not be grown.

2.3.2 Culture of Cell Lines

Seven cell lines from 4 patients were established in liquid culture. The cell line WL2, which will be described in detail in section 2.3.3., was derived from the peripheral blood mononuclear cells of a 78 year old female with follicular small cleaved cell (centroblastic/centrocytic) lymphoma that had transformed to large cell lymphoma and entered a leukaemic phase. One of the cytogenetic abnormalities exhibited by this cell line was t(14;18), a translocation which is characteristic of follicular NHL (Yunis et al, 1987; Weiss et al, 1987). SA4-D8 and SC5-F9 were derived from the peripheral blood mononuclear cells of a 73 year old female with lymphoplasmacytoid lymphoma associated with a λ paraprotein

in the urine. MA2, MB2 and MC5 arose during culture of peripheral blood mononuclear cells from a 64 year old female with relapsed multiple myeloma and circulating malignant cells. CONB5 was derived from the bone marrow mononuclear cells of a 68 year old male with a myeloproliferative disorder and MGUS.

SA4-D8, SC5-F9, MA2, MB2, MC5 and CONB5 were not characterised until they had been stable in culture for some months. These lines were very similar to each other. There was a lag phase of at least 4 weeks after initiation of the cultures before the appearance of proliferating cells. When examined under the inverted microscope, they grew as large cohesive clumps of cells with varying propensities to adhere weakly to the plastic of the tissue culture vessel. Cells at the periphery of the clumps were shaped like a hand-mirror and there were frequently long, thin villi arising from the "handle". Examination of the morphology on May-Grünwald-Giemsa stained preparations revealed heterogeneous lymphoblastoid cells with basophilic cytoplasm. A proportion of cells showed plasmacytoid differentiation. All these lines strongly expressed CD20, CD23 and CD39. There was moderate to strong expression of CD37, CD54 and class II MHC molecules. CD19, CD21, CD22 and CD18 were expressed by the majority of cells in the cell lines and the intensity of expression varied from weak to moderate. CD24 and CD71 were weakly expressed on a minority of cells. CD38 was usually present on the majority of cells but its intensity of expression was always weak. CD9 was weakly expressed by a small number of cells of CONB5. CD14 was weakly expressed on MB2, SC5-F9 and CONB5.

Surface immunoglobulin was only weakly expressed by the cell lines and the isotype was not always concordant with that of the patient's paraprotein (table

2.3), excluding the possibility that the cell line arose from the malignant clone in some cases.

Table 2.3: Comparison of the Surface Immunoglobulin Isotype Expressed by Cell Lines with the Isotype of the Patient's Paraprotein

Cell Line	sIg Expression of Cell Line	Patient's Paraprotein
MA2	IgMλ + IgDλ	IgGκ
MB2	IgDκ	
MC5	IgGκ	
SA4-D8	IgMλ + IgDλ	λ Bence-Jones protein
SC5-F9	IgMκ + IgDκ	
CONB5	IgGλ + IgDλ	IgGκ

To investigate the origin of these cell lines further, immunoglobulin and TCR gene rearrangements were performed on all cell lines and on material from the patients when available. All cell lines were clonal populations and each one had different immunoglobulin J_H rearrangements. The cell lines MA2, MB2, MC5, SA4-D8 and SC5-F9 did not have the same immunoglobulin gene rearrangements as the malignant cells from which they were potentially derived. There was no specimen available for the patient from whom CONB5 was derived but the immunoglobulin light chain isotypes of the cell line and the patient's paraprotein were discordant. The cell lines SA4-D8 and CONB5 had one rearranged allele each for the TCR β gene. All these cell lines expressed the EBNA antigen.

2.3.3 WL2 Lymphoid Cell Line

Patient Details

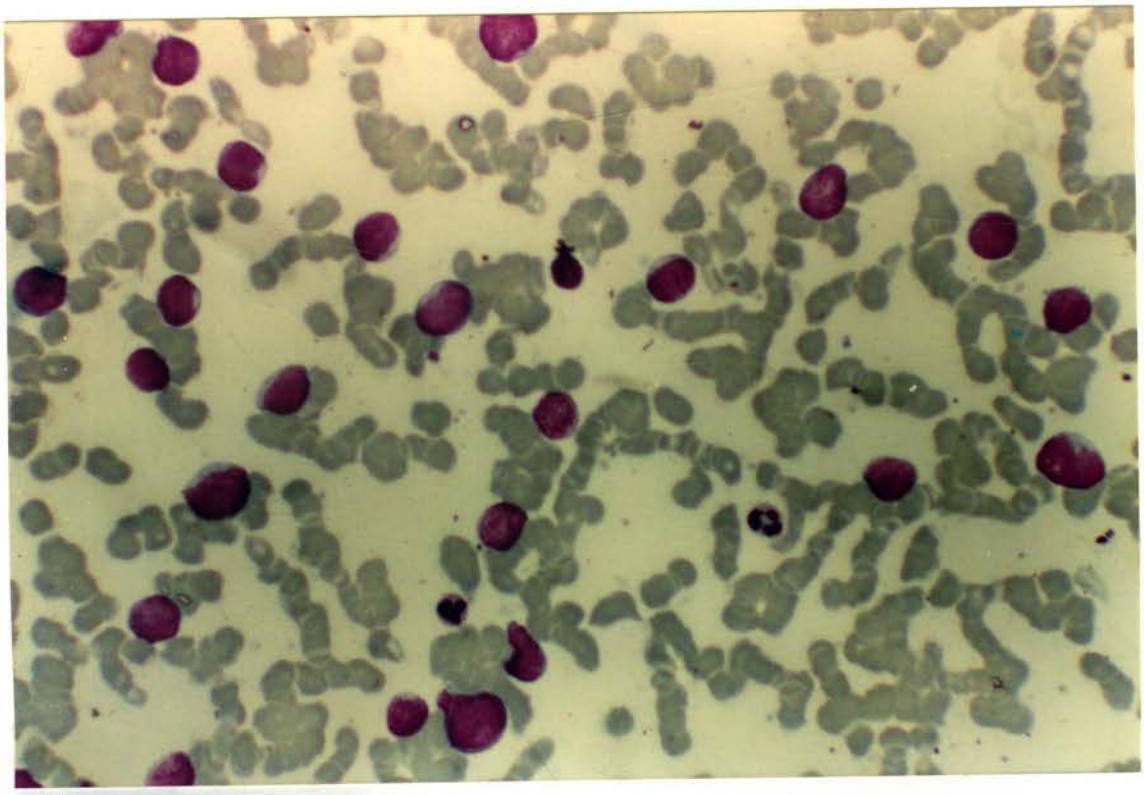
A 78 year old woman presented in September 1987 with bilateral inguinal lymphadenopathy. A diagnosis of follicular small cleaved cell lymphoma (nodular poorly differentiated) was made following lymph node biopsy and she was treated with radiotherapy to both ilio-inguinal regions. Bilateral axillary

lymphadenopathy gradually increased during 1988. Oral cyclophosphamide and prednisone were commenced in September 1988. Although the lymph nodes initially decreased in size following the first course of chemotherapy, they enlarged again prior to commencing the second course. Consequently, bulky lymph nodes in the right axillary and supraclavicular areas were treated with radiotherapy. Circulating lymphoma cells appeared in early December 1988 (figure 2.1). A right breast lump developed in mid December and a fine needle biopsy showed large cleaved cells. She was given 2 mg vincristine and 100 mg hydrocortisone intravenously on 20/12/88 but died on 25/12/88.

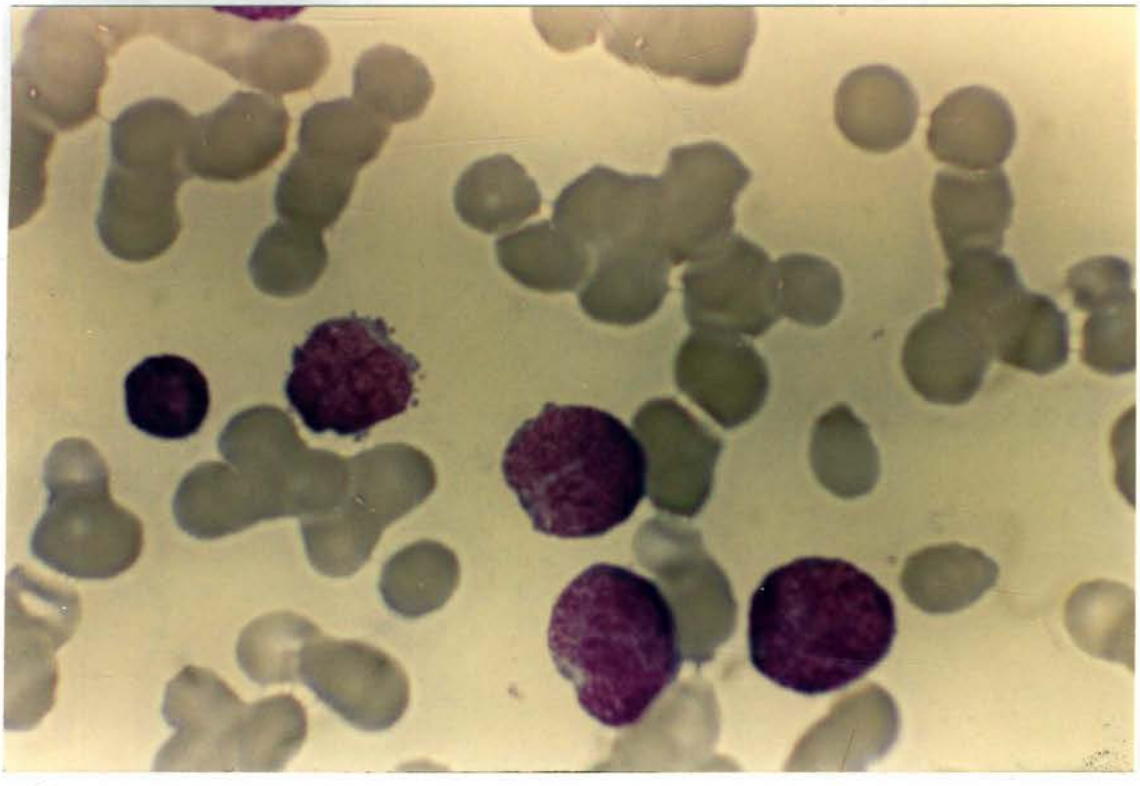
A peripheral blood sample for tissue culture was collected on 20/12/88. At that time, the results of a full blood count were: haemoglobin 9.3 g/dl; white cell count $49.9x10^9/l$; neutrophils $18.4x10^9/l$; lymphocytes $0.0x10^9/l$; blasts $37.4x10^9/l$. The following surface antigens were detected on the lymphoma cells: 88% of the cells were $CD38^+$; 43% were $CD10^+$; 82% were class II MHC^+; 47% were $CD19^+$; 14.2% were $s\lambda^+$. Expression of sIg heavy chain isotypes was not investigated. The karyotype of the cells was complex with the following abnormalities detected: 45, X, -X, -1, -9, +19, +21, i dic (6)(q15), i(17q), t(14;18)(q32;q21), +der 9, t(1;9)(p24;q23), der 1 t(1;?)(p36;?), der 5 t(5;?)(q15;?), der 12 t(12;?3)(p12;?q21), der 14 t(14;?)(q32;?).

Characterisation of the WL2 Cell Line

The mononuclear peripheral blood cells of the above patient began proliferating as soon as they were placed into culture. These cells were not characterised or used in stimulation experiments until they had been in continuous cell culture for 6 months. The WL2 cell line did not express the EBNA antigen. It was



(a)



(b)

Figure 2.1: Morphology of the circulating malignant cells of a patient with follicular small cleaved cell lymphoma that had transformed to large cleaved cell lymphoma. The blood film was stained with May-Grünwald-Giemsa. Light microscopy at (a) 10x10 and (b) 10x100 magnification.

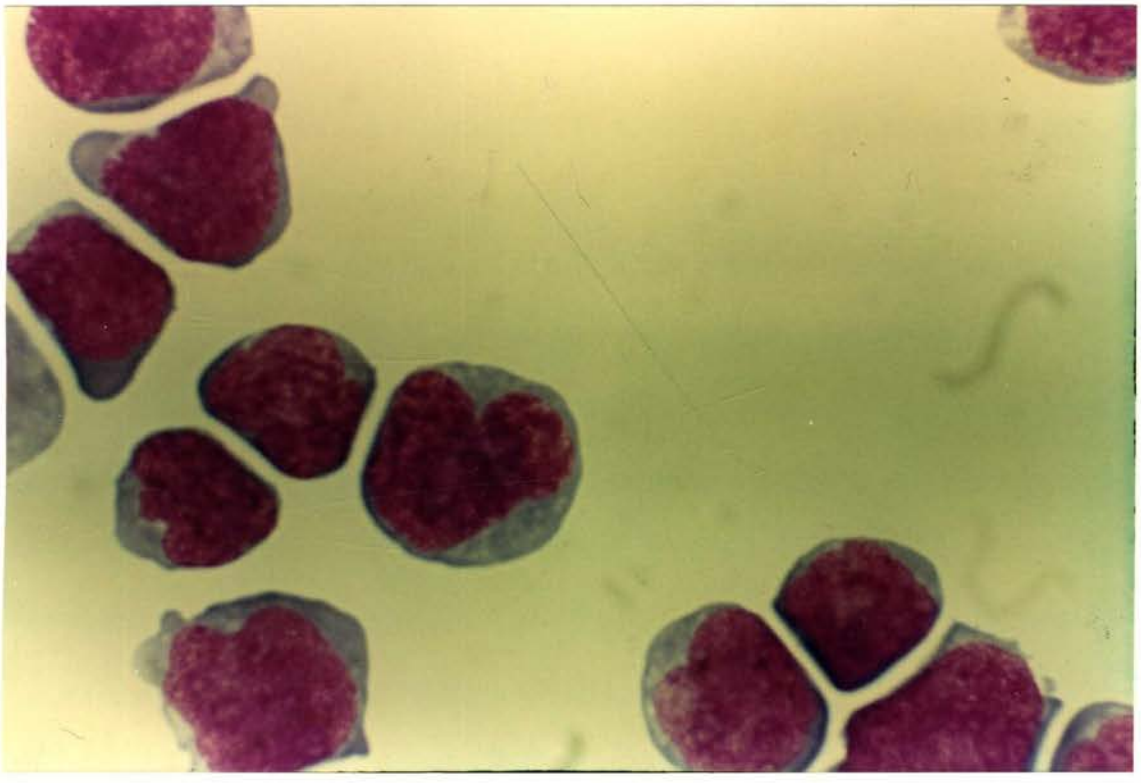


Figure 2.2: Morphology of the WL2 permanent cell line. This cell line was derived from the malignant cells of a patient with follicular NHL in leukaemic phase. The cytospin preparation was stained with May-Grünwald-Giemsa. Light microscopy at 10x100 magnification.

periodically screened for the presence of Mycoplasma infection and was consistently negative.

Cytogenetic studies on the cell line were limited but confirmed that it was derived from the malignant clone. The WL2 line was near triploid. The presence of der 1 t(1;?)(p36;?) was confirmed in the cell line and this abnormality was so distinctive that it was considered a marker chromosome. The translocation t(14;18)(q32;q21) was also present.

When the cells were examined with an inverted microscope, they were seen to be a single cell suspension with no evidence of adherence to the tissue culture flasks. The cellular morphology on May-Grünwald-Giemsa stained preparations was characteristic of centroblastic/centrocytic lymphoma (figure 2.2). The cells were variable in size and many were irregular in outline. Many of the nuclei were indented or cleaved. Multiple nucleoli were present in each nucleus but these were heterogeneous with respect to size, being either large and conspicuous in some cells or small and indistinct in others. The cytoplasm was not deeply basophilic but rather a greyish blue hue.

The phenotype of the cell line was very similar to that of the patient's malignant cells, with concordance of immunoglobulin light chain isotype expression providing further evidence that the cell line was derived from the patient's malignant clone. The cell line had very strong expression of CD38, strong expression of CD10 and CD24, moderate expression of CD19 and weak expression of sIgMλ and class II MHC molecules. There was borderline positive expression of CD9, CD54 and CD71. The percentage of CD9 and CD54 positive cells was low but examination of the fluorescence intensity histograms showed

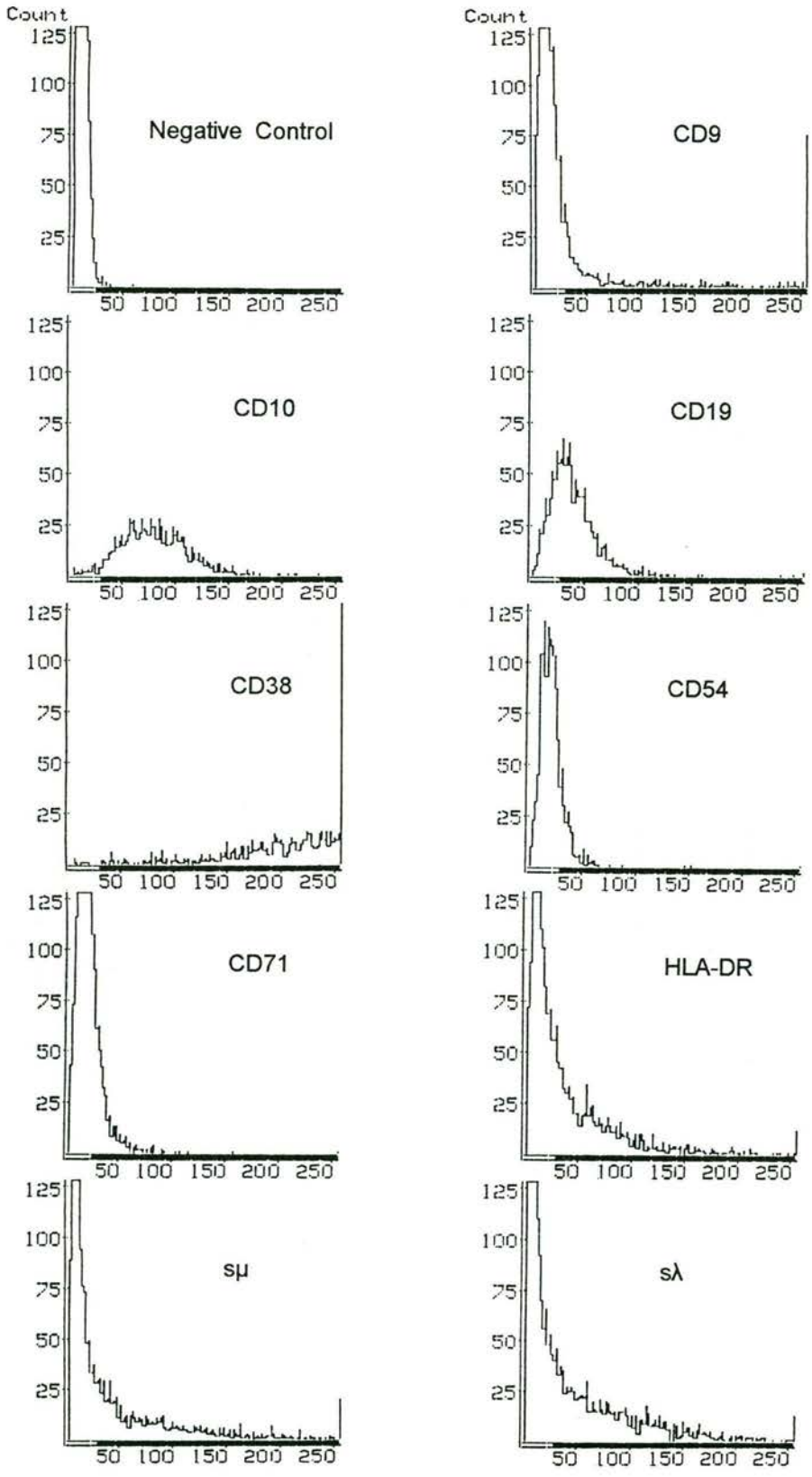


Figure 2.3: Frequency histograms of the fluorescence intensities of the surface antigens expressed by WL2 cells. The shaded zone on the horizontal axis of each histogram indicates the positive analysis region.

Table 2.4: Surface Antigens Expressed by the WL2 Cell Line.

	CD9	CD10	CD19	CD24	CD38	CD54	CD71	HLA-DR	sμ	sλ
Percentage of Positive Cells*	17±7	96±3	53±13	99±1	98±2	15±5	15±10	48±16	39±9	36±10
Fluorescence Intensity of Antigen Expression†‡	29±3	79±15	30±6	N/A-†	219±19	16±3	15±13	36±1	43±7	44±40

N/A-not available; *results are expressed as mean±SD of 6-17 experiments; †fluorescence intensity of the total population (ie. positve and negative cells); ‡fluorescence intensity of negative control was 7±1.

Table 2.5: Effect of TPA on the Proliferation Status and Viability of WL2 Cells.

Time of Harvest	48 Hours			120 Hours		
	LI (%)	Cell Count (x10^6/ml)	Viability (%)	LI (%)	Cell Count (x10^6/ml)	Viability (%)
Control	40.0±7.4*	2.3±0.6	87.3±6.2	45.4±15.4	4.3±0.9	72.6±17.9
TPA 10 ng/ml	8.9±1.3	1.5±0.4	76.8±14.6	20.3±4.6	1.9±0.5	60.7±22.2
Percentage reduction†	23.1±13.3	64.4±11.2	87.8±14.3‡	48.1±13.3	45.7±11.1	80.2±23.0§

*results are expressed as mean±SD of 9-10 experiments; †TPA result expressed as percentage of control result; ‡in only 1/10 experiments was this result ≤80%; §in only 2/10 experiments was this result ≤75%.

that the curves were definitely shifted to the right when compared to the negative control. The intensity of CD9 expression on the cells was highly variable, ranging from weak to very strong. The expression of the CD71 antigen was difficult to assess as it fluctuated. The percentage of positive cells ranged from 0.2 to 37.3%. There was a negative correlation between the percentage of cells in the S phase of the cell cycle and the percentage of $CD71^+$ cells (r=-0.7). CD3, CD5, CD11a, CD11b, CD18, CD20, CD21, CD22, CD23, CD25, CD37, CD39, CD13, CD33, CD36, PCA-1, sIgD, sIgA and sIgG were not detected on the cells. Cytoplasmic immunoglobulin was not detected. Further details of the phenotype are presented in figure 2.3 and table 2.4.

Immunoglobulin and TCR gene rearrangement studies were performed on the cell line but a sample of the patient's cells was not available for analysis (figures 2.4 and 2.5). Both immunoglobulin heavy chain loci were rearranged. Both κ alleles were deleted. One λ immunoglobulin gene was rearranged, while the other remained in germline configuration. Neither TCR β chain gene was rearranged.

2.3.4: Culture of WL2 Cells with Mitogens and Cytokines

Multiple mitogens and cytokines which have been implicated as growth and differentiation factors for normal B cells, myeloma cells or precursor populations were used either singly or in combination to stimulate WL2 cells. IL-3, IL-4, IL-6, GM-CSF and anti-IgM antibodies had no effect on WL2 cells, either as single agents or in any of the combinations listed in the last paragraph of section 2.2.6 (page 76).

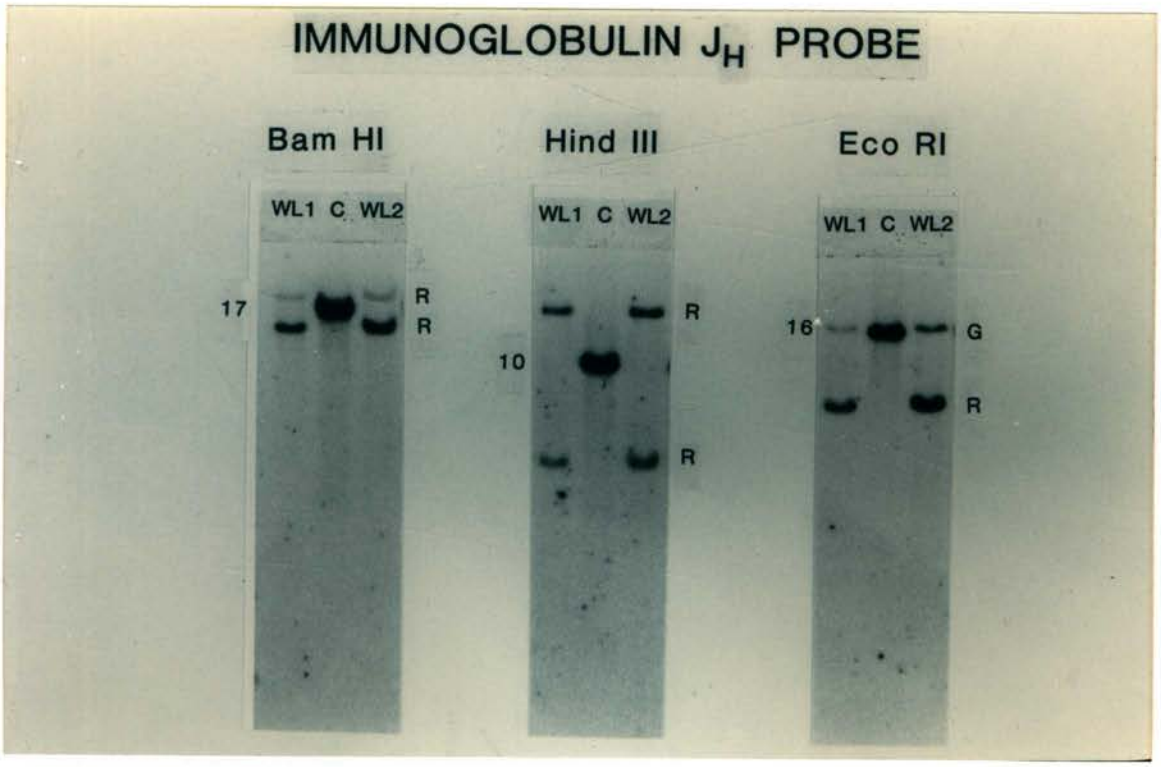


Figure 2.4a: Immunoglobulin gene rearrangements of the WL2 cell line using a J_H probe. Both immunoglobulin heavy chain genes are rearranged. C-negative control. R-rearranged bands. The numbers to the right of each Southern blot indicate the size of the germline band in kilobases. WL1 is another permanent cell line established from the malignant cells of the same patient as WL2.

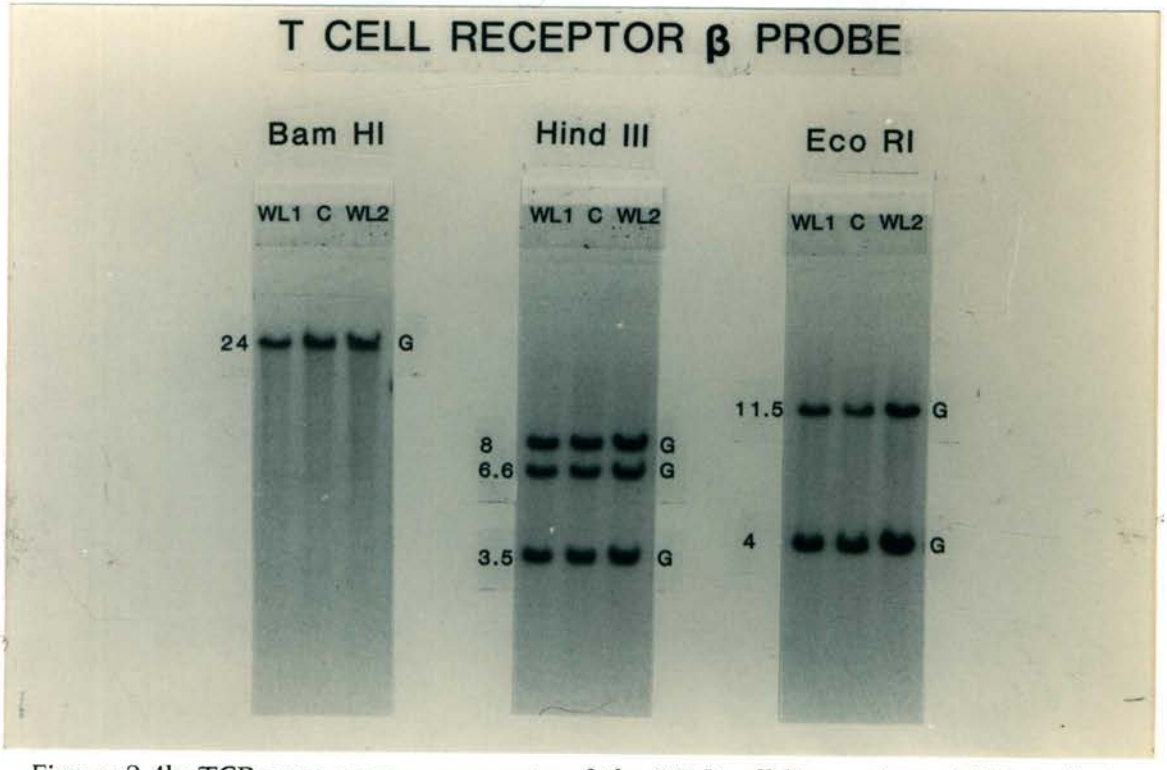


Figure 2.4b: TCR gene rearrangements of the WL2 cell line using a TCR β chain probe. Both alleles are in germline configuration. C-negative control. G-germline bands. The numbers to the right of each Southern blot indicate the size of the germline bands in kilobases. WL1 is another permanent cell line established from the malignant cells of the same patient as WL2.

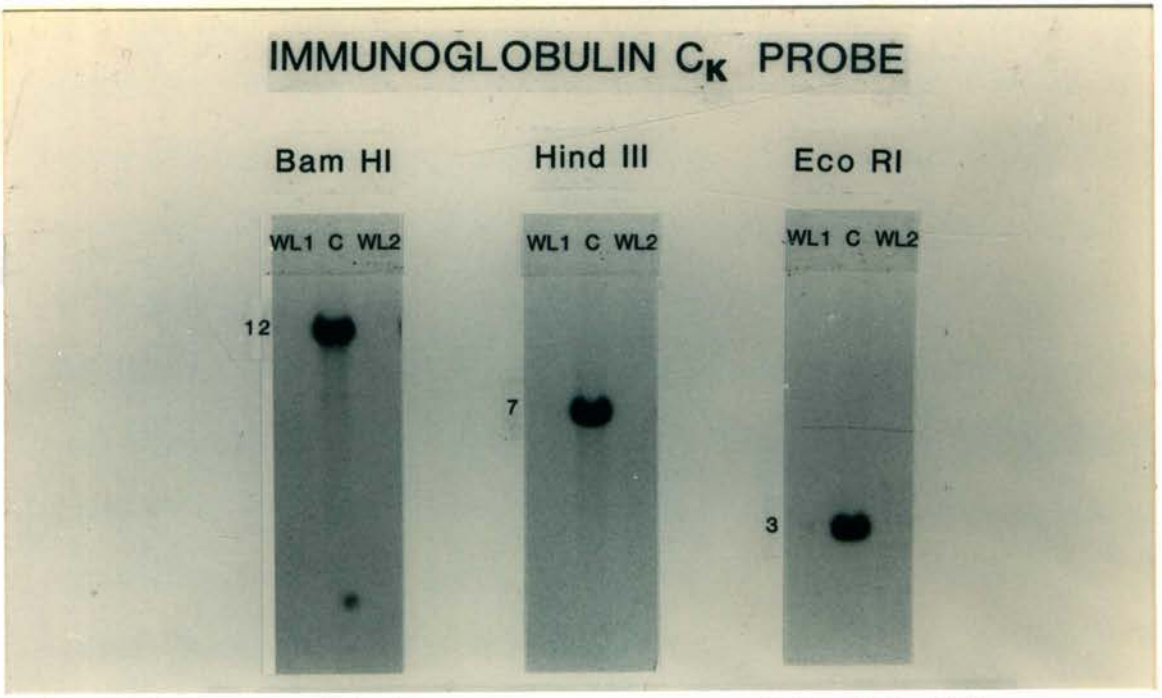


Figure 2.5a: Immunoglobulin gene rearrangements of the WL2 cell line using a C_κ probe. Both κ immunoglobulin light chain genes are rearranged. C-negative control. The numbers to the right of each Southern blot indicate the size of the germline band in kilobases. WL1 is another permanent cell line established from the malignant cells of the same patient as WL2.

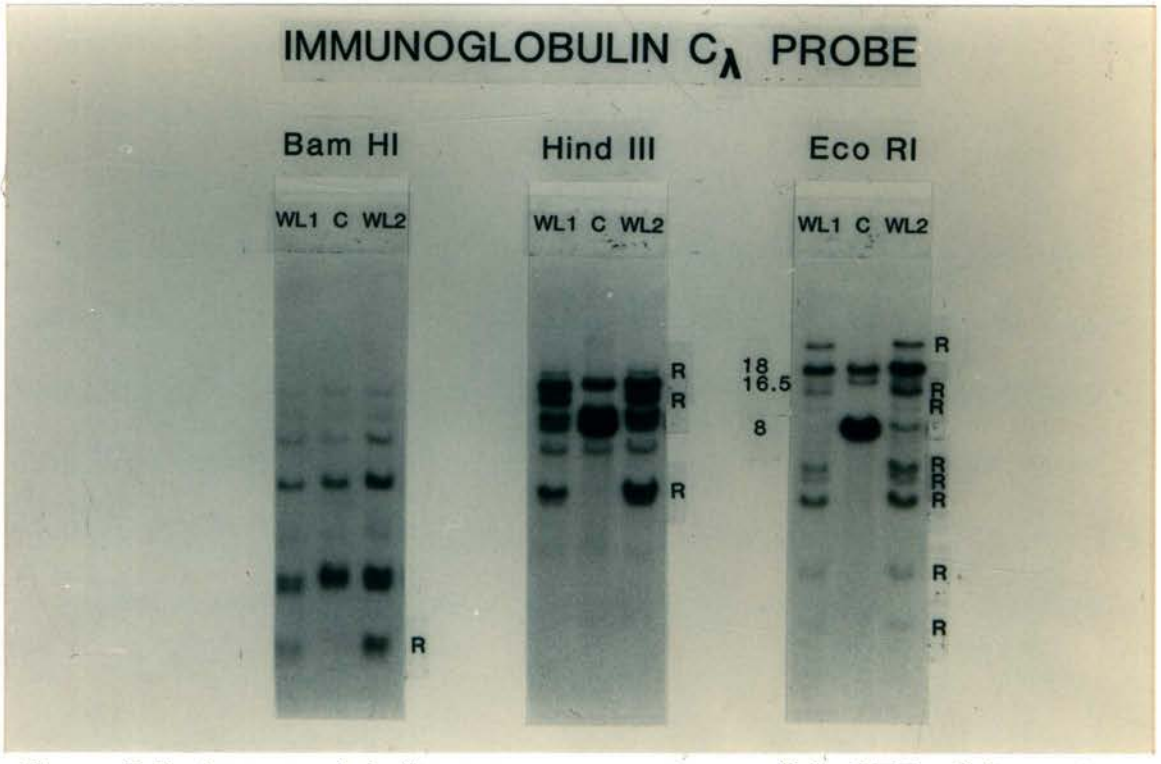


Figure 2.5b: Immunoglobulin gene rearrangements of the WL2 cell line using a C_λ probe. One allele is reaaranged and the other is in germline configuration. C-negative control. R-rearranged bands. The numbers to the right of the Southern blot obtained following digestion of the DNA with the EcoR1 enzyme indicate the size of the germline bands in kilobases. WL1 is another permanent cell line established from the malignant cells of the same patient as WL2.

The most marked changes occurred following stimulation with TPA, with the changes being maximal when TPA was used at 10 ng/ml. The calcium ionophore A23187 did not synergise with TPA. Stimulation with TPA affected the morphology, proliferation and surface antigen expression of WL2 cells. LPS and LCM as single agents had minor effects on the surface antigen expression. Both LPS and IFN-α2 acted in synergy with TPA to produce changes in the immunophenotype of the cells. The combination of TPA and LPS produced distinctive changes in the morphology of WL2 cells.

Growth Characteristics and Morphology

When the cells were viewed with an inverted microscope, they were seen to have lost their angular outline and to have became rounder following culture with TPA. Homotypic adhesion was present in cultures containing TPA plus LPS, as evidenced by the presence of small clusters of cells. Some of these clusters were also weakly adherent to the plastic of the tissue culture vessel.

The addition of TPA resulted in obvious morphological changes as assessed on May-Grünwald-Giemsa stained preparations. The cells were larger with more abundant cytoplasm and a decreased nuclear:cytoplasmic ratio. An occasional cell developed cytoplasmic projections. The nucleoli became more prominent. Following stimulation with TPA and LPS, the cells developed a rounder appearance and the cells with cytoplasmic projections were more abundant.

Proliferation Status

The proportion of WL2 cells in the S phase of the cell cycle was decreased following incubation with TPA or combinations of reagents containing TPA. At 48 and 120 hours, the LI was reduced from a mean value of 40% to 9%

($p<0.0001$) and 45% to 20% ($p=0.0003$) respectively. The effect of TPA on proliferation was confirmed by the cell counts done at the time of harvest. The cell counts were significantly lower in the cultures exposed to TPA than in the control cultures at both 48 ($p<0.0001$) and 120 ($p<0.0001$) hours. Although TPA did result in a significant reduction in the viability of the cells ($p<0.03$ at 48 hours; $p<0.02$ at 120 hours), this was an insufficient explanation for the reduced cell numbers. Details of the LI, cell counts and cell viabilities are given in table 2.5 (page 95). Additions of other reagents to TPA neither augmented nor decreased its effect on the LI.

The LI was also decreased when the cells were incubated with LCM but, based on the results with the control LCM, this was due to residual TPA in the LCM.

Immunophenotypic Analysis

Changes in the surface antigen expression of WL2 cells occurred after incubation with a limited number of reagents. Culture of the cells with TPA resulted in alterations in the expression of 11 antigens. Details of these changes are presented in table 2.6 and figures 2.6 and 2.7. The most obvious alterations were in the expression of CD19, CD20, CD22 and class II MHC molecules. The CD22 antigen, which was not present on unstimulated WL2 cells, was expressed by more than 80% of the cells following stimulation with TPA. CD20 was not detected on unstimulated WL2 cells but was expressed by more than 50% of cells after culture with TPA. The fluorescence intensity of expression on individual cells ranged from weak to very strong. CD20 expression was higher at 48 than 120 hours. For the CD19 and class II MHC molecules, the percentage of cells detected as positive increased from about 50% to at least 80% following incubation with TPA. Although the intensity of expression by individual cells

Table 2.6: Alterations in the Immunophenotype of WL2 Cells Following Culture with TPA.

			CD9	CD18	CD19	CD20	CD21	CD22	CD23	CD39	CD54	CD71	HLA-DR
Percentage of Positive Cells*	Control	48hrs	18±6	2±2	50±16	7±3	1±1	5±3	0±0	1±1	16±6	15±11	43±16
		120hrs	20±6	2±1	42±17	3±2	2±1	9±8	1±0	1±1	13±5	18±7	46±17
	TPA	48hrs	50±8	25±14	96±7	72±9	14±10	88±8	13±9	28±12	22±11	26±12	80±9
		120hrs	46±11	25±16	93±4	60±11	12±9	86±12	13±10	32±14	29±15	38±5	93±4
Fluorescence Intensity of Antigen Expression†‡	Control	48hrs	30±3	8±2	30±7	11±2	7±1	12±1	8±1	9±2	17±3	15±13	36±1
		120hrs	36±4	9±0	26±14	8±1	7±0	11±2	7±1	8±1	15±2	18±0	23±10
	TPA	48hrs	63±5	25±9	154±23	118±25	15±3	68±9	15±7	25±6	23±6	23±5	136±3
		120hrs	62±3	20±9	111±24	74±25	12±3	48±18	14±7	20±7	21±8	29±5	141±41

*results are expressed as mean±SD of 4-10 experiments; †fluorescence intensity of the total population (ie. positve and negative cells); ‡fluorescence intensity of negative control was 7±1.

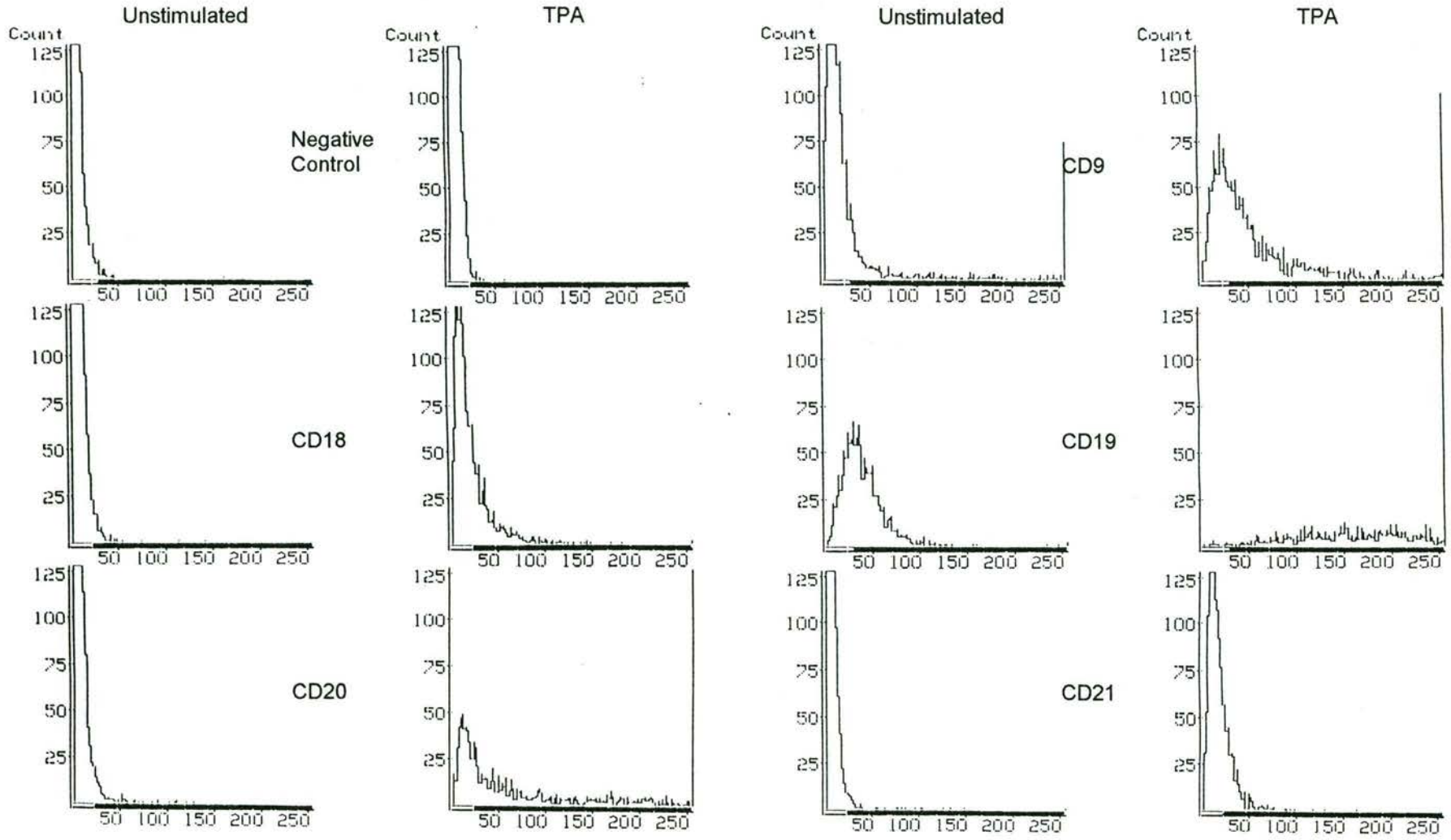


Figure 2.6: Frequency histograms illustrating the differences in the fluorescence intensities of various surface antigens expressed by WL2 cells following culture both with and without TPA. The shaded zone on the horizontal axis of each histogram indicates the positive analysis region.

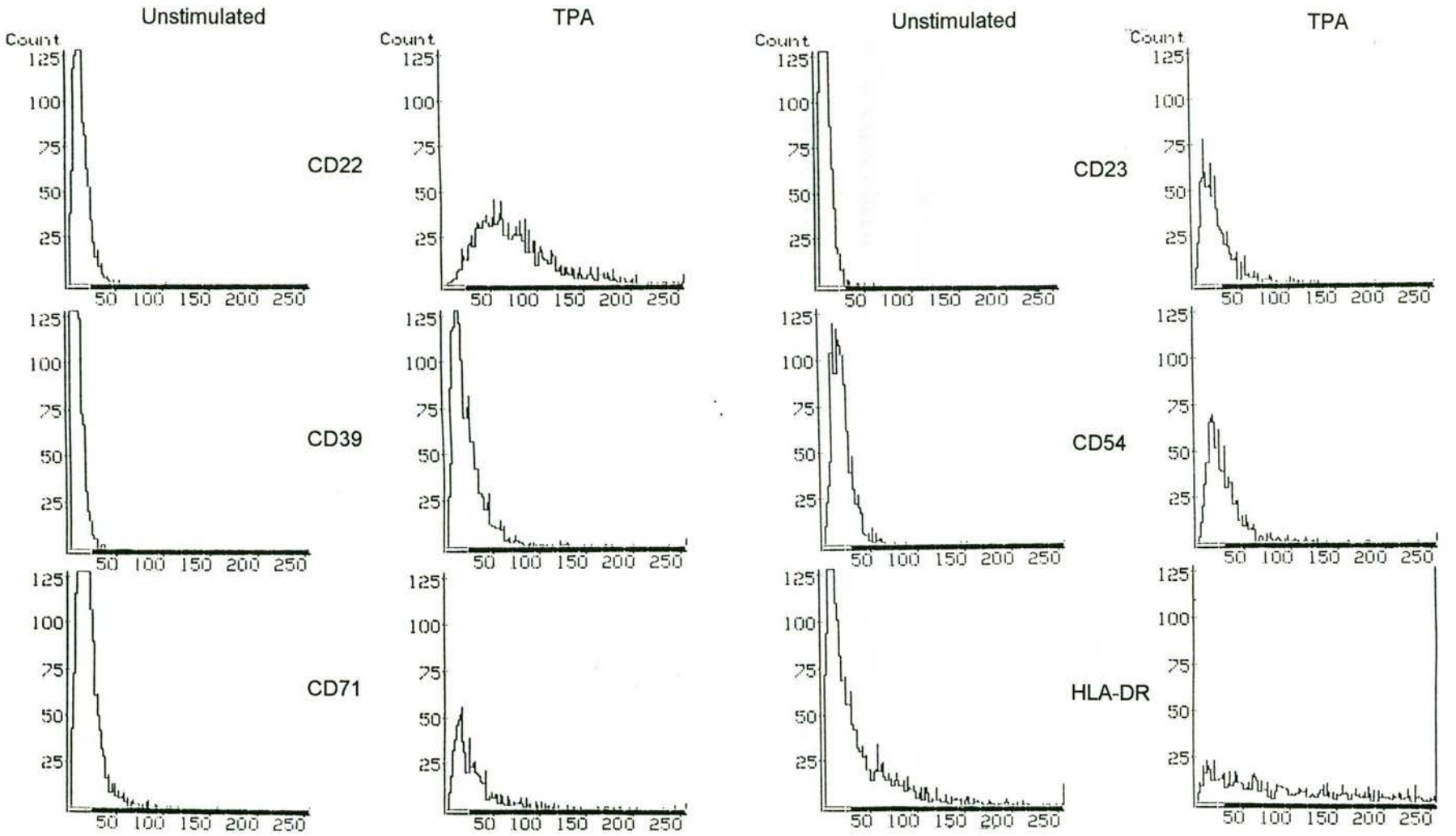


Figure 2.7: Frequency histograms illustrating the differences in the fluorescence intensities of various surface antigens expressed by WL2 cells following culture both with and without TPA.The shaded zone on the horizontal axis of each histogram indicates the positive analysis region. The histograms for the negative controls are shown in figure 2.6.

was extremely variable, ranging from weak to very strong, the fluorescence intensity of the total population was increased compared to the control cultures. The expression of class II MHC molecules was higher at 120 than 48 hours.

The changes in the expression of other antigens was less marked. CD9, CD54 and CD71 were weakly expressed by only 10-20% of unstimulated WL2 cells. Although these antigens were detected on an increased percentage of cells after culture with TPA, they were still expressed by less than 50% of the cells and the intensity of expression remained weak. Furthermore, the fluctuations in the baseline expression of CD71 made assessment of the effect of TPA stimulation at 48 hours difficult. However, the percentage of positive cells at 120 hours, although low, was much less variable than that of the cells in the control cultures. CD18 and CD39 were not detected on unstimulated WL2 cells. Even after TPA stimulation, CD18 and CD39 were still only weakly expressed by a minority of cells. The rapidity of the increase in CD39 expression varied, reaching maximum levels by 48 hours in some experiments but not until 120 hours in others. It may have been interesting to determine the changes in the expression of the surface antigen molecules which form heterodimers with CD18, namely CD11a, CD11b and CD11c. The decision was made not to do so, as the aim of these experiments was to determine which cytokines or mitogens were capable of inducing WL2 cells to mature towards plasma cells. Although TPA did induce changes in WL2 cells, these did not indicate maturation towards plasma cells. The effect of TPA on the expression of CD21 and CD23 was minimal. In some experiments, there was no induction of these surface molecules, whereas in others, there was weak expression by 10-20% of cells.

Culture of the cells with LCM produced changes in the immunophenotype of WL2 cells. Apart from alterations in CD54 expression (figure 2.8), these were similar to those occurring with TPA and, based on the results with control LCM, were due to residual TPA in the supernatant. CD54 was induced on more than 99% of cells, whereas in the experiments using TPA the mean level of positive cells was 22% and 29% on days 2 and 5 respectively, with the maximum level being 46%. Based on the results of experiments using cytokines in combination with TPA, this upregulation was not due to IL-3, IL-4 or IL-6 alone or to a combination of IL-3 and IL-6.

IFN-α2a as a single agent had no effect on the surface antigen expression of WL2 cells but when combined with TPA, there was further upregulation of CD39 and CD71 expression compared to that occurring with TPA as a single agent (figure 2.9).

LPS induced expression of CD20. This was weaker than that induced by TPA. The combination of TPA plus LPS resulted in strong expression of CD20, with both the percentage of positive cells and the fluorescence intensity being higher than with either agent alone (figure 2.10). Culture of WL2 cells with LPS resulted in the upregulation of CD54 expression from borderline to weak positive, a response similar to that occurring with TPA. TPA plus LPS synergised to produce a higher level of CD54 expression than either reagent used singly (figure 2.10).

The isotype of the sIg expressed by the WL2 cells was not altered by any combination of mitogens or interleukins. Detectable amounts of cIg were not induced by any mitogen or cytokine.

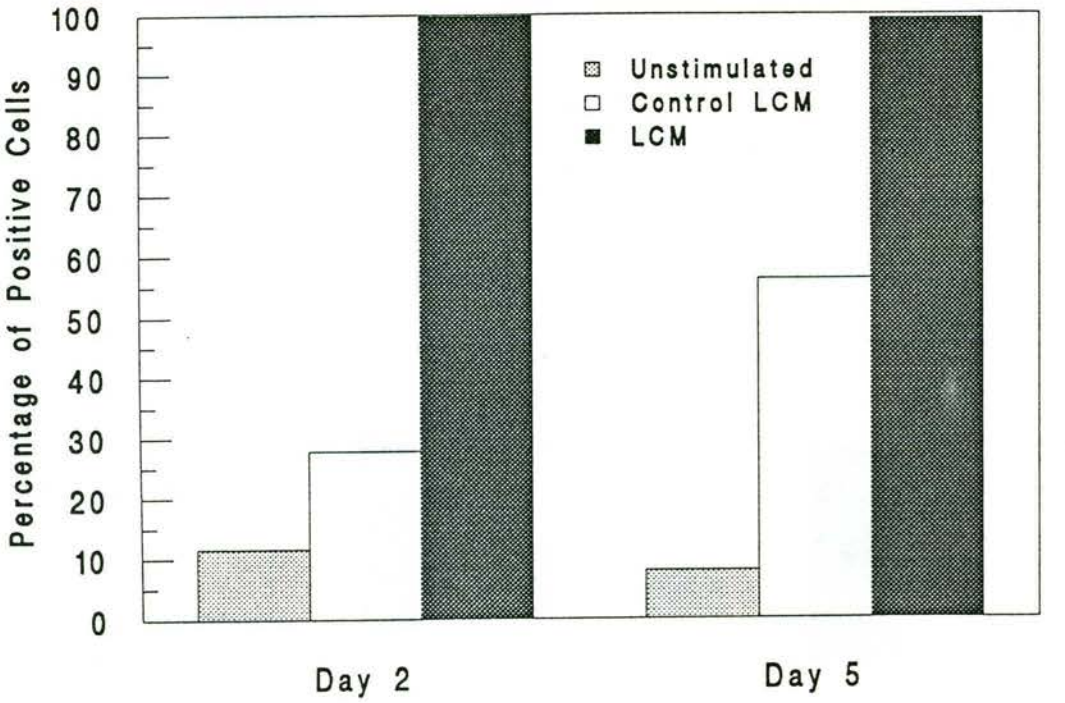


Figure 2.8: Upregulation of CD54 expression on WL2 cells following culture with 20% v/v leucocyte conditioned medium (LCM). The data shown are from a representative experiment.

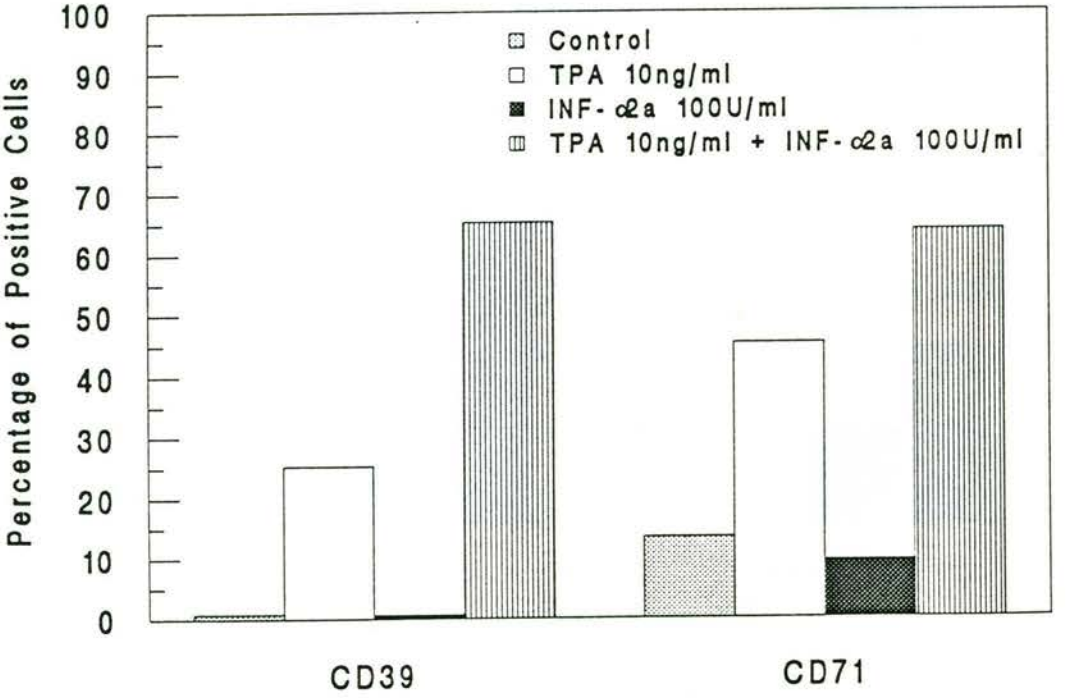


Figure 2.9: Upregulation of CD39 and CD71 expression on WL2 cells following culture with TPA and IFN-α2a. The data shown are from a representative experiment.

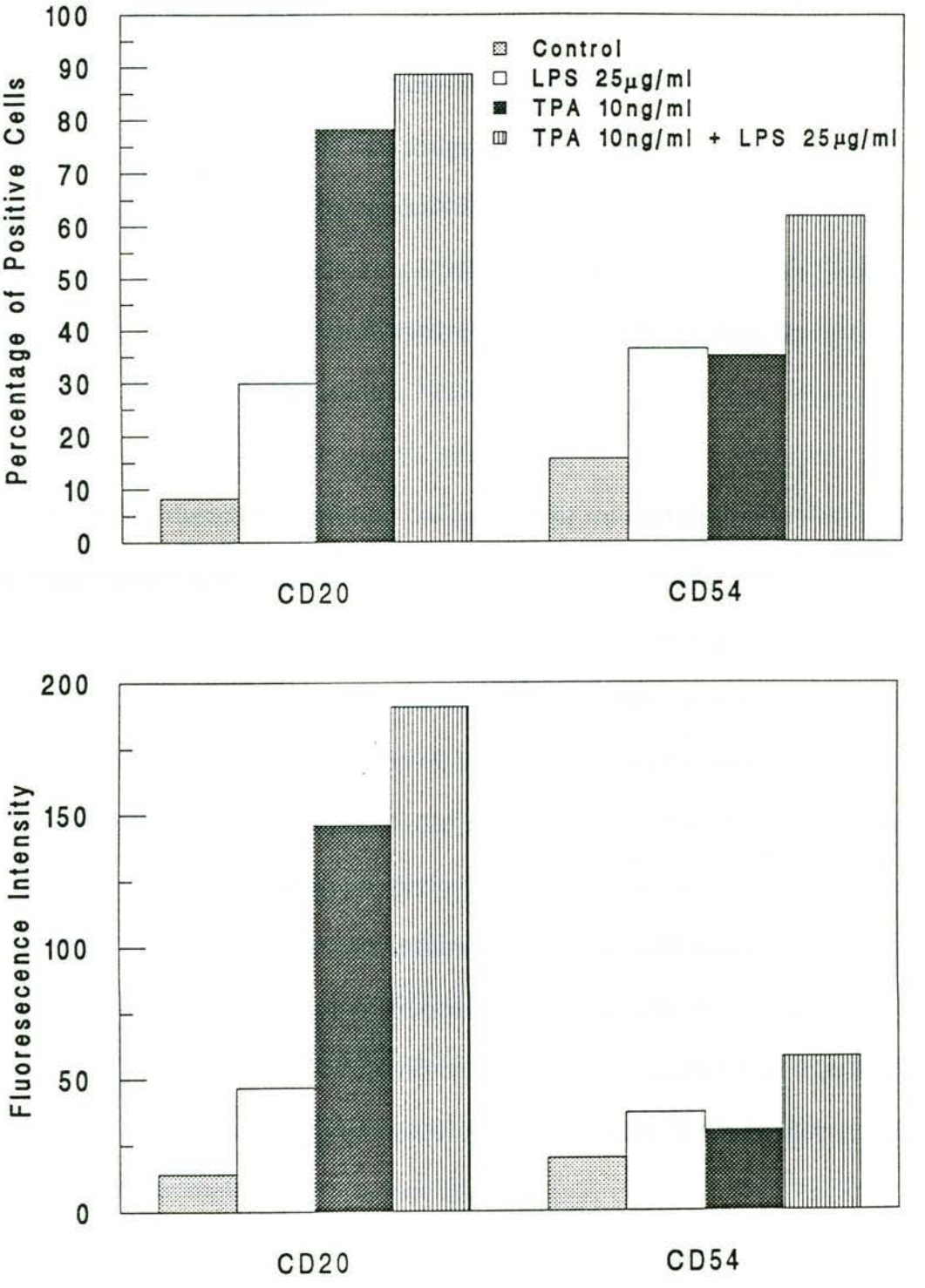


Figure 2.10: Upregulation of CD20 and CD54 expression on WL2 cells following culture with TPA and LPS. The data shown are from a representative experiment.

Immunoglobulin Secretion

In the assays quantifying immunoglobulin in the culture supernatants, there were detectable levels of λ but not κ light chain. Based on the ratio of the amount of free to total λ light chain, only free λ light chain was present in the supernatant of unstimulated cultures. At 48 hours, the quantity of free λ light chain in the supernatant was 119±35 ng/ml/10^6 cells (range: 77-202) and on day 5 was 171±64 ng/ml/10^6 cells (range: 74-322).

Only TPA had any detectable effect on the amount of immunoglobulin detected in the supernatant and only free light chain was detected after the cells were incubated with TPA. After 48 and 120 hours, the amount of light chain in the supernatant was 120±40 and 168±92 ng/ml/10^6 cells respectively in the control cultures and 146±49 and 260±123 ng/ml/10^6 cells in the TPA treated cultures. The amount of immunoglobulin in the supernatants when the cells were incubated with TPA was significantly higher than in control cultures ($p<0.04$ at 48 hours; $p<0.01$ at 120 hours) but the range of assayed light chain was almost identical at 48 hours (94-202 ng/ml/10^6 cells for controls and 96-229 ng/ml/10^6 cells for TPA). The TPA stimulated cultures contained a mean level of free light chain that was only 1.2 (range: 1.0-1.6) and 1.6 (range: 1.2-2.2) times that in the control cultures on days 2 and 5 respectively.

2.4: DISCUSSION

The failure to grow myeloma colonies using the agar/agar or agar/liquid technique remains unexplained. FCS can be considered a "soup" of undefined factors supporting cell growth and there is marked variability in the ability of batches to support proliferation in various cell culture systems. However, three

different batches were used in the myeloma colony assay and these all supported the growth of multiple cell lines and granulocyte-macrophage colonies (GM-CFU_C). The HL-60 cells in the feeder layer were hypothesised by the developers of the method (Millar et al, 1988) to provide a necessary metabolite or growth factor. The HL-60 cell line in our laboratory may be a different subline to that in Millar's laboratory and may not be a source of the required factor. However, the unknown factor is not likely to be uncommon, as the cell lines CCRF-CEM (T cell), EB3p (B cells) and RPMI-8226 (myeloma cells) were also successfully used in the feeder layer by Millar's group.

The results suggest that the technique does not result in myeloma colonies but rather in clumps of either myeloma cells or cells of multiple lineages. The occurrence of pseudocolony formation has been documented in a different myeloma colony culture system (Rhodes at al, 1990). Adhesion of cells from multiple lineages could occur via interactions between ICAM-1 (CD54) and LFA-1 (CD11a/CD18) and homotypic adhesion between myeloma cells has been reported to occur through the interaction of these two molecules (Kawano et al, 1991). There are conflicting results about the expression of LFA-1 by myeloma cells (Barker et al, 1992b) but otherwise it is expressed on all leucocytes (Arnaout, 1990). ICAM-1 is expressed on both normal and malignant plasma cells (Hamilton et al, 1991a; Van Riet et al, 1991; Barker et al, 1992a; Leo et al, 1992). Homotypic adhesion of myeloma cells could also be mediated via the CD56 (N-CAM) molecules present on malignant plasma cells (Van Camp et al, 1990; Drach et al, 1991; Van Riet et al, 1991; Barker et al, 1992a; Harada et al, 1992). Given that the life span of plasma cells in the bone marrow is in the vicinity of 3-4 weeks (Ho et al, 1986), the occurrence of homotypic adhesion between myeloma cells could result in aggregates of cells being interpreted as

colonies. Millar et al (1988) reported that in their culture system there was no evidence of aggregation in cultures of normal bone marrow cells or in cultures of heavily irradiated cells from myeloma patients (Millar et al, 1988) but these heavily irradiated cells may have been rendered non-viable and incapable of adhesion. CD56 is either not or only weakly expressed by normal plasma cells (Van Camp et al, 1991; Van Riet et al, 1991; Barker et al, 1992a; Harada et al, 1992; Leo et al, 1992) and therefore, homotypic adhesion via this mechanism is not possible.

The research groups of Franklin in Birmingham (United Kingdom) and Barlogie in the USA have also failed to reproduce the myeloma culture technique of Millar's group (personal communications). Furthermore, the literature does not contain reports indicating that other research workers have exploited this method in investigating the clonogenic cell in myeloma, as would have been expected if the technique was easily reproducible.

The phenomenon of spontaneously arising EBV^+ lymphoblastoid cell lines in cultures of peripheral blood, bone marrow or lymphoid tissue of healthy individuals or patients with various malignancies is well documented (Nilsson and Klein, 1982). The growth characteristics, morphology and surface immunophenotype of the new cell lines SA4-D8, SC5-F9, MA2, MB2, MC5 and CONB5 were quite characteristic of that previously documented for lymphoblastoid cell lines (Nilsson and Klein, 1982; Ling et al, 1987; Ling et al, 1989; Pezzutto et al, 1989). CD14 has been detected on EBV transformed cell lines with the MY4 monoclonal antibody (Labeta et al, 1991), which was used in this study. These cell lines all expressed CD18 and CD54. The growth of lymphoblastoid cell lines as clumps has been shown to be due to homotypic

adhesion resulting from interaction of LFA-1 (CD11a/CD18) and CD54 molecules (Gregory et al, 1988). The monoclonality of all these newly derived lymphoblastoid cell lines was probably due to two factors. Firstly, the cultures were initiated in small aliquots in 24-well Costar plates and were then split rapidly on the commencement of cell growth. Secondly, the cultures were not characterised until they had been stable in culture for some time. These factors would have allowed the outgrowth of one clone. The possibility that a given patient's malignant clone was EBV^+ and gave rise to an EBV^+ cell line was excluded by comparing immunoglobulin light chain isotype expression and immunoglobulin gene rearrangements.

The failure to establish permanent myeloma cell lines is not surprising. The experience of other workers suggests that this is difficult and such lines have frequently been derived from the cells of patients with more uncommon clinical features such as plasma cell leukaemia or extramedullary disease. The plasma cell lines LB-831, LB-832, NCI-H929, U-1957, U-1958, KHM-1A, KHM-1B, KMS-12-PE and EJM were derived from cells in malignant pleural effusions (Durie et al, 1985; Gazdar et al, 1986; Jernberg et al, 1987; Matsuzakai et al, 1988; Ohtsuki et al, 1989; Hamilton et al, 1990) and U-1996 and LOPRA-1 from cells in ascitic fluid (Jernberg et al, 1987; Lohmeyer et al, 1988). Karpas 620 was derived from peripheral blood cells of a patient with plasma cell leukaemia (Nacheva et al, 1990). JJN-1 and JJN-2 arose from the bone marrow cells of a patient with plasma cell leukaemia (Jackson et al, 1989) and KMOS-12-BM from the bone marrow cells in a patient with a pleural effusion, the cells from which also gave rise to a cell line (Ohtsuki et al, 1988). There is one bone marrow-derived cell line from a patient without plasma cell leukaemia or extramedullary disease (Scibienski et al, 1990). Although cells from two patients with plasma cell

leukaemia were cultured during the period of this study, there were no patients with extramedullary disease.

The WL2 cell line was derived from the circulating neoplastic cells of a terminal patient with a NHL derived from germinal centre cells. The initial histology had shown a follicular small cleaved cell lymphoma but later there was documented histological transformation to large cleaved cell lymphoma. Such transformation is part of the natural history of low-grade NHL and the incidence or timing of this occurrence is not related to therapy (Horning and Rosenberg, 1984). The presence of the t(14;18) translocation was one factor which confirmed that the cell line was derived from the patient's malignant clone. This translocation is present in the majority of follicular NHL and up to 30% of diffuse B cell lymphomas, using both cytogenetic and molecular techniques (Yunis et al, 1987; Weiss et al, 1987). Trisomy 21 is a non-random cytogenetic abnormality associated with follicular large cell lymphoma (Yunis et al, 1987) and was present in this patient at the time of clinical acceleration and histological transformation. The t(14;18) translocation results in rearrangement of the bcl-2 oncogene (Tsujimoto et al, 1985). The WL2 cell line has proved useful to other workers, as it can be used as a positive control for the detection of bcl-2 rearrangement by molecular techniques.

Overall, the surface antigen expression of WL2 cells was consistent with that of a lymphoma arising from germinal centre cells, especially the expression of CD10 and CD38. The presence of sIgMλ indicated that the cells had not undergone isotype switching. Centroblastic/centrocytic lymphomas express either sIgM or sIgG and the absence of sIgD, as seen in WL2 cells, is the usual situation for this type of lymphoma (Stein et al, 1982). Only the B cell antigens

CD19 and CD24 were detected on WL2 cells. Although the literature is not in agreement about the expression of CD24 on normal germinal centre cells (Hsu and Jaffe, 1984; Weinberg et al, 1986; Ling et al, 1987), centroblastic/centrocytic lymphomas do express this antigen, although the absence of CD20 and CD37 is uncommon (Ling et al, 1987). CD23, CD25 and CD39 are not expressed on germinal centre cells (Ling et al, 1987; Gadol et al, 1988; Liu et al, 1989) and were not detected on WL2 cells. Even though LFA-1 (CD11a/CD18) is present on all normal leucocytes (Aranout, 1990), it has been documented to be absent on some cases of NHL (Clayberger et al, 1987). Even though this occurs most commonly on Burkitt's lymphoma, it also occurs on lymphomas derived from germinal centre cells.

Cell lines usually have a higher percentage of cells expressing CD71 than in the WL2 line (Schwarting and Stein, 1989). CD71 is the transferrin receptor and it is an activation antigen that is always expressed on proliferating cells, which have a metabolic requirement for iron, but its presence does not necessarily indicate a proliferating population (Schwarting and Stein, 1989). It has been shown that, in cell lines and mitogen activated T cells, the level of CD71 expression is inversely related to the intracellular iron level (Testa et al, 1985; Pelosi et al, 1986). The addition of iron to culture medium results in increased tritiated thymidine incorporation but decreased expression of transferrin receptors. Many cell lines are grown in media such as RPMI 1640 that does not contain iron salts, thus relying on its presence in the added serum. The WL2 cells were grown in media containing iron salts (Ham's F12) and supplemented with transferrin, as well as a high concentration of serum. The inverse relationship between the percentage of cells in the S phase of the cell cycle and CD71 expression by the WL2 cells was presumably due to modulation of

transferrin receptor expression by the intracellular iron levels but, despite these fluctuations, the expression of CD71 was always low indicating that the intracellular concentration of iron was always relatively high.

Studies on the immunoglobulin in the culture supernatants were consistent with either the exclusive or predominant presence of small quantities of free light chain. Excluding secretion of small amounts of whole immunoglobulin molecules would require specific assay of heavy chains rather than the assay of total and free light chains as was used in this study. The light chains could have been derived from several sources: active secretion, release of cIg from non-viable cells or membrane turnover. Any contribution from membrane turnover must have been minor as complete immunoglobulin molecules were not detected. To demonstrate that the WL2 cells were actively synthesising and secreting free light chain would require incubating the cells with a substance which inhibits protein synthesis (eg. cycloheximide). Secretion of immunoglobulin free light chain, either unaccompanied or in excess of whole immunoglobulin molecules, is a property of both normal and malignant B cells that becomes manifest shortly after productive rearrangement of an immunoglobulin light chain gene (Hannam-Harris and Smith, 1981a; Hannam-Harris and Smith, 1981b; Gordon, 1984; Hopper and Papagiannes, 1986; Hopper et al, 1988). The production of excess free light chain seems to be necessary for the processing of the heavy chain and the assembly of whole immunoglobulin molecules (Gordon, 1984; Hopper and Papagiannes, 1986). Secreted free light chain has been hypothesised to have a role within idiotypic networks that regulate immunoglobulin secretion (Hopper and Papagiannes, 1986; Hopper et al, 1988). In studies of secreted immunoglobulin by lymphomas derived from germinal centre cells, there has been variability in the ratio of light to heavy

chain, ranging from cases with balanced secretion to those with light chain secretion only. The latter situation occurred in some tumours in which centrocytes as opposed to centroblasts were the predominant cell type (Gordon et al, 1978; Hannam-Harris et al, 1982).

Numerous changes were evident in WL2 cells following culture with TPA. TPA is a tumour promoting agent that has a diacylglycerol-like structure in its molecule. Diacylglycerol plus mobilised intracellular calcium activate protein kinase C, which is one component of the intracellular signalling pathways. TPA mimics the effect of diacylglycerol on protein kinase C but without the requirement for calcium. The action of TPA on a cell depends on the lineage of the cell as well as the activation and differentiation status of the cell. This diversity of action may be related to the existence of multiple subspecies of protein kinase C, which have variable distributions in different tissues (Diamond et al, 1980; Nishizuka et al, 1984; Bosca et al, 1989; Polliack et al, 1990).

The changes occurring following the culture of WL2 cells with TPA can be analysed in terms of whether they represent activation or differentiation. The upregulation of CD23, CD25, CD71, CD54 and class II MHC molecules occurs following the activation of B cells (Freedman et al, 1987b; Gordon and Guy, 1987; Boyd et al, 1989; Schwarting and Stein, 1989). There is disagreement in the literature about whether the induction of CD9 on B cells represents differentiation or activation (Zola et al, 1989; Zelenik-Le et al, 1989). The increased expression of CD23, CD54, CD71, class II MHC molecules and possibly CD9 indicate that TPA is acting as an activation factor on WL2 cells.

In B cells, increased secretion of immunoglobulin and the occurrence of immunoglobulin isotype switching are considered to be evidence of differentiation. The increase in the quantity of free light chain in the culture supernatants following TPA stimulation was small in magnitude, reaching a mean of 1.6 and a maximum of 2.2 times the level in control cultures after 5 days. The source of this additional free light chain was not certain. As the viabilities were lower in the TPA cultures, it is conceivable that the extra light chain was derived from the cIg stores of non-viable cells rather than being actively secreted by viable cells. Furthermore, following culture with TPA, WL2 cells did not have detectable cIg, did not secrete complete immunoglobulin molecules into the supernatants and did not undergo isotype switching, as evidenced by the continued detection of sIgM only. The only changes that could be considered evidence that TPA was acting as a differentiating agent on WL2 cells was the decrease in proliferation and possibly the induction of CD9. It is not possible to class the upregulation of CD19, CD20, CD21, CD22 and CD39 as evidence of differentiation, as the phenotype of secondary B blasts in lymphoid follicles or of germinal centre cells rescued *in vitro* is currently unknown. Overall, TPA acted as an activating rather than a differentiating factor on WL2 cells.

The changes resulting from the addition of other mitogens and cytokines to the cultures of WL2 cells were limited. The homotypic adhesion that occurred in the presence of both TPA and LPS was probably mediated via the interaction of the LFA-1 molecule and CD54. Both these molecules were upregulated by TPA but the levels of CD54 were only adequate to allow interaction of this receptor-ligand pair following culture of the cells with both TPA and LPS.

The WL2 cells were unresponsive to most factors that could be considered physiological or to mimic the physiological situation. Among such factors, only IFN-α2a plus TPA and possibly an unidentified component of LCM had any discernible activity. There may be many explanations for the lack of apparent response of a cell to a proliferation or differentiation factor. The factor may not be present at a sufficient concentration but the various cytokines added to cultures of WL2 cells were used across a wide range of concentrations. The cells may lack the receptor or there may be abnormalities of the intracellular signalling mechanisms associated with a given receptor. A factor may be inactive as a single agent but effective when presented as part of a combination. As an example, IFN-α2a had no obvious effect on WL2 cells when used alone but, when combined with TPA, it resulted in the upregulation of CD39 and CD71 expression to higher levels than with TPA alone. The appropriate investigations to detect the activity of a factor may not have been performed. For example, the induction of other cytokine receptors was not assessed in these studies. A cell may both produce and have receptors for a given cytokine and thus not be sensitive to an exogenous source of the cytokine. Such autocrine stimulation has been proposed to be one of the essential factors in tumourigenesis (Lang and Burgess, 1990) and is believed by some workers to be the mechanism by which IL-6 causes proliferation of myeloma cells *in vivo* (Kawano et al, 1988).

Work on the expression of cytokine receptors by WL2 cells was undertaken by other members of the research group at Royal Prince Alfred Hospital. This later research was done independently of the work presented in this thesis but the results of that study can be used to more specifically discuss reasons for the lack of activity of a cytokine on a particular type of cell. The lack of activity of

IL-6 on WL2 cells was not due to lack of expression of the binding ligand of the receptor, as the binding of fluorochrome-labelled IL-6 was detected by flow cytometry (Brown et al, 1992). IL-6 receptors may usually be present on follicular lymphomas, as they have been detected on activated B cells and, in low numbers, on large $sIgD^-$ tonsillar B cells, the fraction containing germinal centre cells (Taga et al, 1987). The IL-6 receptor is a heterodimer of α and β chains with the binding ligand present on the α chain but the β chain is necessary for high affinity binding and functions as the signal transducer (Miyajima et al, 1992; Cushley and Harnett, 1993). Abnormalities of this second chain would result in a non-functional IL-6 receptor. The intracellular signalling mechanisms for the IL-6 receptor have not yet been clarified but activation of protein kinase C has not been reported (Cushley and Harnett, 1993) and hence, the studies with TPA do not assist in determining the reason for the lack of IL-6 activity. As IL-6 does bind to WL2 cells, the possibility of autocrine stimulation must be considered. The published results regarding the expression of the IL-6 gene by cells in the germinal centre are not in agreement. IL-6 production has not been documented for germinal centre cells in normal lymph nodes (Yoshizaki et al, 1989; Butch et al, 1993). IL-6 production has been reported to be absent from germinal centres in hyperplastic lymph nodes (Leger-Ravet et al, 1991), present in occasional hyperplastic lymph nodes (Yoshizaki et al, 1989) or produced by occasional scattered cells (Emilie et al, 1990). In Castleman's disease, studies on tissue sections have shown that cells within the germinal centre produce IL-6. In one study, they were identified as follicular dendritic cells (Leger-Ravet et al, 1991) and, in another, as B cells (Yoshizaki et al, 1989) but this latter study also showed that isolated B cells from such lymph nodes stain with anti-IL-6 antibodies. IL-6 mRNA has been detected in a variety of NHL specimens, including 4/8 follicular small cleaved cell lymphomas (Freeman et al, 1989).

These results are consistent with the possibility that WL2 cells may be subject to autocrine stimulation by IL-6 and not capable of further response to exogenous IL-6.

The experiments with WL2 cells were designed not just to test cytokines involved in B cell activation, proliferation and differentiation but to specifically test the activity of some of the cytokines that exert an *in vitro* effect on myeloma cells or on circulating precursor cells. Stimulation of PBL from myeloma patients with IL-3 and IL-6 leads to the development of monoclonal plasma cells (Bergui et al, 1989). Consequently, this combination was of particular interest but it did not have any effect on WL2 cells, even when combined with TPA or LCM. Even if the hypothesis that the cell in multiple myeloma in which the processes of differentiation and proliferation become uncoupled is either a germinal centre cell (Warburton et al, 1989) or a secondary B blast (MacLennan et al, 1990) is correct, the circulating precursor cells must be further along the differentiation pathway. The cytokines that affect these circulating cells may not exert the same effect on any precursor B cells at an earlier stage of differentiation. The combination of factors that are necessary for the maturation of germinal centre B cells or secondary B blasts to the differentiation stage of the circulating precursor cells may not have been used in this study.

Since these studies on the WL2 cell line were undertaken, *in vitro* studies have identified IL-2, IL-4, IL-1α, recombinant 25 kDA CD23 and signals delivered via the sIg and CD40 receptors as factors involved in the rescue of germinal centre cells from apoptosis and their differentiation into memory or plasmacytoid cells (Liu et al, 1989; Holder et al, 1991; Liu et al, 1991a). Of these agents, only an

anti-IgM reagent and recombinant IL-4 were used in the experiments on the WL2 cells but none had any demonstrable effect. Polyvalent anti-immunoglobulin, anti-IgG but not anti-IgM antibodies are capable of rescuing germinal centre cells (Liu et al, 1989) but the apparent lack of activity of anti-IgM antibodies may have been due to the fact that most of the germinal centre cells expressed sIgG. Only anti-IgM antibodies were used in this study as sIgD, sIgG or sIgA were not detected on resting WL2 cells or after incubation with any of the other reagents used in these experiments. The level of expression of sIgM on most WL2 cells was low and may not have been sufficient to allow cross-linking of these molecules. As IL-4 plus anti-CD40 antibodies causes DNA synthesis in germinal centre cells (Holder et al, 1991), IL-4 receptors are either present on germinal centre cells or can be induced by stimulation of the CD40 antigen. A subsequent study has shown that IL-4 receptors are not expressed by WL2 cells (Brown et al, 1992) but the effect of anti-CD40 antibody on WL2 cells has not been assessed. The lack of expression of IL-4 receptors by WL2 cells may represent an unusual situation for lymphomas arising from germinal centre cells as recombinant IL-4 alone has been shown to decrease thymidine incorporation in follicular and diffuse cleaved cell lymphomas (Taylor et al, 1990). Human peripheral blood mononuclear cells stimulated with PHA produce IL-2 (Morgan et al, 1976). Therefore the LCM would have been expected to contain IL-2 but as the effect of this reagent on WL2 cells was minimal, the cytokines contained in the LCM were not determined. IL-2 exerts an effect only on a subset of germinal centre cells, intimating that this subset does express IL-2 receptors, even though CD25, the α chain of the IL-2 receptor, has not been previously detected on germinal centre cells (Gadol et al, 1988). WL2 cells are unlikely to have represented this subset, as they did not express CD25.

It is conceivable that the WL2 cells would have been unresponsive even if stimulated with all the combinations of factors known to have an effect on germinal centre cells. The inherent abnormalities present in the WL2 cells as a result of their neoplastic nature may render them unresponsive to normal physiological stimuli. The t(14;18) translocation was present in the WL2 cells. This translocation results in juxtaposition of the bcl-2 and immunoglobulin heavy chain genes (Tsujimoto et al, 1985) but the product of the bcl-2/immunoglobulin fusion gene is a normal bcl-2 protein (Cleary et al, 1986). Originally, it was reported that bcl-2 was not expressed in normal lymphoid tissues, including the germinal centre, or by lymphomas lacking the t(14;18) translocation (Ngan et al, 1988) and hence, the expression of bcl-2 in follicular lymphomas was viewed as intrinsically aberrant and involved in the pathogenesis of the malignancy. A subsequent study detected bcl-2 protein in normal T and B cells and in cells from a variety of lymphoproliferative disorders (Pezzella et al, 1990). Immunohistological studies have shown that the bcl-2 protein is expressed by cells in the light zone of the germinal centre (Korsmeyer et al, 1990) and it is expressed following the *in vitro* rescue of germinal centre cells (Liu et al, 1991b). This information allows re-assessment of the role of bcl-2 expression in the pathogenesis of follicular lymphomas. There may be a failure to suppress expression of bcl-2 when a B cell carrying the translocation participates in the formation of a germinal centre. Alternatively, it may be re-expressed when a sIg^- centroblast gives rise to a sIg^+ centrocyte. In either case, the cell has been programmed to survival prior to receiving the normal rescue signals. This may result in the cell being unable to respond to further differentiation signals and consequently, frozen at this point in the differentiation pathway. To provide more information on the response of follicular lymphomas to the differentiation signals for normal germinal centre

cells, it would be useful to examine and compare the expression of cytokine receptors and other important molecules such as CD40 on WL2 cells, specimens of follicular lymphomas and germinal centre cells before and after *in vitro* rescue from apoptosis. Thus, although the WL2 cell line is not suitable for studying the signals which allow differentiation of B cells to plasma cells, it may be able to provide further information on the consequences of the t(14;18) translocation in follicular lymphomas.

SECTION 3: IMMUNOPHENOTYPIC ANALYSIS OF PERIPHERAL BLOOD LYMPHOCYTES EXPRESSING THE CD38 ANTIGEN

3.1: AIMS

The enumeration of $CD38^+$ PBL in patients with multiple myeloma is useful for predicting prognosis and monitoring disease activity. At diagnosis, elevated numbers are associated with a short survival (Omedé et al, 1990; Joshua et al, 1991b). Numbers are higher in progressive disease than during plateau phase (Joshua et al, 1988).

These $CD38^+$ PBL were originally hypothesised to be pre-plasma cells belonging to the malignant clone (Ruiz-Argüelles et al, 1984; Joshua et al, 1988). Two subsequent studies have indicated that the elevated numbers are due to the expression of this antigen by higher than normal numbers of T cells and NK cells rather than pre-plasma cells. Boccadoro et al (1988) performed dual labelling studies on only 4 myeloma patients. They detected increased numbers of $CD4^+38^+$ and $CD8^+38^+$ cells but no clinical details (eg. at diagnosis or later in the course of the disease) were provided. The other study included only untreated patients (Gonzalez et al, 1992). Patients with an elevated proportion of NK ($CD16^+$) cells had a high percentage and absolute number of $CD38^+$ lymphocytes compared to those without an elevated proportion of NK cells. However, some patients without increased NK cell numbers also had high numbers of $CD38^+$ lymphoid cells and detailed results of the dual labelling experiments were not provided.

This study was designed to provide more detailed information on the phenotype of $CD38^+$ lymphocytes in both normal individuals and patients with multiple myeloma and to determine the lymphocyte subsets responsible for the

expansion of the number of $CD38^+$ PBL in myeloma patients. As the previous dual-labelling studies were performed on so few patients, the aim was to determine whether the increased number of CD38+ PBL was due to co-expression on T cells and NK cells in all patients or whether a CD38+ precursor myeloma population could be demonstrated in some patients. Patients from all phases of the disease were studied in order to investigate the possibility that the subset responsible for the expansion of $CD38^+$ cells varies during the course of the disease.

3.2: MATERIALS AND METHODS

3.2.1: Patients

Fifty patients with paraproteins or plasma cell dyscrasias were included in this study. There were 3 patients with MGUS, 43 with multiple myeloma, one with recurrent extramedullary plasmacytomas, 2 with other lymphoproliferative disorders and one with metastatic carcinoma of unknown primary. Multiple myeloma was diagnosed according to the criteria of the Committee of the Chronic Leukemia-Myeloma Task Force (1973). Details of the age, sex, clinical status and current and previous treatment of the patients with MGUS and multiple myeloma are presented in table 3.1. The stage (Durie and Salmon, 1975) and the isotype of the paraproteins are given in table 3.2. The patients with MGUS had been diagnosed 17-186 months previously. The patients with SMM had been diagnosed for periods of 5-38 months and had not received any therapy. All newly diagnosed patients were symptomatic and required therapy. Two of these patients had already commenced treatment for serious complications. One patient with hypercalcaemia had received treatment with steroids for 24 hours. The other had been treated for several days with steroids plus radiotherapy for spinal cord compression. The patients improving on

Table 3.1: Characteristics of Patients with MGUS or Multiple Myeloma

	MGUS 3 patients	SMM 6 patients	Diagnosis 11 patients	Improving On Therapy 5 patients	Plateau 7 patients	Progressive 14 patients
Age (range in years)	65-72	56-84	45-82	36-83	36-81	31-88
Sex						
Male (28 patients)	3	2	8	4	2	9
Female (18 patients)	0	4	3	1	5	5
Treatment Status						
Current	0	0	2*	5	2*	4
Previous	0	0	0	3	7	4 - radiotherapy only 10 - chemotherapy only

*Details of these patients are provided in the text

Table 3.2: Stage (Durie and Salmon System) and Paraprotein Present in Patients with MGUS and Multiple Myeloma.

	MGUS 3 patients	SMM 6 patients	Diagnosis 11 patients	Improving On Therapy 5 patients	Plateau 7 patients	Progressive 14 patients	Total Number 43 patients
Stage							
IA	N/A	4	1	0	0	0	5
IIA	N/A	2	2	1	2	3	10
IIB	N/A	0	1	0	0	0	1
IIIA	N/A	0	5	4	5	9	23
IIIB	N/A	0	2	0	0	2	4
Paraprotein							
IgMκ	0	0	0	0	0	0	0
IgMλ	1	0	0	0	0	0	1
IgAκ	0	0	3	1	0	0	4
IgAλ	0	3	2	1	0	0	6
IgGκ	1	2	4	0	4	4	15
IgGλ	1	0	0	0	2	4	7
κ only	0	0	0	2	0	0	2
λ only	0	0	0	1	1	1	3
Two paraproteins	0	1*	0	0	0	2†	3
No paraprotein	0	0	2	0	0	3	5

*IgGλ+IgAλ; †IgGλ+IgAλ in 1 patient and IgGλ+ IgMκ in the other patient

therapy included two on first-line therapy and three who had been previously treated. Plateau phase disease was defined as clinical and laboratory stability, including transfusion independence, for a period of 6 months (Joshua et al, 1991). In this group, there were 2 patients being treated with IFN-α2: one was participating in a trial of IFN-α2 during plateau phase and the other had initially received the drug for symptomatic disease with continuation of this therapy during plateau phase. Four patients developed progressive disease while on therapy but the other ten patients had been in plateau phase. The patients with multiple myeloma had been treated with various chemotherapy regimens but the commonest combinations were: (1) cyclophosphamide, adriamycin, BCNU and prednisone; (2) melphalan and prednisone.

The blood samples for immunophenotypic analysis were collected at the initial consultation or at the time of routine follow-up appointments. The blood samples or bone marrow samples for other investigations were taken as part of routine management.

The 10 normal controls were healthy subjects from the hospital staff or blood donors at the regional transfusion service. As the upper age limit for blood donation was 65 years, the median age of the normal controls was 53 years with a range of 22-65 years in comparison to a median age of 63.5 years with a range of 20-88 years in the patients. The control and patient groups were matched for sex.

3.2.2: Immunophenotypic Analysis of Peripheral Blood Lymphocytes

Reagents

The co-expression of the CD38 antigen with other surface antigens on PBL was examined using a large panel of antibodies. These antibodies had specificities for antigens expressed by the T-cell, B-cell and myeloid lineages, as well as activation and adhesion molecules. Details of the antibodies are listed in tables 3.3 and 3.4. All antibodies were used at the manufacturer's recommended dilution or had previously been appropriately titrated for routine diagnostic use in the haematology laboratory.

Normal mouse serum was prepared from blood obtained from the DBA2, C57Bl, BALBc and CBA mice strains by brachial artery puncture under ether anaesthesia.

Immunophenotypic Labelling Procedure

Ten millilitres of venous blood were collected into EDTA anticoagulant. One hundred microlitres of whole blood were aliquoted into test tubes and unconjugated antibody was added to each one. The samples were vortexed and incubated at 4°C for 30 minutes. The red blood cells were then lysed by incubation with lysing buffer (0.16 M ammonium chloride, 0.01 M sodium hydrogen carbonate, EDTA dihydrate, pH 7.30-7.35) at room temperature for 10 minutes. The specimens were centrifuged and washed once in PBS at 4°C. Fifty microlitres of a 1:200 dilution of FITC-conjugated affinity-isolated $F(ab)_2$ fragment sheep anti-mouse immunoglobulin (Silenus Laboratories, Hawthorn, Victoria, Australia) were added to the cells, which were then incubated in the dark at 4°C for 30 minutes. The samples were washed twice in PBS at 4°C. Any spare binding sites on the sheep anti-mouse antibody were saturated by

Table 3.3: Specifications of the Non-Conjugated Antibodies Used to Study the Immunophenotype of $CD38^+$ PBL.				
Specificity	Commercial Name	Clone	Isotype	Source
CD1a	T6	SFCI19Thy1A8	IgG1κ	Coulter Immunology, Hialeah, Florida
CD2	T11	SFCI3Pt2H9	IgG1κ	Coulter Immunology, Hialeah, Florida
CD3	T3	SFCIRW2-8C8($T3_c$)	IgG1κ	Coulter Immunology, Hialeah, Florida
CD4	T4	SFCI12T4D11	IgG1κ	Coulter Immunology, Hialeah, Florida
CD8	T8	SFCI21Thy	IgG1κ	Coulter Immunology, Hialeah, Florida
CD9	-	BU-16	IgG2a	Gift: G.D. Johnson, Department of Immunology, University of Birmingham, U.K.
CD10	Anti-CALLA	W8E7	IgG2aκ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD11a	DAKO-CD11a	MHM24	IgG1κ	Dakopatts A/S, Glostrup, Denmark
CD13	MY7	366	IgG1κ	Coulter Immunology, Hialeah, Florida
CD16	Leu-11b	G022	IgMκ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD18	DAKO-CD18	MHM23	IgG1κ	Dakopatts A/S, Glostrup, Denmark
CD19	B4	89B	IgG1κ	Coulter Immunology, Hialeah, Florida
CD20	B1	H299	IgG2aκ	Coulter Immunology, Hialeah, Florida
CD21	-	BU-34	IgG1	Gift: G.D. Johnson, Department of Immunology, University of Birmingham, U.K.
CD22	Leu-14	SHCL-1	IgG2bκ	Becton Dickinson Immunocytometry Systems, San Jose, California
CD23	Dako-CD23	MHM6	IgG1	Dakopatts A/S, Glostrup, Denmark
CD24	IOB3	ALB9	IgG1κ	Immunotech S.A., Marseille, France

Table 3.3: Continued.

Specificity	Commercial Name	Clone	Isotype	Source
CD25	Anti-Interleukin-2 Receptor	2A3	IgG1κ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD33	MY9	906	IgG2bκ	Coulter Immunology, Hialeah, Florida
CD34	Anti-HPCA-1	My10	IgG1κ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD36	OKM5	-	IgG1	Ortho Diagnostic Systems, Raritan, New Jersey
CD38	OKT10	-	IgG1	Ortho Diagnostic Systems, Raritan, New Jersey
CD39	-	AC2	IgG1	Gift: Department of Immunology, University of Birmingham, U.K.
CD44	IOL44	J-173	IgG1	Immunotech S.A., Marseille, France
CDw49d	IOP49d	HP2/1	IgG1	Immunotech S.A., Marseille, France
CD54	-	ICAM-1	IgG2a	Gift: A. Boyd, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
CD56	NKH-1	N901	IgG1κ	Coulter Immunology, Hialeah, Florida
CD71	OKT9	-	IgG1	Ortho Diagnostic Systems, Raritan, New Jersey
HLA-DR framework antigen	OKIa*1	-	IgG2	Ortho Diagnostic Systems, Raritan, New Jersey
Unclustered†	PCA-1	138	IgG2a	Coulter Immunology, Hialeah, Florida
Isotype Control	-	L7	IgG1	Gift: Clinical Immunology and Research Centre, University of Sydney, Australia ‡
Isotype Control	MsIgG2a	7T4-1F5	IgG2aκ	Coulter Immunology, Hialeah, Florida
Isotype Control	MsIgG2b	MPC-11	IgG2bκ	Coulter Immunology, Hialeah, Florida
Isotype Control	MsIgM	R4A3-22-12	IgMκ	Coulter Immunology, Hialeah, Florida

†Anderson et al, 1983; ‡mouse anti-Mycobacterium Leprae (Britton et al, 1985).

Table 3.4: Specifications of the Conjugated Antibodies Used to Study $CD38^+$ PBL.

Specificity	Commercial Name	Clone	Conjugate	Isotype*	Source
CD38	Anti-Leu-17	HB-7	RPE	IgG1κ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD38	IOB6	T16	FITC	IgG1	Immunotech S.A., Marseille, France
Kappa Light Chains	-	-	FITC	Polyclonal rabbit $F(ab')_2$	Dakopatts A/S, Glostrup, Denmark
Lambda Light Chains	-	-	FITC	Polyclonal rabbit $F(ab')_2$	Dakopatts A/S, Glostrup, Denmark
Isotype Control	-	DAK-GO1	FITC	IgG1κ	Dakopatts A/S, Glostrup, Denmark
Isotype Control	-	DAK-GO1	RPE	IgG1κ	Dakopatts A/S, Glostrup, Denmark
Isotype Control	-	-	FITC	Polyclonal rabbit $F(ab')_2$	Dakopatts A/S, Glostrup, Denmark

*antibodies are mouse monoclonals unless otherwise indicated; rabbit polyclonal antibodies are affinity isolated.

incubation with a 1:30 dilution of normal mouse serum at 4°C for 10 minutes. The PE-conjugated anti-CD38 antibody was then added for another 30 minute incubation in the dark at 4°C. The specimens were washed twice in PBS at 4°C and then resuspended in 0.2-1 ml PBS depending on the lymphocyte count.

For the detection of sIg light chains, the specimens were diluted with PBS and centrifuged to separate the cells from the serum. In order to remove cytophilic immunoglobulin, the cells were incubated with PBS at 37°C for 20 minutes. The samples were then centrifuged and the supernatants were aspirated. This procedure was repeated 3 times. The FITC-conjugated polyclonal $F(ab)_2$ fragment rabbit anti-human light chain antibody and PE-conjugated anti-CD38 monoclonal antibody were added simultaneously to the test tubes, which were incubated in the dark at 4°C for 30 minutes. The red blood cells were then lysed and the cells washed as described above.

All specimens were processed and analysed by flow cytometry on the day of collection.

Flow Cytometry Analysis

The specimens were analysed using a Coulter Epics Profile II flow cytometer (Coulter Electronics Inc., Hialeah, Florida), which is equipped with an air-cooled argon laser which emits at 488 nm with a power of 15 mW. Forward angle light scatter is detected by a silicone photodiode after passing through a neutral density filter (optical density of 1.0). Right angle (90°) light scatter is reflected to a S-20 photomultiplier tube by a 488 nm dichroic 90° light scatter filter. A 457-502 nm laser blocking filter is used to prevent scattered laser light from reaching the fluorescence detectors. FITC fluorescence is split off to the first

high performance photomultiplier tube using a 550 nm long pass dichroic mirror and is collected through a 525 nm band pass filter. PE fluorescence is directed to the third high performance photomultiplier tube with a 600 nm short pass dichroic mirror and collected through a 575 nm band pass filter. The signals from the photomultiplier tubes detecting fluorescence are processed using a four decade logarithmic amplification system.

Quality control procedures were undertaken each day prior to the analysis of patient samples. The operation of the fluidic systems and the optical alignment of the system were checked using Immuno-Check Beads (Coulter Corporation, Hialeah, Florida). Fluorescence intensity standardisation was performed using Standard-Brite Beads (Coulter Corporation, Hialeah, Florida). Colour compensation, which corrects for the overlap of the emission spectra of FITC and PE, was determined by dual staining of a biological sample with FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 antibodies.

The lymphocytes were identified as the population of interest by setting a gate over this population based on its forward angle and right angle light scatter properties. The fluorescence data from 5,000 lymphocytes were collected for each sample.

The results were analysed using the inbuilt Epics Profile Software versions 2.02 and 2.2. Appropriate isotypic negative controls were processed and the positive analysis regions were determined automatically by the computer software so that ≥98% of lymphocytes were negative for both fluorochromes and <2% of cells were positive for either FITC or PE fluorescence.

3.2.3: Other Investigations

The results of several other investigations were used in this study. These tests were used for the routine monitoring of myeloma patients and were performed by staff in the haematology laboratory.

Full Blood Count

A full blood count, including a lymphocyte count, was performed on a 5 ml sample of venous blood anticoagulated with EDTA using a Technicon H.1 automated analyser (Technicon Instruments Corporation, Tarrytown, New York, USA). The differential white cell count was checked to exclude the presence of abnormal cell populations by examination of a blood film stained with May-Grünwald-Giemsa.

Serum β2 Microglobulin

Serum β2M was determined by radioimmunoassay using a commercially available kit (Pharmacia β_2-micro RIA, Pharmacia, Uppsala, Sweden). The β2M in the sample being assayed competed with a fixed amount of 125-I labelled β2M for the binding sites on a sheep anti-β2M antibody. Bound and free β2M were separated by the addition of horse anti-sheep IgG coupled to sepharose, followed by centrifugation and decantation of the supernatant containing the free β2M. The radioactivity in the remaining pellet was inversely proportional to the quantity of β2M in the unknown sample. The normal range was ≤2.4 mg/l in patients aged under 60 years and ≤3 mg/l in those aged 60 years or over.

Serum Thymidine Kinase

STK was determined by radioenzyme assay, using an in-house method which was based on that used in the Prolifigen radioenzyme kit (Sangtec Medical, Sweden). 5-[^{125}I]Iodo-2-deoxyuridine (Amersham International, Amersham, Buckinghamshire, England) acted as a substrate which was phosphorylated by thymidine kinase in the patient's serum. Phosphorylated nucleotides were then bound to ion-exchange cellulose paper and the unbound labelled nucleotide was washed away using dilute ammonium formate. The amount of bound nucleotide was counted on a gamma counter. The thymidine kinase concentration in the patient's serum was calculated from a calibration curve. The results were expressed as units per litre of serum, where 1 U is defined as 1.2×10^{-12} katal. The normal range was ≤ 5 U/l.

Serum Immunoglobulin Levels

Serum immunoglobulins (IgA, IgG and IgM) were precipitated with the appropriate rabbit anti-human immunoglobulin antiserum (F. Hoffmann-La Roche Ltd. Diagnostics Division, Basel, Switzerland). Quantitation of the serum immunoglobulins was then determined turbidimetrically by the fixed time method at 340 nm using a Cobas Fara II analyser (Roche Diagnostics, Basel, Switzerland). The normal ranges were 0.6-4.0 g/l for IgA, 6.5-15.0 g/l for IgG and 0.5-3.2 g/l for IgM.

Labelling Index of Bone Marrow Plasma Cells

The LI of the bone marrow plasma cells was determined using the method described by Greipp et al (1985). Bone marrow samples were collected into 1 ml RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) containing 100 U preservative free porcine heparin sodium (Fisons Pharmaceuticals, Thornleigh,

NSW, Australia). A single cell suspension was prepared and the mononuclear cell fraction was obtained by Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, New Jersey) separation. After washing 3 times in RPMI 1640, the cells were resuspended at 10^7 cells/ml. The method from this point has already been described in section 2.2.7. However, in this case, the staining with anti-immunoglobulin light chain reagents enabled the plasma cells to be identified among the various cell types present in the preparation. The slides were examined using an Axioscope 20 microscope (Zeiss, West Germany) equipped with an HBO 50 high pressure mercury lamp as the light source for incident light fluorescence (epi-fluorescence) and filter sets suitable for detecting FITC and TRITC fluorescence. Five hundred plasma cells (cIg^+ cells) were counted and the LI calculated as the percentage of cells in S-phase.

3.2.4: Statistical Analysis

Patient Groupings

The analysis was performed by grouping the patients in two different ways: the first set of subgroups was based on disease status, namely normal controls, MGUS, SMM, active myeloma at diagnosis, myeloma improving on therapy, myeloma in plateau phase and myeloma with progressive disease; the second set was based on the number of $CD38^+$ PBL (subsequently referred to as "CD38 status"), namely normal controls, MGUS, untreated patients (SMM and at diagnosis) with elevated $CD38^+$ cell numbers, untreated patients without elevated $CD38^+$ cell numbers, treated patients (on treatment, plateau phase and progressive) with elevated $CD38^+$ cell numbers and untreated patients without elevated $CD38^+$ cell numbers. Patients who had either a relative or absolute number of $CD38^+$ PBL above the upper limit of the normal range were considered to have elevated numbers in the latter analysis. Two patients with

progressive myeloma were excluded from the statistical analysis: one had plasma cell leukaemia and the other concurrent lung carcinoma. These patients are discussed in the results section.

Statistical Methods

Analysis was performed with the results expressed as both a percentage and an absolute number per litre. The normality of the data sets was determined using the normal probability plot correlation coefficient (Filliben, 1975). The majority of the data sets were not normal distributions, so non-parametric statistical techniques were employed and the data were summarised as median (range). When more than two groups are being compared with respect to any given variable, as in this study, it is not statistically valid to perform a test of significance between each possible pairing. It is necessary to initially perform a test which analyses all groups simultaneously to determine if any one group comes from a different population to the other groups. If this test is significant, then it is permissible to perform a test of significance between paired groups of interest (Daniel, 1978; Rimm et al, 1980). In this study the Kruskal-Wallis one-way analysis of variance by ranks (two-tailed) was used to simultaneously compare the differences in the expression of each lymphocyte antigen by the various subgroups. If this test was statistically significant for a particular lymphocyte subset, then comparisons between the normal controls and each of the subgroups were performed using the Mann-Whitney-U test (two-tailed). In certain instances, comparisons between patient groups were also performed. As there were few patients in some subgroups, Fisher's exact test, rather than the χ^2 test, was used to compare the difference between two proportions (Rimm et al, 1980). Correlation analysis was performed using the Spearman rank correlation test. Results were considered statistically significant if $p \leq 0.05$. These

analyses were performed using the statistical software packages Abstat version 4.05 (Anderson-Bell Co.) and Minitab version 8.2 (Minitab Inc., State College, PA, USA).

3.3: RESULTS

3.3.1: CD38 Antigen Expression on Peripheral Blood Lymphocytes

Based on the surface antigen expression of the $CD38^+$ cells and the fluorescence intensity of the CD38 antigen on the cells of the controls and patients in comparison to that on myeloma cells (table 3.5), the cells in the peripheral blood expressing the CD38 antigen were lymphocytes and not plasma cells.

Table 3.5: Mean Fluorescence Intensity of CD38 Antigen Expression on Different Populations of Cells*

Peripheral Blood Cells of Controls	Peripheral Blood Cells of Patients	Bone Marrow Myeloma Cells†
3.23 (2.47--4.19)‡	3.49 (1.93-6.72)	197.3 (54.1-416.3)

*all tests were done using the same PE-conjugated anti-CD38 antibody and the same flow cytometer;
†the bone marrow myeloma cells were not from the same patients as the peripheral blood cells;
‡results expressed as median (range)

In the control group, the median values for the number of $CD38^+$ lymphocytes were $0.84x10^9/l$ with a range of $0.52\text{-}1.00x10^9/l$ and 50.1% with a range of 39.0-62.2% when the results were expressed in absolute and relative numbers respectively. These normal values are higher than those previously reported in the literature but the values in those studies have been quite variable. An upper limit of $0.15x10^9/l$ for the OKT10 antibody was used by Joshua et al (1988). Omedé et al (1990), using the HAN-PC1 antibody, established a median value of $0.24x10^9/l$ with a range of $0.04\text{-}0.48x10^9/l$. Gonzalez et al (1992) reported values of $0.47\pm0.18x10^9/l$ (mean±SD) and 18±5% for absolute and relative numbers respectively. Boccadoro et al (1988) found values of 8.7±6.2% (mean±SD) for the TEC-T10 antibody and 10.8±6.7% for HAN-PC1. In the IVth Leucocyte Typing

Workshop (Pezzutto et al, 1989), the mean percentages of peripheral blood lymphocytes and T cells expressing CD38 were 36% and 31% respectively.

Jackson et al (1990) encountered and investigated a similar phenomenon when studying the expression of the CD25 antigen by PBL. In their study, the mean number of $CD25^+$ cells was 31% with a range of 18-38% and these values are very similar to those obtained in this study (table 3.20). Earlier studies had usually found that <10% of PBL express the CD25 antigen. Multiple technical reasons were found to account for this increase in the number of $CD25^+$ PBL over time. Modern flow cytometers have more sensitive photomultiplier tubes. In our laboratory, the normal range for $CD38^+$ PBL was higher using a modern flow cytometer (Coulter Epics Profile II) than using an older machine (Ortho Spectrum III) (unpublished data). Furthermore, even among modern flow cytometers, there were marked differences in the number of cells detected as expressing the CD25 antigen. Differences were also noted for the same antibody clone labelled with different fluorochromes, with PE-conjugates giving higher values than FITC-conjugates.

The expression of the CD38 antigen by PBL in this study was complex in pattern as defined in the IIIrd Leucocyte Typing Workshop (Gotch, 1987). Thus, although there was a significant percentage of positive cells, the positive and negative populations were not clearly separable. This pattern of expression has been noted previously for the CD38 antigen (Cobbold et al, 1987). Consequently, the normal range will vary depending on the setting of the positive analysis region on the negative control.

To examine the variation in the number of $CD38^+$ PBL obtained with different anti-CD38 reagents, the percentage of $CD38^+$ cells in 5 control subjects and 7 patients was determined on the same sample using 3 different antibodies: (1) OKT10 detected by indirect immunofluorescence with a FITC-conjugated second layer antibody; (2) HB-7 directly conjugated to PE; (3) T16 directly conjugated to FITC. The results are shown in table 3.6. Usually, the lowest percentage of $CD38^+$ cells was obtained with the OKT10 antibody, whereas the highest was with the T16 antibody. Eleven of the 12 subjects had a higher percentage with the HB-7 than the OKT10 antibody. Ten had higher values with FITC-conjugated T16 than PE-conjugated HB-7. All subjects had a higher percentage of $CD38^+$ PBL detected by the T16 compared to the OKT10 antibody.

Table 3.6: Comparison of the Percentage of $CD38^+$ PBL Detected by Different Monoclonal Antibodies.

	OKT10	HB-7	T16
Normal	42.0	58.1	43.8
Normal	45.8	46.1	68.6
Normal	48.6	60.7	64.4
Normal	48.8	53.1	55.5
Normal	51.8	55.6	74.4
Diagnosis	43.1	39.0	44.3
Diagnosis	59.8	71.4	82.7
Progressive	61.4	74.3	81.0
Diagnosis	63.5	72.5	80.3
Diagnosis	64.2	72.4	81.8
Carcinoma with paraprotein	66.2	71.8	76.7
Improving on treatment	73.8	79.3	85.6

For any given antigen, differences in the percentage of positive cells or the fluorescence intensity of the antigen on positive cells may be due to (i) the binding of the antibodies to different epitopes, (ii) varying affinities of the antibodies for the same epitope or (iii) the use of different fluorochromes to detect the bound antibodies. For the CD38 antigen with its complex pattern of

expression, these factors will lead to differences in both the percentage of positive cells and the fluorescence intensity of the antigen on positive cells. For a molecule such as CD4 which has distinct negative and positive populations, only the fluorescence intensity of expression is likely to be affected. The differences due to the binding of antibodies to different epitopes may be increased by the presence of structural polymorphism linked to the expression on different cell populations but the CD38 molecule does not exhibit such polymorphism (Alessio et al, 1990). The antibodies HB-7 and T-16 have been shown to react with the same or closely associated epitopes but OKT10 was not included in the study (Schwartz-Albiez and Moldenhauer, 1989). For the OKT10 antibody, the differences in the percentage cells detected as positive compared to the other antibodies may have been due to any of the factors outline above. For the T16 and HB-7 antibodies, the differences are likely to have been due to varying affinities for the same epitope as, if all other factors are equal, PE-conjugates give higher values than FITC-conjugates.

The complex pattern of CD38 expression and the fact that various lymphocyte subsets express the CD38 antigen at different intensities mean that the differences between individuals for any two of the anti-CD38 antibodies will not be constant. In a subject with a large proportion of lymphocytes expressing lower levels of this antigen, the variation will be greater than in a patient in whom the majority of cells expressing higher levels of the antigen.

3.3.2: Identity of Lymphoid Cells Expressing the CD56 Antigen

The expression of CD16 and CD56 by PBL was included in this study as both these antigens are expressed by NK cells. However, there was a significant difference between groups for the $CD56^+$ and $CD56^+38^+$ but not the $CD16^+$ and

$CD16^+38^+$subsets. These findings raise the issue of the identity of the cells expressing CD56 and especially those co-expressing CD56 and CD38. CD38 is expressed by multiple cell lineages including normal and neoplastic plasma cells (Hercend et al, 1981; Bhan et al, 1981; Foon et al, 1982; Aisenberg and Wilkes, 1983; Bhan et al, 1984; Hsu and Jaffe, 1984; Tedder et al, 1984) and 60-90% of NK cells (Ortaldo et al, 1981; Ritz et al, 1988; Robertson et al, 1990). The intensity of CD38 is an order of magnitude higher on plasma cells in comparison to other cells and this has been exploited to study the phenotype of both malignant and normal plasma cells, even when they are present only in low numbers (Terstappen et al, 1990; Harada et al, 1992). On the lymphocytes of normal individuals, CD56 is expressed by >95% of all NK cells and by a subpopulation of T cells which constitute <5% of PBL (Lanier et al, 1986; Schmidt et al, 1986). Approximately 10% of NK cells are $CD16^-56^+$ (Lanier et al, 1986). However, CD56 is strongly expressed by the majority of plasma cells in cases of multiple myeloma but is either absent or occasionally weakly expressed by normal plasma cells (Van Camp et al, 1990; Drach et al, 1991; Van Riet et al, 1991; Barker et al, 1992a; Leo et al, 1992). CD56 has not yet been identified on any putative precursor populations. CD16 is a marker of NK cells that is not expressed on myeloma cells (Van Camp et al, 1990; Drach et al, 1991; Leo et al, 1992). Thus, there are three alternative $CD56^+$ populations, which may contain varying proportions of cells co-expressing CD38: $CD3^+16^-56^+$ T cells; $CD3^-16^-56^+$ NK cells; or neoplastic $CD56^+$ plasma cells or their precursors. To accurately discriminate $CD3^+16^-56^+$ T cells from the other two possibilities would require a triple labelling procedure to study the co-expression of CD3, CD56 and CD38 on lymphoid cells.

Determining whether the $CD56^+$ and in particular the $CD56^+38^+$ cells are neoplastic cells is more problematical. The percentage of $CD56^+38^+$ cells in the normal controls was 5.5% (3.3-10.7%) and in the patients was 12.0% (3.9-31.1%). Thus the $CD56^+38^+$ cells constitute a significant proportion of PBL but none of the patients had circulating plasma cells as determined by examining a stained blood film. This implies that if a large proportion of the $CD56^+38^+$ cells are neoplastic, then they are not plasma cells but precursor myeloma cells that are morphologically indistinguishable from normal lymphocytes. More convincing is data based on the fluorescence intensity of the CD38 antigen on $CD16^+$ and $CD56^+$ PBL (table 3.7).

Table 3.7: Fluorescence Intensity of the CD38 Antigen on $CD3^+38^+$, $CD16^+38^+$ and $CD56^+38^+$ PBL Compared to Bone Marrow Plasma Cells

	Peripheral Blood			Bone Marrow* Plasma Cells
	CD3	CD16	CD56	
Normals	2.56 (2.11-3.24)† n=10	6.43 (3.93-10.18) n=10	6.04 (2.82-10.23) n=10	312.4 n=1
Patients	2.49 (1.43-5.20) n=43 NS	6.69 (2.51-30.73) n=42 NS	6.84 (2.89-22.63) o>39 NS	197.3 (54.1-416.3)‡ n=13

NS-not significant; *The bone marrow samples were not obtained from the same normals and patients as the peripheral blood samples; †Results are expressed as median (range); ‡In only 2/13 patients was the mean fluorescence intensity of bone marrow plasma cells <100.

Within the normal group, the fluorescence intensity of CD38 on T ($CD3^+$) cells was significantly lower than that on either $CD16^+$ or $CD56^+$ PBL ($p<0.0001$ and $p=0.0004$ respectively) but was not significantly different on $CD16^+$ compared to $CD56^+$ cells. The same pattern was present in the patient group ($p<0.0001$ for both $CD16^+$ and $CD56^+$ cells compared to $CD3^+$ PBL). The intensity of CD38 on CD56+ cells was not significantly different in the controls compared to the patients. This was also the case for $CD16^+$ PBL. There was a strong correlation between the intensity of CD38 on $CD16^+$ and $CD56^+$ cells in the patient group ($r_s=0.91$, $p<0.001$). In ten of the 39 patients the intensity of the CD38 antigen

on $CD56^+$ cells was higher than in any of the controls but the intensity was clearly lower than that documented on plasma cells using the same PE-conjugated anti-CD38 antibody and flow cytometer (see table 3.7). This data on the intensity of CD38 on $CD56^+$ PBL suggests that the majority of $CD56^+38^+$ cells in peripheral blood are not neoplastic plasma cells. However, it does not exclude the possibility that a small number of the $CD56^+38^+$ cells are neoplastic precursor cells, if it is assumed that the intensity of CD38 on such cells is much lower than on the malignant plasma cells. However, CD56 has not yet been demonstrated on putative precursor populations by any other research groups.

3.3.3: Analysis of Results by Disease Status

The p values obtained for each lymphocyte subset using the Kruskal-Wallis analysis of variance when the patients were divided into subgroups on the basis of disease status are detailed in table 3.8. Results were considered significant if $p \leq 0.05$ but, in this and subsequent tables, the exact p values are also given if there was a trend towards significance ($p \leq 0.1$ but >0.5).

CD38 Antigen Expression on Lymphocytes (Table 3.9)

The percentage and absolute number of $CD38^+$ PBL were not statistically different from the normals at any phase of the disease. All patients with MGUS had a normal percentage and absolute number of $CD38^+$ lymphocytes. The number of $CD38^+$ cells was quite variable in the other groups, with patients having values below and above the normal range, irrespective of whether the results were expressed as a percentage or absolute number. The existence of patients with decreased numbers of $CD38^+$ PBL has not been noted previously. The higher values for the number of positive lymphocytes in normal controls in this study than in previous studies may have allowed this phenomenon to

Table 3.8: Results (p values) of Kruskal-Wallis Analysis of Variance for Patient Groups Based on Disease Status.

Lymphocyte Subset	%	A	Lymphocyte Subset	%	A
Lymphocyte Count	<0.06		$CD38^+$	NS	NS
$CD1a^+$	NS	NS	$CD1a^+38^+$	NS	NS
$CD2^+$	NS	**<0.05**	$CD2^+38^+$	NS	NS
$CD3^+$	NS	<0.06	$CD3^+38^+$	NS	NS
$CD4^+$	**0.003**	**0.001**	$CD4^+38^+$	**0.02**	**0.008**
$CD8^+$	**<0.03**	NS	$CD8^+38^+$	**<0.04**	NS
$CD16^+$	NS	NS	$CD16^+38^+$	NS	NS
$CD56^+$	<0.06	NS	$CD56^+38^+$	**<0.04**	NS
CD4:CD8 Ratio	**0.004**		κ:λ Ratio	NS	
$CD19^+$	**0.007**	**0.005**	$CD19^+38^+$	**<0.02**	**0.008**
$CD20^+$	**<0.02**	**0.01**	$CD20^+38^+$	<0.06	<0.06
$CD21^+$	**<0.02**	**<0.02**	$CD21^+38^+$	**<0.02**	**<0.02**
$CD22^+$	**0.007**	**0.01**	$CD22^+38^+$	**0.02**	**<0.02**
$CD23^+$	**<0.04**	**<0.05**	$CD23^+38^+$	**<0.02**	**<0.03**
$CD24^+$	**0.009**	**<0.02**	$CD24^+38^+$	**<0.03**	**0.009**
$CD39^+$	**<0.04**	**<0.04**	$CD39^+38^+$	<0.09	<0.06
$HLA\text{-}DR^+$	NS	NS	$HLA\text{-}DR^+CD38^+$	NS	NS
$CD25^+$	NS	**0.006**	$CD25^+38^+$	<0.1	**0.002**
$CD9^+$	NS	NS	$CD9^+38^+$	<0.09	NS
$CD71^+$	NS	NS	$CD71^+38^+$	NS	NS
$PCA1^+$	NS	NS	$PCA1^+CD38^+$	NS	NS
$CD10^+$	NS	NS	$CD10^+38^+$	NS	NS
$CD13^+$	NS	NS	$CD13^+38^+$	NS	NS
$CD33^+$	NS	NS	$CD33^+38^+$	NS	NS
$CD34^+$	NS	NS	$CD34^+38^+$	NS	NS
$CD36^+$	NS	NS	$CD36^+38^+$	NS	NS
$CD11a^+$	**0.002**	**<0.04**	$CD11a^+38^+$	NS	NS
$CD18^+$	<0.07	**<0.05**	$CD18^+38^+$	NS	NS
$CDw49d^+$	<0.1	<0.08	$CDw49d^+38^+$	NS	NS
$CD54^+$	NS	NS	$CD54^+38^+$	<0.1	NS
$CD44^+$	NS	<0.09	Intensity of $CD11a^+$ Cells*	**<0.02**	
Intensity of $CD18^+$ Cells	**<0.02**		Intensity of $CD44^+$ Cells	NS	
Intensity of $CDw49d^+$ Cells	**<0.05**		Intensity of $CD54^+$ Cells	<0.07	
Serum ß2 Microglobulin	NS		Serum Thymidine Kinase	NS	
Labelling Index	NS		Serum IgM	NS	

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$);
NS-not significant; *fluorescence intensity of adhesion molecule expression.

Table 3.9: Lymphocyte Count and CD38 Antigen Expression on PBL for Patient Groups Based on Disease Status.

		Normals 10 subjects	MGUS 3 subjects	SMM 6 subjects	Diagnosis 11 subjects	On Treatment 5 subjects	Plateau 12 subjects	Progressive
Lymphocytes ($x10^9$/L)		1.7 (1.3-2.2)	1.5 (1.5-1.8)	2.0 (1.9-2.9)	1.5 (0.5-2.5)	1.4 (0.9-1.5)	1.4 (0.5-1.8)	1.1 (0.4-2.7)
$CD38^+$	%	50.1 (39.0-62.2)	53.1 (40.0-56.1)	35.3 (25.5-53.6)	60.3 (29.1-70.8)	46.0 (16.5-73.5)	57.6 (17.5-70.7)	55.0 (21.7-70.3)
	A	0.84 (0.52-1.0)	0.84 (0.60-0.96)	0.74 (0.63-1.02)	0.88 (0.15-1.68)	0.41 (0.23-1.03)	0.53 (0.26-1.18)	0.50 (0.18-1.47)

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9$/l); there were no significant differences between groups.

become apparent. A more detailed discussion of these changes will be presented when the results are analysed on the basis of $CD38^+$ cell numbers.

Lymphocyte Counts (Table 3.9)

The Kruskal-Wallis test showed a trend towards a significant difference in the lymphocyte counts of the various groups ($p<0.06$). The patients with progressive disease had a median lymphocyte count below the normal range. No patient with MGUS or SMM had a lymphocyte count below the normal range but lymphopenia was documented in 5/11 patients at diagnosis, 2/5 currently receiving therapy, 2/7 in plateau phase and 7/12 with progressive disease. Two of the 5 newly diagnosed patients with low lymphocyte counts had received steroids prior to the study.

T Lymphocytes and NK Cells (Table 3.10)

Lymphocytes expressing T cell antigens and the ratio of CD4:CD8 lymphocytes were normal in patients with MGUS but there was an increase in the percentage of $CD56^+38^+$ cells. In SMM, there were decreases in the proportion of $CD4^+$ and $CD4^+38^+$ lymphocytes but normal absolute numbers. There was a trend towards a decreased CD4:CD8 ratio ($p<0.06$).

In newly diagnosed patients, the $CD4^+$, $CD4^+38^+$, $CD8^+$ and $CD8^+38^+$ subsets were normal for both the percentage and absolute results but the ranges for most of these subsets were wider than in the normal controls, suggesting heterogeneity of expression in this group. The CD4:CD8 ratio was normal but the range was wider than in the normals, again emphasising the heterogeneity within this group. There was a trend towards an increased percentage of $CD56^+38^+$ lymphocytes.

Table 3.10: T Cell and NK Cell Antigen Expression on PBL for Patient Groups Based on Disease Status.

		Normals	MGUS	SMM	Diagnosis	On Treatment	Plateau	Progressive
$CD2^+$	A	1.42 (0.96-1.96)	1.35 (1.26-1.58) NS	1.72 (1.52-2.71) **p<0.05**	1.24 (0.43-2.01) NS	1.01 (0.72-1.29) NS	1.24 (0.44-1.31) p<0.08	0.93 (0.28-2.16) **p<0.04**
	No.	10	3	5	11	2	5	10
$CD4^+$	%	55.3 (44.3-69.2)	59.9 (51.5-63.7) NS	44.1 (30.9-58.3) **p<0.03**	52.3 (27.0-72.6) NS	38.7 (20.7-47.8) **p<0.006**	39.6 (14.9-58.5) **p<0.02**	37.0 (21.4-54.5) **p=0.0005**
	A	0.92 (0.69-1.21)	0.96 (0.77-1.08) NS	0.88 (0.59-1.46) NS	0.58 (0.20-1.63) NS	0.42 (0.29-0.67) **p<0.003**	0.40 (0.21-0.88) **p<0.003**	0.45 (0.13-0.95) **p=0.0004**
	No.	10	3	6	11	5	7	12
$CD4^+38^+$	%	23.9 (12.0-41.4)	23.4 (19.2-24.0) NS	12.0 (8.0-32.6) **p<0.04**	16.9 (6.2-52.4) NS	11.0 (7.4-18.6) **p<0.02**	12.2 (4.6-21.8) **p<0.03**	13.0 (3.5-25.4) **p<0.02**
	A	0.42 (0.16-0.63)	0.36 (0.29-0.42) NS	0.24 (0.21-0.62) NS	0.27 (0.07-0.88) NS	0.12 (0.10-0.28) **p<0.006**	0.14 (0.06-0.37) **p<0.003**	0.13 (0.07-0.41) **p=0.004**
	No.	10	3	6	11	5	7	10
$CD8^+$	%	28.7 (14.4-35.6)	19.0 (17.7-31.4) NS	35.8 (20.9-52.5) NS	28.0 (10.8-50.9) NS	32.4 (26.2-73.3) NS	44.5 (28.9-53.5) **p<0.03**	36.7 (25.3-65.9) **p=0.005**
	No.	10	3	6	11	5	7	12
$CD8^+38^+$	%	8.0 (3.6-18.7)	15.4 (4.6-19.5) NS	10.5 (7.4-15.4) NS	12.0 (2.7-18.6) NS	19.2 (7.8-57.2) **p<0.04**	18.0 (8.5-42.8) **p<0.02**	19.2 (8.2-38.4) **p<0.005**
	No.	10	3	6	11	5	7	11
$CD56^+38^+$	%	5.5 (3.3-10.7)	14.5 (9.4-17.8) **p<0.04**	8.1 (6.4-13.5) NS	10.2 (3.9-22.9) p<0.08	16.7 (4.6-28.8) NS	8.0 (5.5-12.2) NS	14.4 (5.4-31.1) **p<0.003**
	No.	10	3	6	11	2	6	11
CD4:CD8 Ratio		1.92 (1.27-4.42)	3.15 (1.64-3.60) NS	1.13 (0.71-2.79) p<0.06	2.09 (0.53-6.03) NS	1.19 (0.28-1.82) **p<0.02**	1.01 (0.28-1.79) **p=0.004**	0.97 (0.42-2.15) **p<0.002**
	No.	10	3	6	11	5	7	12

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); No.-number of subjects tested; NS-not significant.

There were decreases of both the proportion and absolute number of $CD4^+$ and $CD4^+38^+$ lymphoid cells in patients on therapy, in plateau phase or with progressive disease. The relative numbers of $CD8^+$ and $CD8^+38^+$ cells were significantly increased in plateau phase and progressive disease but, in patients who were currently receiving therapy, only the percentage of $CD8^+38^+$ cells was increased. In all the groups of treated patients, there was a decrease in the CD4:CD8 ratio. The patients with progressive disease had an increased percentage of $CD56^+38^+$ cells, whereas it was normal in those on treatment or in plateau phase.

B Lymphocytes (Table 3.11)

There were significant differences between groups for the majority of B cell subsets. The exceptions were the $CD20^+38^+$ and $CD39^+38^+$ subsets, as well as the $\kappa:\lambda$ ratio.

The CD39 antigen is expressed by 12-17% of PBL (Pezzutto et al, 1989), with the B cells identified as those expressing this antigen. In this study, the pattern of changes in the expression of the CD39 antigen usually reflected that of the other B cell antigens, whether the patient groups were based on disease status or $CD38^+$ cell numbers, but the median values and ranges for the $CD39^+$ and $CD39^+38^+$ populations were higher than for the other B cell antigens. Although not previously documented, it seems likely that low levels of the CD39 antigen were being detected on lymphocytes other than B cells as a result of the increasingly sensitive technology discussed previously. Possible candidates are activated T cells or NK cells. These cells have been stimulated *in vitro* to express

Table 3.11: B Cell Antigen Expression on PBL for Patient Groups Based on Disease Status.

		Normals	MGUS	SMM	Diagnosis	On Treatment	Plateau	Progressive
$CD19^+$	%	9.6 (8.3-14.3)	3.8 (3.6-8.2) **p<0.02**	5.6 (3.4-8.5) **p=0.002**	5.3 (3.2-16.1) **p<0.05**	2.1 (1.3-6.3) **p<0.003**	4.6 (1.7-23.6) p<0.07	3.6 (1.8-15.9) **p<0.007**
	A	0.16 (0.12-0.27)	0.06 (0.06-0.12) **p<0.02**	0.12 (0.06-0.16) **p<0.04**	0.06 (0.03-0.40) **p<0.05**	0.03 (0.01-0.09) **p<0.003**	0.05 (0.02-0.42) **p<0.02**	0.05 (0.01-0.28) **p<0.007**
	No.	10	3	6	11	5	7	12
$CD19^+38^+$	%	7.1 (5.5-8.8)	2.7 (2.5-3.9) **p<0.02**	3.1 (1.9-6.5) **p=0.004**	3.4 (1.7-12.2) p<0.08	1.2 (0.5-4.9) **p<0.003**	3.1 (0.8-19.1) NS	2.1 (0.8-14.0) **p<0.009**
	A	0.11 (0.08-0.17)	0.05 (0.04-0.06) **p<0.02**	0.07 (0.04-0.12) **p=0.01**	0.04 (0.01-0.31) p<0.07	0.01 (0.01-0.07) **p<0.003**	0.03 (0.01-0.34) **p<0.02**	0.02 (0.01-0.18) **p<0.02**
	No.	10	3	6	11	5	7	11
$CD20^+$	%	9.2 (7.2-14.0)	4.9 (4.4-8.2) **p<0.04**	6.1 (4.1-8.5) **p<0.009**	5.8 (3.2-13.4) NS	2.8 (1.7-5.8) **p<0.003**	4.8 (1.7-18.8) p<0.07	3.4 (2.6-14.8) **p<0.02**
	A	0.15 (0.11-0.27)	0.09 (0.07-0.12) **p<0.04**	0.12 (0.08-0.16) NS	0.08 (0.03-0.24) NS	0.04 (0.02-0.09) **p<0.003**	0.05 (0.02-0.34) **p<0.02**	0.06 (0.01-0.25) **p<0.009**
	No.	10	3	5	8	5	7	11
$CD21^+$	%	10.0 (7.6-12.7)	4.5 (3.2-5.8) **p<0.02**	6.6 (4.0-8.2) **p<0.006**	6.2 (2.9-17.6) NS	3.4 (1.5-6.5) **p<0.003**	6.0 (2.1-25.0) **p<0.04**	3.5 (1.8-14.9) **p<0.006**
	A	0.17 (0.11-0.24)	0.08 (0.05-0.09) **p<0.02**	0.13 (0.08-0.16) NS	0.08 (0.02-0.44) NS	0.05 (0.01-0.10) **p<0.003**	0.05 (0.03-0.45) **p<0.02**	0.05 (0.02-0.23) **p<0.006**
	No.	10	3	5	8	5	7	11
$CD21^+38^+$	%	7.1 (5.2-8.9)	3.2 (2.1-3.5) **p<0.02**	2.3 (2.1-5.7) **p<0.006**	3.9 (1.6-13.2) NS	1.8 (0.9-5.9) **p=0.004**	3.3 (1.2-21.8) p<0.1	2.0 (0.8-13.2) **p<0.007**
	A	0.11 (0.09-0.17)	0.05 (0.03-0.06) **p<0.02**	0.07 (0.04-0.11) p<0.06	0.06 (0.01-0.33) NS	0.02 (0.01-0.09) **p<0.004**	0.03 (0.02-0.39) **p<0.02**	0.02 (0.01-0.17) **p<0.02**
	No.	10	3	5	8	5	7	11
$CD22^+$	%	10.1 (8.4-12.4)	7.4 (4.1-9.0) **p<0.03**	6.3 (3.2-8.3) **p<0.003**	5.6 (4.5-15.1) NS	3.0 (1.6-5.4) **p<0.003**	4.4 (2.0-23.6) **p<0.03**	3.5 (1.7-14.3) **p<0.009**
	A	0.17 (0.13-0.24)	0.13 (0.06-0.14) NS	0.13 (0.07-0.16) p<0.09	0.10 (0.03-0.38) NS	0.04 (0.01-0.08) **p<0.003**	0.05 (0.03-0.42) **p<0.02**	0.04 (0.02-0.25) **p=0.008**
	No.	10	3	5	8	5	7	11

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); No.-number of subjects tested; NS-not significant.

Table 3.11: Continued.

		Normals	MGUS	SMM	Diagnosis	On Treatment	Plateau	Progressive
CD22$^+$38$^+$	%	6.7 (5.3-9.2)	5.6 (3.2-6.3) p<0.07	2.3 (1.8-5.7) **p<0.008**	3.8 (1.9-11.0) NS	2.0 (0.8-4.5) **p<0.004**	3.1 (1.2-20.1) P<0.1	3.1 (0.5-12.4) **p<0.008**
	A	0.12 (0.09-0.16)	0.08 (0.05-0.11) p<0.06	0.05 (0.04-0.11) **p<0.03**	0.07 (0.01-0.28) NS	0.02 (0.01-0.07) **p<0.004**	0.04 (0.02-0.36) **p<0.02**	0.03 (0.01-0.16) **p<0.02**
	No.	9	3	5	8	5	7	10
CD23$^+$	%	8.0 (6.2-11.6)	4.7 (2.6-5.7) **p<0.02**	3.3 (2.6-5.0) **p<0.006**	4.2 (2.5-13.9) NS	2.6 (0.6-7.1) **p<0.006**	4.0 (2.8-20.6) **p<0.04**	3.7 (2.0-12.8) **p<0.03**
	A	0.14 (0.09-0.22)	0.08 (0.04-0.09) **p<0.03**	0.08 (0.05-0.10) **p<0.04**	0.06 (0.02-0.35) NS	0.04 (0.01-0.11) **p<0.009**	0.05 (0.03-0.37) **p<0.02**	0.05 (0.01-0.25) **p=0.008**
	No.	10	3	4	8	5	7	11
CD23$^+$38$^+$	%	6.3 (4.9-8.9)	3.2 (1.8-3.7) **p<0.02**	1.6 (1.4-3.3) **p<0.006**	3.1 (0.9-11.7) NS	1.1 (0.3-5.8) **p<0.009**	2.8 (1.4-18.4) p<0.08	2.0 (0.7-12.0) **p<0.02**
	A	0.11 (0.07-0.17)	0.05 (0.03-0.07) **p<0.03**	0.04 (0.03-0.07) **p<0.009**	0.05 (0.01-0.29) NS	0.02 (0.00-0.09) **p<0.009**	0.03 (0.02-0.33) **p<0.02**	0.02 (0.01-0.17) **p<0.02**
	No.	10	3	4	8	5	7	11
CD24$^+$	%	9.7 (7.7-13.2)	4.9 (4.4-7.7) **p<0.02**	6.6 (3.8-9.3) **p<0.02**	6.1 (3.7-15.0) NS	2.9 (1.5-5.6) **p<0.003**	4.7 (2.2-20.7) **p<0.04**	3.5 (2.9-14.8) **p<0.02**
	A	0.16 (0.11-0.25)	0.09 (0.07-0.12) **p<0.03**	0.14 (0.07-0.18) NS	0.08 (0.03-0.38) NS	0.04 (0.01-0.08) **p<0.003**	0.04 (0.03-0.37) **p<0.02**	0.06 (0.01-0.26) **p=0.009**
	No.	10	3	5	8	5	7	11
CD24$^+$38$^+$	%	6.5 (4.7-9.0)	3.6 (3.3-3.9) **p<0.02**	2.4 (2.0-7.2) **p<0.05**	4.3 (1.3-11.0) NS	1.6 (0.8-4.2) **p<0.003**	2.5 (1.3-15.5) NS	2.1 (0.9-12.3) **p<0.003**
	A	0.11 (0.06-0.15)	0.06 (0.05-0.06) **p<0.03**	0.06 (0.04-0.14) NS	0.05 (0.01-0.28) NS	0.01 (0.01-0.06) **p=0.003**	0.03 (0.02-0.28) **p<0.02**	0.04 (0.00-0.16) **p<0.003**
	No.	10	3	5	8	5	7	11
CD39$^+$	%	17.8 (10.9-20.8)	11.2 (7.8-11.9) **p<0.04**	10.1 (6.7-15.7) **p<0.02**	12.5 (4.9-17.5) **p<0.03**	9.3 (4.8-13.6) **p<0.009**	17.9 (8.6-31.1) NS	14.6 (4.6-22.5) p<0.1
	A	0.29 (0.14-0.35)	0.17 (0.14-0.18) **p<0.05**	0.26 (0.13-0.31) NS	0.15 (0.04-0.36) NS	0.11 (0.04-0.19) **p<0.005**	0.18 (0.12-0.51) p<0.08	0.15 (0.03-0.44) **p=0.009**
	No.	10	3	5	8	5	7	11

%-results expressed as a percentage; A-results expressed as an absolute number (x10^9/l); No.-number of subjects tested; NS-not significant.

CD39 as a late appearing and persistent activation antigen (Gregory et al, 1987; Aversa and Hall, 1989; Kansas et al, 1991).

Most B cell subsets were significantly decreased in MGUS. All patients had a decreased percentage and absolute number of $CD19^+$ lymphocytes. None had a $\kappa:\lambda$ ratio outside the normal range.

In SMM, all the B cell populations were significantly decreased compared to the controls in the analysis of percentage results but this was the situation for only half the subsets in the absolute analysis, as these patients had lymphocyte counts either in the high normal range or above it. Five and 3 of the 6 patients in the percentage and absolute analyses respectively had $CD19^+$ cell numbers below the normal range. The remainder had normal numbers. All patients in this group had a normal $\kappa:\lambda$ ratio.

In patients with active disease at diagnosis, there were decreases in the relative and absolute numbers of $CD19^+$ cells with a trend in that direction for the $CD19^+38^+$ cells. Eight of the 11 patients had a low proportion and absolute number of $CD19^+$ cells. Despite the fact that the median values for the subsets delineated by the other B cell antigens were lower than those in the normals, the differences did not reach the level of significance. This was probably due to the fact that CD19 was the only B cell antigen studied in 3 patients and these patients all had $CD19^+$ and $CD19^+38^+$ cells below the normal range for both the percentage and absolute results. Only one patient had an abnormal $\kappa:\lambda$ ratio and this indicated the presence of LCIS.

In the analysis of absolute results, the B cell populations were decreased in patients on treatment, in plateau or with progressive disease. In the percentage analysis, all the B cell subsets were decreased in those individuals on therapy or with progressive disease. In the patients in plateau phase, the median values for the B cell populations, with the exception of the $CD39^+$ subset, were lower than in the controls but there were significant decreases in only 4/12 B cell subsets with a trend in the same direction in a further 5/12 subsets. All patients currently receiving treatment had relative and absolute $CD19^+$ cell numbers below the normal range. For the percentage and absolute results respectively, 5 and 6/7 patients in plateau phase plus 9 and 10/12 with progressive disease had lower numbers of $CD19^+$ cells than any of the normals. One individual on treatment, one in plateau and three with progressive disease had abnormal κ:λ ratios that were consistent with clonal excess. In addition, one patient with non-secretory myeloma and progressive disease had a κ:λ ratio below the normal range. There was no significant difference between the number of patients with stable (on treatment and plateau groups) as opposed to progressive disease who had clonal excess.

Lymphocytes Expressing Activation Antigens (Table 3.12)

When the results were expressed as percentages, there were no differences between groups for any of the populations defined by activation antigens.

In the absolute analysis, the number of $CD25^+$ cells was normal in those with MGUS and SMM but reduced in the other groups. The number of $CD25^+38^+$ cells was decreased in those on treatment, in plateau or with progressive disease.

Table 3.12: Activation Antigen Expression on PBL for Patient Groups Based on Disease Status.

		Normals	MGUS	SMM	Diagnosis	On Treatment	Plateau	Progressive
$CD25^+$	A	0.51 (0.32-0.67)	0.53 (0.38-0.76) NS	0.47 (0.25-0.60) NS	0.27 (0.08-0.77) **p<0.04**	0.28 (0.10-0.51) **p<0.02**	0.23 (0.15-0.37) **p<0.007**	0.24 (0.11-0.59) **p<0.003**
	No.	10	3	5	11	5	7	12
$CD25^+38^+$	A	0.16 (0.08-0.27)	0.19 (0.13-0.27) NS	0.12 (0.06-0.22) NS	0.11 (0.02-0.39) NS	0.05 (0.03-0.12) **p<0.009**	0.06 (0.04-0.21) **p=0.008**	0.06 (0.03-0.13) **p=0.0006**
	No.	10	3	5	11	5	7	12

A-results expressed as an absolute number ($x10^9/l$); No.-number of subjects tested; NS-not significant.

Lymphocytes Expressing Adhesion Molecules (Table 3.14)

There were several problems encountered with the study of the adhesion molecules. Firstly, the commercial supply of the antibodies against CD44 and CDw49d was unreliable, so the expression of these antigens was not studied in all patients.

Secondly, the antibodies against the CD11a, CD18 and CD44 antigens seemed to interfere with the binding of the anti-CD38 antibody. The percentage of $CD38^+$ PBL for each subject was calculated by averaging the values obtained for each dual-labelled sample. For most antibody combinations, the individual values for the percentage of $CD38^+$ PBL were closely clustered around the mean. However, in the samples testing the co-expression of CD38 with the CD11a, CD18 or CD44 antigens, the value for the percentage of $CD38^+$ PBL was not uncommonly markedly below the mean. This is illustrated in table 3.13, which compares the number and percentage of samples with a CD38 value below the mean for the samples testing the co-expression of CD38 with CD19, CD11a, CD18 or CD44. Only one patient sample had a CD38 value more than 5 percentage points below the mean for the CD19 antigen. In contrast, 25-30% of the patient samples had values more than 10 percentage points below the mean for the dual-labelled CD11a and CD18 samples. For the CD44 antigen, more than 60% of both the control and patient samples were more than 10 percentage points below the mean. As a consequence, analysis of the $CD44^+38^+$ population was not possible. In the case of the anti-CD11a and anti-CD18 antibodies, the dual-labelled population was excluded from the analysis in those patients in whom the number of $CD38^+$ cells in the sample was more than 10 percentage points below the mean. This interference may have been due to stearic hindrance. The CD11a, CD18 and CD44 antigens are present on virtually all cells

Table 3.13: Comparison of the Number and Percentage of Samples with a Value for the Percentage of $CD38^+$ PBL Below the Mean Value for Samples Dual-Labelled for the Co-Expression of CD38 with CD19, CD11a, CD18 or CD44.

		0-5 Percentage Points Below Mean		>5-≤10 Percentage Points Below Mean		>10-≤20 Percentage Points Below Mean		>20 Percentage Points Below Mean	
Antigen		Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
CD19	Normals (n=10)	4	40	0	0	0	0	0	0
	Patients (n=44)	18	41	1	<1	0	0	0	0
CD11a	Normals (n=10)	6	60	4	40	0	0	0	0
	Patients (n=40)	16	40	10	25	10	25	2	5
CD18	Normals (n=10)	6	60	4	40	0	0	0	0
	Patients (n=40)	13	33	15	38	8	20	2	5
CD44	Normals (n=10)	1	10	2	20	5	50	2	20
	Patients (n=33)	4	12	6	18	18	55	3	9

at moderate to high fluorescence intensity and the antibodies used to detect these antigens were not directly conjugated. The problem may not have occurred if these antibodies, as well as the anti-CD38 antibody, were directly conjugated.

The percentage of cells expressing CD11a was decreased in patients with MGUS and myeloma patients at diagnosis, on treatment, in plateau or with progressive disease. There was a trend towards a decrease in SMM. The percentage of $CD18^+$ cells was not significantly different at the various phases of the disease. When the results were expressed in absolute numbers, $CD11a^+$ and $CD18^+$ cells were decreased in those patients on therapy or in plateau (table 3.14).

Fluorescence Intensity of Adhesion Molecule Expression (Table 3.15)

The median fluorescence intensities of CD11a and CD18 expression were increased in MGUS, SMM and newly diagnosed patients but the values were not statistically different from the normals. The intensities of these molecules were significantly increased in patients on treatment, in plateau or with progressive disease. The intensity of CDw49d expression was increased in plateau phase and progressive disease. There were insufficient data for analysis in the patients receiving therapy.

Summary

In the analysis based on disease status, the numbers of lymphocytes and $CD38^+$ PBL were no different in the patient groups than in the normals. B cell numbers were reduced in all patient groups. T cell subsets were normal in patients with MGUS. The numbers of $CD4^+$ and $CD4^+38^+$ PBL were reduced in all groups of treated patients (on treatment, plateau phase and progressive disease). The

Table 3.14: Adhesion Molecule Expression on PBL for Patient Groups Based on Disease Status.								
		Normals	MGUS	SMM	Diagnosis	On Treatment	Plateau	Progressive
CD11a$^+$	%	99.1 (97.3-99.6)	96.9 (92.6-97.8) **p<0.03**	94.6 (82.0-99.8) p<0.08	95.7 (73.5-99.2) **p<0.006**	91.6 (75.3-98.1) **p=0.004**	91.9 (87.2-95.9) **p=0.0008**	97.9 (38.8-99.2) **p<0.009**
	A	1.68 (1.26-2.17)	1.47 (1.45-1.67) NS	1.81 (1.56-2.80) NS	1.48 (0.48-2.33) NS	1.05 (0.75-1.37) **p<0.02**	1.28 (0.48-1.65) **p<0.02**	0.99 (0.40-2.65) p<0.1
	No.	10	3	5	9	5	7	11
CD18$^+$	A	1.63 (1.16-2.17)	1.47 (1.42-1.70) NS	1.85 (1.77-2.80) p<0.08	1.51 (0.49-2.29) NS	1.20 (0.73-1.38) **p<0.04**	1.19 (0.48-1.72) **p<0.03**	1.08 (0.38-2.66) NS
	No.	10	3	5	9	5	7	11
%-results expressed as a percentage; A-results expressed as an absolute number (x10^9/l); No.-number of subjects tested; NS-not significant.								

Table 3.15: Fluorescence Intensity of Adhesion Molecule Expression on PBL for Patient Groups Based on Disease Status.								
		Normals	MGUS	SMM	Diagnosis	On Treatment	Plateau	Progressive
CD11a$^+$	I	13.88 (7.19-20.96)	21.07 (13.50-26.98) NS	22.10 (12.74-30.41) p<0.1	19.67 (5.10-27.23) NS	24.40 (18.88-49.95) **p<0.006**	28.91 (18.38-43.85) **p<0.002**	20.41 (7.58-36.10) **p<0.03**
	No.	10	3	5	9	5	7	11
CD18$^+$	I	12.38 (8.95-19.84)	18.87 (12.32-22.07) NS	20.46 (12.10-26.31) NS	13.88 (4.93-24.07) NS	20.97 (16.31-48.92) **p<0.006**	27.62 (16.02-41.82) **p<0.003**	16.64 (9.09-30.87) NS
	No.	10	3	5	9	5	7	11
CDw49d$^+$	I	2.82 (2.01-3.66)	3.35 (2.37-3.59) NS	4.11 (2.35-5.87) NS	3.32 (1.43-5.93) NS	Insufficient Data	6.20 (5.10-7.14) **p<0.007**	4.15 (3.18-4.99) **p<0.02**
	No.	9	3	2	8	-	4	6
I-fluorescence intensity; No.-number of subjects tested; NS-not significant.								

percentages of $CD8^+$ and $CD8^+38^+$ cells were increased and the CD4:CD8 ratio was decreased in these same patient groups. Similar changes in the T cell subsets were present in individual patients with untreated myeloma (SMM and active disease) but they usually did not reach the level of significance in these groups. This was due to marked heterogeneity in the size of these subsets in the untreated patients, especially those with active disease. The percentage of $CD56^+38^+$ cells, which as discussed in section 3.3.2 are probably either NK cells or a subset of T cells, was increased in patients with MGUS and progressive myeloma. Alterations in the expression of activation and adhesion molecules, including the fluorescence intensities of the latter, were most pronounced in the groups of treated patients.

3.3.4: Analysis of Results Based on CD38 Status

The results (p values) obtained with the Kruskal-Wallis analysis of variance test when the patients were divided on the basis of $CD38^+$ cell numbers are presented in table 3.16.

CD38 Antigen Expression on Lymphocytes (Table 3.17)

No patient with MGUS had an elevated percentage or absolute number of lymphocytes expressing the CD38 antigen.

Lymphoid cells expressing the CD38 antigen were elevated above the percentage and absolute normal ranges in 4 (24%) and 5 (29%) of the 17 untreated patients respectively. No patient with SMM had an elevated percentage of $CD38^+$ PBL but one patient had an increased absolute number. This latter patient was the only one with SMM who had a normal as opposed to a low percentage of $CD38^+$ cells. In the other 5 patients with SMM, the percentage of $CD38^+$ cells was below the

Table 3.16: Results (p values) of Kruskal-Wallis Analysis of Variance for Patient Groups Based on the Number of $CD38^+$ PBL.

Lymphocyte Subset	%	A	Lymphocyte Subset	%	A
Lymphocyte Count	NS	**0.003**	$CD38^+$	**<0.001**	**<0.001**
$CD1a^+$	NS	**0.002**	$CD1a^+38^+$	NS	**0.002**
$CD2^+$	NS	**0.006**	$CD2^+38^+$	**0.008**	**0.001**
$CD3^+$	NS	**0.003**	$CD3^+38^+$	**<0.04**	**<0.001**
$CD4^+$	**<0.001**	**<0.001**	$CD4^+38^+$	**<0.001**	**<0.001**
$CD8^+$	**0.001**	**<0.04**	$CD8^+38^+$	**0.002**	NS
$CD16^+$	NS	NS	$CD16^+38^+$	NS	NS
$CD56^+$	**<0.03**	<0.1	$CD56^+38^+$	**<0.03**	**<0.04**
CD4:CD8 Ratio	**<0.001**	**<0.001**	κ:λ Ratio	NS	NS
$CD19^+$	**0.001**	**<0.001**	$CD19^+38^+$	**<0.001**	**<0.001**
$CD20^+$	**0.001**	**<0.001**	$CD20^+38^+$	**0.002**	**<0.001**
$CD21^+$	**<0.001**	**<0.001**	$CD21^+38^+$	**<0.001**	**<0.001**
$CD22^+$	**0.001**	**<0.001**	$CD22^+38^+$	**0.001**	**<0.001**
$CD23^+$	**0.001**	**<0.001**	$CD23^+38^+$	**<0.001**	**<0.001**
$CD24^+$	**0.001**	**<0.001**	$CD24^+38^+$	**<0.001**	**<0.001**
$CD39^+$	**<0.03**	**0.001**	$CD39^+38^+$	**0.001**	**<0.001**
HLA-DR^+	**0.002**	**<0.04**	HLA-DR^+38^+	**0.001**	**<0.02**
$CD25^+$	**<0.03**	**0.001**	$CD25^+38^+$	**<0.02**	**<0.001**
$CD9^+$	NS	**<0.03**	$CD9^+38^+$	**0.001**	**0.001**
$CD71^+$	**<0.05**	**0.003**	$CD71^+38^+$	**0.003**	**0.001**
$PCA1^+$	NS	NS	$PCA1^+CD38^+$	**<0.03**	**<0.04**
$CD10^+$	NS	NS	$CD10^+38^+$	NS	<0.09
$CD13^+$	NS	**<0.02**	$CD13^+38^+$	NS	**0.003**
$CD33^+$	NS	<0.09	$CD33^+38^+$	NS	<0.08
$CD34^+$	NS	**<0.03**	$CD34^+38^+$	NS	**<0.02**
$CD36^+$	NS	NS	$CD36^+38^+$	**<0.03**	<0.1
$CD11a^+$	**0.007**	**0.005**	$CD11a^+38^+$	**0.01**	**<0.001**
$CD18^+$	NS	**0.002**	$CD18^+38^+$	**0.004**	**<0.001**
$CDw49d^+$	<0.07	**<0.03**	$CDw49d^+38^+$	**0.004**	**<0.04**
$CD54^+$	NS	**<0.05**	$CD54^+38^+$	**<0.001**	**<0.02**
$CD44^+$	NS	**0.002**	Intensity of $CD11a^+$ Cells*	**0.002**	**0.002**
Intensity of $CD18^+$ Cells	**0.005**	**0.009**	Intensity of $CD44^+$ Cells	NS	NS
Intensity of $CDw49d^+$ Cells	**0.005**	**<0.02**	Intensity of $CD54^+$ Cells	**<0.02**	**<0.03**
Serum ß2 Microglobulin	NS	NS	Serum Thymidine Kinase	NS	NS
Labelling Index	NS	NS	Serum IgM	0.1	<0.09

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$);
NS-not significant; *fluorescence intensity of adhesion molecule expression.

Table 3.17: Lymphocyte Counts and CD38 Antigen Expression On PBL for Patient Groups Based on the Number of CD38$^+$ PBL.							
		Normals	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
Lymphocytes	%	1.7 (1.3-2.2) 10*	1.5 (1.5-1.8) 3	1.8 (1.5-2.5) 4	1.9 (0.5-2.9) 13	1.3 (0.4-2.1) 9	1.1 (0.4-2.7) 15
	A	1.7 (1.3-2.2) 10	1.5 (1.5-1.8) NS 3	1.9 (1.5-2.5) NS 5	1.8 (0.5-2.9) NS 12	2.1 (1.4-2.3) NS 5	1.1 (0.4-2.7) **p=0.002** 19
CD38$^+$	%	50.1 (39.0-62.2) 10	53.1 (40.0-56.1) NS 3	67.8 (62.4-70.8) **p<0.006** 4	37.2 (25.5-62.2) **p<0.05** 13	70.2 (63.9-73.5) **p=0.0003** 9	37.7 (16.5-59.0) NS 15
	A	0.84 (0.52-1.00) 10	0.84 (0.60-0.96) NS 3	1.13 (1.02-1.68) **p<0.003** 5	0.64 (0.15-0.97) **p<0.05** 12	1.24 (1.03-1.47) **p<0.003** 5	0.41 (0.18-0.96) **p<0.002** 19
%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.							

normal range but the absolute number was within the normal range. Four newly diagnosed patients had elevations of both the relative and absolute numbers of $CD38^+$ cells. Neither of the recently diagnosed patients who had received steroids prior to testing had elevated values. The untreated patients with an increased number of $CD38^+$ PBL will be referred to as the "untreated CD38hi group" and those without an increased number as the "untreated CD38lo group". In the untreated CD38lo groups, there were 3/13 and 4/12 individuals in the percentage and absolute analyses respectively in whom the number of $CD38^+$ cells was below the normal range. Consequently, the untreated CD38lo groups actually had significantly reduced $CD38^+$ cell numbers compared to the normals.

There was no statistical difference in the distribution of patients with SMM and at diagnosis between the untreated CD38hi and CD38lo groups for either the percentage or absolute results. The presence or absence of BJP excretion at diagnosis discriminated between those in the untreated CD38hi and CD38lo groups. Three of the 4 patients with an elevated percentage of $CD38^+$ cells but no patient without an elevated percentage excreted BJP ($p<0.02$). Three of the 5 patients with a raised absolute number of $CD38^+$ lymphocytes but none of those without an elevated absolute $CD38^+$ count excreted BJP ($p<0.03$). The Durie and Salmon stage (stage I and II versus III) or the distribution of paraprotein isotypes was not significantly different between the untreated CD38hi and CD38lo groups.

In the untreated patients, there was a negative correlation between the number of $CD38^+$ PBL and the age of the patients in both the percentage ($r_s=-0.70$, $p<0.005$) and absolute ($r_s=-0.65$, $p<0.005$) analyses. There was no correlation

between the percentage or absolute number of $CD38^+$ cells and the STK, the sβ2M, the plasma cell LI or the amount of serum paraprotein.

Nine of the 24 (38%) previously treated patients had an elevated percentage of $CD38^+$ lymphocytes but only five (21%) had an increased absolute number of $CD38^+$ PBL. Only 3 patients had both an elevated percentage and absolute number of $CD38^+$ cells. All patients with elevated absolute $CD38^+$ cell numbers had normal lymphocyte counts. On the other hand, 3/6 patients who had an elevated percentage but not an elevated absolute number of $CD38^+$ cells had lymphocyte counts $<1.0x10^9/l$. The treated patients with an increased number of $CD38^+$ PBL will be referred to as the "treated CD38hi group" and those without an increased number as the "treated CD38lo group". In the treated CD38lo percentage group, there were 8/15 patients in whom the relative number of $CD38^+$ cells was below the normal range, although the group did not have a significantly decreased percentage of $CD38^+$ cells. In the treated CD38lo absolute group, the number of $CD38^+$ PBL was below the normal range in 12/19 individuals and this group did have a significantly lower number of $CD38^+$ cells than the controls.

The treated CD38hi percentage group comprised 2/5 patients currently receiving chemotherapy, 3/7 with plateau phase disease and 4/12 with progressive disease. The treated CD38hi absolute group comprised one patient on treatment, one in plateau phase and 3 with progressive disease. When the treated patients were divided into those with non-progressive disease (on treatment and plateau phase) versus progressive disease, there was no statistical difference in the distribution of the patients between the treated CD38hi or CD38lo groups in either the percentage or absolute analysis. Similarly, the

treated CD38hi groups could not be discriminated from the CD38lo groups on the basis of BJP excretion, the isotype of the paraprotein or the Durie and Salmon stage (stage I and II versus III), either at the time of diagnosis or at the time of the study.

In the treated patients the number of $CD38^+$ PBL positively correlated with both the STK (r_s=0.35, $p<0.05$ for both the percentage and absolute analyses) and the sβ2M (r_s=0.36, $p<0.05$ for the percentage analysis; r_s=0.44, $p<0.025$ for the absolute analysis). There was no correlation between the number of $CD38^+$ cells and the plasma cell LI, the age of the patient or the quantity of serum paraprotein.

Lymphocyte Counts (Table 3.17)

There was a significant difference in lymphocyte counts between groups only for the absolute results. The treated CD38lo patients as a group had significant lymphopenia. Patients with MGUS had lymphocyte counts within the normal range. The five newly diagnosed patients with active disease and $<1.3x10^9/l$ lymphocytes had neither an elevated percentage nor absolute number of $CD38^+$ cells. Three of the nine patients in the treated CD38hi percentage group had lymphopenia compared to none in the treated CD38hi absolute group. In the treated CD38lo groups, 8/15 and 11/19 patients in the percentage and absolute analyses respectively had low lymphocyte counts (table 3.17, page 161).

T Lymphocytes and NK Cells (Table 3.18)

The T cell subsets and CD4:CD8 ratio in patients with MGUS were normal, whether the results were expressed as percentages or absolute numbers. In

Table 3.18: T Cell and NK Cell Antigen Expression on PBL for Patient Groups Based on the Number of $CD38^+$ PBL.

		Normals	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
$CD2^+$	A	1.42 (0.96-1.96) 10*	1.35 (1.26-1.58) NS 3	1.52 (1.24-2.01) NS 5	1.48 (0.43-2.71) NS 11	1.84 (1.24-2.16) NS 3	0.82 (0.28-1.31) **p=0.0008** 14
$CD2^+38^+$	%	39.9 (25.1-48.7) 8	44.2 (26.7-61.6) NS 2	54.2 (46.4-61.3) **p<0.03** 4	31.7 (19.1-49.2) NS 11	51.0 (40.2-61.7) **p<0.02** 6	38.8 (14.4-51.4) NS 10
	A	0.62 (0.34-0.83) 8	0.66 (0.40-0.92) NS 2	0.92 (0.77-1.30) **p<0.007** 5	0.63 (0.15-0.78) NS 10	0.92 (0.72-1.13) p<0.06 3	0.35 (0.12-0.73) **p<0.02** 13
$CD3^+$	A	1.39 (0.91-1.87) 10	1.12 (1.11-1.22) NS 3	1.46 (0.93-1.85) NS 5	1.17 (0.39-2.55) NS 12	1.48 (1.11-1.95) NS 5	0.78 (0.19-2.47) **p<0.002** 19
$CD3^+38^+$	%	34.9 (17.3-56.4) 9	31.3 (22.0-33.1) NS 3	47.5 (30.0-54.8) NS 4	21.1 (14.1-45.4) **p<0.03** 12	39.7 (8.5-70.2) NS 8	26.5 (11.0-46.0) p<0.1 13
	A	0.60 (0.22-1.01) 9	0.47 (0.33-0.60) NS 3	0.86 (0.48-1.14) NS 5	0.41 (0.11-0.58) **p<0.03** 11	0.86 (0.54-1.47) NS 5	0.27 (0.08-0.78) **p<0.005** 16
$CD4^+$	%	55.3 (44.3-69.2) 10	59.9 (51.5-63.7) NS 3	67.3 (52.3-72.6) NS 4	42.6 (27.0-67.9) **p<0.02** 13	32.8 (20.7-42.1) **p=0.0003** 9	46.2 (14.9-58.5) **p<0.002** 15
	A	0.92 (0.69-1.21) 10	0.96 (0.77-1.08) NS 3	1.11 (0.84-1.63) NS 5	0.59 (0.20-1.46) **p<0.02** 12	0.62 (0.29-0.95) **p<0.03** 5	0.40 (0.13-0.88) **p<0.0001** 19
$CD4^+38^+$	%	23.9 (12.0-41.4) 10	23.4 (19.2-24.0) NS 3	38.9 (28.0-52.4) **p<0.05** 4	13.8 (6.2-32.9) **p<0.02** 13	16.5 (5.9-22.7) **p<0.05** 9	11.0 (3.5-25.4) **p<0.002** 13
	A	0.42 (0.16-0.63) 10	0.36 (0.29-0.42) NS 3	0.79 (0.45-0.88) **p<0.02** 5	0.24 (0.07-0.33) **p<0.004** 12	0.31 (0.14-0.41) NS 5	0.11 (0.06-0.28) **p=0.0001** 17

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.

Table 3.18: Continued.

		Normals	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
$CD8^+$	%	28.7 (14.4-35.6) 10*	19.0 (17.7-31.4) NS 3	16.8 (10.8-23.2) **p<0.03** 4	35.5 (20.9-52.5) p<0.07 13	36.7 (28.9-73.3) **p<0.02** 9	36.7 (25.3-65.9) **p<0.02** 15
	A	0.46 (0.19-0.73) 10	0.34 (0.27-0.47) NS 3	0.37 (0.23-0.40) NS 5	0.56 (0.19-1.47) NS 12	1.03 (0.52-1.45) **p<0.02** 5	0.41 (0.11-1.62) NS 19
$CD8^+38^+$	%	8.0 (3.6-18.7) 10	15.4 (4.6-19.5) NS 3	8.35 (5.0-18.3) NS 4	12.0 (2.7-18.6) NS 13	27.5 (12.9-57.2) **p=0.0006** 8	14.4 (7.8-38.4) **p<0.02** 15
	A	0.14 (0.07-0.41) 10	0.28 (0.07-0.29) 3	0.14 (0.13-0.29) 5	0.19 (0.02-0.37) 12	0.63 (0.23-0.84) 4	0.17 (0.03-0.56) 19
$CD56^+$	%	10.5 (5.0-20.8) 10	20.3 (14.0-25.6) p<0.06 3	9.6 (5.4-25.2) NS 4	21.3 (9.5-33.4) **p<0.003** 13	18.1 (6.4-35.2) p<0.09 7	16.7 (5.7-23.3) p<0.07 12
$CD56^+38^+$	%	5.5 (3.3-10.7) 10	14.5 (9.4-17.8) **p<0.04** 3	7.0 (3.9-22.8) NS 4	10.2 (4.0-22.9) **p<0.03** 13	15.5 (5.5-31.1) **p<0.008** 7	8.4 (4.6-15.2) **p<0.04** 12
	A	0.10 (0.04-0.16) 10	0.22 (0.14-0.32) **p=0.05** 3	0.13 (0.06-0.36) NS 5	0.18 (0.03-0.37) p<0.08 12	0.32 (0.22-0.33) **p<0.02** 3	0.12 (0.03-0.43) NS 16
CD4:CD8 Ratio	%	1.92 (1.27-4.42) 10	3.15 (1.64-3.60) NS 3	4.28 (2.25-6.03) **p<0.03** 4	1.18 (0.53-2.79) **p<0.02** 13	0.77 (0.28-1.20) **p=0.0003** 9	1.17 (0.28-2.15) **p=0.003** 15
	A	1.92 (1.27-4.42) 10	3.15 (1.64-3.60) NS 3	3.77 (2.25-6.03) **p<0.03** 5	1.12 (0.53-2.45) **p<0.007** 12	0.56 (0.28-1.20) **p<0.003** 5	1.15 (0.28-2.15) **p=0.0007** 19

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.

these patients, there was a tendency towards an increase in the percentage of $CD56^+$ cells. The $CD56^+38^+$ subset was significantly expanded in both analyses.

The changes in the untreated patients were similar irrespective of whether the analysis was based on the percentage or absolute results. The CD38hi groups had expanded $CD4^+38^+$ populations, although the total $CD4^+$ populations were normal. The patients with an elevated percentage of $CD38^+$ PBL had a decreased percentage of $CD8^+$ but not $CD8^+38^+$ cells. However, the $CD8^+$ lymphoid cells were normal in those with an absolute $CD38^+$ count above the normal range. The CD4:CD8 ratio was elevated in both analyses, reflecting T cell subset imbalance, even in the absolute CD38hi group in which neither the $CD4^+$ nor $CD8^+$ counts were significantly altered. The untreated CD38hi groups had normal NK cell populations.

In the untreated CD38lo patients, the $CD3^+CD38^+$ counts were reduced due to decreased $CD4^+CD38^+$ counts. The $CD4^+$ cell numbers were also decreased. The $CD8^+$ and $CD8^+38^+$ subsets were unaltered. As a result of these alterations, the CD4:CD8 ratio was reduced. The $CD56^+$ and $CD56^+38^+$ cell numbers were increased in the CD38lo percentage group but there was only a trend towards increased $CD56^+38^+$ numbers in the CD38lo absolute group.

There was no overlap in the ranges for the $CD4^+38^+$ populations in the untreated CD38hi and CD38lo groups in the absolute analysis. The overlap in the ranges for the $CD4^+38^+$ subsets in the analysis of percentage results was due to the single patient with SMM who had a normal percentage of $CD38^+$ PBL but an elevated absolute number. Consequently, for both the percentage and absolute results in the untreated patients, there were significant correlations

between the numbers of $CD38^+$ and $CD4^+38^+$ PBL (r_s=0.85, p<0.001 and r_s=0.90, p<0.001 respectively) and the numbers of $CD38^+$ and $CD4^+$ cells (r_s=0.49, p<0.025 and r_s=0.81, p<0.001 respectively). In contrast, there was no correlation between the numbers of $CD38^+$ and $CD8^+38^+$ cells in either analysis.

The alterations in the T cell subsets for the percentage results were similar in both the treated CD38hi and CD38lo patients. Both groups had decreased percentages of $CD4^+$ and $CD4^+38^+$ cells but increased percentages of $CD8^+$ and $CD8^+38^+$ cells compared to the normals. However, the percentage of $CD8^+38^+$ cells was significantly higher in the CD38hi than in the CD38lo patients (p<0.02). The CD4:CD8 ratio was decreased in both groups. There was an increased percentage of $CD56^+38^+$ lymphocytes in both groups but only a trend towards an increased percentage of $CD56^+$ PBL. The number of $CD56^+38^+$ cells in the CD38hi group was almost significantly higher than that in the CD38lo group (p<0.06).

When the absolute results were analysed, the alterations were different in the treated CD38hi and CD38lo groups. The $CD2^+$, $CD2^+38^+$, $CD3^+$ and $CD3^+38^+$ subsets were reduced in the CD38lo patients, as this group had significantly reduced numbers of both total and $CD38^+$ lymphocytes. Both the treated CD38hi and CD38lo groups had decreased $CD4^+$ subsets. The number of $CD4^+38^+$ cells was normal in the CD38hi group but decreased in the CD38lo group, with the results being significantly different between these groups (p<0.005). The CD38hi group had significantly elevated $CD8^+$ numbers compared to both the controls (p<0.02) and the CD38lo group (p<0.006). However, the $CD8^+$ lymphocytes were the only T cell subset present in normal numbers in the untreated CD38lo group. The Kruskal-Wallis test did not detect

a significant difference in the absolute analysis of $CD8^+38^+$ cells but the median value for this subset in the treated CD38hi group was $0.63x10^9/l$ compared to $0.14x10^9/l$ in the normals. The analysis of this subset in the CD38hi group included the results for only 4/5 patients, of whom 3 had elevated $CD8^+38^+$ numbers. The missing result was omitted as the $CD38^+$ value from the dual labelling study of the CD8 and CD38 antigens was >10% below the mean $CD38^+$ value for that patient. Nevertheless, this patient still clearly had an elevated absolute number of $CD8^+38^+$ cells at $0.84x10^9/l$. Thus, 4/5 patients in the treated CD38hi group had elevated $CD8^+38^+$ counts. The range of values in the treated CD38lo group was similar to the normals and only 1/19 patients had an elevated number of $CD8^+38^+$ PBL. The CD4:CD8 ratio was decreased in both the treated CD38hi and CD38lo groups. $CD56^+38^+$ cell numbers were significantly elevated in the CD38hi patients but normal in the CD38lo group with a significant difference between these groups ($p<0.03$).

For both the percentage and absolute results in treated patients, there were significant correlations between the numbers of $CD38^+$ and $CD8^+CD38^+$ cells ($r_s=0.80$, $p<0.001$ and $r_s=0.89$, $p<0.001$ respectively), $CD38^+$ and $CD4^+38^+$ cells ($r_s=0.47$, $p<0.025$ and $r_s=0.64$, $p<0.001$ respectively), and $CD38^+$ and $CD56^+38^+$ cells ($r_s=0.44$, $p<0.05$ and $r_s=0.69$, $p<0.001$ respectively). For the percentage results, there was a negative correlation between the numbers of $CD38^+$ and $CD4^+$ PBL ($r_s=-0.42$, $p<0.025$). For the absolute results, there was a positive correlation between $CD38^+$ and $CD8^+$ cell numbers ($r_s=0.71$, $p<0.001$).

B Lymphocytes (Table 3.19)

The only B cell data set for which there was not a significant difference between groups was the $\kappa:\lambda$ ratio.

Table 3.19: B Cell Antigen Expression on PBL for Patient Groups Based on the Number of CD38$^+$ PBL.

		Normal	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
CD19$^+$	%	9.6 (8.3-14.3) 10*	3.8 (3.6-8.2) **p<0.02** 3	11.4 (5.0-16.1) NS 4	5.1 (3.2-8.5) **p=0.0001** 13	4.6 (1.8-23.6) **p<0.05** 9	3.5 (1.3-12.2) **p=0.0008** 15
	A	0.16 (0.12-0.27) 10	0.06 (0.06-0.12) **p<0.02** 3	0.20 (0.08-0.40) NS 5	0.06 (0.03-0.15) **p=0.0003** 12	0.06 (0.04-0.42) NS 5	0.04 (0.01-0.21) **p=0.0001** 19
CD19$^+$38$^+$	%	7.1 (5.5-8.8) 10	2.7 (2.5-3.9) **p<0.02** 3	9.0 (3.3-12.2) NS 4	2.9 (1.7-6.5) **p=0.0001** 13	3.5 (2.1-19.1) p<0.09 8	1.6 (0.5-8.6) **p<0.002** 15
	A	0.11 (0.08-0.17) 10	0.05 (0.04-0.06) **p<0.02** 3	0.16 (0.05-0.31) NS 5	0.04 (0.01-0.08) **p=0.0001** 12	0.11 (0.04-0.34) NS 4	0.02 (0.01-0.18) **p=0.0001** 19
CD20$^+$	%	9.2 (7.2-14.0) 10	4.9 (4.4-8.2) **p<0.04** 3	9.6 (9.6-13.4) NS 3	5.5 (3.2-8.5) **p=0.0004** 10	4.5 (2.6-18.8) p<0.09 8	3.2 (1.7-11.0) **p=0.0007** 15
	A	0.15 (0.11-0.27) 10	0.09 (0.07-0.12) **p<0.04** 3	0.20 (0.16-0.24) NS 4	0.08 (0.03-0.14) **p<0.003** 9	0.07 (0.06-0.34) NS 5	0.04 (0.01-0.19) **p=0.0001** 18
CD20$^+$38$^+$	%	4.2 (3.2-5.3) 10	2.0 (1.3-2.4) **p<0.02** 3	5.7 (3.9-7.6) NS 3	1.4 (0.8-5.1) **p<0.003** 10	2.5 (0.8-12.0) p<0.1 8	0.7 (0.2-7.5) **p<0.003** 15
	A	0.07 (0.04-0.09) 10	0.04 (0.02-0.04) **p<0.03** 3	0.11 (0.10-0.12) **p<0.006** 4	0.02 (0.01-0.06) **p=0.0005** 9	0.03 (0.01-0.22) NS 5	0.01 (0.00-0.13) **p=0.0002** 18

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.

Table 3.19: Continued.

		Normal	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
CD21⁺	%	10.0 (7.6-12.7) 10*	4.5 (3.2-5.8) **p<0.02** 3	13.1 (10.8-17.6) **p<0.04** 3	5.8 (2.9-8.2) **p=0.0003** 10	4.8 (2.4-25.0) p<0.09 8	3.5 (1.5-10.0) **p=0.0002** 15
	A	0.17 (0.11-0.24) 10	0.08 (0.05-0.09) **p<0.02** 3	0.22 (0.15-0.44) NS 4	0.08 (0.02-0.16) **p<0.004** 9	0.06 (0.05-0.45) NS 5	0.04 (0.01-0.19) **p=0.0001** 18
CD21⁺38⁺	%	7.1 (5.2-8.9) 10	3.2 (2.1-3.5) **p<0.02** 3	10.9 (8.7-13.2) **p<0.03** 3	2.7 (1.6-5.7) **p=0.0003** 10	3.4 (1.3-21.8) NS 8	1.9 (0.8-8.3) **p=0.0007** 15
	A	0.11 (0.09-0.17) 10	0.05 (0.03-0.06) **p<0.02** 3	0.17 (0.11-0.33) p<0.07 4	0.04 (0.01-0.11) **p=0.001** 9	0.04 (0.03-0.39) NS 5	0.02 (0.01-0.17) **p=0.0001** 18
CD22⁺	%	10.1 (8.4-12.4) 10	7.4 (4.1-9.0) **p<0.03** 3	11.6 (10.5-15.1) NS 3	5.3 (3.2-8.3) **p=0.0002** 10	3.7 (1.8-23.6) p<0.09 8	4.4 (1.6-10.8) **p=0.0002** 15
	A	0.17 (0.13-0.24) 10	0.13 (0.06-0.14) NS 3	0.20 (0.16-0.38) NS 4	0.08 (0.03-0.16) **p<0.002** 9	0.04 (0.04-0.42) NS 5	0.05 (0.01-0.19) **p=0.0001** 18
CD22⁺38⁺	%	6.7 (5.3-9.2) 9	5.6 (3.2-6.3) p<0.07 3	10.1 (8.1-11.0) p<0.1 3	2.9 (1.8-5.7) **p=0.0005** 10	3.1 (1.2-20.1) NS 7	2.7 (0.5-8.4) **p=0.0005** 15
	A	0.12 (0.09-0.16) 9	0.08 (0.05-0.11) p<0.06 3	0.16 (0.11-0.28) NS 4	0.04 (0.01-0.11) **p=0.001** 9	0.09 (0.02-0.36) NS 4	0.02 (0.01-0.16) **p=0.0002** 18
CD23⁺	%	8.0 (6.2-11.6) 10	4.7 (2.6-5.7) **p<0.02** 3	11.2 (8.7-13.9) NS 3	3.3 (2.5-5.0) **p=0.0003** 9	4.4 (2.6-20.6) NS 8	3.1 (0.6-10.8) **p=0.0009** 15
	A	0.14 (0.09-0.22) 10	0.08 (0.04-0.09) **p<0.03** 3	0.18 (0.17-0.35) NS 3	0.05 (0.02-0.10) **p<0.002** 9	0.08 (0.04-0.37) NS 5	0.04 (0.01-0.17) **p=0.0001** 18

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.

Table 3.19: Continued.

		Normal	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
$CD23^+38^+$	%	6.3 (4.9-8.9) 10*	3.2 (1.8-3.7) **p<0.02** 3	9.7 (7.3-11.7) p<0.06 3	2.0 (0.9-3.3) **p=0.0003** 9	3.0 (1.9-18.4) NS 8	1.4 (0.3-7.3) **p=0.001** 15
	A	0.11 (0.07-0.17) 10	0.05 (0.03-0.07) **p<0.03** 3	0.16 (0.15-0.29) **p<0.05** 3	0.03 (0.01-0.07) **p=0.0004** 9	0.05 (0.02-0.33) NS 5	0.02 (0.00-0.16) **p=0.0002** 18
$CD24^+$	%	9.7 (7.7-13.2) 10	4.9 (4.4-7.7) **p<0.02** 3	11.0 (9.4-15.0) NS 3	5.3 (3.7-9.3) **p=0.0008** 10	4.5 (2.9-20.7) p<0.09 8	3.1 (1.5-11.4) **p=0.0005** 15
	A	0.16 (0.11-0.25) 10	0.09 (0.07-0.12) **p<0.03** 3	0.19 (0.18-0.38) NS 4	0.07 (0.03-0.14) **p<0.003** 9	0.07 (0.06-0.37) NS 5	0.04 (0.01-0.19) **p=0.0001** 18
$CD24^+38^+$	%	6.5 (4.7-9.0) 10	3.6 (3.3-3.9) **p<0.02** 3	8.9 (7.5-11.0) p<0.08 3	2.6 (1.3-7.2) **p<0.002** 10	3.9 (1.8-15.5) p<0.09 8	2.1 (0.8-7.9) **p=0.0003** 15
	A	0.11 (0.06-0.15) 10	0.06 (0.05-0.06) **p<0.03** 3	0.15 (0.14-0.28) **p<0.03** 4	0.04 (0.01-0.10) **p=0.0009** 9	0.05 (0.04-0.28) NS 5	0.03 (0.00-0.16) **p=0.0002** 18
$CD39^+$	%	17.8 (10.9-20.8) 10	11.2 (7.8-11.9) **p<0.04** 3	16.6 (14.4-17.5) NS 3	10.1 (4.9-16.7) **p<0.002** 10	14.1 (9.3-28.5) NS 8	12.2 (4.6-31.1) p<0.07 15
	A	0.29 (0.14-0.35) 10	0.17 (0.14-0.18) **p<0.05** 3	0.31 (0.19-0.36) NS 4	0.13 (0.04-0.31) **p<0.02** 9	0.23 (0.10-0.51) NS 5	0.14 (0.03-0.27) **p=0.0002** 18
$CD39^+38^+$	%	9.7 (8.2-12.1) 10	6.3 (5.9-6.6) **p<0.05** 2	11.8 (10.6-13.0) NS 3	4.7 (2.2-8.4) **p=0.0003** 10	10.6 (6.8-21.4) NS 8	4.8 (1.2-20.7) **p<0.02** 15
	A	0.16 (0.11-0.23) 10	0.11 (0.10-0.11) **p<0.05** 2	0.23 (0.15-0.27) NS 4	0.08 (0.02-0.17) **p=0.002** 9	0.15 (0.06-0.39) NS 5	0.07 (0.02-0.14) **p<0.0001** 18

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.

In MGUS, the number of B cells was significantly decreased for the vast majority of antigens in the panel. All patients had a low percentage and absolute number of $CD19^+$ cells and none had B cells above the normal range in any of the other subsets. No patient had an abnormal $\kappa:\lambda$ ratio. Despite the low B cell numbers, no patient had serum levels of the non-clonal immunoglobulin isotypes below the normal ranges.

In the untreated CD38hi groups, the B cell populations were either normal or, in a few instances, significantly increased. For the CD19 antigen in both the percentage and absolute analyses, one patient had a value above the normal range, three within the normal range and one below the normal range. One newly diagnosed individual had LCIS but the other untreated CD38hi patients had $\kappa:\lambda$ ratios within the normal range.

The untreated CD38lo patients had decreased B cell populations. No patient had a value above the normal range for any B cell antigen. Only 1/13 and 2/12 patients had normal numbers of $CD19^+$ cells in the percentage and absolute results respectively. No patient had an abnormal $\kappa:\lambda$ ratio.

In the untreated patients, there were significant positive correlations for both percentage and absolute results between the numbers of $CD38^+$ and both $CD19^+$ ($r_s=0.50$, $p<0.025$ and $r_s=0.86$, $p<0.001$ respectively) and $CD19^+38^+$ cells ($r_s=0.50$, $p<0.025$ and $r_s=0.84$, $p<0.001$ respectively). Using the Kruskal-Wallis test, there was no difference in serum IgM levels between the groups in either analysis. However, this analysis was complicated by two factors: (1) serum IgM levels were not measured in the normal controls; (2) 12 patients had serum IgM results

issued as <0.2g/l (entered as 0.19 for statistical analysis) but 3 had results of 0.1g/l. This latter problem resulted from interassay variability for low levels of serum IgM. As the median values for serum IgM in the untreated CD38hi groups were higher than the other groups, an alternative approach was adopted. In the untreated patients, there were positive correlations between $CD38^+$ cell numbers and serum IgM (r_s=0.42, $p<0.05$ for percentage results and r_s=0.64, $p<0.005$ for absolute results) and between $CD19^+$ cell numbers and serum IgM (r_s=0.71, $p<0.001$ and r_s=0.63, $p<0.005$ respectively).

In the treated CD38hi groups, the median values for the B cell subsets were lower than in the normal controls, with the exception of the $CD39^+38^+$ subset in the percentage results. Despite this, only the percentage of $CD19^+$ lymphoid cells was significantly reduced, as for each B cell subset there were patients in whom the number of positive cells was below, within and above the normal range. Seven of the nine patients had a decreased proportion and 3/5 a decreased absolute number of $CD19^+$ lymphocytes. The other individuals had $CD19^+$ cell numbers above the normal range. Three individuals, who all had decreased numbers of B lymphocytes, had abnormal κ:λ ratios. Two patients who had both elevated percentages and absolute numbers of $CD38^+$ cells had clonal excess. One patient with non-secretory myeloma and an increased percentage of $CD38^+$ PBL had a κ:λ ratio below the normal range.

The treated CD38lo patients had significantly reduced B cell subsets. For the percentage and absolute results respectively, 3/15 and 1/19 patients had normal $CD19^+$ numbers, whereas the others had decreased numbers. Four patients in these groups had abnormal κ:λ ratios plus low B cell numbers. The same 3 patients in both analyses had clonal excess. The other patient with an

abnormal κ:λ ratio had non-secretory myeloma and was the patient mentioned in the preceding paragraph.

In the treated patients, there were positive correlations between the numbers of $CD38^+$ and both $CD19^+$ (r_s=0.53, $p<0.005$) and $CD19^+38^+$ (r_s=0.60, $p<0.005$) cells for the absolute results. There were no such correlations for the percentage results. There was no correlation between $CD38^+$ cell numbers and serum IgM in either analysis but there was a significant positive correlation in both the percentage and absolute analyses between $CD19^+$ cell numbers and serum IgM (r_s=0.42, $p<0.025$ in both analyses). There was no significant difference in the distribution of patients with clonal excess between the treated CD38hi and CD38lo groups.

Lymphocytes Expressing Activation Markers (Table 3.20)

Apart from the results for the $CD25^+$ and $CD25^+38^+$ populations, the alterations in expression of activation markers were similar in both the percentage and absolute analyses for most patient groups.

The MGUS and untreated CD38hi patients had no alteration in activation marker expression. The untreated CD38lo groups had decreased numbers of $CD9^+38^+$ and $CD71^+38^+$ PBL in both analyses plus decreased $CD71^+$ cell numbers for the percentage results and a trend in the same direction for the absolute results. The $CD25^+$ and $CD25^+38^+$ subsets were normal in the percentage analysis but significantly reduced in the absolute analysis.

CD38hi treated patients had expanded $HLA\text{-}DR^+$, $CD38^+HLA\text{-}DR^+$, $CD9^+38^+$ and $CD38^+PCA\text{-}1^+$ populations for both the percentage and absolute results. There

Table 3.20: Activation Antigen Expression on PBL for Patient Groups Based on the Number of CD38⁺ PBL.							
		Normal	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
HLA-DR⁺	%	17.5 (11.1-38.1) 10*	17.4 (16.8-24.5) NS 3	16.1 (9.3-21.8) NS 4	18.9 (8.7-36.8) NS 13	47.8 (20.4-77.9) **p=0.0007** 9	17.8 (7.8-35.3) NS 15
	A	0.29 (0.16-0.65) 10	0.26 (0.25-0.44) NS 3	0.32 (0.14-0.55) NS 5	0.30 (0.07-1.04) NS 12	0.71 (0.45-1.27) **p=0.004** 5	0.27 (0.05-0.82) NS 19
HLA-DR⁺CD38⁺	%	8.9 (7.3-10.6) 10	9.5 (8.6-14.3) NS 3	11.8 (5.9-15.2) NS 4	9.3 (4.0-18.0) NS 13	34.8 (13.4-59.0) **p=0.0003** 9	11.3 (2.2-22.5) NS 14
	A	0.15 (0.10-0.19) 10	0.14 (0.13-0.26) NS 3	0.24 (0.09-0.37) NS 5	0.13 (0.03-0.30) NS 12	0.50 (0.29-1.06) **p<0.003** 5	0.11 (0.03-0.59) NS 18
CD25⁺	%	30.0 (16.6-39.4) 10	35.4 (25.4-42.1) NS 3	27.6 (18.1-30.7) NS 4	21.9 (7.4-41.5) p<0.07 12	18.3 (7.3-31.7) **p=0.008** 9	25.6 (13.5-36.6) NS 15
	A	0.51 (0.32-0.67) 10	0.53 (0.38-0.76) NS 3	0.47 (0.27-0.77) NS 5	0.27 (0.08-0.60) **p<0.02** 11	0.30 (0.10-0.59) p<0.09 5	0.23 (0.11-0.51) **p=0.0003** 19
CD25⁺38⁺	%	9.4 (4.5-21.0) 10	12.5 (8.8-15.2) NS 3	12.5 (8.9-15.7) NS 4	6.6 (1.9-11.6) NS 12	7.8 (1.4-17.1) NS 9	5.2 (1.7-14.4) **p<0.006** 15
	A	0.16 (0.08-0.27) 10	0.19 (0.13-0.27) NS 3	0.22 (0.13-0.39) NS 5	0.09 (0.02-0.18) **p<0.02** 11	0.08 (0.03-0.21) NS 5	0.05 (0.04-0.12) **p<0.0001** 19
%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.							

Table 3.20: Continued.

		Normal	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
$CD9^+$	A	0.22 (0.15-0.52) 10^*	0.27 (0.27-0.36) NS 3	0.25 (0.20-0.60) NS 4	0.21 (0.08-0.56) NS 11	0.34 (0.18-0.48) NS 5	0.18 (0.07-0.26) p<0.08 19
$CD9^+38^+$	%	8.4 (6.5-15.7) 10	10.8 (10.4-11.3) NS 3	10.7 (7.0-17.2) NS 3	6.4 (3.1-8.3) **p<0.003** 11	13.9 (8.4-22.3) **p<0.02** 8	8.5 (2.3-18.0) NS 15
	A	0.14 (0.08-0.27) 10	0.16 (0.16-0.20) NS 3	0.17 (0.14-0.43) NS 4	0.08 (0.03-0.19) **p<0.009** 10	0.29 (0.18-0.36) **p<0.05** 4	0.08 (0.04-0.20) **p<0.006** 19
$CD71^+$	%	5.1 (2.8-8.7) 10	4.4 (2.9-5.2) NS 3	6.2 (6.1-6.9) NS 3	3.6 (1.9-5.1) **p<0.02** **10**	4.1 (2.5-8.8) NS 8	4.3 (1.4-6.5) p<0.1 15
	A	0.09 (0.04-0.19) 10	0.08 (0.04-0.08) NS 3	0.12 (0.09-0.16) NS 4	0.06 (0.02-0.12) p<0.08 9	0.08 (0.04-0.14) NS 5	0.04 (0.01-0.12) **p<0.003** 18
$CD71^+38^+$	%	3.4 (1.9-6.0) 10	2.4 (2.2-3.1) NS 3	4.8 (4.6-4.9) NS 2	1.6 (0.7-2.9) **p=0.0009** 10	2.8 (1.5-7.1) NS 7	2.1 (0.7-4.8) **p<0.02** 15
	A	0.06 (0.02-0.13) 10	0.04 (0.03-0.06) NS 3	0.10 (0.06-0.12) NS 3	0.02 (0.01-0.06) **p<0.009** 9	0.06 (0.03-0.13) NS 4	0.02 (0.01-0.06) **p=0.0005** 18
$PCA1^+38^+$	%	6.0 (3.8-12.1) 10	12.3 (4.2-18.6) NS 3	6.6 (6.5-7.6) NS 4	6.7 (3.2-13.2) NS 12	16.2 (5.8-26.1) **p<0.003** 8	7.5 (2.9-26.2) NS 15
	A	0.10 (0.07-0.17) 10	0.18 (0.06-0.33) NS 3	0.10 (0.09-0.17) NS 5	0.09 (0.03-0.19) NS 11	0.24 (0.20-0.30) **p<0.006** 4	0.10 (0.04-0.24) NS 19
$CD36^+38^+$	%	6.3 (4.6-10.9) 8	12.9 (6.5-19.3) NS 2	6.5 (6.2-8.0) NS 3	6.0 (3.6-10.9) NS 9	11.8 (6.3-21.5) **p<0.04** 8	5.7 (1.4-20.4) NS 13

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.

was also an increased percentage of $CD36^+38^+$ cells. There were decreased $CD25^+$ but normal $CD25^+38^+$ subsets in the percentage analysis, whereas both these subsets were normal in the absolute analysis.

In the CD38lo treated patients, a decrease in the number of $CD71^+38^+$ PBL was present in both analyses. For the absolute results, there was also a reduction in the $CD71^+$ and $CD9^+38^+$ subsets. The $CD25^+38^+$ population was decreased in the percentage results but both the $CD25^+$ and $CD25^+38^+$ subsets were decreased in the absolute results.

Lymphocytes Expressing Adhesion Molecules (Table 3.21)

The adhesion molecules CD11a, CD18, CD44 and CDw49d were expressed on the majority of PBL in the normal controls (75.8-94.1% for CDw49d; >90% for the other 3 antigens). For the percentage results, there was a significant difference between groups only for the CD11a antigen. The CD38hi untreated group had a normal proportion of $CD11a^+$ cells, whereas the other groups, including MGUS, had slightly but significantly decreased percentages. In the absolute results, the numbers of $CD11a^+$, $CD18^+$, $CD44^+$ and $CDw49d^+$ cells were decreased in the treated CD38lo group, which was the only significantly lymphopenic group. The alterations in the $CD11a^+38^+$, $CD18^+38^+$ and $CDw49d^+38^+$ subsets reflected the number of $CD38^+$ PBL in each of the patient groups in both the percentage and absolute analyses.

The CD54 antigen was present on fewer cells (23.9-57.0%) in the controls than the other adhesion molecules. The proportion of $CD54^+38^+$ lymphoid cells was increased in untreated and treated CD38hi groups. In the absolute analysis, the

Table 3.21: Adhesion Molecule Expression on PBL for Patient Groups Based on the Number of $CD38^+$ PBL.							
		Normals	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
$CD11a^+$	%	99.1 (97.3-99.6) 10*	96.9 (92.6-97.8) **p<0.03** 3	93.3 (92.8-99.2) NS 3	95.7 (73.5-99.8) **p<0.004** 11	93.7 (38.8-98.5) **p=0.0009** 8	94.5 (75.3-99.2) **p<0.002** 15
	A	1.68 (1.26-2.17) 10	1.47 (1.45-1.67) NS 3	1.89 (1.48-2.33) NS 4	1.54 (0.48-2.80) NS 10	1.65 (0.81-2.25) NS 5	1.02 (0.40-2.65) **p=0.0007** 18
$CD11a^+38^+$	%	42.2 (34.2-59.0) 10	Insufficient Data	59.1 (57.3-63.4) **p<0.03** 3	27.6 (16.1-57.5) p<0.09 8	64.9 (59.9-69.8) **p<0.05** 2	33.2 (13.3-55.7) NS 18
	A	0.77 (0.46-0.92) 10		1.10 (1.01-1.43) **p<0.006** 4	0.47 (0.11-0.73) **p<0.02** 7	1.19 (1.14-1.23) **p<0.05** 2	0.29 (0.15-0.90) **p=0.0005** 13
$CD18^+$	A	1.63 (1.16-2.17) 10	1.47 (1.42-1.70) NS 3	1.89 (1.51-2.29) NS 4	1.68 (0.49-2.80) NS 10	1.72 (1.37-2.23) NS 5	1.05 (0.38-2.66) **p=0.0009** 18
$CD18^+38^+$	%	42.9 (33.9-52.5) 10	Insufficient Data	58.4 (56.6-65.1) **p<0.02** 3	30.1 (17.4-56.1) NS 10	58.1 (57.6-70.0) **p<0.02** 3	30.1 (13.9-54.3) NS 13
	A	0.74 (0.44-0.91) 10		1.12 (1.04-1.42) **p<0.006** 4	0.56 (0.12-0.82) **p<0.04** 9	1.10 (1.05-1.15) **p<0.05** 2	0.32 (0.14-0.86) **p=0.0004** 14
%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.							

Table 3.21: Continued.

		Normals	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
CD44⁺	A	1.63 (1.25-2.20) 10*	1.49 (1.41-1.78) NS 3	1.93 (1.54-2.49) NS 4	1.60 (0.50-2.87) NS 9	2.20 (1.78-2.25) p<0.10 3	1.03 (0.39-1.47) **p=0.0008** 14
CDw49d⁺	A	1.29 (1.02-1.79) 9	1.30 (1.12-1.51) NS 3	1.66 (1.32-1.99) NS 2	1.23 (0.42-2.48) NS 8	Insufficient Data	0.80 (0.33-1.23) **p<0.002** 10
CDw49d⁺38⁺	%	43.8 (34.8-52.1) 9	48.0 (36.0-50.2) NS 3	58.1 (55.0-61.1) **p<0.05** 2	32.4 (21.2-59.2) NS 8	66.9 (63.9-71.7) **p<0.004** 5	39.0 (30.4-55.3) NS 5
	A	0.70 (0.45-0.86) 9	0.72 (0.54-0.90) NS 3	1.18 (0.98-1.38) **p<0.05** 2	0.60 (0.13-0.93) NS 8	Insufficient Data	0.36 (0.16-0.92) p<0.06 9
CD54⁺	A	0.77 (0.31-1.08) 10	0.81 (0.46-0.88) NS 3	0.68 (0.59-1.04) NS 3	0.78 (0.10-2.39) NS 10	1.04 (0.98-1.88) **p<0.009** 5	0.52 (0.22-1.71) NS 18
CD54⁺38⁺	%	16.5 (11.2-20.1) 10	22.6 (11.3-23.2) NS 3	24.1 (24.0-24.1) **p<0.05** 2	16.0 (7.1-28.3) NS 11	45.8 (27.3-61.0) **p=0.0004** 8	18.4 (3.8-48.9) NS 14
	A	0.26 (0.15-0.34) 10	0.34 (0.17-0.42) NS 3	0.39 (0.25-0.60) NS 3	0.22 (0.07-0.57) NS 10	0.69 (0.44-1.28) **p<0.003** 5	0.20 (0.05-0.74) NS 17

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); * number of subjects tested; NS-not significant.

$CD54^+$ and $CD54^+38^+$ subsets were significantly expanded only in the treated CD38hi group.

Fluorescence Intensity of Adhesion Molecule Expression (Table 3.22)

Only the fluorescence intensity of CD44 antigen expression was not different between groups. Patients with MGUS exhibited no alterations in the fluorescence intensities of any of the adhesion molecules.

The results in untreated patients were similar in both the percentage and absolute analyses. The CD38hi groups had decreased intensity of CDw49d expression on PBL. The intensities of the other adhesion molecules were unaltered. The CD38lo groups had increased intensities of CD11a and CD18 expression. The fluorescence intensities of CDw49d and CD54 expression were normal.

In the analysis of percentage results in treated patients, there were increases in the fluorescence intensities of the CD11a, CD18, CDw49d and CD54 antigens, irrespective of the percentage of $CD38^+$ cells. In the absolute analysis, the treated CD38hi patients exhibited a trend towards increased intensities of CD11a, CD18 and CD54 expression. The treated CD38lo patients had increased intensities of expression of all the adhesion molecules.

Lymphocytes Expressing Other Antigens (Table 3.23)

In the absolute analysis, there were differences between groups for several other antigens. Treated CD38lo patients had decreased populations of $CD1^+$, $CD1^+38^+$, $CD13^+$ and $CD13^+38^+$ cells. These alterations may merely be a reflection of the lymphopenia in this group. $CD34^+$ cell numbers were significantly increased in

Table 3.22: Fluorescence Intensity of Adhesion Molecule Expression on PBL for Patient Groups Based on the Number of $CD38^+$ PBL.

		Normals	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
$CD11a^+$	%	13.88 (7.19-20.96) 10*	21.07 (13.5-26.98) NS 3	6.16 (5.10-15.86) NS 3	21.01 (12.74-30.41) **p<0.03** 11	23.03 (18.38-49.95) **p<0.002** 8	24.40 (7.58-34.64) **p<0.004** 15
	A			10.67 (5.10-15.86) NS 4	21.06 (12.74-30.41) **p<0.02** 10	33.81 (7.58-49.95) p<0.1 5	23.48 (13.14-43.85) **p=0.0004** 18
$CD18^+$	%	12.38 (8.95-19.84) 10	18.87 (12.32-22.07) NS 3	5.73 (4.93-12.94) NS 3	18.71 (11.34-26.31) **p<0.05** 11	25.00 (16.02-48.92) **p<0.003** 8	19.85 (9.09-30.87) **p<0.03** 15
	A			9.34 (4.93-12.97) NS 4	19.59 (11.34-26.31) **p<0.04** 10	25.17 (9.09-48.92) p<0.1 5	20.41 (10.03-41.82) **p<0.004** 18
$CDw49d^+$	%	2.82 (2.01-3.66) 10	3.35 (2.37-3.59) NS 3	1.57 (1.43-1.71) **p<0.05** 2	3.76 (1.89-5.93) NS 8	6.18 (4.20-7.14) **p<0.004** 5	3.69 (3.18-5.10) **p<0.04** 6
	A			1.57 (1.43-1.71) **p<0.05** 2	3.76 (1.89-5.93) NS 8	Insufficient Data	4.25 (3.18-7.14) **p<0.004** 10
$CD54^+$	%	0.92 (0.73-1.28) 10	0.92 (0.77-1.03) NS 3	0.88 (0.79-0.97) NS 2	1.15 (0.63-2.02) p<0.1 11	1.32 (0.89-1.45) **p<0.009** 8	1.35 (0.83-1.64) **p<0.008** 14
	A			0.97 (0.79-1.10) NS 3	1.16 (0.63-2.02) NS 10	1.31 (0.83-1.45) p<0.08 5	1.34 (0.88-1.64) **p<0.003** 18

%-results expressed as a percentage; A-results expressed as an absolute number ($x10^9/l$); *number of subjects tested; NS-not significant.

Table 3.23: Miscellaneous Antigen Expression on PBL for Patient Groups Based on the Number of CD38⁺ PBL.

	Normals	MGUS	CD38hi Untreated	CD38lo Untreated	CD38hi Treated	CD38lo Treated
CD1a⁺	0.05 (0.03-0.11)*	0.04 (0.04-0.04)‡	0.06 (0.04-0.07)	0.03 (0.01-0.11)	0.05 (0.03-0.07)	0.02 (0.01-0.06)
			NS	NS	NS	**p=0.0009**
	10†	3	4	9	5	18
CD1a⁺38⁺	0.03 (0.02-0.07)	0.03 (0.01-0.03)	0.05 (0.03-0.05)	0.02 (0.00-0.07)	0.04 (0.03-0.06)	0.02 (0.00-0.03)
		NS	NS	NS	NS	**p<0.003**
	10	3	4	9	4	16
CD34⁺	0.05 (0.03-0.08)	0.10 (0.06-0.14)	0.07 (0.06-0.07)	0.05 (0.02-0.13)	0.08 (0.06-0.14)	0.04 (0.01-0.14)
		p<0.05	NS	NS	p<0.06	NS
	10	3	4	9	4	18
CD34⁺38⁺	0.02 (0.01-0.04)	0.03 (0.02-0.05)	0.04 (0.03-0.05)	0.02 (0.01-0.07)	0.06 (0.03-0.08)	0.01 (0.01-0.08)
		NS	**p<0.03**	NS	**p<0.03**	NS
	10	3	4	9	3	17
CD13⁺	0.08 (0.03-0.10)	0.08 (0.08-0.17)	0.07 (0.05-0.10)	0.05 (0.01-0.18)	0.09 (0.04-0.16)	0.04 (0.02-0.11)
		NS	NS	NS	NS	**p<0.006**
	10	3	4	9	5	18
CD13⁺38⁺	0.06 (0.02-0.07)	0.07 (0.05-0.15)	0.06 (0.04-0.09)	0.03 (0.01-0.13)	0.07 (0.02-0.13)	0.02 (0.01-0.06)
		NS	NS	NS	NS	**p<0.002**
	10	3	4	9	5	17

*all results are expressed in absolute numbers ($x10^9$/l); †number of subjects tested; ‡all 3 values were identical and hence computation could not be performed; NS-not significant.

MGUS. The number of $CD34^+38^+$ cells was increased in both untreated and treated CD38hi groups.

Summary

In the analysis based on the number of $CD38^+$ PBL, the patients with MGUS had normal numbers of $CD38^+$ cells, decreased B cell numbers, normal serum IgM levels, increased $CD56^+38^+$ cells, which are likely to be either NK cells or a minor subset of T cells (section 3.3.2) but otherwise normal T cell subsets. The changes in myeloma patients were more complicated. Those myeloma patients without increased numbers of $CD38^+$ PBL actually had $CD38^+$ cell numbers below the normal range. $CD4^+$ cells were normal in the untreated CD38hi groups but decreased in all the other groups. $CD4^+38^+$ cells were increased in the untreated CD38 hi group but usually reduced in the other groups. The $CD8^+$ and $CD8^+38^+$ subsets were increased in the treated patients, especially those in the CD38hi group, but were normal in the untreated patients. The CD56+ and CD56+38+ cells were normal in the untreated CD38hi group but there was either a trend towards or a significant increase in most of the other groups. The untreated CD38hi group had normal B cell numbers and serum IgM levels at the lower limit of the normal range. The other groups had decreased B cell numbers and markedly reduced serum IgM levels. Only the treated CD38hi group had increased expression of activation markers on PBL. The intensity of adhesion molecule expression was increased in both the treated CD38hi and CD38lo groups and, to a lesser extent, in the untreated CD38lo group.

3.3.5: Excluded Patients

Six patients were excluded from the statistical analysis: one with progressive myeloma plus carcinoma of the lung; one with progressive myeloma manifest as plasma cell leukaemia; one with recurrent extramedullary plasmacytomas; two with lymphoproliferative disorders associated with monoclonal paraproteins; one with metastatic carcinoma of unknown primary and an IgGκ paraprotein.

In the patient with plasma cell leukemia, the lymphocytes and plasma cells could not be analysed separately by selective gating. Absolute results for the principal lymphocyte populations could be calculated from the flow cytometry results by combining the lymphocyte and plasma cell counts of the full blood count differential. The alterations in the lymphocyte subsets were typical of a myeloma patient with progressive disease.

Both the patient with concurrent progressive myeloma and lung carcinoma and the one with recurrent extramedullary plasmacytomas had normal relative but low absolute numbers of $CD38^+$ PBL. The results for the lymphocyte subsets were similar to those in other myeloma patients with progressive disease or belonging to the treated CD38lo groups.

One patient with a lymphoproliferative disorder had a $s\kappa^+$ B cell clone in the peripheral blood but the relative and absolute number of $CD38^+$ PBL were normal. The other results were similar to those in the patients with MGUS. The other patient, who was not definitively diagnosed prior to death, had abdominal lymphadenopathy and an IgGκ paraprotein. The proportion and absolute number of $CD38^+$ cells were above the normal range. The alterations in the

lymphoid populations were different to those in untreated CD38hi myeloma patients.

The patient with disseminated carcinoma had both a higher percentage and absolute number of $CD38^+$ cells than any other patient in this study. The changes in lymphocyte subsets were similar but not identical to those in untreated CD38hi patients.

3.3.6: Follow-up Studies

Follow-up studies were performed on one patient with SMM and 5 patients who were evaluated at diagnosis and subsequently commenced therapy. These follow-up studies were not included in the main statistical analysis.

The patient with SMM was restudied after a period of 6 months. The percentage and absolute number of $CD38^+$ cells remained within the normal range. There were no major changes in the lymphocyte subsets when the results were compared to the original analyses based on disease status and $CD38^+$ cell numbers.

Three patients who at diagnosis had an increased percentage and absolute number of $CD38^+$ PBL were restudied: two prior to the third course of chemotherapy and one prior to the sixth course. Two patients belonging to the untreated CD38lo groups in both the percentage and absolute analyses were studied prior to the third treatment course. The most obvious alteration in the patients who originally had high numbers of $CD38^+$ cells was the development of low numbers of $CD19^+$ and $CD19^+38^+$ cells, in contrast to the normal or high levels present prior to therapy. The rapidity of this change in $CD19^+$ cell

numbers suggests it may have been a direct effect of therapy. No obvious pattern emerged for the alterations in the relative and absolute numbers of $CD38^+$ PBL or in the T cell or NK cell subsets following therapy. It is therefore not possible to draw any conclusions about the impact of treatment on the majority of lymphocyte subsets, as opposed to any effects resulting directly from interactions between the myeloma clone and the immune system.

Most of these patients had not developed the lymphocyte subset abnormalities present in the on treatment group in the main analysis but there may be a simple explanation for this. In the on treatment group, 3/5 patients had received both prior chemotherapy and radiotherapy. Thus, the results in these patients may not be representative of those occurring in individuals on first-line therapy.

3.4: DISCUSSION

The percentage and absolute number of $CD38^+$ PBL in normal individuals were higher than documented previously. This rise in the number of cells detected as positive for a given antigen is not unprecedented, having been observed for the CD25 antigen (Jackson et al, 1990). As already discussed (section 3.3.1), the factors which influence the number of cells detected as expressing an antigen include the flow cytometer, the fluorochrome, the pattern of antigen expression and the affinity of the antibody. These observations emphasise the necessity for each laboratory to determine the normal range for its particular set of conditions, rather than relying on published normal values.

When the patients were divided into groups based on disease status, there was no phase during the course of the disease when the percentage or absolute

number of $CD38^+$ lymphocytes differed statistically from the control group. Apart from those patients with MGUS, there were patients at each phase of the disease who had $CD38^+$ cell numbers below, within and above the normal range. When the patients were divided into 4 groups on the basis of treatment status and the number of $CD38^+$ PBL, it became apparent that elevated numbers of $CD38^+$ cells were associated with different alterations in other lymphocyte subsets compared to those occurring in patients without elevated numbers. Furthermore, the changes in the lymphocyte subsets in patients with $CD38^+$ cell numbers above the normal range differed depending on whether they had received prior therapy.

When the patients were grouped according to disease status, the principal alterations documented in the T lymphocyte subsets were a decreased percentage and absolute number of $CD4^+$ and $CD4^+38^+$ cells, an increased percentage of $CD8^+$ and $CD8^+38^+$ cells, and a reduced CD4:CD8 ratio. In any of the groups in which the patients had been exposed to therapy, these alterations were statistically significant. There was marked heterogeneity within these populations in the untreated patients but the abnormalities mentioned above were present in some patients. For example, 4/6 patients with SMM and 5/11 with active disease at diagnosis had low CD4:CD8 ratios. The occurrence of these changes in SMM suggest that they may not be unfavourable prognostic factors in untreated patients. The fact that there are similar alterations in patients in plateau phase or with progressive disease also argues against a simple link with active disease. However, patients with an absolute $CD4^+$ count $<0.7x10^9/l$ at diagnosis have been reported to have a significantly shorter survival than those with higher counts (San Miguel et al, 1992). In that study, 57% of patients were in this poor prognostic group. In this current study, 41%

of patients had a $CD4^+$ count $<0.7x10^9/l$, including 1/6 (17%) patients with SMM and 6/11 (55%) patients with active disease at diagnosis. None of the patients with a $CD4^+$ count $<0.7x10^9/l$ had an increased absolute number of $CD38^+$ PBL, another known poor prognostic factor (Boccadoro et al, 1988).

No differences in the relative and absolute numbers of NK cells were detected between patients at various phases of the disease. This contrasts with the study of Österborg et al (1990) but agrees with the findings of Gonzalez et al (1992), who studied only untreated patients. The discrepancy between this study and that of Österborg and co-workers may be related to several factors: their study had a larger number of patients, divided newly diagnosed patients into Durie and Salmon stage I and II versus III and employed an invalid statistical analysis. When multiple groups are being analysed for differences with respect to a single factor, it is correct statistical procedure to perform an analysis of variance which must be significant before comparisons can be made between any pairs of interest (Daniel, 1978; Rimm et al, 1980). This was not done in the study of Österborg et al (1990). Applying the appropriate procedures to the analysis based on $CD38^+$ cell numbers in this study did allow the identification of a group with a significant increase in $CD56^+$ cells, namely untreated CD38lo patients, although it is not clear from this study whether the majority of these cells were NK or $CD56^+$ T cells.

In all the groups based on disease status, the majority of individuals had B cell numbers below the normal range. However, there were some patients at diagnosis, in plateau or with progression who had normal to elevated B cell numbers.

Care was taken when enumerating $s\kappa^+$ and $s\lambda^+$ lymphocytes. The cells were extensively washed to remove cytophilic immunoglobulin from non B cells. The antibodies were $F(ab')_2$ fragments and were thus not capable of binding to Fc receptors themselves. The nature of this study precluded two colour analysis using antibodies against a specific B cell antigen and sIg, as recommended by King and Radicchi (1992). Despite this limitation, only one patient with LCIS and 5 patients with clonal excess were detected. The incidence of LCIS was similar to that reported by Bagg et al (1989) and the incidence of clonal excess was similar to that reported by Büchi et al (1990). Most other studies have documented markedly higher incidences of these phenomena (Pettersson et al, 1981; Wearne et al, 1984; Joshua et al, 1987; Österborg et al, 1987; Bagg et al, 1989; Büchi et al, 1990; Oritani et al, 1990). The disagreement in the literature regarding the existence and the frequency of LCIS and clonal excess indicates that further large studies of these phenomena, employing the recommendations of King and Radicchi (1992), are required to clarify the situation.

The abnormalities detected in the T and B lymphoid subsets when the patients were divided on the basis of disease status are consistent with those previously reported (see Introduction section 1.8). The diversity of the reported abnormalities of lymphoid subsets in myeloma patients and the heterogeneity exhibited by some groups in this study, especially the newly diagnosed patients with active disease, were clarified by the analysis based on $CD38^+$ cell numbers. The results of that analysis are summarised in table 3.25 (page 200).

The untreated CD38hi patients as a group had normal numbers of $CD4^+$ PBL, high normal to elevated numbers of $CD4^+38^+$ cells, an increased CD4:CD8 ratio,

normal numbers of total and $CD38^+$ B cells and IgM levels just below or within the normal range. Although these patients had normal B cell numbers, the absence of clonal excess excluded the presence of a large number of cells belonging to the malignant clone. On an individual basis only the results in one patient were not consistent with this pattern. This person had low B cell numbers and a markedly decreased serum IgM but did have an elevated number of $CD4^+38^+$ cells. The untreated CD38lo patients had decreased $CD4^+$ cells, $CD4^+38^+$ cells, CD4:CD8 ratio, B cells, $CD38^+$ B cells and serum IgM levels but increases in some of the $CD56^+$ subsets (ie. NK cells or $CD56^+$ T cells). The different abnormalities of the lymphocyte subsets in the untreated CD38hi and CD38lo patients account for the heterogeneity of the results when the untreated patients were analysed as patients with SMM versus newly diagnosed patients with active disease.

Some of the changes in the lymphoid subsets were common to the untreated CD38lo, treated CD38hi and treated CD38lo groups. They had reduced $CD4^+$ subsets, reduced or normal $CD4^+38^+$ subsets, decreased CD4:CD8 ratios and increases in some of the $CD56^+$ subsets (ie. NK cells or $CD56^+$ T cells). The expansion of the $CD56^+38^+$ subset was most marked in the treated CD38hi group. Serum IgM levels were markedly reduced in all these groups but the B cell numbers were statistically normal rather than decreased in the treated CD38hi group, as a few patients had increased B cell numbers, although the majority had reduced numbers. Clonal excess was present only in patients with low B cell numbers, reconfirming that any circulating malignant $CD38^+$ B cell population must be numerically small.

There were additional abnormalities in the untreated CD38hi and CD38lo groups that were not present in the untreated CD38lo groups. The $CD8^+$ and $CD8^+38^+$ subsets were expanded in the untreated CD38hi patients. These same subsets were increased in the analysis of the percentage results in the treated CD38lo group but were normal in the absolute analysis. On an individual basis, the most consistent alteration in the untreated CD38hi patients was the increase in $CD8^+38^+$ cells.

When the results based on the number of $CD38^+$ PBL are examined, possible explanations for some of the variability in the results of previous studies which divided the patients only into untreated and treated groups are apparent. The results for the $CD4^+$ subset and the CD4:CD8 ratio in untreated patients would depend on the proportion of CD38hi versus CD38lo patients in a particular study. This could be influenced by the referral pattern to a particular institution. Similarly, the results for absolute $CD8^+$ counts in treated patients may be high or normal depending on the balance between CD38lo and CD38hi patients. Moreover, it is conceivable that the $CD8^+$ counts may even be decreased if the study included an even larger proportion of markedly lymphopenic patients. Although not examined in this study, the degree of lymphopenia may be related to the amount of previous therapy or the use of particular chemotherapy drugs.

The significance of changes in the expression of activation and adhesion molecules by PBL are also more easily understood in the analysis based on $CD38^+$ cell numbers than that on disease status. The adhesion molecules CD11a, CD18, CD44 and CDw49d were expressed on the majority of PBL in the normal controls. In the analysis based on disease status, there was either a trend

towards or a significant decrease, albeit quantitatively small, in the percentage of cells expressing the CD11a antigen in all patient groups. The analysis based on CD38 status demonstrated that only untreated CD38hi patients had a normal proportion of cells expressing this antigen. These changes were not accompanied by a decreased percentage of $CD18^+$ cells. As CD18 forms heterodimers with the CD11a, CD11b and CD11c molecules, the discrepancy between the CD11a and CD18 results suggests that the lymphoid cells with decreased expression of CD11a express CD11b or CD11c. NK cells or $CD8^+11b^+$ T cells are possible candidates but this study was not designed to resolve this question and it is not clear why these cells would have downregulated CD11a expression. The absolute numbers of $CD11a^+$ and $CD18^+$ cells were decreased only in the groups containing treated patients. This was related predominantly to the lymphopenia present in some patients, as confirmed by decreased expression of these antigens in the untreated CD38lo group, which was the only significantly lymphopenic group. Alterations in the numbers of $CD44^+$ and $CDw49d^+$ cells similarly reflected lymphopenia.

Alterations in the populations co-expressing CD38 with either CD11a, CD18 or CDw49d were present only in the analyses based on $CD38^+$ cell numbers and reflected the variation in the proportion or absolute number of $CD38^+$ PBL in the various patient groups.

The antigens CD11a, CD18, CD44 and CD29 are adhesion molecules whose expression is upregulated on memory cells as compared to naive T cells (Sanders et al, 1988a). CD29 is the common β subunit for a family of molecules known as very late antigens. CDw49d associates with CD29 to form the VLA-4 molecule but it is only one of the α subunits associated with CD29 on

lymphocytes (Hemler, 1990; Hemler et al, 1990). The interpretation in the following paragraph of the alterations in the fluorescence intensities of CD11a, CD18, CD44 and CDw49d, involves the assumption that these changes occur predominantly on a single lymphoid subset. As shown in table 3.24, these adhesion molecules are expressed on the majority of PBL in the control subjects. T cells (CD3+) are the major lymphoid subset in peripheral blood. With the exception of the percentage of $CD11a^+$ cells (discussed in the preceding paragraph), the percentage of cells expressing any of these adhesion molecules or CD3 was not significantly different in the control and patient groups in either the analysis based on disease status or the number of $CD38^+$ PBL. Therefore the assumption has been made that changes in the fluorescence intensity of these molecules on the total lymphoid population predominantly reflect changes in the fluorescence intensity on T cells.

Table 3.24: Total T cells and Lymphocytes Expressing Adhesion Molecules Expressed as a Percentage of PBL.

	CD3	CD11a	CD18	CD44	CDw49d
Normals	81.9 (70.1-85.5)* n=10	99.1 (97.3-99.6) n=10	98.3 (88.9-99.8) n=10	99.1 (94.2-99.8) n=10	84.6 (75.8-94.1) n=9
Patients	75.8 (35.2-92.9) 60-<70% - 10/44† <60% -3/44	95.1 (38.8-99.8) >90% - 33/40	95.2 (74.7-99.4) >88% - 35/40	98.8 (89.6-99.9) >90% - 33/33	86.1 (63.4-95.5) >75% - 21/24

*results expressed as median (range); †number of patients out of total number tested for that antigen

The fluorescence intensities of CD11a, CD18 and CDw49d expression on PBL either exhibited a trend towards or were significantly increased in all previously treated patients, irrespective of disease or CD38 status. In patients with SMM or at diagnosis, there were increased intensities of CD11a and CD18 expression in some patients, although there were not statistically significant alterations in these groups. When the untreated patients were divided on the basis of $CD38^+$ cell numbers, the CD38lo but not the CD38hi groups had a significant increase

in the fluorescence intensities of CD11a and CD18. However, the intensity of CDw49d was decreased in the untreated CD38hi groups and normal in the untreated CD38lo groups. Overall, these results are suggestive of a decrease in the ratio of naive:memory T cells in myeloma, as previously reported by some (Serra et al, 1988; Massaia et al, 1991) but not all investigators (Shapira et al, 1989). However, this study identified a group of myeloma patients which does not have this altered ratio, namely untreated CD38hi patients. The lack of difference in the intensity of CD44 antigen expression between groups does not invalidate this hypothesis, as CD44 by itself is not a good discriminator of naive and memory cells (Sanders et al, 1988a). The intensity of CDw49d expression in untreated patients did not follow the pattern exhibited by CD11a and CD18. This is not surprising for several reasons. CDw49d is not as obviously biphasic in intensity in single parameter flow cytometry analysis as some of the other molecules which form heterodimers with CD29 (Hemler, 1990). Hence, it may not be the most sensitive discriminator of naive and memory cells. Furthermore, as CDw49d is not the only molecule that forms heterodimers with CD29, changes in the fluorescence intensity of CDw49d may have implications apart from merely reflecting the absence or presence of memory. This interpretation of the alterations in the fluorescence intensities of these adhesion molecules requires confirmation and the easiest approach would be to assess the co-expression of CD3 and the isoforms of the CD45 molecule (ie. CD45RO and CD45RA), rather than investigating changes in the fluorescence intensity of adhesion molecules.

CD54 is an adhesion molecule that serves as both a marker of activation and of memory cells. It is present at low levels on approximately 30% of memory cells (Boyd et al, 1989; Wallace and Beverley, 1990). It is not present on resting naive

T lymphocytes but is rapidly upregulated to high levels following *in vivo* activation of either naive or memory cells. Unlike the situation for the other adhesion molecules, the numbers of $CD54^+$ and $CD54^+38^+$ cells did not simply reflect lymphopenia or varying $CD38^+$ cell numbers. Markedly expanded $CD54^+$ and $CD54^+38^+$ subsets were present in the treated CD38hi groups. There was a significant increase of fluorescence intensity of CD54 in both the treated CD38hi and CD38lo groups. The combination of these two results suggests that CD54 is acting as an activation marker in the CD38hi patients. It seems probable that CD54 is being co-expressed by an increased number of $CD8^+$ PBL irrespective of whether or not the latter cells co-express CD38. Dual labelling for the CD8 and CD38 antigens would be required to confirm this hypothesis. The hypothesised reduced naive:memory T cell ratio in untreated CD38lo patients was not confirmed by the presence of an increased fluorescence intensity of CD54 but this antigen is not present on all memory cells and is thus a less accurate discriminator of these two cell subsets than CD11a or CD18. The increase in the fluorescence intensity of CD54 to levels comparable to those in the treated CD38hi patients suggest that the $CD54^+$ cells in the treated CD38lo group may also be activated and not just resting memory cells.

The CD25 antigen is expressed on multiple populations of PBL. Two thirds of $CD25^+$ lymphocytes are $CD4^+$, 10% are $CD19^+$, 18% are $CD8^+$ and 5% are $CD16^+$. Almost 100% of the $CD4^+25^+$ lymphocytes are memory cells (Jackson et al, 1990). In this study, the $CD25^+$ and $CD25^+38^+$ subsets were either normal or decreased. Decreased $CD19^+$ and $CD19^+38^+$ populations, which were present in many groups, would have been a contributing factor to these alterations in CD25 antigen expression. In some groups with decreased $CD25^+$ subsets, the decrement was larger than that in the $CD19^+$ subsets. $CD4^+$ lymphocytes were

also decreased in many of these groups, suggesting that there may have been a decrease in the number of $CD4^+$ memory cells in at least some patients, especially when the results were expressed as absolute numbers. This is not inconsistent with the presence of a decreased naive:memory T cell ratio, as this requires only a more marked depletion of naive than memory cells. Indeed, Serra et al (1988b) noted that some myeloma patients had a decreased percentage of $CD4^+$ memory cells but even in these patients the naive:memory cell T ratio was usually reduced.

Significantly, CD25 expression was normal in the untreated CD38hi patients in whom the $CD4^+$ and $CD19^+$ subsets were also normal. These groups did have increased $CD4^+38^+$ but normal $CD25^+38^+$ populations, suggesting that the increased expression of CD38 on helper T cells is not accompanied by co-expression of the CD25 antigen. Furthermore, none of the other activation antigens accompanied this increased expression of CD38 antigen by $CD4^+$ cells.

Expression of the CD25 antigen was not increased in the treated CD38hi groups. However, these groups did have markedly expanded HLA-DR^+ and HLA-DR^+CD38^+ subsets, suggesting that a large proportion of $CD8^+$ and possibly $CD56^+$ lymphoid cells expressed class II MHC molecules. Resting NK cells do not express class II MHC antigens but are induced to do so in short term tissue culture (Anegón et al, 1988). Even if the $CD56^+$ cells did have increased expression of MHC Class II molecules, the increments in the HLA-DR^+ subsets were larger than those in the $CD56^+$ ones, implying co-expression of HLA-DR on $CD8^+56$- cells.

CD9, CD36, CD71 and PCA-1 are other antigens which are definite or possible markers of activation. The CD9 antigen is expressed on platelets, monocytes, B cells at various stages of differentiation but only weakly on B cells in peripheral blood, and on 26% of peripheral blood T cells (Horton and Hogg, 1987; Ling et al, 1987; Van Dem Borne and Modderman, 1989a). CD9 is present on T cell clones derived from both normal individuals and patients with myeloma (Pilarski et al, 1985a) and on increased numbers of T cells in some myeloma patients (Pilarski et al, 1989b). The CD36 antigen is expressed on monocytes, platelets and weakly on B cells (Horton and Hogg, 1987; Van Dem Borne and Modderman, 1989b). However, the IVth Leucocyte Typing Workshop reported that 10% of peripheral blood T cells and 16% of PHA activated T cells express this antigen (Van Dem Borne and Modderman, 1989b). CD71, which is the transferrin receptor, is upregulated in all lymphocyte activation models and, although associated with proliferation, not all cells expressing this antigen are proliferating (Schwarting and Stein, 1989). The antibody against PCA-1 was originally described as reacting with malignant plasma cells but not peripheral blood T or B cells, although it was reactive with PHA activated T cells and T cell lines expressing Class II MHC molecules (Anderson et al, 1983). Subsequently, the PCA-1 antigen has been detected on some PBL, predominantly B cells, in normal individuals (Boccadoro et al, 1988). In myeloma patients, there is a statistically significant correlation between the numbers of $CD38^+$ and $PCA\text{-}1^+$ PBL, suggesting co-expression of these antigens (Boccadoro et al, 1988; Omedé et al, 1990) and this has been confirmed by dual colour analysis in a few patients (Omedé et al, 1990). The expanded $PCA\text{-}1^+$ lymphoid population in myeloma patients comprised not just B cells but also approximately 20% of both $CD4^+$ and $CD8^+$ T cells (Boccadoro et al, 1988). In this study, there was also an increased percentage of $CD11b^+$ cells expressing PCA-1 but, as increased

numbers of both $CD4^+11b^+$ and $CD8^+11b^+$ cells have been reported in myeloma (Massaia et al, 1986; Dianzani et al, 1988; Massaia et al, 1988b; Massaia et al, 1990), it is not possible to be certain whether any NK cells, which are known to express CD11b (Ortaldo et al, 1981; Griffin et al, 1983; Perussia et al, 1983; Ritz et al, 1988; Robertson and Ritz, 1990), also express PCA-1.

The treated CD38hi groups had expanded $PCA\text{-}1^+38^+$, $CD9^+38^+$ and $CD36^+38^+$ but not $CD71^+38^+$ subsets. Although dual labelling of cells for these activation markers and either the CD8 or CD56 antigens was not performed, it seems probable that the $CD8^+$ and/or $CD56^+$ lymphocytes expressing the CD38 antigen also expressed the CD9, CD36 and PCA-1 antigens.

Both the untreated and treated CD38lo groups had diminished $CD9^+38^+$ and $CD71^+38^+$ populations. As the $CD4^+38^+$ and $CD19^+38^+$ cells were decreased in these groups, it seems likely that some of the cells in these subsets normally express CD9 and CD71. In the treated CD38hi groups, increased numbers of $CD8^+38^+$ or $CD56^+38^+$ cells co-expressing the CD9 antigen offset any decrease resulting from the decreased numbers of $CD4^+38^+$ and, in the majority of patients, also $CD19^+38^+$ cells. In contrast, any increased expression of CD71 by $CD8^+38^+$ or $CD56^+38^+$ cells was sufficient only to maintain $CD71^+38^+$ cell numbers within the normal range.

Combining the results for the B cell and major T cell populations with those for the expression of activation and adhesion molecules allows the emergence of a more detailed overview of the changes in lymphocyte subsets that occur in myeloma patients. These results are summarised in table 3.25.

Table 3.25: Summary of Changes in Lymphocyte Subsets in Myeloma.				
	Untreated		Treated	
	CD38hi	CD38lo	CD38hi	CD38lo
$CD4^+$ Cells (T Cell Subset)	N	↓	↓	↓
$CD4^+38^+$ Cells	↑	↓	↓ or N*	↓
$CD8^+$ Cells (T Cell Subset)	↓ or N*	N	↑	↑ or N*
$CD8^+38^+$ Cells	N	N	↑	↑ or N*
CD4:CD8 Ratio	↑	↓	↓	↓
$CD56^+$ Cells (NK Cells or T Cell Subset)	N	↑ or N*	N	N
$CD56^+38^+$ Cells	N	↑ or N*	↑	↑ or N*
B Cells	N	↓	N (V†)	↓
$CD38^+$ B Cells	N	↓	N (V†)	↓
Serum IgM	Low normal	↓	↓	↓
Ratio of Naive:Memory T Cells	N	↓	↓	↓
Activated Subsets	No	No	Yes	Possibly
↑-increased; ↓-decreased; N-normal; V-variable; *percentage and absolute results differed; †most patients had reduced numbers but two had elevated numbers.				

The untreated patients with increased numbers of $CD38^+$ PBL had normal numbers of $CD4^+$ and $CD8^+$ lymphocytes but a high CD4:CD8 ratio, high normal to elevated numbers of $CD4^+38^+$ cells, normal numbers of B cells and $CD38^+$ B cells, IgM levels just below or within the normal range, normal $CD56^+$ subsets, which are likely to be either NK cells or a minor subset of T cells (section 3.3.2) and possibly a normal ratio of naive:memory T cells. The elevated number of $CD38^+$ cells was predominantly due to an expansion of the $CD4^+38^+$ population with maintenance of normal $CD38^+$ B cell numbers. In two patients, an expanded $CD56^+38^+$ population also contributed to the increased number of $CD38^+$ cells. Although the CD38 antigen is an activation antigen on T cells (Terhorst et al, 1981; Hercend et al, 1981), its function is not known. The expression of none of the other activation antigens was increased, suggesting that the $CD4^+38^+$ cells did not co-express these antigens, although this requires further dual labelling experiments for confirmation. This conclusion is not

necessarily inconsistent with previous data. Although $CD4^+HLA\text{-}DR^+$ cells were increased when myeloma patients at all phases of the disease were studied as a single group (Massaia et al, 1988b; Massaia et al, 1990), this population was not significantly expanded when patients were grouped according to disease status, despite increased median values (Dianzani et al, 1988)

The untreated CD38lo, treated CD38hi and treated CD38lo groups shared some abnormalities in lymphocyte populations: reduced $CD4^+$ subsets; reduced or normal $CD4^+38^+$ subsets; decreased CD4:CD8 ratios; expansion of some $CD56^+$ subsets, which are likely to represent either NK cells or a minor subset of T cells (section 3.3.2); decreased B cell numbers; low serum IgM levels; and a possible reduction of the naive:memory T cell ratio. Both treated groups had alterations in $CD8^+$ and $CD8^+38^+$ populations but the expansions of these subsets were greater in the CD38hi patients.

In the treated CD38hi group, the elevated number of $CD38^+$ PBL was due to elevated numbers of $CD8^+$ T cells and, depending on the identity of $CD56^+$ cells, possibly NK cells co-expressing this antigen. In the absolute analysis, there was also a contribution from $CD38^+$ helper T cells which were present in normal numbers. In two patients, including the only one with a normal number of $CD8^+38^+$ cells, there was also a contribution from an expanded $CD19^+38^+$ population. There was evidence of activated lymphoid populations in the treated CD38hi patients. Although the appropriate dual labelling studies were not performed, the pattern of alterations was consistent with a high level of co-expression of the class II MHC and CD54 antigens by $CD8^+$ T cells, irrespective of their expression of CD38. Some $CD56^+$ cells may also have expressed these antigens.

Despite the failure to detect increased numbers of lymphocytes expressing activation antigens in the treated CD38lo patients, the high intensity of expression of the CD54 antigen suggests the possible presence of some activated subsets. The expression of class II MHC molecules and their co-expression with CD38 was normal in this group as a whole, although clearly elevated in some individuals. Given that B cells, which constitutively express class II MHC antigen, are decreased in this group, it is conceivable that there may be increased expression of these molecules by other subsets. Further two colour immunophenotypic studies would be required to investigate this possibility.

Given the strong positive correlations between the numbers of $CD38^+$ and both $CD19^+$ and $CD19^+38^+$ lymphocytes, as well as those between serum IgM levels and both $CD19^+$ and $CD38^+$ numbers, the high number of $CD38^+$ PBL and the associated lymphoid subset alterations in some untreated patients may reflect a less marked disturbance of humoral immunity than is present in other patients. As a high number of $CD38^+$ PBL is an adverse prognostic factor at diagnosis in multiple myeloma (Omedé et al, 1990; Joshua et al, 1991b), the presence of near normal polyclonal immunoglobulin levels may be associated with a short survival duration. The maintenance of relatively normal humoral immunity could be via either a passive or active mechanism.

In the untreated CD38hi patients, there may be a failure to activate a mechanism which suppresses the malignant clone and at the same time suppresses normal humoral immunity. Several groups have identified T cells with specificity for the idiotypic determinants on the paraprotein but there is disagreement about the fluctuations in the number of these cells with disease

activity. Österborg et al (1991) reported the presence of such cells in 3 patients with either MGUS or SMM but it is not clear whether they studied any untreated patients with active disease. Nelson et al (1993) found that idiotype-reactive T cells were more numerous in patients in plateau phase than with progressive disease. In contrast, Dianzani et al (1988) reported the highest numbers in those patients with active disease and a poor prognosis but they did not provide details on the numbers of untreated versus treated patients in the study. Idiotype-reactive T cells may result in suppression of normal B cells, as well as the malignant clone, as they may recognise public as well as private idiotopes. It has been proposed that the decreased naive:memory T cell ratio in myeloma patients reflects an altered T cell repertoire resulting from the presence of large numbers of idiotype-reactive T cells (Massaia et al, 1991).

The data in this study suggests that there may be a normal naive:memory T cell ratio in the untreated CD38hi patients, with the inference that these patients have not responded to the malignant clone by developing T cells involved in idiotypic networks. In contrast, the data suggests that the untreated CD38lo groups and both the treated CD38hi and CD38lo groups have a decreased naive:memory T cell ratio, with the inference being that idiotype-reactive T cells have developed. Although the purpose of these cells would be to suppress the malignant clone, the activity of these cells may also have resulted in the suppression of normal B cell numbers and polyclonal immunoglobulin production documented in these patient groups. The mechanism of action of idiotype-reactive T cells is not clear. If one such mechanism was via an effect on helper T ($CD4^+$) cells, this may account for the decreased numbers of $CD4^+$ cells in these patients.

The above theory is one possible explanation for the normal B cell subsets, normal $CD4^+$ cell numbers and low normal serum IgM levels in untreated CD38hi patients but fails to address the issue of the increased numbers of $CD4^+$ cells co-expressing CD38. The increased numbers of $CD4^+38^+$ PBL may indicate the presence of an active mechanism for maintaining humoral immunity. The nature of such a mechanism is not apparent. Possibly there are intrinsic properties of the malignant clone which allow it to be seen as more abnormal by the immune system compared to the clone in the untreated CD38lo patients. Interestingly, all the patients with BJP excretion at diagnosis were in the CD38hi treated groups. Secretion of excessive amounts of free light chain is a property of the malignant clone and BJP excretion is indicative of a poor prognosis (Durie and Salmon, 1975; San Miguel et al, 1989). The immune response to the most abnormal clones could be specifically designed to maintain humoral immunity or could be just an incidental outcome of a non-specific immune response. Why this response should be different to that in the untreated CD38lo patients, rather than being a more pronounced response of the same nature (ie. a more expanded population of idiotype-reactive T cells), is unclear.

The assumption has been made in the above discussions that the specificities of the B cells and the serum IgM in the untreated CD38hi patients are the same as those in normal individuals. This may not be correct. An increased proportion of B cells with anti-idiotypic specificities have been detected in both untreated and treated myeloma patients (Bergenbrant et al, 1991; Pilarski et al, 1985b; Pilarski et al, 1989b). Bergenbrant et al (1991) found the highest production of anti-idiotypic antibodies in patients with MGUS or stable myeloma but Pilarski's group (1985b; 1989b) found the highest levels in those with active disease. $CD5^+$ B cells are the major source of autoantibodies and anti-idiotypic

antibodies (Cassali et al, 1987; Raveche, 1990). These antibodies are predominantly IgM. One possibility is that the normal B cell numbers in the untreated CD38hi patients are due to an increased number of $CD5^+$ B cells and that the serum IgM is enriched for anti-idiotypic antibodies. This seems unlikely for several reasons. The increased proportion of B cells with anti-idiotypic specificities has been found in patients with both relatively normal and severely reduced total B cell numbers (Pilarski et al, 1985b; Pilarski et al, 1989b). There is no correlation between the production of anti-idiotypic antibodies *in vitro* by EBV transformed B cells from myeloma patients and the serum immunoglobulin levels (Bergenbrant et al, 1991). If these cells are able to secrete *in vivo*, immunosuppression of polyclonal immunoglobulin production would be anticipated but the untreated CD38hi patients had normal levels of the other non-malignant immunoglobulin isotypes as well as IgM. Extensive studies of $CD5^+$ B cells in myeloma patients have shown that these cells do have immunosuppressive properties *in vitro* (Paglieroni and MacKenzie, 1977; Paglieroni and MacKenzie, 1980; MacKenzie et al, 1987; Mackenzie et al, 1991).

Helper T cells expressing the CD11b antigen are increased in myeloma patients at diagnosis (Dianzani et al, 1988). It would be interesting to determine whether expansion of this population is confined to the untreated CD38hi patients. This population represents a minor subset of $CD4^+$ cells in normals. The function of these cells is unclear but, unlike $CD4^+$ cells as a whole, they do not provide help for B cell proliferation and differentiation in the usual *in vivo* assay systems (Velardi et al, 1985b; Morshita et al, 1986). The morphology, function and immunophenotype of these cells is similar to a population of helper T cells present in the germinal centres of secondary lymphoid tissue (Velardi et al, 1986a; Velardi et al, 1986b). These cells have been hypothesised to provide

support for the B cell maturation processes occurring at these sites (Velardi et al, 1986b; Bowen et al, 1991).

The number of $CD38^+$ PBL in untreated patients did not correlate with the majority of other prognostic factors at diagnosis, namely sβ2M, STK or plasma cell LI, indicating that it is an independent prognostic factor. The lack of correlation with the STK or plasma cell LI suggests that the poor prognosis in patients with an increased number of $CD38^+$ PBL is not related just to uncontrolled proliferation of the malignant clone.

All untreated patients who excreted BJP had high numbers of $CD38^+$ cells. The presence of malignant light chains in the urine has been identified as a poor prognostic factor by most investigators (Durie and Salmon, 1975; Simonsson et al, 1988; San Miguel et al, 1989) and patients with urinary light chain excretion are less likely to reach plateau (Durie et al, 1988; Joshua et al, 1991). It is difficult to reconcile these observations with some of the proposed functions of free immunoglobulin light chains. There is ample evidence that secretion of free light chains is a property of normal B cells (Hannam-Harris and Smith, 1981a; Hannam-Harris and Smith, 1981b; Gordon, 1984; Hopper and Papagiannes, 1986). One hypothesised role for free light chains is interaction within an autologous idiotypic network which serves to regulate clonal immunoglobulin secretion (Hopper and Papagiannes, 1986; Hopper et al, 1988). There is also experimental evidence that free light chains may regulate immunoglobulin secretion via an isotype specific mechanism and this mechanism may be responsible for the phenomenon of LCIS (Ioannidis et al, 1989). The operation of either of these mechanisms would predict that the elevated secretion of light chain by myeloma cells in those patients excreting

BJP would result in immunoregulation of the malignant clone. This is clearly not the case. Either these immune mechanisms are ineffective in suppressing the malignant clone or the excessive quantities of free light chains in patients with BJP excretion do not behave in the same manner as the free light chains in normal individuals and fail to trigger the usual immunoregulatory circuits. As discussed earlier, the evidence infers that the untreated CD38hi patients do not have idiotype-reactive T cells.

The finding of a significant negative correlation between age and $CD38^+$ cell numbers in the untreated patients was unexpected, as increasing age has been reported as an adverse prognostic factor (Simonsson et al, 1988; Greipp et al, 1988; Cavo et al, 1989).

The various correlations between the numbers of $CD38^+$, $CD19^+$ and $CD19^+38^+$ cells and the serum IgM levels were either weaker or non-existent in the treated patients when compared to those in the untreated patients. This suggests that in treated patients the elevation of $CD38^+$ PBL is not primarily related to maintaining normal polyclonal immunoglobulin levels, as has been hypothesised to be the situation in the untreated patients. Treatment seems to have abolished or severely depressed such an immune response. This may be reflected in the rapid fall in the B cell populations after patients commence treatment. As discussed earlier, the data tends to suggest that the treated CD38hi and CD38lo patients had a decreased naive:memory T cell ratio, possibly indicating the presence of idiotype-reactive T cells (Massaia et al, 1991). The $CD8^+$HLA-DR^+ T cell population in myeloma is enriched for cells recognising such idiotypic determinants (Dianzani et al, 1988). As the elevated number of $CD38^+$ PBL in treated patients was due to increased expression of this antigen

by an expanded and activated $CD8^+$ population, increased $CD38^+$ cell numbers in these patients may reflect not just the presence of but a markedly increased number of activated $CD8^+$ idiotype-reactive T cells.

In the treated patients, the number of $CD38^+$ PBL correlated with sβ2M and STK, which are prognostic factors used to monitor disease activity in individual patients. The correlation with sβ2M suggests that the expansion of idiotype-reactive T cells in treated patients is related to tumour cell mass. Moreover, as sβ2M continues to predict survival duration during the course of the disease (Cuzick et al, 1990), it implies that elevated expression of the CD38 antigen in treated patients also indicates a poor survival, as has been previously suggested (Ruiz-Argüelles et al, 1984; Joshua et al, 1988). The fact that this study did not find an increased proportion of patients with progressive disease in the CD38hi groups may be related to the fact that this was a cross-sectional rather than a longitudinal study. The presence of the most marked expansion of the idiotypic network in those individuals with the poorest prognosis suggests that this mechanism of controlling the malignant clone is either ineffective per se or that the development of resistance to this mechanism is one mode of escape of the malignancy from immune surveillance. The latter explanation seems more probable, as the data suggest the presence of idiotype-reactive T cells, albeit in lower numbers and in a less activated state, in both untreated and treated patients without increased numbers of $CD38^+$ PBL (ie. good prognosis patients). The development of resistance to the activity of anti-idiotypic T cells would result in increasing numbers of such cells in the presence of an increasing tumour cell mass.

Although the number of $CD38^+$ cells correlated with STK, there was no correlation with the plasma cell LI. This discordance between the two measures of proliferation has been noted previously (Brown et al, 1989) and was attributed to the STK reflecting proliferation in both the plasma cell and precursor compartments, whereas the LI reflects proliferation only in the plasma cell compartment. The results of this study suggest an alternative explanation. The STK may also be reflecting proliferation of $CD38^+$ PBL, which would not be unexpected considering the activated state of the $CD8^+$ cells and possibly the NK cells in the CD38hi patients. This concept is supported by previous work showing that the LI of $CD2^+$ PBL correlates with the proportion of HLA-DR^+ cells within the $CD8^+$ population (Massaia et al, 1988b).

The group of patients with MGUS was too small to draw definitive conclusions. However, the lack of multiple abnormalities in comparison to those present in the untreated myeloma patients, including those with SMM, and the obvious dichotomy between reduced B cell numbers and normal polyclonal immunoglobulin levels in the patients with MGUS serve to emphasise the fact that MGUS is a separate disease entity to myeloma and is probably accompanied by different host-tumour interactions.

In summary, precursor myeloma cells do not significantly contribute to the increased number of CD38+ PBL in myeloma patients, although this study has not excluded the possibility of such cells in small numbers. The lymphocyte populations which contribute to the elevated number of $CD38^+$ PBL are predominantly T cell subsets and possibly NK cells, as indicated in the earlier studies of smaller patient numbers (Boccadoro et al, 1988; Gonzalez et al, 1992). However, this study highlights the fact that these subsets are different in

untreated and treated patients, as are the correlations between $CD38^+$ counts and other prognostic and monitoring parameters. The obvious conclusion is that the way in which the immune system is able to interact with the tumour is at least partially dependent on the treatment status of the patient. As evidence for a mechanism attempting to maintain normal humoral immunity was detected only in untreated patients, it may be that some immune responses to the tumour are inactivated by therapy. Intrinsic properties of the malignant clone would also be expected to influence the interaction between the immune system and the tumour. The development of resistance to the activity of idiotype-reactive T cells is an example of this. The follow-up studies are too preliminary to indicate whether untreated patients continue to have elevated $CD38^+$ numbers despite therapy or to accurately delineate the time course of the alterations in the lymphocyte subsets occurring with therapy.

SECTION 4: IMMUNOPHENOTYPIC ANALYSIS OF PERIPHERAL BLOOD LYMPHOCYTES EXPRESSING THE CD10 ANTIGEN

4.1: AIMS

Small increases in the number of PBL expressing the CD10 antigen in patients with multiple myeloma were initially identified in single colour immunophenotypic studies (Wearne et al, 1987a; Joshua et al, 1988). The phenotype of these cells and their precise relationship to the malignant clone was not elucidated, although the presence of an increased absolute number did correlate with the absence of LCIS but not with the presence of an elevated sβ2M level (Wearne et al, 1987a). There is also an increased number of $CD10^+$ lymphocytes in the bone marrow of patients with myeloma (Shimazaki et al, 1990).

The CD10 antigen has been detected on the malignant plasma cells of patients with multiple myeloma (Ruiz-Argüelles et al, 1984; Caligaris-Cappio et al, 1985; Durie and Grogan, 1985; San Miguel et al, 1986; Epstein et al, 1988; Jackson et al, 1988; Grogan et al, 1989; Epstein et al, 1990; Shimazaki et al, 1990; Van Camp et al, 1990; Drach et al, 1991; Hamilton et al, 1991a; San Miguel et al, 1991; Leo et al, 1992) and on normal plasma cells (Caligaris-Cappio et al, 1985; Tominaga et al, 1989; Terstappen et al, 1990). It is therefore not surprising that it has been detected on both peripheral blood and bone marrow cells which have been identified as precursor cells by various techniques (Caligaris-Cappio et al, 1985; Durie and Grogan, 1985; Grogan et al, 1987; Epstein et al, 1988; Bergui et al, 1989; King and Nelson, 1989; Jensen et al, 1991; Pilarski and Jensen, 1992). Caligaris-Cappio et al (1985) reported the phenotype of putative $CD10^+$ precursor cells but they studied principally B cell antigens. Extensive immunophenotypic analysis of precursor cells has recently been performed by

one research group (Jensen et al, 1991; Pilarski et al, 1992) but these precursor cells were identified within the total peripheral blood mononuclear fraction rather than just within the lymphoid population.

The aims of this study of $CD10^+$ PBL were two-fold:

(1) The percentage and absolute number of $CD10^+$ PBL were enumerated in a series of patients at various stages of the disease and compared to those in normal individuals. Correlations with known prognostic and monitoring factors were also undertaken to ascertain whether the number of these cells fluctuates with the activity of the disease. If $CD10^+$ PBL are precursor cells, it is conceivable that increasing numbers may be indicative of progressive disease.

(2) Immunophenotypic analysis of $CD10^+$ PBL was performed in order to confirm the B cell lineage of these cells, to ascertain whether the non-B cell antigens (eg. myelomonocytic) detected on myeloma cells and normal plasma cells were present on this population and to compare the phenotype of these cells with that of other previously reported putative precursor populations.

4.2: MATERIALS AND METHODS

4.2.1: Patients

In the first part of the study, the percentage and absolute number of $CD10^+$ PBL were enumerated in normal individuals and patients with plasma cell dyscrasias. This was done as part of the study of $CD38^+$ PBL described in section 3. Ten controls, 3 patients with MGUS and 39 with multiple myeloma were studied. The patients with myeloma included 6 with SMM, 9 at diagnosis, 5 responding to therapy, 7 in plateau phase and 12 with progressive disease. Further details on these patients, the normal controls and the collection of specimens are given in section 3.2.1.

Detailed immunophenotypic analysis of $CD10^+$ PBL in 7 normal individuals and 8 myeloma patients comprised the second part of this study. The control and patient groups were matched for age and sex. The 7 controls were all involved in the previously described study of $CD38^+$ PBL (section 3.2.1). The myeloma patients were not chosen at random. Samples from myeloma patients were initially screened by staining an aliquot of whole blood with the PE-conjugated anti-CD10 antibody and only those with $\geq 1.5\%$ $CD10^+$ lymphocytes were studied further. This was done on the assumption that specimens with higher relative numbers of $CD10^+$ cells would contain a lower proportion of $CD10^+$ cells that had non-specifically labelled with the antibody directed against the other antigen of interest.

The myeloma patients comprised 3 newly diagnosed patients, 3 in plateau phase and 2 with progressive disease. All of the newly diagnosed patients had active disease requiring therapy. Two were studied prior to any treatment but one had received steroids as therapy for hypercalcaemia for a period of 24 hours prior to the study. Two of the patients in plateau phase were receiving IFN-α2: one was participating in a trial of IFN-α2 during plateau phase and the other had initially received the drug for symptomatic disease with continuation of this therapy during plateau phase. One patient with progressive disease had received prior treatment with radiotherapy only, whereas the other had received systemic chemotherapy. Three patients had IgGκ paraproteins and one each had IgGλ, IgAκ, IgAλ and λ light chain only paraproteins. One patient had non-secretory myeloma. At the time of the study, one patient had Durie and Salmon stage IA disease, one stage IIA, 4 stage IIIA and 2 stage IIIB.

4.2.2: Immunophenotypic Analysis of Peripheral Blood Lymphocytes

Reagents

The antibodies used in this study had the following specificities: the B cell antigens CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD37, CD39, κ and λ immunoglobulin light chains; the T cell and NK cell antigens CD1, CD2, CD3, CD4, CD5, CD7, CD8 and CD16; the myelomonocytic antigens CD13, CD33 and CD36; the progenitor cell antigen CD34; the non-lineage antigens CD9, CD25, CD38, CD71, PCA-1 and class II MHC molecules; the adhesion and homing molecules CD11a, CD18, CD44, CDw49d and CD54. Studying the expression of CD56 was deemed to be particularly important. Not only is it a marker of NK cells but it has also recently been described as being strongly expressed by neoplastic plasma cells in contrast to normal plasma cells, which either do not or occasionally weakly express this antigen (Van Camp et al, 1990; Drach et al, 1991; Van Riet et al, 1991; Barker et al, 1992a; Leo et al, 1992). The specifications of these antibodies are listed in tables 4.1 and 4.2. Other reagents used in the surface antigen labelling procedure were the same as those used in the study of $CD38^+$ PBL and are described in section 3.2.2.

Immunophenotypic Labelling Procedure

This assay was almost identical to that described in section 3.2.2., as it involved the use of a PE-conjugated antibody to detect the CD10 antigen in combination with an indirect detection method for the other antigen of interest. Spare binding sites on the FITC-conjugated affinity-isolated $F(ab)_2$ fragment sheep anti-mouse antibody were blocked by incubation with a 1:10 rather than a 1:30 dilution of normal mouse serum. In some cases, the antibodies detecting the T cell antigens CD2, CD3, CD4 and CD8 were FITC-conjugated. The rationale for this will be explained in the results (section 4.3.2). The FITC-conjugated anti-T

Table 4.1: Specifications of the Non-Conjugated Antibodies Used to Study the Immunophenotype of CD10+ PBL.				
Specificity	Commercial Name	Clone	Isotype	Source
CD1a	T6	SFCI19Thy1A8	IgG1κ	Coulter Immunology, Hialeah, Florida
CD2	T11	SFCI3Pt2H9	IgG1κ	Coulter Immunology, Hialeah, Florida
CD3	T3	SFCIRW2-8C8($T3_C$)	IgG1κ	Coulter Immunology, Hialeah, Florida
CD4	T4	SFCI12T4D11	IgG1κ	Coulter Immunology, Hialeah, Florida
CD5	Anti-Leu-1	L17F12	IgG2aκ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD7	Anti-Leu-9	4H9	IgG2aκ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD8	T8	SFCI21Thy	IgG1κ	Coulter Immunology, Hialeah, Florida
CD9	-	BU-16	IgG2a	Gift: G.D. Johnson, Department of Immunology, University of Birmingham, U.K.
CD10	Anti-CALLA	W8E7	IgG2aκ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD11a	DAKO-CD11a	MHM24	IgG1κ	Dakopatts A/S, Glostrup, Denmark
CD13	MY7	366	IgG1κ	Coulter Immunology, Hialeah, Florida
CD16	Leu-11b	G022	IgMκ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD18	DAKO-CD18	MHM23	IgG1κ	Dakopatts A/S, Glostrup, Denmark
CD19	B4	89B	IgG1κ	Coulter Immunology, Hialeah, Florida
CD20	B1	H299	IgG2aκ	Coulter Immunology, Hialeah, Florida
CD21	-	BU-34	IgG1	Gift: G.D. Johnson, Department of Immunology, University of Birmingham, U.K.
CD22	Leu-14	SHCL-1	IgG2bκ	Becton Dickinson Immunocytometry Systems, San Jose, California
CD23	DAKO-CD23	MHM6	IgG1	Dakopatts A/S, Glostrup, Denmark
CD24	IOB3	ALB9	IgG1κ	Immunotech S.A., Marseille, France

Table 4.1: Continued.

Specificity	Commercial Name	Clone	Isotype	Source
CD25	Anti-Interleukin-2 Receptor	2A3	IgG1κ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD33	MY9	906	IgG2bκ	Coulter Immunology, Hialeah, Florida
CD34	Anti-HPCA-1	My10	IgG1κ	Becton Dickinson Immunocytometry Systems, Mountain View, California
CD36	OKM5	-	IgG1	Ortho Diagnostic Systems, Raritan, New Jersey
CD37	IOB1	BL14	IgG1	Immunotech S.A., Marseille, France
CD38	OKT10	-	IgG1	Ortho Diagnostic Systems, Raritan, New Jersey
CD39	-	AC2	IgG1	Gift: Department of Immunology, University of Birmingham, U.K.
CD44	IOL44	J-173	IgG1	Immunotech S.A., Marseille, France
CDw49d	IOP49d	HP2/1	IgG1	Immunotech S.A., Marseille, France
CD54	-	ICAM-1	IgG2a	Gift: A. Boyd, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia
CD56	NKH-1	N901	IgG1κ	Coulter Immunology, Hialeah, Florida
CD71	OKT9	-	IgG1	Ortho Diagnostic Systems, Raritan, New Jersey
HLA-DR framework antigen	OKIa*1	-	IgG2	Ortho Diagnostic Systems, Raritan, New Jersey
Unclustered†	PCA-1	138	IgG2a	Coulter Immunology, Hialeah, Florida
Isotype Control	-	L7	IgG1	Gift: Clinical Immunology and Research Centre, University of Sydney, Australia‡
Isotype Control	MsIgG2a	7T4-1F5	IgG2aκ	Coulter Immunology, Hialeah, Florida
Isotype Control	MsIgG2b	MPC-11	IgG2bκ	Coulter Immunology, Hialeah, Florida
Isotype Control	MsIgM	R4A3-22-12	IgMKκ	Coulter Immunology, Hialeah, Florida

†Anderson et al, 1983; ‡mouse anti-Mycobacterium Leprae (Britton et al, 1985).

Table 4.2: Specifications of Conjugated Antibodies Used to Study the Immunophenotype of CD10+ PBL.					
Specificity	Commercial Name	Clone	Conjugate	Isotype*	Source
2	DAKO-CD2	MT910	FITC	IgG1κ	Dakopatts A/S, Glostrup, Denmark
3	DAKO-CD3	UCHT1	FITC	IgG1κ	Dakopatts A/S, Glostrup, Denmark
4	DAKO-CD4	MT310	FITC	IgG1κ	Dakopatts A/S, Glostrup, Denmark
8	DAKO-CD8	DK25	FITC	IgG1κ	Dakopatts A/S, Glostrup, Denmark
10	DAKO-CD10	SS2/36	PE	IgG1κ	Dakopatts A/S, Glostrup, Denmark
Kappa Light Chains	-	-	FITC	Polyclonal rabbit $F(ab')_2$	Dakopatts A/S, Glostrup, Denmark
Lambda Light Chains	-	-	FITC	Polyclonal rabbit $F(ab')_2$	Dakopatts A/S, Glostrup, Denmark
Isotype Control	-	DAK-GO1	FITC	IgG1κ	Dakopatts A/S, Glostrup, Denmark
Isotype Control	-	DAK-GO1	PE	IgG1κ	Dakopatts A/S, Glostrup, Denmark
Isotype Control	-	-	FITC	Polyclonal rabbit $F(ab')_2$	Dakopatts A/S, Glostrup, Denmark
*antibodies are mouse monoclonals unless otherwise indicated; rabbit and sheep polyclonal antibodies are affinity isolated.					

cell and the PE-conjugated anti-CD10 antibodies were added simultaneously and the specimen was incubated in the dark at 4°C for 30 minutes. The red blood cells were then lysed and the remaining white cells were washed and resuspended in PBS. The method for detecting cells expressing sIg light chain is detailed in section 3.2.2.

Flow Cytometry Analysis

The flow cytometry analysis of the specimens is described in section 3.2.2. The only difference was that the positive analysis regions determined automatically by the inbuilt software following the analysis of the appropriate isotypic controls were adjusted so that ≤0.5% of lymphocytes were positive for the PE-conjugated IgG1 antibody serving as the negative control for the PE-conjugated anti-CD10 antibody.

4.2.3: Other Investigations

The procedures for determining the full blood count, sβ2M, STK, serum immunoglobulin levels and bone marrow plasma cell LI are outlined in section 3.2.3.

4.2.4: Statistical Analysis

$CD10^+$ PBL were considered to be negative for a given antigen if <30% of these cells expressed that antigen. This cut-off value was chosen to allow for the relatively higher contribution of non-specifically stained cells when the population of interest (ie. $CD10^+$) is numerically small. The expression of an antigen was classed as "positive" if ≥30% but <60% of the cells expressed the antigen and "strongly positive" if ≥60% bore the antigen. Data were summarised as median (range). The Kruskal-Wallis one-way analysis of variance by ranks

(two-tailed) was used to detect a difference in the number of $CD10^+$ PBL between normals, patients with MGUS and myeloma patients. The percentage of $CD10^+$ PBL expressing each antigen in the normal individuals was compared to that in the myeloma patients using the two-tailed Mann-Whitney-U test. For each antigen expressed by the $CD10^+$ PBL, the fluorescence intensity of that antigen on both $CD10^+$ and $CD10^-$ cells in the normals was compared to that in the patients using the Mann-Whitney-U test. As there were no significant differences in the fluorescence intensity of each antigen in the normals compared to the patients, the results in the controls and the myeloma patients were pooled and the fluorescence intensity of each antigen on the $CD10^+$ cells was compared to that on the $CD10^-$ cells using the Wilcoxon signed-rank test for paired samples. Correlation analysis was performed using the Spearman rank correlation test. For comparing the difference between two proportions, either the χ^2 test or Fisher's exact test was used depending on the number of patients in each subgroup (Rimm et al, 1980). Results were considered statistically significant if $p \leq 0.05$. The analyses were performed using the statistical software packages Abstat version 4.05 (Anderson-Bell Co.) and Minitab version 8.2 (Minitab Inc., State College, PA, USA).

4.3: RESULTS

4.3.1: CD10 Antigen Expression on Peripheral Blood Lymphocytes

The percentage and absolute number of PBL expressing the CD10 antigen were not significantly different in normal individuals, patients with MGUS and patients with multiple myeloma (table 4.3). There were also no significant differences between the normals or any subgroup of myeloma patients when the latter were divided into the following categories based on disease activity: SMM; at diagnosis; on treatment; plateau phase; progressive disease.

Table 4.3: Number of PBL Expressing the CD10 Antigen.			
	Normals 10 subjects	MGUS 3 patients	Multiple Myeloma 39 patients
Percentage	1.3 (0.5-2.3)	1.1 (0.8-1.6)	1.3 (0.4-3.5)
Absolute Number ($x10^9/l$)	0.02 (0.01-0.04)	0.02 (0.01-0.02)	0.02 (0.01-0.06)
There were no significant differences between groups for either the percentage or absolute results			

No patient with MGUS had an elevated percentage or absolute number of $CD10^+$ cells. The percentage and absolute number of $CD10^+$ PBL were above the range found in the normal individuals in 6/39 (15%) and 2/39 (5%) myeloma patients respectively. The percentage was raised in 0/6 patients with SMM, 1/9 at diagnosis, 2/5 on treatment, 2/7 in plateau phase and 1/12 with progressive disease. The single untreated patient with an increased percentage of $CD10^+$ PBL had a normal percentage of $CD38^+$ PBL. Of the 5 previously treated patients with an elevated percentage of $CD10^+$ lymphoid cells, three had an elevated percentage and two had a normal percentage of $CD38^+$ PBL. The absolute number of $CD10^+$ PBL was raised in 0/6 SMM, 1/9 at diagnosis, 0/5 on treatment, 1/7 in plateau and 0/12 with progression. Both of the patients with an increased absolute number of $CD10^+$ PBL had an increased absolute number of $CD38^+$ PBL. One patient in plateau phase had both an elevated percentage and absolute number of $CD10^+$ cells. In neither untreated nor previously treated patients was there any relationship between the presence or absence of elevated numbers of $CD10^+$ cells and the presence or absence of increased numbers of $CD38^+$ PBL. However, there was a weak but statistically significant correlation between the absolute numbers of $CD10^+$ and $CD38^+$ cells ($r=0.47$, $p=0.004$).

In both the analysis of relative and absolute numbers, patients with elevated numbers of $CD10^+$ cells could not be discriminated from those with normal numbers on the basis of SMM versus active disease in untreated patients or

stable (improving on treatment and in plateau) versus progressive disease in treated patients. Neither could they be discriminated on the basis of Durie and Salmon stage (I and II versus III), excretion of BJP, presence of a κ:λ ratio outside the normal range or paraprotein isotype.

In myeloma patients, there were no correlations between the percentage or absolute number of $CD10^+$ PBL and any of the following factors: sβ2M; STK; bone marrow plasma cell LI; serum IgM level, which was used as a reflection of polyclonal immunoglobulin production; amount of serum paraprotein; patient age.

4.3.2: Immunophenotype of $CD10^+$ Peripheral Blood Lymphocytes

In this section of the study, the lymphocyte counts in the controls ranged from 1.3-1.9x10^9/l with 1.1-2.1% of these cells expressing the CD10 antigen. In the myeloma patients, the lymphocyte counts ranged from 0.5-2.5x10^9/l with 1.6-4.6% being $CD10^+$. As explained in the materials and methods (section 4.2.1), the myeloma patients were not a random sample and hence the proportion of $CD10^+$ PBL in these groups can not be compared, apart from stating that some myeloma patients have a higher percentage of $CD10^+$ PBL than normal individuals.

The surface antigen expression of the $CD10^+$ lymphocytes was similar in both the normal individuals and patients with multiple myeloma. The results for the various antigens are given in table 4.4.

Table 4.4: Surface Antigens Expressed on CD10⁺ PBL.				
	Controls		Patients	
	Percentage of CD10⁺ PBL Expressing the Antigen	No. of Cases	Percentage of CD10⁺ PBL Expressing the Antigen	No. of Cases
B Cell Antigens				
CD19	54.6 (41.7-92.3)*	7	56.8 (25.0-92.9)	8
CD20	33.3 (20.0-70.0)	7	32.3 (6.7-75.0)	8
CD21	50.0 (37.5-76.2)	7	61.3 (16.0-80.9)	8
CD22	69.2 (45.5-85.0)	7	61.7 (29.4-83.8)	8
CD23	50.0 (33.3-69.6)	7	53.9 (17.1-62.5)	7
CD24	40.0 (18.2-66.7)	7	55.0 (33.3-87.5)	8
CD37	50.0 (12.5-70.0)	7	44.4 (12.5-80.0)	7
CD39	46.2 (40.0-60.0)	7	46.9 (26.2-50.0)	8
sIg	29.7 (14.3-53.0)	7	36.5 (4.4-74.9)	7
κ/λ Ratio	1.5 (0.0-2.1)	7	2.6 (0.9-5.3)	7
Non-Lineage Antigens				
CD9	32.0 (25.0-60.0)	7	45.8 (35.3-64.7)	7
CD25	16.7 (8.3-25.0)	7	13.3 (8.0-25.8)	8
CD38	70.0 (50.0-84.6)†	7	80.9 (71.4-94.7)†	8
CD71	10.5 (0.0-14.3)	7	17.4 (5.6-36.4)	8
HLA-DR	75.0 (27.3-84.6)	7	70.0 (40.0-87.5)	8
PCA-1	10.0 (4.0-15.0)	7	19.3 (5.9-48.0)	6
Myelomonocytic and Progenitor Cell Antigens				
CD13	7.1 (5.0-16.7)	7	13.3 (4.6-21.4)	7
CD33	6.7 (0.0-14.3)	7	14.6 (2.3-22.7)	8
CD34	11.1 (4.4-33.3)	7	9.4 (3.5-17.7)	8
CD36	23.1 (13.0-30.0)	5	15.5 (9.1-69.2)	8
T Cell and NK Cell Antigens				
CD1a	7.7 (3.5-15.4)	7	13.2 (4.3-29.6)	8
CD2	18.2 (16.7-27.3)	3	27.5 (11.6-43.5)	2
CD3	32.2 (13.3-66.7)	4	15.0 (11.5-15.8)	3
CD4	32.0 (13.6-83.3)	6	20.8 (11.1-30.4)	2
CD5	53.9 (33.3-76.9)	7	53.8 (17.2-73.3)	8
CD7	25.0 (13.0-33.3)	5	19.1 (8.5-42.4)	7
CD8	16.1 (7.1-17.4)	4	14.6 (7.0-22.2)	2
CD16	15.4 (11.5-25.0)	7	17.4 (7.1-23.1)	8
CD56	7.1 (0.0-8.3)	7	10.3 (4.2-25.0)	8
Adhesion and Homing Molecules				
CD11a	100.0 (100.0-100.0)	6	91.7 (79.3-100.0)	7
CD18	100.0 (95.5-100.0)	6	85.6 (66.0-100.0)	8
CD44	100.0 (100.0-100.0)	7	99.1 (72.7-100.0)	7
CDw49d	94.4 (86.7-100.0)	7	86.7 (72.2-100.0)	7
CD54	63.6 (40.0-75.0)	7	57.7 (28.2-81.8)	7
*results expressed as median (range); †p=0.001 - no significant differences for other antigens.				

B Cell Antigens

The B cell antigens studied were CD19, CD20, CD21, CD22, CD23, CD24, CD37 and CD39. The proportion of sIg^+ cells was determined by adding together the percentage of $s\kappa^+$ and $s\lambda^+$ cells. In each individual, the B cell antigen present on the highest proportion of $CD10^+$ cells was expressed on ≥50% of these cells. The antigen with the highest expression was in the "strong positive" category in 4/7 normal individuals and in three was present on ≥80% of the cells. In the myeloma patients, the antigen with the highest expression was classed as "strong positive" in all individuals and in two was expressed on ≥80% of the cells.

There were no significant differences between the percentage of positive cells in the normals compared to the myeloma patients for any B cell antigen. The percentage of $CD10^+$ cells expressing the various B cell antigens is illustrated in figure 4.1.

The median percentage of $CD22^+$ cells within the $CD10^+$ lymphoid population was higher than that of the other B cell antigens in both the controls and myeloma patients with 4 and 7 individuals respectively having expression of this antigen in the "strong positive" category. In the normals, CD19 was present on the highest proportion of $CD10^+$ cells in 3 cases and CD22 in 4 cases, with one individual in this latter group having the same percentage of cells positive for CD22 and CD23. In myeloma, the B cell antigen occurring on the highest percentage of $CD10^+$ cells was more variable: CD19 in one case, CD21 in 2, CD22 in 2 and CD24 in 3. With the exception of CD20 and sIg, which are discussed below, the percentage of $CD10^+$ cells expressing a given B cell antigen fell within the "negative" category in no more than 2 subjects. The expression of CD21,

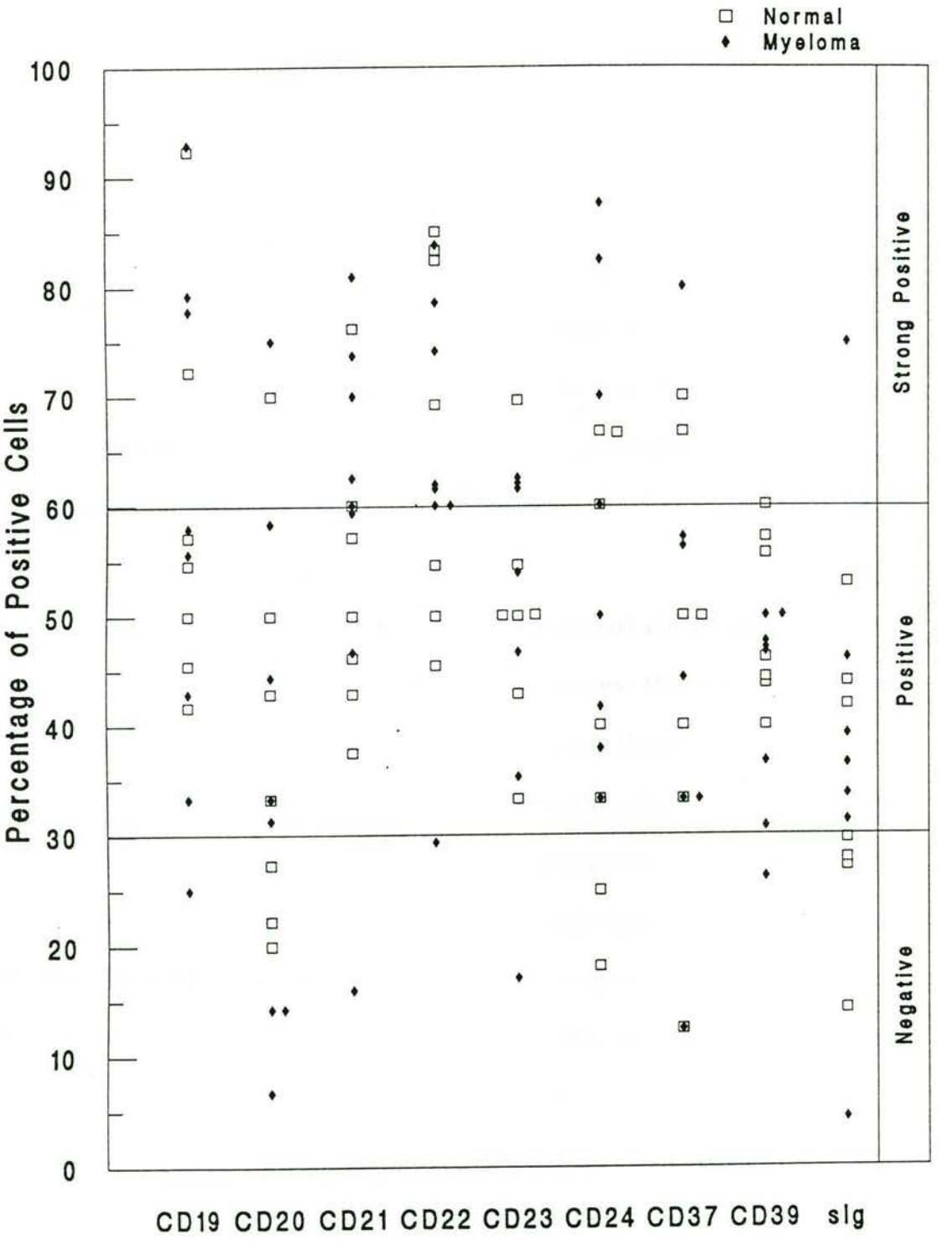


Figure 4.1: Expression of B cell antigens on $CD10^+$ peripheral blood lymphocytes in normal individuals and patients with multiple myeloma.

CD22 and CD24 were classed as "strong positive" in at least half the positive cases, whereas for CD19, CD23, CD37 and CD39, the majority of positive cases were classed as "positive".

Among the B cell antigens, the median percentage of $CD20^+$ cells within the $CD10^+$ subset was the lowest in both normals and myeloma patients, with lack of expression in 3/7 controls and 3/8 patients. It was either the antigen present on the lowest or the second lowest proportion of $CD10^+$ PBL in 6 controls and 7 myeloma patients. Its expression was classed as "strong positive" in only one normal and one patient.

When analysing the relative fluorescence intensity of the B cell antigens on $CD10^+$ and $CD10^-$ PBL, only cases considered to express the antigen of interest were included. For the CD20 and CD24 antigens, the fluorescence intensity of expression on the $CD10^+$ PBL was significantly higher than on the $CD10^-$ cells ($p<0.03$ for CD20; $p<0.02$ for CD24). The differences in the fluorescence intensity of CD19, CD23 and CD39 expression were quite small in magnitude but the direction of these differences was consistent. Consequently, the intensity of each of these antigens was significantly lower on the $CD10^+$ than the $CD10^-$ cells ($p<0.02$ for CD19; $p=0.002$ for CD23; $p=0.007$ for CD39).

The median percentage of $CD10^+$ PBL expressing sIg was similar to that of the CD20 antigen, being slightly lower in the normal individuals and slightly higher in the myeloma patients. In order to assess sIg expression by $CD10^+$ B cells, the ratio of sIg^+ cells to $CD10^+$ B cells, when the latter was defined by the B cell antigen present on the highest percentage of $CD10^+$ PBL, was calculated. In 4 controls and 4 myeloma patients, this ratio was between 0.4-0.6, with only one

normal and 2 myeloma patients having a ratio higher than 0.6. There was no easy method for assessing the relative fluorescence intensity of sIg on $CD10^+$ and $CD10^-$ cells, as the percentage of sIg^+ cells was determined by the addition of $s\kappa^+$ and $s\lambda^+$ cells. By merely inspecting the results, the fluorescence intensity of either light chain isotype was only markedly higher on $CD10^+$ cells in 2 normals and one myeloma patient. More commonly the fluorescence intensity of light chain isotype expression was obviously lower on the $CD10^+$ PBL.

The $\kappa:\lambda$ ratio was examined in an attempt to determine whether the $CD10^+$ B cells were a monoclonal population. Based on the results in the study of $CD38^+$ PBL (section 3), the $\kappa:\lambda$ ratio within the total PBL population is 0.9-4.5 but there is no evidence that this would necessarily be applicable to any B cell subpopulation. Nevertheless, 6/7 normals and 5/7 myeloma patients had ratios within this range. One control had only $s\lambda$ expression on $CD10^+$ PBL. Two myeloma patients, both with κ paraproteins, had ratios ≥ 5.0. In the normals, the upper limit of the ratio within the $CD10^+$ population was 2.1. Four of the myeloma patients, including the two mentioned above, had a ratio above this limit but the other 2 patients had λ paraproteins.

Non-Lineage Antigens

The percentage of $CD10^+$ cells expressing each non-lineage antigen is illustrated in figure 4.2. The CD38 antigen was invariably expressed by $CD10^+$ PBL and in only one normal individual was the proportion of positive cells not in the "strong positive" category. In 2 normals and 5 myeloma patients, the percentage of $CD38^+$ cells within the $CD10^+$ lymphoid subset was higher than that of any of the B cell antigens. The percentage of $CD10^+$ cells bearing the CD38 antigen was significantly higher in the myeloma patients than in the normals ($p<0.02$).

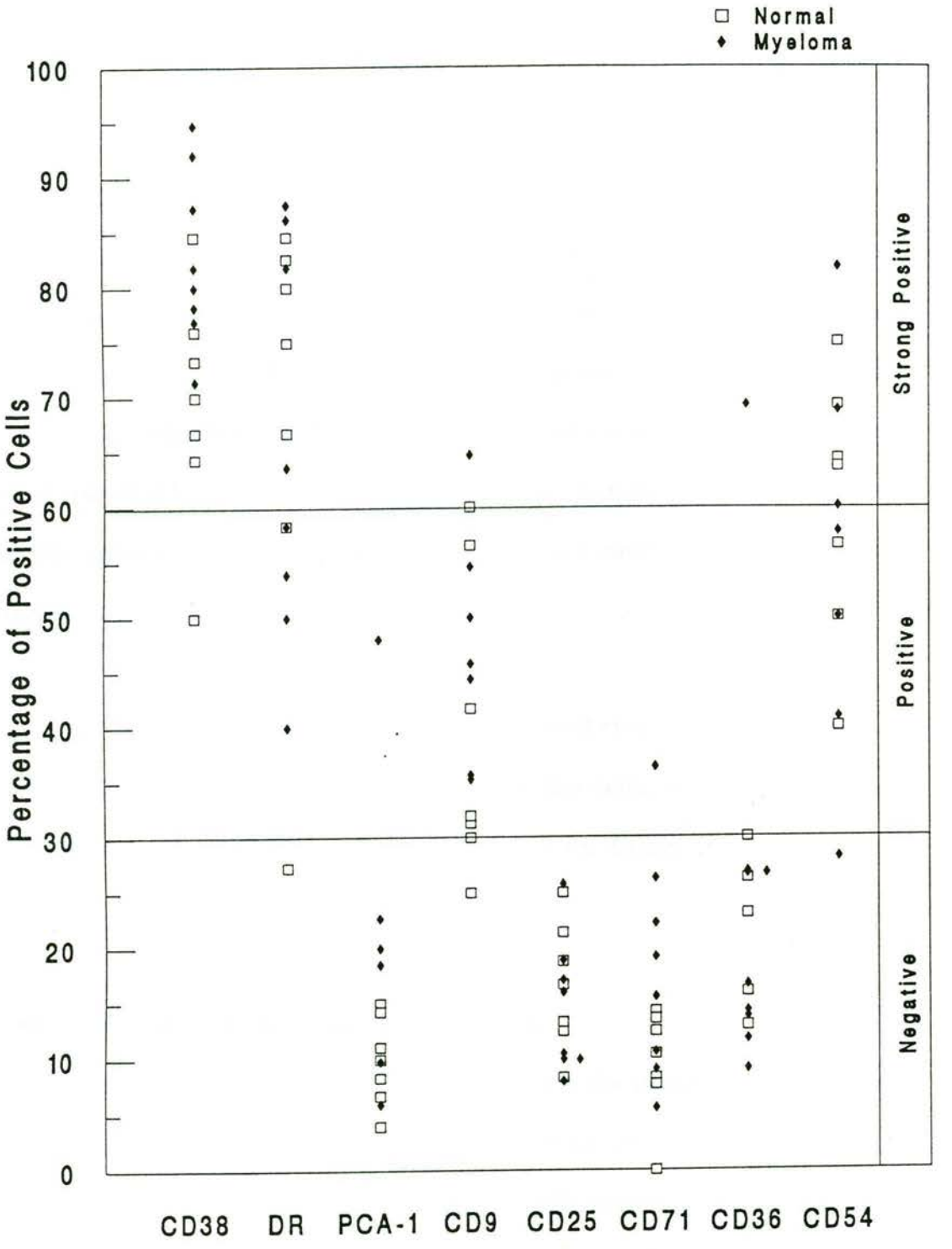


Figure 4.2: Immunophenotype of CD10+ peripheral blood lymphocytes in normal individuals and patients with multiple myeloma.

In contrast, the other antigen frequently used as a marker of plasma cells, PCA-1, was detected on the cells of only one myeloma patient.

The CD9 antigen was not present on the $CD10^+$ PBL in only one normal individual. In one myeloma patient, the percentage of $CD9^+$ cells fell in the "strong positive" range, whereas in the other subjects it fell in the "positive" range. The activation marker CD25 was not detected on the $CD10^+$ cells of any individual and the CD71 antigen was present in only one myeloma patient. Class II MHC molecules, which are a marker of B cells and an activation marker on cells of other lineages, was absent on the $CD10^+$ lymphocytes of one control but the proportion of positive cells in the other cases was quite variable, ranging from 40-88%.

The fluorescence intensities of CD38, CD9 and class II MHC molecule expression were significantly higher on the $CD10^+$ than the cells, although the actual differences were small in magnitude ($p=0.001$ for CD38; $p=0.004$ for CD9; $p=0.01$ for HLA-DR).

Myelomonocytic and Progenitor cell Antigens

The myelomonocytic antigens CD13 and CD33 and the progenitor cell antigen CD34 were not expressed on $CD10^+$ PBL. No normal individual had $CD10^+36^+$ cells but in 1 myeloma patient 69% of $CD10^+$ cells were positive for the CD36 antigen (figure 4.2). This patient was the same one that had $CD10^+$ cells expressing PCA-1.

T Cell and NK Cell Antigens

The significance of the results obtained following the dual labelling of lymphocytes with anti-CD10 antibodies and either antibodies directed against CD2, CD3, CD4 or CD8 are difficult to assess. There were obviously false positive results with these anti-T cell antibodies, as the percentage of $CD10^+$ PBL was frequently elevated in comparison to the values obtained on the other samples from that individual (eg. a value of 69.5% for the number of $CD10^+$ PBL on the aliquot labelled with anti-CD3 antibody compared to a mean of 2.9% with antibodies against non-T cell antigens). This technical problem did not result from the presence of binding sites on the sheep anti-mouse second layer antibody that had not been saturated by the 1:10 dilution of normal mouse serum, as the problem persisted despite the use of directly conjugated anti-T cell antibodies. It would seem unlikely that the colour compensation for spectral overlap of the fluorochromes was inadequate, as this was checked daily by calibrating the flow cytometer with a normal specimen labelled with both anti-CD4 and anti-CD8 antibodies. Furthermore, this technical problem only occurred with the antibodies mentioned above and not with the antibodies directed against the T cell antigens CD5 and CD7.

Apparently adequate results for the CD2, CD3, CD4 and CD8 antigens were available in 3, 4, 6 and 4 controls and 2, 3, 2 and 2 myeloma patients respectively. $CD10^+$ cells were negative for CD8. In contrast, the cells of one myeloma patient expressed CD2 and those of 2 controls expressed CD3. In 3 normals and a single myeloma patient, CD4 was detected on the $CD10^+$ PBL. These results are illustrated in figure 4.3.

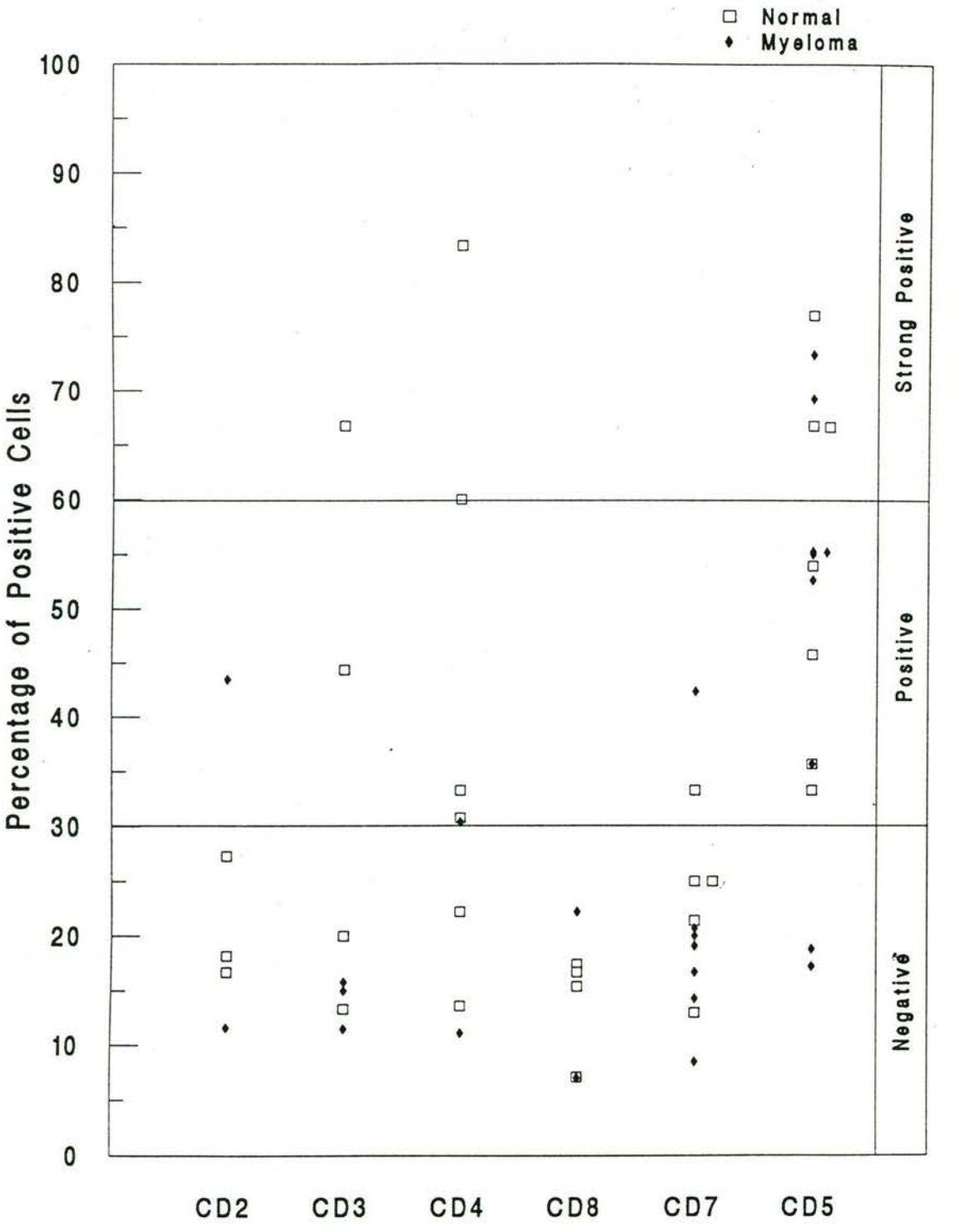


Figure 4.3: Expression of T cell antigens on $CD10^+$ peripheral blood lymphocytes in normal individuals and patients with multiple myeloma.

Four of the five studied normal individuals and 6/7 of the myeloma patients did not express the CD7 antigen on the $CD10^+$ cells. In the other 2 individuals, 33% and 42% of the cells were positive for this antigen. The results for CD5 were markedly different, with this antigen being detected on the cells of all normals and 6/8 patients. The proportion of positive cells was quite variable, ranging from 33-77%. The fluorescence intensity of CD5 expression was significantly lower on $CD10^+$ than $CD10^-$ PBL ($p=0.002$).

As the nature of the technical problem affecting the results with the antibodies against CD2, CD3, CD4 and CD8 remained unclarified, it is difficult to be certain whether the apparently unaffected results were really valid reflections of the immunophenotype of the $CD10^+$ lymphoid cells. This is complicated by the fact that all patients who had "valid" positive expression of one of these T cell antigens had results for at least one of the other T cell antigens excluded due to technical difficulties. This makes it difficult to determine whether any of these patients had either $CD10^+$ cells with a phenotype consistent with that of T lineage cells or aberrant expression of T cell antigens on another cell lineage, or whether all the results with antibodies against the CD2, CD3, CD4 and CD8 antigens were false positives. Table 4.5 compares the percentage of $CD10^+$ PBL expressing the various T cell antigens, apart from CD5, with the highest percentage of positive cells for a B cell antigen in patients who had $CD10^+$ cells with "valid" expression of one of the T cell antigens. In control 3 and patient 4, the percentage of $CD4^+$ cells was just high enough to be classified in the "positive" category and the expression of the other T cell antigens was classified as "negative". Control 7 had isolated expression of CD4 on 83.3% of cells with the fluorescence intensity of CD4 being much lower on the $CD10^+$ cells (2.73) than on the $CD10^-$ ones (16.25). Control 5 had "strong positive" expression of

both CD3 and CD4 with the fluorescence intensity of both these antigens being similar on $CD10^+$ and $CD10^-$ cells. However, the $CD10^+$ cells did not express CD7. Control 6 had expression in the "positive" category for CD3, CD4 and CD7 with the maximum expression of any B cell antigen being only 50%, suggesting that in this patient there may be $CD4^+$ T cells expressing the CD10 antigen.

The thymocyte antigen CD1 and the NK cell antigens CD16 and CD56 were not present on $CD10^+$ PBL.

Table 4.5: T Cell and Maximum B Cell Antigen Expression in Subjects with $CD10^+$ PBL Expressing a T Cell Antigen.

		CD2	CD3	CD4	CD8	CD7	B Cell
Controls	C3	27.3*	-	30.7	-	27.0	83.3
	C5	-	66.7	60	-	21.4	69.2
	C6	-	44.4	33.3	16.7	33.3	50.0
	C7	18.2	20.0	83.3	15.4	-	54.6
Patients	P1	43.5	-	-	22.2	20.7	82.4
	P2	-	-	-	-	42.4	70.0
	P4	-	15.0	30.4	-	16.7	60.0

C-control number; P-patient number; *results expressed as a percentage of total $CD10^+$ PBL.

Adhesion and Homing Molecules

The CD11a, CD18, CD44 and CDw49d antigens were expressed by >85% of $CD10^+$ lymphocytes in the controls. These antigens were detected on a lower proportion of cells in the myeloma patients but the expression was still "strong positive" in all cases. The percentage of $CD10^+$ cells expressing CD54 was more variable in both controls and myeloma patients (figure 4.2, page 227). Nevertheless, in only one myeloma patient were the $CD10^+$ PBL negative for this adhesion molecule.

Although the fluorescence intensities of the CD11a, CD18 and CD44 expression were highly variable between individuals, they were always weaker on the $CD10^+$ than the $CD10^-$ cells, irrespective of whether the individual was normal or had myeloma. These differences were statistically significant ($p=0.002$ for CD11a; $p=0.001$ for CD18; $p=0.002$ for CD44). There was much less inter-individual variability in the fluorescence intensity of CD54 expression. The difference in intensity of CD54 on the $CD10^+$ and $CD10^-$ cells was small in magnitude and much less than that for the antigens discussed above, but it was higher on the $CD10^+$ PBL in all controls and 7/8 myeloma patients, thus reaching the level of significance ($p=0.002$). The intensity of CDw49d expression was also relatively uniform between individuals with no difference in intensity on $CD10^+$ as compared to $CD10^-$ cells.

4.4: DISCUSSION

Despite the difficulties obtaining reliable results with most antibodies directed against T cell antigens, the classification of the $CD10^+$ PBL as either B or T lymphocytes was possible using results obtained with an anti-CD7 antibody. The CD7 antigen is expressed on the majority of peripheral T lymphocytes (Reiter, 1989). Table 4.6 summarises the results obtained for T and B cell antigens, as well as other salient features of the immunophenotype of $CD10^+$ PBL.

The results are consistent with a B cell lineage for the majority of $CD10^+$ PBL in both normal individuals and patients with myeloma, as the values obtained for co-expression of CD10 and B cell antigens were higher than those for co-expression of CD10 and CD7. There was no individual in whom all the $CD10^+$ cells expressed a B cell antigen or in whom all the $CD10^+$ B cells expressed sIg.

The surface antigen expression on the $CD10^+$ PBL in most subjects can be summarised as follows: (1) a high proportion of the $CD10^+$ cells were $CD21^+$, $CD22^+$ and $CD24^+$, with a lower percentage expressing the CD19, CD23, CD37 and CD39 antigens; (2) the CD20 antigen was either absent or present on a low percentage of these cells; (3) other antigens consistently expressed by $CD10^+$ PBL were CD38, class II MHC molecules and CD9, as well as the adhesion and homing molecules CD11a, CD18, CD44, Cdw49d and CD54; (4) the CD5 antigen was expressed; (5) the following antigens were not detected: PCA-1, CD1, CD16, CD56, CD25, CD71, CD13, CD33, CD34, CD36.

Table 4.6: Summary of Immunophenotypic Characteristics of PBL Expressing CD10.

		$CD7^+10^{+*}$	$CD10^+$ B Cells*† (range)	Ratio of $CD10^+sIg^+$:$CD10^+$B Cells‡	$CD5^+10^{+*}$	Ratio of $CD10^+5^+$:$CD10^+$B Cells‡
Controls	C1	-§	43.8-92.3	0.57	76.9	0.83
	C2	25.0	33.3-54.6	0.54	35.7	0.65
	C3	25.0	50.0-83.3	0.50	66.7	0.80
	C4	13.0	42.9-85.0	0.32	45.8	0.54
	C5	21.4	18.2-69.2	0.21	66.7	0.96
	C6	33.3	12.5-50.0	0.56	33.3	0.67
	C7	-§	27.3-54.6	0.81	53.9	0.99
Patients	P1	20.7	30.8-82.4	0.91	55.0	0.67
	P2	42.4	6.7-70.0	0.48	17.2	-¶
	P3	14.3	14.3-62.5	0.50	35.7	0.57
	P4	16.7	31.3-60.0	0.65	55.2	0.92
	P5	19.1	12.5-61.9	0.07	52.6	0.85
	P6	8.5	26.2-87.5	0.42	18.7	-¶
	P7	20.0	44.4-70.0	-	69.2	0.99
	P8	-§	33.3-92.9	0.50	73.3	0.79

C-control number; P-patient number; *results expressed as a percentage of total $CD10^+$ PBL; †the range reprsents the results for the B cell antigens expressed by the least and the most $CD10^+$ cells; ‡ratio was calculated by using the result for the B cell antigen expressed on the highest percentage of $CD10^+$ cells; §an acceptable result was available for the anti-CD3 antibody in these 3 individuals-it was not expressed by the $CD10^+$ cells: ¶there is no ratio as CD5 was not expressed by the $CD10^+$ cells.

The CD5 antigen was originally described as a pan-T cell antigen. It is also expressed by a minor subset of normal B cells in peripheral blood and

peripheral lymphoid tissues but the expression is much weaker than on T cells (Caligaris-Cappio et al, 1982; Ault et al, 1985; Plater-Zyberk et al, 1985; Hardy et al, 1987). There is *in vitro* evidence that CD5 may be an activation antigen on B cells (Freedman et al, 1987b; Freedman et al, 1989; Werner-Favre et al, 1989; Visser et al, 1990). CD5 was detected on a variable percentage of the $CD10^+$ PBL in 13/15 individuals. The markedly and significantly lower fluorescence intensity on $CD10^+5^+$ cells in comparison to $CD10^-5^+$ cells, the overwhelming majority of which are T cells, is consistent with the conclusion that the CD5 antigen is being expressed on B cells within the $CD10^+$ population. In the $CD5^+$ cases, the ratio of $CD5^+$ cells to B cells was greater than 0.5 in all cases and greater than 0.75 in 8 cases.

In 2 patients, the CD5 antigen was not expressed by the $CD10^+$ PBL. In one of these cases (P6), the phenotype of the $CD10^+$ cells otherwise corresponded to that described above. The absence of the CD5 antigen may have been a technical artefact in this case. In the $CD5^+$ cases, the median fluorescence intensity of CD5 on the $CD10^+$ cells was 2.32 with a range of 1.42-7.24, whereas that on the $CD5^+10^-$ cells was 16.26 with a range of 12.65-21.68. However, in this patient the intensity on the $CD5^+10^-$ cells was only 5.78, suggesting that there may have been insufficient antibody to saturate the binding sites on T cells or to detect the low level of CD5 on the $CD10^+$ cells. This was not the explanation in the other patient (P2) whose $CD10^+$ lymphocytes did not express the CD5 antigen. The phenotype of the cells in this individual was different to that documented in the other subjects and will be discussed later.

$CD10^+$ PBL have previously been identified as being increased in some patients with multiple myeloma (Wearne et al, 1987a; Joshua et al, 1988). The surface

antigen expression of these cells was analysed in this study not only to ascertain their lineage but also to investigate the possibility that they are a malignant precursor population, especially as the precursor populations identified in peripheral blood and bone marrow of myeloma patients have almost invariably been identified as expressing CD10 (Caligaris-Cappio et al, 1985; Durie and Grogan, 1985; Grogan et al, 1987; Epstein et al, 1988; Bergui et al, 1989; King and Nelson, 1989; Jensen et al, 1991; Pilarski and Jensen, 1992). The phenotype of the $CD10^+$ PBL in this study was identical in both normal individuals and myeloma patients. Thus, if the cells in those with myeloma are malignant precursors, then there is a detectable equivalent polyclonal B cell population in normal individuals. As recent studies have demonstrated few phenotypic differences distinguishing myeloma cells from normal plasma cells, it would not be unreasonable to hypothesise that the pre-plasma cells in normal individuals would have a similar surface antigen expression to those in myeloma patients. If the $CD10^+$ cells in the normals are circulating pre-plasma cells or plasmablasts homing to the bone marrow, the $CD10^+$ population in myeloma patients may consist of varying proportions of both neoplastic and normal cells and it may be difficult to detect the presence of monoclonal cells using the $\kappa:\lambda$ ratio. In this study, there was no convincing evidence of monoclonality based on this ratio. Assessing the significance of this ratio was further complicated by two facts. Firstly, the results in the normals suggested that the normal range for $CD10^+$ PBL may not be the same as that for the total peripheral B cell population. In one control, only λ light chains were detected on the surface of the $CD10^+$ cells. Consequently, undue emphasis cannot be placed on the apparent predominance of $s\kappa^+CD10^+$ cells suggested by a $\kappa:\lambda$ ratio >5.0 in 2 patients with κ paraproteins, especially as the percentage of $CD10^+$ PBL was not increased in either of these patients. Secondly, in many individuals

there was no detectable sIg on about half the CD10$^+$ cells. It is therefore difficult to definitively exclude a monoclonal component in this population in the myeloma patients. This issue could be resolved by detecting immunoglobulin gene rearrangements concordant with those in the myeloma cells in highly purified CD10$^+$ PBL using sensitive techniques such as the polymerase chain reaction.

We have previously hypothesised that the precursor cell in myeloma, defined as the cell in which there is uncoupling of the usual controls of proliferation and differentiation, is of germinal centre origin and possibly represents an antigen-rescued cell of the germinal centre reaction (Warburton et al, 1989). An alternative hypothesis is that the secondary follicular B blasts are a more likely candidate than centroblasts or centrocytes, as centroblasts have the capacity for self-renewal for a period of only 3 weeks but secondary follicular B blasts have the capacity for long-term renewal (MacLennan, 1992). Irrespective of these alternative hypotheses, it is known that bone marrow plasma cells are derived from blast cells which migrate to that site from peripheral lymphoid tissue (Benner et al, 1981) and isolated germinal centre cells can be induced *in vitro* to develop plasmacytoid features (Liu et al, 1991a). If the CD10$^+$ PBL were this pre-plasma cell blast population, it would be detectable in normal individuals, albeit probably in lower numbers than in myeloma patients.

The phenotype of germinal centre cells is known (see Introduction section 1.6.2) but that of the secondary follicular B cells blasts or the rescued germinal centre cell with features of plasmacytoid differentiation is not. Of prime importance in relationship to the phenotype of the CD10$^+$ PBL is that germinal centre cells express both CD10 and CD38. In all cases, the CD38 antigen was detected on

the $CD10^+$ PBL. In 2/7 normals and 5/8 myeloma patients, this antigen was present on a higher proportion of $CD10^+$ lymphocytes than any of the B cell antigens. Moreover, the expression of CD38 is so characteristic of both normal and malignant plasma cells that it has been used to identify small numbers of these cells in immunophenotypic studies (Terstappen et al, 1990; Harada et al, 1992). CD10 is also present on normal plasma cells (Caligaris-Cappio et al, 1985) and one detailed study reported that a mean of 26% (range: 4-55%) of normal bone marrow plasma cells express this antigen (Terstappen et al, 1990). Consequently, a precursor population, derived from germinal centre cells and homing to the bone marrow where it undergoes terminal differentiation to plasma cells, would be expected to express both CD10 and CD38. Both germinal centre cells and the $CD10^+$ PBL also express CD19, CD20, CD21, CD22, CD37, CD9, CD54 and class II MHC antigens. The $CD10^+$ cells are $CD24^+$ but there is disagreement about expression of this antigen by germinal centre cells. CD25 is present on neither population. The presence of sIg on only about half the $CD10^+$ cells in most subjects could be consistent with a population which is switching from the expression of sIg to the cytoplasmic expression and secretion of immunoglobulin. Studying the heavy chain immunoglobulin isotypes on the $CD10^+$ PBL would be useful, as it would be predicted to be predominantly IgG and IgA as opposed to IgM and IgD.

There are some discrepant features between the phenotype of the $CD10^+$ PBL and germinal centre cells. CD23 and CD39 have not been detected on germinal centre cells but were present on the $CD10^+$ PBL. The converse is true for the CD71 antigen. CD44 is either absent or weakly expressed on germinal centre cells. It was present on the $CD10^+$ PBL but its intensity of expression on these cells was significantly less than on the $CD44^+10^-$ population. As the $CD10^+$ PBL

may be a cell derived from a germinal centre cell rather than an actual germinal centre cell and would be expected to be homing to the bone marrow, some differences in immunophenotype would not be unexpected, especially the expression of adhesion and homing molecules. The adhesion and homing molecules CD44, CDw49d and CD54 have been detected on both normal and myeloma plasma cells (Hamilton et al, 1991a; Van Riet et al, 1991; Barker et al, 1992a; Harada et al, 1992; Kawano et al, 1992) and CD39 is expressed by a small proportion of normal plasma cells (Ling et al, 1987).

The CD5 antigen is generally not considered to be part of the phenotype of germinal centre cells but a few $CD3^{-}5^{+}$ cells are located in the germinal centre (Caligaris-Cappio et al, 1982) and, although >80% of $CD5^{+}$ cells in suspensions of tonsillar B cells have the phenotype of follicular mantle cells, about 6% express CD10 (Defrance et al, 1992). The histological location of this latter population was not investigated but a germinal centre origin is one possibility. The function of $CD5^{+}$ B cells in the germinal centre is not known but, as they are a minor population, they may represent cells which have already received rescue signals and are about to leave the germinal centre. CD5 has been described on other putative precursor populations (Pilarski and Jensen, 1992) and may be an activation antigen on B cells rather than a marker of a separate B cell lineage (Freedman et al, 1987b; Freedman et al, 1989; Werner-Favre et al, 1989; Visser et al, 1990).

It is also of interest to compare the phenotype of these $CD10^{+}$ PBL with that of other putative precursor populations. The lymphoid population identified by Caligaris-Cappio and colleagues (1985) in the bone marrow of all and in the peripheral blood of half of those with myeloma cells expressing the CD10

antigen was $CD10^+$, $CD38^+$, HLA-DR^+ and sIg^-. However, these cells were markedly different from the current population, as they were negative for the B cell antigens CD19, CD20, CD21, CD22 and CD24, as well as CD9.

The initial population identified by Pilarski and co-workers (1985c) had no features which identified it as monoclonal and the phenotype was dissimilar to the cells in this study. This group of workers (Jensen et al, 1991; Pilarski and Jensen, 1992) recently described precursor cells which expressed CD19, CD20, CD24, CD10, PCA-1, CD11b, CD44, CD29, CDw49b, CDw49d, CDw49f, VLA-5, LAM-1 and RHAMM. A variable proportion were $CD5^+$ and $CD9^+$, whereas CD38 was present at low density on a small subset. CD21, CD34 and CD56 were not detected. There are some phenotypic similarities between these cells and the $CD10^+$ PBL described in this study. Both are B cells expressing CD5 and CD9. Some of the discrepancies may relate to differences in the peripheral blood populations chosen for study. In Pilarski's studies, the precursor cells were both morphologically and phenotypically heterogeneous and were present in the total mononuclear population, whereas only cells within the lymphoid gate were analysed in this current study. In the precursor population, only a low proportion of the small cells (ie. predominantly lymphoid) expressed PCA-1 and CD38. Although the low expression of PCA-1 by the small cells is consistent with the phenotype of $CD10^+$ PBL in this current study, this is not the situation for the CD38 antigen. Furthermore, although both these populations express adhesion and homing molecules, there are indications that the intensities of expression of these antigens may be different. The malignant precursor cells had a moderate to high intensity of CD44 expression, whereas the $CD10^+$ PBL had a significantly lower CD44 intensity than the $CD10^-44^+$ PBL and, on an individual basis, these differences in intensity were large.

One study of the phenotype of Id^+ peripheral blood cells in 2 patients detected CD38, CD21 and HLA-DR but CD20 was present in only one case (Bloem et al, 1988). Although this phenotype is limited, it is not inconsistent with that of the $CD10^+$ lymphocytes.

Despite some similarities in the phenotype of the $CD10^+$ PBL in comparison to germinal centre cells and some of the putative precursor populations, the presence of some differences and the failure to demonstrate monoclonality in the former requires the consideration of an alternative hypothesis for the nature of these B cells. The $CD10^+$ PBL may be a non-malignant B cell population which becomes expanded in some patients with multiple myeloma. $CD5^+$ B cells (B-1a cells) are a subpopulation of B cells in the peripheral blood of adults. These B cells are known to have weak or absent expression of sIg (Caligaris-Cappio et al, 1982; Freedman et al, 1987a). The detection of sIg on only a proportion of $CD10^+$ B cells is thus consistent with their expression of the CD5 antigen, especially as the ratio of sIg^+:B cells was always less than the ratio of $CD5^+$:B cells. Hypothesising that the $CD10^+$ PBL are $CD5^+$ PBL is attractive because these cells have anti-idiotypic activity (Raveche, 1990). In myeloma patients, there is an increase in the number of B cells which have anti-idiotypic specificity (Pilarski et al, 1985b; Pilarski et al, 1989b; Bergenbrant et al, 1991).

A B cell with immunosuppressive properties has been extensively studied in myeloma patients (Paglieroni and MacKenzie, 1977; Paglieroni and MacKenzie, 1980; MacKenzie et al, 1987; MacKenzie et al, 1991; Paglieroni et al, 1992a; Paglieroni et al, 1992b). These cells have not been demonstrated to be related to the malignant clone but have been identified as $CD5^+$ B cells (MacKenzie et al, 1987). In contrast to the situation in myeloma patients, normal $CD5^+$ B cells

do not express CD14 or have immunosuppressive properties (MacKenzie et al, 1991). The expression of the CD14 antigen in myeloma patients was detected with the MY4 antibody. It does not necessarily represent aberrant expression of a monocyte antigen, as the CD14 antigen has been detected on normal B cells (Ziegler-Heithbrock et al, 1988; Labeta et al, 1991), B-CLL (Morabito et al, 1987; Labeta et al, 1991) and EBV transformed B cells (Labeta et al, 1991) using the MY4 antibody. The MY4 antibody detects the same antigen on monocytes and B cells and the CD14 antigen on B cells is easily detected with MY4, weakly with MEM18 but not at all with Mo2 and Leu M3 antibodies (Labeta et al, 1991). The presence of CD14 on $CD5^+$ B cells in myeloma may reflect a difference in activation status in comparison to the equivalent cells in normal individuals. The expression of CD14 by the $CD10^+$ PBL was not examined in this study.

Many features of the phenotype of the $CD10^+$ PBL correspond to that of the $CD5^+$ B cells in both normal individuals and myeloma patients (see Introduction section 1.8.2). Both are $CD19^+$, $CD20^+$, $CD21^+$, $CD22^+$, $CD24^+$, HLA-DR^+ and weakly sIg^+. The CD20 antigen was expressed on only about half the immunosuppressive $CD5^+$ B cells in myeloma (MacKenzie et al, 1987) and was the B cell antigen present on the lowest percentage of $CD10^+$ PBL. However, when present, its intensity of expression was higher than on other B cells and this has been reported to be the situation for the $CD5^+$ B cells that occur in increased numbers following bone marrow transplantation (Ault et al, 1985). Expression of the CD23 antigen has been reported in only one study of normal $CD5^+$ B cells (Caligaris-Cappio et al, 1989). It was present on a mean of 30% of cells with a range of 12-63%. This same study reported that CD25 and CD71 were present on 19% (range: 7-35%) and 21% (range: 11-37%) of cells respectively. The cut-off value for positive as opposed to absent expression of an antigen by

the CD10$^+$ PBL was 30% and thus the lack of expression of CD25 and CD71 is not inconsistent with the findings of Caligaris-Cappio et al (1989).

For the other antigens that were detected on the CD10$^+$ PBL, there are no published results pertaining to the expression of these antigens on CD5$^+$ B cells. The cells in CLL are considered to be the malignant counterpart of CD5$^+$ B cells (Caligaris-Cappio et al, 1982; Freedman et al, 1987a) and, apart from CDw49d, the expression of the other antigens detected on CD10$^+$ PBL has been investigated on CLL. The B cell antigens CD37 and CD39 are expressed on CLL cells (Ling et al, 1987; Pezzutto et al, 1989) and CD9 is expressed by the cells of >50% of cases (Ling et al, 1987). CD11a/CD18 (LFA-1) is expressed on the cells of the majority of CLL cases but the expression is weak in about half the cases (Palleson et al, 1989). The intensities of CD11a and CD18 expression on the CD10$^+$ PBL were significantly lower than on the CD10$^-$ PBL. CD44 is present on the cells of all CLL cases (Pezzutto et al, 1989). CD54 is present on the cells of <50% of CLL cases but the precise proportion of positive cases has varied between studies (Boyd et al, 1989; Pezzutto et al, 1989; Maio et al, 1990).

The major difficulty with the hypothesis that the CD10$^+$ PBL are CD5$^+$ B cells is the expression of both CD38 and CD10 itself. The presence of the CD38 antigen has not been studied on CD5$^+$ B cells in myeloma patients. It has been investigated in only one study of normal CD5$^+$ B cells and was found to be absent (Caligaris-Cappio et al, 1989). However, CD38 has been detected on the cells of a minority of cases of CLL (Ling et al, 1987; Pezzutto et al, 1989).

CD10 has not been detected on the CD5$^+$ immunosuppressive B cells in myeloma patients (MacKenzie et al, 1987) or on normal CD5$^+$ B cells in most studies (Ault et al, 1985; Antin et al, 1986; Caligaris-Cappio et al, 1989). A recent

study of the surface antigen expression of $CD5^+$ tonsillar B cells using flow cytometry found that 6% of these cells were $CD10^+$ but >80% had the phenotype of follicular mantle cells (Defrance et al, 1992). Although the expression of CD10 may indicate a germinal centre origin for this $CD5^+10^+$ population, it may also represent a subpopulation of the follicular mantle $CD5^+$ B cells. Assuming that the $CD5^+$ B cells identified in this study represent a subset of the total $CD5^+$ population, the expression of CD10 and CD38 by these cells may indicate either an activated state or an evolving intermediate phenotype following an undefined stimulus. $CD5^+$ B cells isolated from cord blood and cultured with IL-2 and IL-4 become $CD5^-$, $sIgD^-$, $CD10^+$ and $CD38^+$ (Caligaris-Cappio et al, 1989).

The explanation for the failure to detect CD10 on $CD5^+$ cells in the majority of studies may be related to the choice of antibody clones used to detect the CD10 antigen. A study demonstrating that $CD10^+$ PBL exhibit circadian rhythms noted that the number of positive cells was consistently and markedly higher with the J5 than with the VIL-A1 antibody (Canon et al, 1985). In a comparative study of 5 anti-CD10 monoclonal antibodies, the results with all the antibodies were similar in cases of ALL. The J5 antibody reacted with a variable proportion of the cells in cases of CLL, prolymphocytic leukaemia and NHL by immunofluorescence but not immunoperoxidase techniques. The OKB-cALLA antibody reacted with a lower proportion of the cells in these chronic lymphoproliferative disorders than J5. The Nu-N1, Nu-N2 and VIL-A1 antibodies gave negative results (Haralambidou et al, 1987). There is not yet a definitive explanation for these results. It may reflect different binding affinities of the antibodies. Alternatively, the antibodies may detect different epitopes. Differences in the epitopes expressed by various cells may result from modifications in transcription or translation of the gene, or post-translational

modifications of the gene product, including glycosylation differences. The original studies of $CD10^+$ PBL in myeloma used the J5 antibody (Wearne et al, 1987a; Joshua et al, 1988). Defrance et al (1992) did not specify the anti-CD10 antibody clone used in their study of $CD5^+$ tonsillar B cells but the other studies of $CD5^+$ B cells all used the W8E7 antibody. We used this same antibody clone to enumerate the $CD10^+$ PBL in the controls and the myeloma patients but the SS2/36 clone was used to study the phenotype of $CD10^+$ cells. Neither W8E7 nor SS2/36 were used in the comparative study by Haralambidou et al (1987), so it is not possible to be certain whether this explanation does account for the failure to detect the CD10 antigen on even a subset of $CD5^+$ B cells in most previous studies.

Although no functional studies of the $CD10^+$ PBL were undertaken, there was no circumstantial evidence that these cells had immunosuppressive properties. The percentage or absolute number of $CD10^+$ PBL in myeloma patients did not correlate with serum IgM levels. However this is almost certainly a too simplistic approach to the problem, as the suppression of polyclonal immunoglobulin production in myeloma patients is multifactorial.

As mentioned previously, the phenotype of the $CD10^+$ PBL in one myeloma patient differed significantly from that in the other individuals. The expression of the CD24, CD38, CD36, CD11a, CD18 and CDw49d antigens was in the "strong positive" category and that of the CD7, class II MHC and PCA-1 antigens was in the "positive" category. CD9, CD37, CD39 and sIg were expressed on just enough $CD10^+$ PBL to be classified as "positive'. The CD5 antigen was absent. This patient was the only one with cells expressing CD36 and PCA-1 but not CD54. The phenotype of these cells does not correspond to that of any of the

putative precursor populations described by other workers. The strong co-expression of CD10 and CD38 is still suggestive of a cell originating in the germinal centre and destined to become a plasma cell in the bone marrow. Such a population may be differentiating while still in the circulation and, in that case, the phenotype of $CD10^{+}38^{+}$ cells may vary between individuals. To determine the proportion of patients and normal individuals in whom the phenotype of the $CD10^{+}$ PBL varies from the usual one would require a larger study. Many of the antigens expressed by the $CD10^{+}$ cells in this particular patient have been described on normal plasma cells, myeloma cells and precursor populations (see sections 1.5.2, 1.5.3, 1.4.6 and table 1.1). CD10, CD38, CD24, CD37, CD39, CD7, CD9, class II MHC molecules, PCA-1, CD11a, CD18 and CDw49d have all been detected on such populations. Results for the expression of CD36 have not been reported but other myelomonocytic antigens have been detected on normal bone marrow plasma cells (Terstappen et al, 1990).

It is difficult to propose another cell type which would account for most aspects of this particular phenotype. When using flow cytometry analysis, small numbers of monocytes can be included in the lymphoid gate. The CD9, CD36, CD37, CD38, CD11a, CD18, CDw49d and PCA-1 antigens have all been detected on a variable proportion of monocytes (Anderson et al, 1983; Horton and Hogg, 1987; Ling et al, 1987; Dörken et al, 1989; Modderman, 1989; Von Dem Borne and Modderman, 1989a; Von Dem Borne and Modderman, 1989b; Aranout, 1989; Hemler et al, 1990; Terstappen et al, 1990). CD10, CD24 and CD39 are not present on monocytes. CD10, CD24 and PCA-1 but not the other antigens may be expressed by granulocytes (Anderson et al, 1983; Pesando et al, 1983; Ling et al, 1987; Dörken et al, 1989; LeBien and McCormack, 1989) but mature

granulocytes would not be expected to be present in the lymphoid gate. Possibly the $CD10^+$ PBL in this one patient comprise cells of multiple lineages.

In conclusion, $CD10^+$ PBL are present in both normal individuals and myeloma patients. The number of these cells, although raised in individual patients, is not significantly elevated in the myeloma patients as a group. Based on surface antigen expression, these cells are B cells which express the CD5 antigen. This population may be either a minor subset of $CD5^+$ B cells or may be a circulating pre-plasma cell population originating from a population of B cells present in secondary lymphoid follicles, namely rescued germinal centre cells or secondary B blasts. Further studies are required to distinguish between these possibilities. Attempts to identify a monoclonal component within the $CD10^+$ population in myeloma patients must be undertaken. Studies of the heavy chain immunoglobulin isotype on the surface of these cells would be helpful, as non-malignant $CD5^+$ B cells should be $sIgM^+$ and $sIgD^+$ but precursor cells should be $sIgG^+$ and $sIgA^+$. Purifying these cells in order to examine the morphology and expression of cIg would also be useful. Examining the ability of these $CD10^+$ B cells to suppress normal immunoglobulin secretion *in vitro* would determine whether there was a relationship to the immunosuppressive $CD5^+$ B cells in myeloma patients described by MacKenzie's group.

SECTION 5: CONCLUSIONS AND SUMMARY

A significant amount of research on multiple myeloma has been directed at proving the existence of precursor (pre-plasma) cells, especially in the peripheral blood compartment, and subsequently at determining the characteristics of these cells. There is debate regarding the maturation status of the precursor cells. Some investigators have proposed that there are haemopoietic stem cells or pre-B cells belonging to the malignant clone but others believe that precursor cells arise at a later stage in B cell ontogeny. The proponents of the former view have based their arguments principally on the expression of CD10 and myelomonocytic antigens by malignant plasma cells, myeloma cells and putative precursor populations (Durie and Grogan, 1985; Epstein et al, 1988; Barlogie et al, 1989; Epstein et al, 1990). It is now known that CD10 expression within the B cell lineage is not confined to pre-B cells. It is present on germinal centre B cells (Hsu and Jaffe, 1984; Weinberg et al, 1986; Ling et al, 1987; Gadol et al, 1988; Liu et al, 1989) and some normal plasma cells (Caligaris-Cappio et al, 1985; Terstappen et al, 1990). Myelomonocytic antigens are also present on normal plasma cells (Terstappen et al, 1990).

There are now several studies which support the view that precursor cells arise at a later stage in B cell ontogeny. It has been shown that the number of pre-B cells is not increased in either the bone marrow or peripheral blood of patients with multiple myeloma (Zhang et al, 1988; Duperray et al, 1991). On the basis of both PCA-1 and CD45 isoform expression, the cells of a putative precursor population have been shown to be late B cells or pre-plasma cells rather than pre-B cells (Jensen et al, 1991; Pilarski and Jensen, 1992). Sequencing of immunoglobulin V_H genes in myeloma patients has demonstrated that these genes have undergone somatic mutation but that there is no evidence of

ongoing mutation as the disease progresses (Bakkus et al, 1992; Ralph et al, 1993). Somatic mutation has been shown to occur during the germinal centre reaction (Jacob et al, 1991). It is not possible to believe that precursor cells sited at the pre-B cell stage would give rise to progeny whose immunoglobulin genes undergo identical somatic mutation during the differentiation process.

The studies described in this thesis were based on the hypothesis that the earliest stage in B cell ontogeny at which oncogenic transformation (ie. the point at which the processes of differentiation and proliferation become uncoupled) can give rise to a malignant clone manifest as multiple myeloma is a germinal centre B cell (Warburton et al, 1989). This does not exclude the possibility that at least some of the oncogenic insults occur in haemopoietic stem cells or early in B cell ontogeny. This hypothesis was based on the requirement of the precursor cell to have stable immunoglobulin gene rearrangements, to have been exposed to antigen, to have undergone somatic mutation and to have undergone immunoglobulin isotype switching. Studies on the precursor cells in myeloma were approached in several ways: (1) a clonogenic assay for myeloma cells; (2) development of a germinal centre B cell line which was used to study the differentiation of germinal centre cells to plasma cells; (3) immunophenotypic analysis of $CD10^+$ or $CD38^+$ PBL.

Millar et al (1988) have described a clonogenic assay for myeloma cells. They reported that myeloma colonies could be reproducibly grown from the majority of bone marrow aspirates and about half the peripheral blood samples from myeloma patients, irrespective of the stage of the disease or the degree of plasma cell infiltration in the bone marrow. The colonies were composed of either large plasmacytoid cells, small lymphoid cells or both cell types. This

suggested that the colonies arose from malignant cells at different differentiation stages, including ones representing pre-plasma cell stages. Despite multiple modifications to the published technique and a visit to Millar's laboratory, attempts to reproduce this assay were unsuccessful. The results suggested that the technique does not result in myeloma colonies but that apparent colonies are due to clumps of either myeloma cells or cells of multiple lineages. The occurrence of pseudocolony formation has been documented previously in a different myeloma colony culture system (Rhodes at al, 1990). Homotypic adhesion between myeloma cells has been reported to occur through the interaction of LFA-1 and CD54 (Kawano et al, 1991) and could also be theoretically mediated via the CD56 molecules present on malignant plasma cells (Van Camp et al, 1990; Drach et al, 1991; Van Riet et al, 1991; Barker et al, 1992a; Harada et al, 1992). The life span of plasma cells in the bone marrow is in the vicinity of 3-4 weeks (Ho et al, 1986), so the occurrence of homotypic adhesion between myeloma cells could result in aggregates of cells being interpreted as colonies.

Bone marrow and peripheral blood mononuclear cells from myeloma patients were placed into liquid culture in an attempt to establish permanent cell lines. This attempt was based on the premise that cell lines are representative of the proliferating precursor cells rather than the end-stage plasma cells. A permanent cell line would enable more detailed studies of proliferation and differentiation in a given patient than would be possible with a single specimen studied in the clonogenic assay. The experience of other workers has been that permanent myeloma cell lines are difficult to establish and have usually been derived from the cells of patients with more uncommon clinical features such as plasma cell leukaemia or extramedullary disease. This proved to be the case

and no new myeloma cell lines were established. Six cell lines were obtained but they were all positive for the EBNA antigen. The growth characteristics, morphology and surface antigen expression were typical of EBV transformed cell lines. The phenomenon of spontaneously arising EBV^+ lymphoblastoid cell lines in cultures of peripheral blood, bone marrow or lymphoid tissue of healthy individuals or patients with various malignancies is well documented (Nilsson and Klein, 1982).

In order to demonstrate that germinal centre B cells can mature into plasma cells and to study the differentiation signals for this process, a germinal centre B cell line (WL2) was established from the malignant circulating cells of a patient with follicular small cleaved cell lymphoma which had transformed to large cleaved cell lymphoma. The morphology and phenotype of the WL2 cell line were consistent with that of germinal centre cells. Culture of these cells with the non-physiological reagent TPA resulted in alterations of the surface antigen expression and a significant decrease in the proliferation of the cells. Overall, the results were more consistent with TPA acting as an activation rather than a differentiation factor. Minor changes occurred with LPS and the combinations of TPA plus LPS and TPA plus IFN-α2a. Apart from the very limited activity of IFN-α2a, culture of the WL2 cells with physiological stimuli such as interleukins produced no detectable changes. Thus, the WL2 cell line was not a useful model for differentiation studies on germinal centre B cells. This may have been related to the inherent malignant nature of the cell line. In particular, the presence of the t(14;18) translocation and the resultant constitutive expression of the bcl-2 protein may have resulted in these cells being unable to respond to differentiation signals.

The studies of $CD10^+$ and $CD38^+$ were prompted by the knowledge that both these antigens are expressed on germinal centre B cells and both have been associated with precursor populations in multiple myeloma. CD38 is expressed on both normal and malignant plasma cells (Hercend et al, 1981; Bhan et al, 1981; Foon et al, 1982; Hsu and Jaffe, 1984; Tedder et al, 1984; Aisenberg and Wilkes, 1983; Bhan et al, 1984). Increased numbers of $CD38^+$ PBL in myeloma patients are associated with a poor prognosis at all stages of the disease (Ruiz-Argüelles et al, 1984; Joshua et al, 1988; Omedé et al, 1990; Joshua et al, 1991b). It was originally proposed that these $CD38^+$ cells are precursor cells (Ruiz-Argüelles et al, 1984; Joshua et al, 1988). More recent, albeit limited, studies have suggested that CD38 is expressed on increased numbers of T cells and NK cells rather than precursor cells (Boccadoro et al, 1988; Gonzalez et al, 1992). These findings were confirmed in this study on a larger series of patients but it was demonstrated that the lymphocyte subsets responsible for the elevated numbers of $CD38^+$ PBL were different in the untreated and the treated patients.

In untreated patients, CD38 is expressed on increased numbers of helper T cells and was associated with normal B cell numbers and serum IgM levels in the low normal range. As an increased number of $CD38^+$ PBL is an adverse prognostic factor, the presence of comparatively normal humoral immunity is associated with a poor prognosis. The immune mechanism underlying this phenomenon requires further investigation. There were data which indirectly suggested that, in untreated patients without increased numbers of $CD38^+$ PBL, there are idiotype-reactive T cells. Such cells would be theoretically capable of causing immunosuppression. The data were not consistent with the presence of these idiotype-reactive T cells in the patients with increased numbers of $CD38^+$ PBL. Alternatively, a more active mechanism may be responsible for this

phenomenon. This is suggested by the presence of increased as opposed to normal numbers of $CD38^+$ helper T cells.

In treated patients, there were increased numbers of activated suppressor/cytotoxic T cells and NK cells expressing CD38. Other investigators have found that the $CD8^+HLA\text{-}DR^+$ T cell population in myeloma is enriched for idiotype-reactive T cells (Dianzani et al, 1988) and the presence of a decreased naive:memory cell ratio has been hypothesised to indicate the presence of idiotype-reactive T cells (Massaia et al, 1991). As this study produced indirect evidence that the naive:memory cell ratio was reduced in these patients, it seems probable that the activated $CD8^+38^+$ cells were idiotype-reactive T cells. Although there was evidence of similar T cells in both untreated and treated myeloma patients without elevated numbers of $CD38^+$ PBL, this population was not as expanded nor as activated as it was in the treated patients with increased $CD38^+$ cell numbers. The presence of increasing numbers of activated idiotype-reactive T cells is a poor prognostic sign and may reflect resistance of the tumour to this form of immune surveillance.

This study also provided an explanation for the previously noted discordance between STK and LI in individual myeloma patients (Brown et al, 1989). The number of $CD38^+$ PBL in treated patients correlated with STK but not plasma cell LI. This indicates that the STK is reflecting proliferation of $CD38^+$ PBL and, by deduction, activated $CD8^+$ T cells as well as myeloma cells. This is supported by a study showing that the LI of $CD2^+$ PBL correlates with the proportion of $HLA\text{-}DR^+$ cells within the $CD8^+$ population (Massaia et al, 1988b).

There are increased numbers of PBL expressing CD10 in some patients with myeloma (Wearne et al, 1987a; Joshua et al, 1988). The CD10 antigen has been detected on the malignant plasma cells of patients with multiple myeloma and has also been consistently reported as being expressed by populations of putative precursor cells (Ruiz-Argüelles et al, 1984; Caligaris-Cappio et al, 1985; Durie and Grogan, 1985; San Miguel et al, 1986; Epstein et al, 1988; Grogan et al, 1987; Jackson et al, 1988; Bergui et al, 1989; Grogan et al, 1989; King and Nelson, 1989; Epstein et al, 1990; Shimazaki et al, 1990; Van Camp et al, 1990; Drach et al, 1991; Hamilton et al, 1991a; Jensen et al, 1991; San Miguel et al, 1991; Leo et al, 1992; Pilarski and Jensen, 1992). It is also present on normal plasma cells (Caligaris-Cappio et al, 1985; Tominaga et al, 1989; Terstappen et al, 1990). In this study, the $CD10^+$ PBL were shown to be $CD5^+$ B cells that also expressed CD38. These same cells were detected in normal individuals, although their numbers were clearly elevated in some myeloma patients. Furthermore, on the basis of the $\kappa:\lambda$ ratio within the $CD10^+$ population, these cells were not monoclonal. However, about half the $CD10^+$ cells had no detectable sIg. It is therefore still possible that some of the cells in this population were malignant precursors, if the assumption is made that the $CD10^+$ PBL in the normals are plasma cell precursors and that the population in the myeloma patients is composed of both normal and malignant precursors. The immunophenotype of these cells was similar but not identical to germinal centre B cells and other putative precursor populations. Alternatively, these cells may be related to the previously described non-malignant immunosuppressive $CD5^+$ B cells in myeloma patients (Paglieroni and MacKenzie, 1977; Paglieroni and MacKenzie, 1980; MacKenzie et al, 1987; Mackenzie et al, 1991). Further studies are required to discriminate between these two possibilities.

In conclusion, these experiments were not able to determine the stage in B cell ontogeny at which oncogenic transformation is capable of giving rise to a myeloma clone. The majority of $CD38^+$ PBL are not malignant precursors but it is still possible that there are a small numbers of circulating precursor cells which co-express CD10, CD38 and CD5. The results of the study of $CD38^+$ PBL emphasise the importance of host-tumour interactions in myeloma.

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