THE PHENOTYPE AND IN VITRO CULTURE CHARACTERISTICS OF HUMAN MULTIPLE MYELOMA CELLS

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SUMMARY

Studies were undertaken to define the malignant cell, and in particular the clonogenic progenitor cell, in multiple myeloma (MM). The aim was to find ways of removing these cells from bone marrow for use in autologous bone marrow transplantation.

Using monoclonal antibodies (MoAb) defined at the IIIrd International Workshop on Human Leucocyte Differentiation Antigens, myeloma plasma cells were found to lack expression of the B-cell antigens CD19-23, 37, 39 and w40. They expressed CD38 and the epitope of CD24 which reacts with a MoAb called HB8. Weak expression of CD9 and CD10 was also occasionally seen. No plasma cell-specific MoAb, that might have been useful for 'purging' myeloma bone marrow, were identified. Cellular DNA analysis showed about half the cases of MM to be aneuploid.

Short-term cultures were found to be unsatisfactory for the study of the clonogenic cell. MM cells tended to aggregate into clumps, which were initially mistaken for colonies. Short-term incubation with PHA and interleukin 2 (IL2) induced expression of IL2 receptors (IL2R) on the surface of MM cells, suggesting that the clonogenic cell may be able to respond to IL2. However, the possibility that these IL2R were soluble IL2R released from T cells and taken up by the MM cells could not be excluded.

MM plasma cells remained viable in simple liquid culture media for several weeks, but no long-term cell lines were produced by this method. Experiments to fuse human MM cells with a mouse plasmacytoma produced, from one patient, a number of hybridomas which secreted human immunoglobulin (Ig) for six to ten weeks. In another case the fusion failed, but an unfused human plasma cell line was produced and which secreted Ig. A subclone of this line was produced that was dependent on interleukin 6 (B cell stimulatory factor 2).

ABBREVIATIONS

ABCM	adriamycin, BCNU, cyclophosphamide, melphalan
ABMT	autologous bone marrow transplantation
AET	5.2 aminoethylisothiouronium bromide hydrobromide
ALL	acute lymphoblastic leukaemia
AML	acute myeloblastic leukaemia
B cell	the human lymphocyte corresponding to the chicken lymphocyte originating in the Bursa of Fabricius
$ß_2M$	beta$_2$microglobulin
BCGF	B cell growth factor
BCNU	bischloroethyl nitrosurea
BM	bone marrow
BMT	bone marrow transplantation
BrdU	bromodeoxyuridine
BSF-2	B cell stimulatory factor 2
c	cytoplasmic
°C	degrees Celsius
C3	third component of complement
CaI	calcium ionophore
CALLA	common acute lymphoblastic leukaemia antigen
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea
CD	cluster of differentiation
CFU-GM	colony forming units granulocyte/macrophage
CFU-Mix	colony forming units mixed (i.e. granulocyte, erythroid, macrophage, megakaryocyte)
CGL	chronic granulocytic leukaemia
cGy	centiGray
CLL	B cell chronic lymphocytic leukaemia
CR	complete remission
CV	coefficient of variation
DEAE	diethylaminoethyl cellulose
del	deleted chromosome
Dep	department
der	derived chromosome
dl	decilitre
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
E$^-$cells	fraction of cells which had had the T cells removed by SRBC rosetting and density centrifugation
ESG	Ewing's sarcoma cell line supernatant
FACS	fluorescence-activated cell sorter
Fc	crystallizable fragment of Ig
FCS	fetal calf serum
g	gram
G_0	resting phase of the cell cycle
G_1	growth phase of the cell cycle
G_2	second growth phase of the cell cycle
G6PD	glucose-6-phosphate dehydrogenase
^3H-	tritiated
4HC	4-hydroperoxycyclophosphamide
HAT	hypoxanthine, aminopterin, thymidine
HDM	high dose melphalan
Hepes	N-2-Hydroxyethylpiperazine-N'-2 ethanesulfonic acid
Hepes/2%	Hepes buffered RPMI-1640 medium, pH=7.3 with 2% FCS

ABBREVIATIONS (Cont'd)

HGPRT	hypoxanthine-guanine-phosphoribosyl transferase
HLA	human leucocyte antigen
Id	idiotype
Ig	immunoglobulin
IL	interleukin
IL2R	interleukin 2 receptor
Isc/Ham	a 1:1 mixture of Iscove's and Ham's media
i.u.	international units
kDa	kiloDalton
l	litre
LI	labelling index
LTBMC	long term bone marrow culture
M	molar
M(phase)	mitotic phase of the cell cycle
MDS	myelodysplastic syndrome
MEM	minimal essential medium
mg	milligram
MGUS	monoclonal gammopathy of undetermined significance
MHC	major histocompatibility complex
ml	millilitre
MM	multiple myeloma
mmol	millimole
μCi	microcurie
μg	microgram
μl	microlitre
μmol	micromole
MoAb	monoclonal antibody
MRC	Medical Research Council
My-CFU	myeloma colony forming units
PBS	phosphate buffered saline
PC	plasma cell
PCL	plasma cell leukaemia
%	percentage
PEG	polyethylene glycol
PHA	phytohaemagglutinin
PHA-TCM	PHA-T-cell conditioned medium
PHSC	pluripotential haemopoietic stem cell
PI	propidium iodide
PTFE	polytetrafluoroethylene
r	recombinant
RNA	ribonucleic acid
rpm	revolutions per minute
s	surface
S	DNA-synthesizing phase of the cell cycle
SAC	staphylococcus aureus Cowan I
SRBC	sheep red blood cells
T cell	lymphocyte of thymic origin
t	translocation
TdT	terminal deoxynucleotidyl transferase
TNF	tumour necrosis factor
TPA	12.0 tetradecanoylphorbol-13-acetate
VAD	vincristine, adriamycin, dexamethasone
vol	volume
w/v	weight/volume

CONTENTS

CHAPTER 1

INTRODUCTION

This thesis describes work carried out *in vitro* on multiple myeloma (MM) and plasma cell leukaemia (PCL). These are neoplastic disorders of the B lymphocyte lineage, characterised by an excess of monoclonal plasma cells in the bone marrow (MM), and also the blood in PCL. These conditions have a poor prognosis and are incurable by conventional treatment. These studies were undertaken initially to explore the possibility of performing autologous bone marrow transplants in MM, with the malignant cells removed from the autologous graft. The surface antigen phenotype of the malignant plasma cells (PC) was therefore investigated, and short- and long-term culture studies were performed to study the dividing cell population.

The Introduction will discuss the following 5 subjects:

1. The physiology of normal B cell differentiation.
2. Clinical and laboratory features of MM.
3. The treatment of MM.
4. Autologous bone marrow transplantation.
5. The immediate background to the studies undertaken:
    (a) The phenotype of MM cells,
    (b) The clonogenic progenitor cell(s) in MM, including cell culture studies.

1.1 THE PHYSIOLOGY OF NORMAL B CELL DIFFERENTIATION.

1.1.1 The origin of B cells

Normal bone marrow plasma cells are at the terminal stage of B lymphocyte differentiation, which starts from the pluripotential haemopoietic stem cell (PHSC). The evidence for a common lymphoid and haemopoietic progenitor cell is based mainly on isoenzyme studies of malignant cells from females suffering from Chronic Granulocytic Leukaemia (CGL). Granulocytes, erythrocytes, platelets, macrophages and Epstein-Barr virus transformed B cell lines from patients heterozygous for

the X-linked marker Glucose-6-phosphate Dehydrogenase (G6PD) express only one of the two G6PD isoenzymes (Fialkow et al, 1977; Martin et al, 1980). The Philadelphia chromosome has been demonstrated in B cell precursors of CGL patients (Martin et al, 1980; Bernheim et al, 1981). Thus, at least in CGL, the B cells and myeloid/erythroid cells appear to arise from a common progenitor, probably at or near the PHSC.

1.1.2 B cell differentiation

The earliest identifiable immunoglobulin(Ig)-producing cells are rapidly dividing pre-B cells which express cytoplasmic Ig μ heavy chains and nuclear terminal deoxynucleotidyl transferase (TdT). By this stage, the Ig heavy chain genes have undergone their initial rearrangements to bring the various gene segments together to form transcribable units of precise antigenic specificity. Ig gene rearrangement of itself cannot be taken to be indicative of inevitable commitment to the B cell lineage, for in a significant minority of cases of acute lymphoblastic leukaemia rearrangement of both heavy chain genes and T cell receptor genes has taken place.

Recent work has highlighted the role of bone marrow stromal cells in the generation of B progenitors (Dorshkind, 1987). Stromal cell lines from long term bone marrow cultures (LTBMC) secrete factors which stimulate the formation of pre-B cells in short term bone marrow cultures (Landreth et al, 1985), but as yet these factors are poorly characterised. The intimate association between stromal and haemopoietic cells in vivo and in LTBMC suggests that direct cell-cell contact may also be important at certain stages of differentiation. Pre-B cells mature through a small non-dividing pre-B stage into surface IgM expressing 'virgin' B cells (i.e. not yet activated by antigen).

Newly formed virgin B cells can be activated by T-cell dependent antigens in periods immediately following exposure to

antigen, and can give rise to short-lived IgM-, IgG- or IgA-secreting plasma cells or memory B cells (Maclennan & Gray, 1986). In established immune responses to these antigens IgM production ceases and the antibody produced is mainly IgG and IgA. The plasmablasts at this stage of the response either migrate to the bone marrow where they become long-lived plasma cells, or recirculate as memory B cells. This is summarised in Figure 1.1. The antigen specificity can undergo minor change during secondary activation, probably within the germinal centre. This is brought about by somatic mutation within the rearranged immunoglobulin genes (Griffiths et al, 1984).

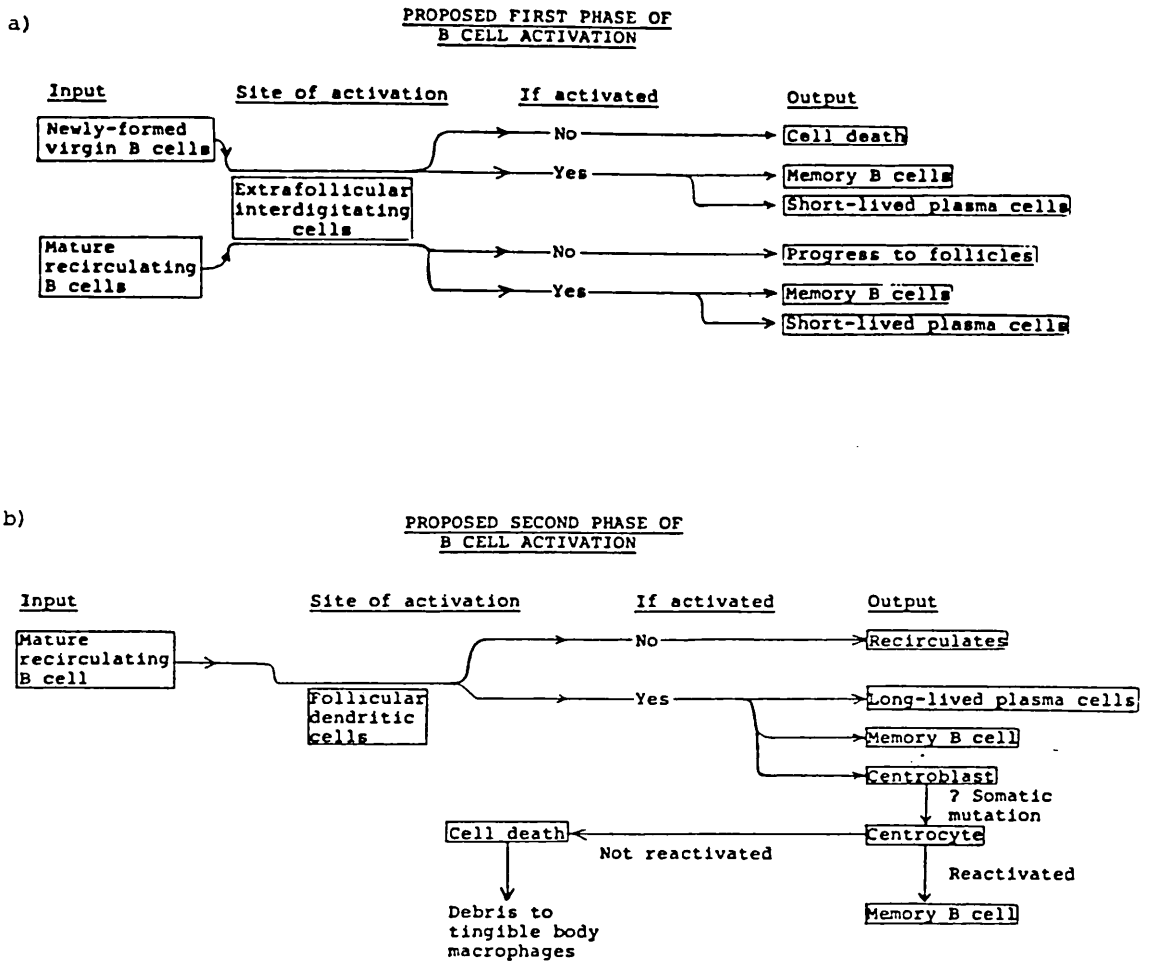


Figure 1.1 An hypothetical model of two phases of B cell activation, based on knowledge at the end of 1985 (Maclennan & Gray, 1986; with permission).

1.1.3 B cell growth factors

Several humoral factors are now known to be involved in the growth and differentiation of B cells *in vitro*. The majority of experiments have been performed with B cells isolated from lymph nodes, tonsils, or spleen, which contains a heterogeneous population of predominantly mature recirculating B cells rather than newly-produced virgin B cells. The B cell response to antigen in these cells occurs in 3 phases (Gordon & Guy, 1987):

(a) Activation. This is defined as the entry of resting (i.e. G_0) cells into cell cycle, including G_1 and S phase. B cell 'activators' are subdivided into:

(i) Activation factors, which can act on completely resting cells to enhance responsiveness to B cell growth factors acting via surface immunoglobulin (i.e. anti-Ig, or antigen). One major activation factor is B cell growth factor (BCGF) I (also called B cell stimulatory factor I, or Interleukin 4), a 20kiloDalton (kDa) T cell product (Rabin *et al*, 1985).

(ii) Progression factors, which can drive activated B cells through S and into the G_2 phase of the cycle, without necessarily proceeding to mitosis, e.g. low molecular weight BCGF ($BCGF_{low}$), a 12kDa T cell product (Sharma *et al*, 1986).

(b) Replication. This is the phase of the response involving mitosis, and factors acting at this stage include a high molecular weight (60kDa) BCGF ($BCGF_{high}$), interleukin 2 and gamma-interferon (Ambrus & Fauci, 1985; Ralph *et al*, 1984; Romagnani, 1986).

(c) Differentiation. Some activated cells do not continue dividing, but leave the cell cycle and differentiate into antibody-secreting cells. Most, if not all, replication

factors also appear to promote Ig secretion. B cell stimulatory factor 2 (BSF-2, also known as hybridoma growth factor, B cell differentiation factor, Interferon β_2, 26k protein or Interleukin 6), which is produced by many cells including T cells and fibroblasts, also promotes both growth and differentiation (Billiau, 1987; Wong & Clark, 1988).

B cells also produce their own autocrine stimulatory factors including part of a surface receptor recognized by CD23 antibodies (see below), which is released after the binding of $BCGF_{low}$ (Guy & Gordon, 1987).

Thus, a large number of B cell 'growth' factors has been identified, although most also have actions on cells other than B cells (Gordon & Guy, 1987). This implies that, in vivo, control must also be regulated by other mechanisms, such as the short half-life of these factors, the location of the cells and their antigen. The proximity to and/or contact with other cell types such as T cells, and other accessory cells such as extrafollicular interdigitating cells and follicular dendritic cells, is also important.

1.1.4 B cell differentiation antigens

A large number of antigens have been described whose expression characterises different stages of B cell differentiation. Groups of antibodies reacting with a single antigen have been defined and 'clustered' into CD groups (i.e. clusters of differentiation) at international workshops (Bernard et al, 1984; Reinherz et al, 1986; McMichael et al, 1987). These are summarised in Figure 1.2 (page 8), but as noted above, B cell differentiation is not purely linear. It involves branching pathways and recirculation of both memory B cells and mature virgin B cells.

The precise function of most of these antigens is unknown, although some of them are undoubtedly involved in the regula-

tion of B cell growth and differentiation. The CD20 molecule may provide an alternative receptor for activation in place of antigen triggering (Golay et al, 1985), and antibody binding to CD19 inhibits B cell activation (Pezzutto et al, 1987a). Both CD21 and CD22 mediate signals that potentiate B cell responses (Nemerow et al, 1985; Pezzutto et al, 1987b). The CD23 molecule is cleaved when low molecular weight BCGF binds on or near it, and a 35kDa molecule (or a 25kDa product of further proteolysis) that is released may act as an autocrine B cell stimulant (Gordon & Guy, 1987). Signals through the CDw40 (p50) antigen can provide powerful co-stimulation when the initial triggering stimulus acts through surface Ig (Clark & Ledbetter, 1986). It will be noted that most of the antigens found on peripheral B cells, such as CD19, 20, 21, 22, 24, and 37 become absent at the secretory B cell stage. Precise growth regulation may no longer be required at this stage: antibody synthesis could be regulated by varying the rate at which these terminally-differentiated cells with a finite life span, are produced.

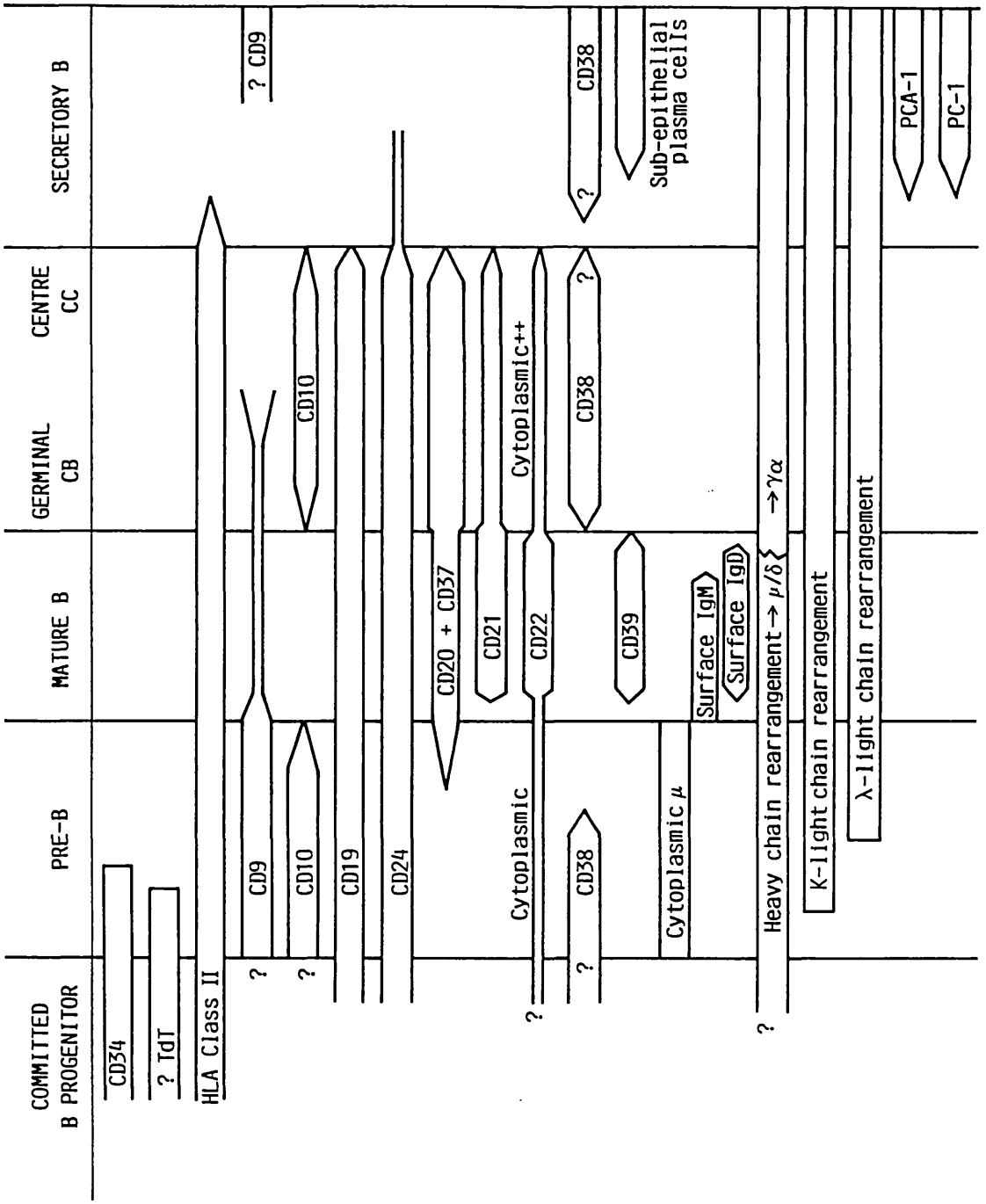


Figure 1.2 A diagram to show the expression of a number of differentiation antigens during B cell ontogeny (CD= cluster of differentiation).

1.2 CLINICAL AND LABORATORY FEATURES OF MULTIPLE MYELOMA.

William MacIntyre (1850) was the first to describe multiple myeloma as a 'Case of mollities and fragilitas ossium accompanied with urine strongly charged with animal matter', and the important clinical and pathological features were described by him and the pathologist J.Dalrymple (1848). Bence Jones described the excretion of the urinary protein named after him, which is now known to be immunoglobulin light chain, in 1848.

1.2.1 Epidemiology

The U.K. incidence of recognized myeloma is approximately 2 per 100,000 (Segi, 1977). This has increased due to improved case detection, and to the population living longer, leading to a greater number of older people at risk for this disease, which predominantly affects the elderly (Cuzick, 1986). A recent increase in American blacks suggests that there is also a real increase in the incidence of MM (Blattner *et al*, 1980). There is indeed a higher incidence in negroes and a slight excess in males (Blattner, 1980). Only 2% of cases appear before the age of 40 years, the mean age at diagnosis being approximately 60 years (Kyle, 1975).

1.2.2 Diagnostic criteria

Patients can be diagnosed as having multiple myeloma if they have at least two of the following three criteria (Medical Research Council (MRC) working party on leukaemias in adults, 1985):

(a) Bone marrow sections or smears showing the presence of a neoplastic plasma cell infiltrate and/or microplasmacytomas. Plasma cell infiltrates alone should either amount to >20% of marrow nucleated cells, or if <20%, objective evidence of monoclonality of plasma cells should be observed.

(b) A paraprotein present in blood or urine.
(c) Definite lytic bone lesions (not just osteoporosis).

A patient may be described as having equivocal myelomatosis if they have MM as described above, but also fulfil all the following criteria (MRC VIth trial protocol):

(a) No symptoms or minimal symptoms
(b) Pretransfusion haemoglobin >100g/l (10g/dl)
(c) Post-rehydration blood urea <8mmol/l, and creatinine <130μmol/l
(d) No osteolytic lesions other than minimal lesions which do not threaten pathological fracture and are not associated with pain.
(e) Plasma cells <30% of marrow nucleated cells. All normal marrow elements should be present in a marrow which has normal cellularity.
(f) Serum β_2 microglobulin <4mg/l
(g) Less than 1 unit of free light chain (=1g polyclonal standard) excretion per gram of urinary creatinine.
(h) There are no objective factors which indicate that the patient has progressive myelomatosis.

These patients, who are normally observed for progression of disease rather than treated initially, are roughly comparable to those with 'smouldering' or 'indolent' multiple myeloma (Kyle & Greipp, 1980; Conklin & Alexanian, 1975). However, about half progress within a short time to frank MM (MRC trial data; ICM Maclennan, personal communication).

Monoclonal gammopathy of undetermined significance (MGUS), sometimes called 'Benign monoclonal gammopathy', is defined as (Kyle, 1984):

(a) Paraprotein level less than 30g/l
(b) Plasma cells <5%

(c) Absence of anaemia and osteolytic lesions
(d) Normal serum albumin
(e) Absence of Bence-Jones proteinuria.

1.2.3 Aetiology

The only aetiological agent identified with any certainty is radiation: for example atomic bomb survivors receiving more than 100cGy of irradiation had an incidence 4.7 times a control unexposed population, with a latent period of at least 15 years in most cases (Ichimaru *et al*, 1979). Other postulated aetiological agents include chemicals (Greene, 1982), and certain occupations (Cuzick, 1986). Copplestone *et al*, (1986) report 9 patients with coexisting myeloma and myelodysplasia (MDS) and 11 others with other B cell malignancies and MDS. They speculate that the two diseases could be part of the same neoplastic process originating in a stem cell giving rise to abnormal differentiation along a number of different pathways. However, the fact that commitment to a particular idiotype does not occur until the cell has differentiated to the pre-B stage makes it unlikely that the myeloma cell has its clonogenic precursor at such an early stage. It is possible that a cellular event occurs at an early stage of haemopoeisis which is not of itself sufficient to induce neoplasia, but which predisposes to neoplastic change in more differentiated cells. It is presumed that the last 'oncogenic' event only occurs after cells have rearranged their Ig genes. Alternatively, one neoplasm could predispose to the development of the other through an associated immune deficiency.

1.2.4 Prognostic features and staging systems

There is a very wide range of survival times of patients with MM, from a few days to more than 10 years. Both for the management of the individual patient, and for the conduct of meaningful clinical trials, it is important to have ways of assessing prognosis from the presenting features of the disease. Hansen and Galton (1985) reviewed the many series of

myeloma patients that have assessed prognostic variables by multivariate analysis. Renal function was clearly the most important independent variable, closely followed by haemoglobin concentration and performance status. Other important variables included serum calcium concentration and the presence of Bence-Jones proteinuria. Patients without a heavy chain component to their paraprotein ('Bence-Jones myeloma') have a much poorer prognosis, especially lambda-light chain disease (Durie & Salmon, 1982). The MRC working party on leukaemia in adults (1980) devised a simple 3 stage system based on the blood urea and haemoglobin (after rehydration) and the performance status:

MRC STAGING FOR MYELOMA

Group	Prognosis		Criteria
I	Good		1. Blood urea <8mmol/l
			2. Haemoglobin >100g/l
		and	3. Minimal symptoms or asymptomatic
II	Intermediate		All those not in I or II
III	Poor		1. Blood urea >8mmol/l
		or	2. Haemoglobin <75g/l
		and	3. Restricted activity or bedridden.

The validity of this system has been confirmed both retrospectively (applied to the first and second MRC trials) and prospectively in the fourth trial (MRC working party on leukaemia in adults, 1985). However, more than half the patients fall into the 'intermediate' prognosis group, and most clinical staging systems result in unevenly sized groups. The staging system of Durie and Salmon (1975) is based on the haemoglobin, calcium, creatinine, serum and urine paraprotein levels, and the presence or absence of 'advanced' lytic bone

lesions. This widely-used system correlates well with the total body myeloma cell number as calculated from in vitro studies, but the discrimination in survival between stages II and III is poor, and the majority of patients are placed in stage III. A recent comparison of 7 clinical staging systems found the MRC classification to provide the best separation into 3 groups, but wide differences in survival are still evident within each group (Gassmans et al, 1985).

The failure of clinical features to provide accurate prognostic information has led to studies of the biology of the malignant cells themselves, rather than just their quantity, and some of these studies have demonstrated features of independent prognostic importance.

(a) The serum β_2-microglobulin. β_2-microglobulin (β_2M) is an 11 kilodalton protein linked to Class I HLA (human leucocyte antigen) molecules on the surface of most cells. β_2M molecules are released into the blood and the rate of release gives some indication of cell turnover. Elevated serum levels are found in a number of malignant conditions, and in renal failure. Most studies have demonstrated that the pre-treatment β_2M level is the single most powerful predictor of survival (Child et al, 1986; Scarffe et al, 1983; Cuzick et al, 1985; MRC Fifth Trial, unpublished data). However, one small study has disputed whether it is independent of other factors, especially renal function (Van Dobbenburgh et al, 1985).

(b) Kinetics of tumour growth. The percentage of plasma cells synthesizing DNA, as measured by ^{3}H-thymidine incorporation in vitro and autoradiography - the plasma cell labelling index (LI) - has been taken to be a measure of the growth fraction of the tumour and correlates well with prognosis. In particular, a subgroup with a high LI has a particularly poor prognosis (Durie et al, 1980), whereas those with a very low LI may have 'smouldering' myeloma (Kyle & Griepp, 1980; Boccadoro et al,

1984). Similar information is obtainable using bromodeoxyuridine (BrdU) incorporation and staining with an anti-BrdU monoclonal antibody (MoAb), or even by staining cells directly with the MoAb Ki-67 which is reactive with the nuclei of proliferating cells (Lokhorst et al, 1987).

(c) Karyotypic abnormalities. Chromosomal analyses in myeloma are difficult to perform because of the low numbers of proliferating cells. Karyotypic abnormalities may be missed in the myeloma cells as the metaphases examined may not be from cells of the neoplastic clone. The metaphases may be from other cell lineages - e.g. myeloid - thus producing results not representative of the MM clone. The presence of chromosomal abnormalities might reflect an associated myelodysplastic syndrome (Dewald et al, 1985). However, careful analysis has shown a number of karyotypic abnormalities that may be associated with the plasma cell proliferation itself: abnormalities of chromosomes 1, 3 and 14 especially 14q32. The translocation t(11:14)(q13:q32) which may be specific for B cell malignancies is particularly common in plasma cell leukaemia (Philip, 1980). Abnormalities of 6q are associated with enhanced production of the osteoclast activating factor, Lymphotoxin (tumour necrosis factor (TNF)-β) (Durie et al, 1986). The breakpoints on the chromosomes show a good correlation with known fragile sites and oncogene locations (Chen et al, 1986). Chromosomal abnormalities at presentation are associated with a worse prognosis (Dewald et al, 1985), but whether they add information to other prognostic indicators has not been investigated. Hypodiploidy indicates a very poor prognosis (Chen et al, 1986). Cytogenetic studies may also help differentiate patients who have a pancytopenia because of an evolving therapy-associated leukaemia (who may have structural abnormalities/losses of chromosomes 5 and 7) from those with progressive marrow infiltration with plasma cells (Dewald et al, 1985). However, this will normally be clear from the morphology of a bone marrow aspirate and trephine biopsy.

(d) Cellular DNA content abnormalities. Flow cytometry using propidium iodide as a DNA stain can identify abnormalities of total cellular DNA content even in the absence of proliferating cells. Aneuploidy can be shown in 60-80% of myeloma cases and correlates with resistance to chemotherapy and a poor prognosis. Hypodiploidy and biclonal stem lines are associated with a particularly poor outlook (Barlogie et al, 1983 & 1985). Aneuploidy was found to be a more useful prognostic indicator than the Durie/Salmon stage by Bunn et al (1982). The measurement of cellular RNA content appears to correlate with cellular 'maturity' - i.e. low RNA content is associated with poorly-differentiated 'anaplastic' myeloma cells. A low RNA content is a good predictor of a poor response to chemotherapy, but DNA content abnormalities and measures of tumour load correlate better with survival (Barlogie & Alexanian, 1986).

1.3 THE TREATMENT OF MULTIPLE MYELOMA

Current therapy for MM is not curative for the vast majority of patients - treatment is aimed at controlling symptoms and prolonging survival if possible, usually by trying to achieve a 'plateau phase' i.e an unmaintained remission or partial remission. If the patient is asymptomatic and free of complications, treatment is usually delayed until obvious progression occurs as there is no evidence that early treatment is beneficial. Attention must be given to general management as well as chemotherapy, including the treatment of hypercalcaemia, hyperuricaemia, renal impairment, infections, bone pain, hyperviscosity and psychological problems, but these will not be dealt with further here (see Kyle, 1986).

1.3.1 Single alkylating agents and combination chemotherapy

Prior to the introduction of alkylating agents, patients with MM had a median survival of less than 12 months (e.g. Feinleib & MacMahon, 1960), although it is likely that only the more severe forms of the disease were recognized. The median survival using melphalan or cyclophosphamide as single agents, given either continuously in low dose or intermittently in higher dose, has remained constant over the last 20 years at approximately 24 months from initiation of therapy, and 30-36 months from diagnosis (reviewed in Love, 1986). The addition of conventional dose prednisolone (e.g. 40mg/m^2/day for 4 days) causes greater and more rapid reductions in serum paraprotein, possibly by increasing the rate of immunoglobulin catabolism, but survival is not prolonged. Combination chemotherapy, usually involving melphalan (M) or cyclophosphamide (C) together with drugs such as vincristine, prednisolone, adriamycin (A) and Bischloroethyl nitrosurea, BCNU, (B), appears to result in higher initial response rates, especially in patients with a high tumour load. However, whether this improves survival has been controversial until recently. Many studies have been too small to achieve significant results, but the

results of the MRC's Fifth Trial showed a significant survival benefit for those treated with a combination of ABCM over those treated with M alone (median survival 29 versus 24 months, $p<0.0004$; 31% versus 16% alive at 4 years, Maclennan et al, 1988). It will be noted that median survivals may not be the most appropriate parameter to compare as the divergence is most marked after the median survival has been reached (Figure 1.3). Surprisingly, the differences in survival between the ABCM and melphalan treated groups were actually greater in the good prognostic groups, i.e. low β_2M, normal haemoglobin, normal creatinine (ICM Maclennan, personal communication). The current MRC trial is examining whether the addition of Prednisolone to ABCM, which appears to improve response rates, will improve survival still further.

1.3.2 Plateau Phase

Approximately 60% of patients reach a stable plateau phase in which continuing chemotherapy over a 6 month period does not result in a further fall in paraprotein and in which the patient is stable haematologically and clinically (Medical Research Council Vth trial, ABCM arm). Further chemotherapy at this stage is unhelpful, and may in fact result in poorer survival because of an increased incidence of infection, refractory pancytopenia and acute leukaemia (Alexanian et al, 1975 and 1978; MRC working party on leukaemia in adults, 1985). Mandelli and co-workers (Mandelli et al, 1988) have shown, in a randomized study of 60 patients, a significant lengthening of plateau phase in patients treated with α-interferon 3×10^6 i.u./m^2 three times per week compared to those receiving no treatment in plateau phase. Larger trials (e.g. under the auspices of the MRC) are now under way to confirm this and to show whether this benefit also leads to lengthened survival.

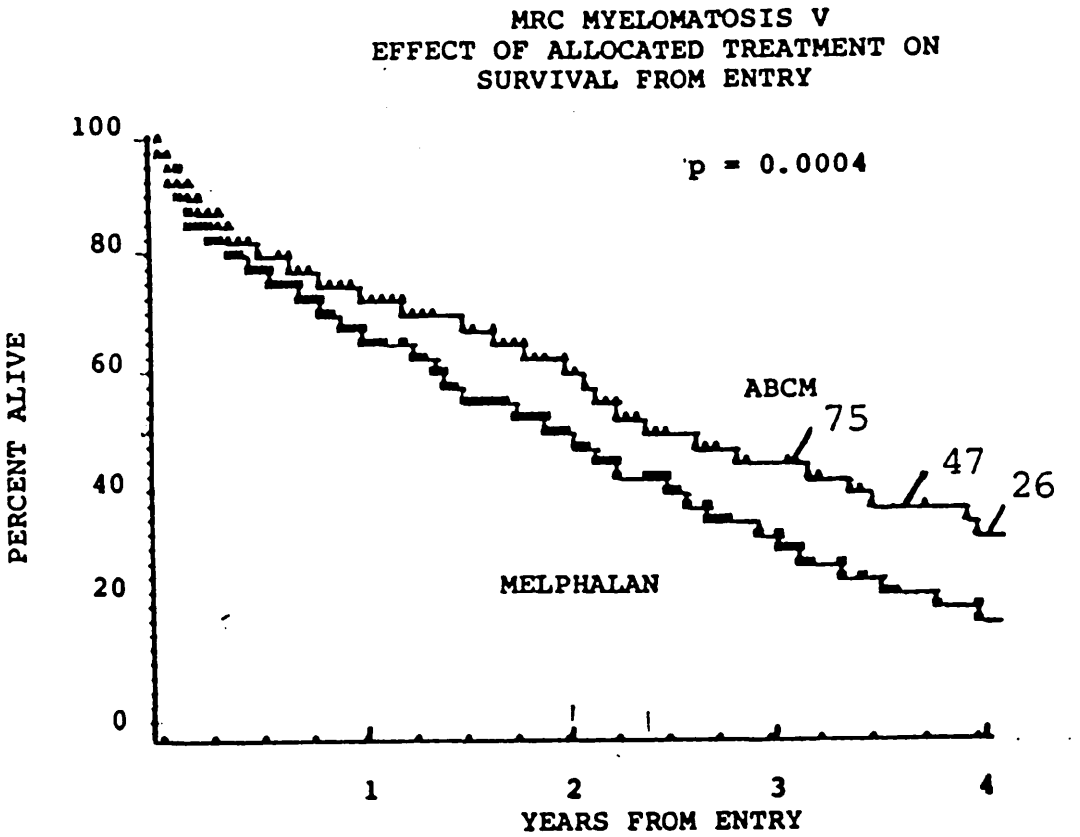


Figure 1.3 Actuarial survival curves for patients in the MRC Vth Myelomatosis trial at September 1987. This analysis is based on all 312 eligible patients allocated to melphalan and the 315 eligible patients allocated to ABCM. Numbers on the life tables indicate the number of patients alive in each treatment arm at the times from entry indicated (Maclennan et al, 1988).

1.3.3 Relapse

Most patients achieving a 50-75% reduction in paraprotein remain in remission for an average of 3 years (Durie & Salmon, 1982), but virtually all patients will relapse eventually, with <5% surviving 10 years (Kyle, 1983). Buzaid and Durie (1988) have recently reviewed the management of relapsed and initially

refractory myeloma. Their conclusions were summarised as follows:

'1. For truly resistant patients, i.e. those who do not respond to initial therapy, high dose or pulsed glucocorticosteroid is the best treatment with an expected response rate (≥50% reduction in paraprotein) of 40%;
2. For patients who relapse during therapy or relapse within 6 months of stopping the initial treatment, the VAD regime (vincristine, adriamycin, dexamethasone, Barlogie et al, 1984) is the most effective therapy resulting in a response rate of approximately 75%.
3. For patients who relapse more than 6 months after stopping therapy, reinitiation of the same first regimen leads to re-control in 60-70% cases. If no response is observed, or if there is response and then relapse, VAD chemotherapy can again be employed; and
4. Patients who fail second line salvage therapy should enter well designed clinical trials to evaluate new treatment modalities. If this is not feasible, interferon alpha-2a or 'systemic' radiotherapy are recommended in selected cases.'

While the responses to VAD are impressive, the side effects, especially infection, are severe (Sheehan et al, 1986), and the median duration of response is only 8-9 months (Monconduit et al, 1986). Although interferon initially appeared promising (Mellstedt et al, 1979), more recently response rates have been less than 25% in untreated patients and around 10% in relapsing patients (Quesada et al, 1984).

1.3.4 Newer approaches to treatment

While regimes such as ABCM show some improvement over previous therapies, the treatment is still palliative, and this approach is unsatisfactory particularly for younger patients. More aggressive forms of therapy with the aim of achieving cures have been explored.

(a) High dose melphalan (HDM). HDM treatment (140mg/m^2) has resulted in 'complete remission' (CR) in 11/41 (27%) of previously untreated patients, where CR is defined as a normal bone marrow morphologically and absence of the paraprotein (not stated whether this was in both blood and urine) (Selby et al, 1987). Partial remission was achieved in 21/41 (51%) patients. The median duration of remission was 19 months but only 1 patient remained in CR more than 2 years from treatment. An encouraging feature of the remissions was that normal polyclonal immunoglobulin levels were restored in a third of cases, which is rarely seen in 'plateau phase' of conventionally treated MM. However, relapsing/resistant patients responded less well to HDM, with all such patients relapsing in less than 1 year. There was a high early death rate mainly due to infection, but increasing experience with the procedure had reduced this. The MRC VIth trial is now comparing ABCM with HDM, with or without high dose methyl prednisolone, for the treatment of newly diagnosed patients with MM. From their early results Selby's team have not found any significant difference between HDM with or without high dose methyl prednisolone, or if the HDM is proceeded by several courses of 'VAMP' (vincristine, adriamycin, methyl prednisolone) (Selby et al, 1988). Barlogie et al (1986) report using HDM (80-100mg/m^2) in patients resistant to standard melphalan and VAD. Responses (>75% reduction in paraprotein) occurred in 6/16 patients, and although none achieved CR, 2 patients were alive at 13 and 20 months post treatment. Toxicity was again severe with a 25% procedural mortality.

(b) Bone marrow transplantation (BMT). A small number of syngeneic transplants have been performed for MM but they have not been very successful. Of 13 cases in the literature (Thomas, 1983; Fefer et al, 1982; Osserman et al, 1982; Kyle, 1986), only 7 were still alive at the time of reporting, and only 3 patients had not relapsed post BMT. However, most of these cases were transplanted when the disease had become refractory to normal chemotherapy. Gahrton et al (1987) reported 14 patients aged 29-46 who had undergone allogeneic marrow transplantation from HLA-identical siblings for MM. Ten of these patients were alive at 6 to 34 months post-transplantation, but only 5 were without any signs of disease. Amongst the 5 disease-free patients were 2 patients previously refractory to chemotherapy. The majority were conditioned with cyclophosphamide 120mg/kg and total body irradiation (9.5-12 Gray), and some received additional cytotoxic drugs (melphalan, BCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea (CCNU), vincristine or prednisolone). This shows that in some cases the disease can be eliminated with sufficient doses of chemo-radiotherapy, although longer follow-up is required to be sure that late relapses will not occur.

(c) Autologous bone marrow transplantation (ABMT). Because of the age of most patients with MM, and the lack of compatible donors for many young patients, massive chemoradiotherapy with ABMT could be a more widely applicable form of therapy than allogeneic BMT. Initial results using HDM with ABMT in refractory patients showed good tumour responses but the patients relapsed again very quickly (Barlogie et al 1986). Other studies are continuing, treating patients in plateau phase or in some form of remission (Selby et al, 1988). Two major problems appear to exist for this form of treatment. First, MM is difficult to eradicate, even with large doses of radiotherapy and currently available chemotherapy (as the allogeneic/syngeneic BMT results show). Secondly, MM cells and their progenitors theoretically contaminate the reinfused

marrow, even if they are below the level of morphological, or even immunological, detection. They may need to be removed before the marrow is reinfused into the patient. These two problems were the main starting points for the experimental work described in this thesis:

(1) to understand the nature of the myeloma cell and its progenitor(s), in order that more effective ways of eradicating the disease can be designed; and

(2) to study ways of selectively removing myeloma cells and their progenitors from bone marrow leaving sufficient normal haemopoietic stem cells to reconstitute haemopoiesis in the patient.

The second half of the introduction will survey ABMT in general (section 1.4) and then describe the immediate background to the studies undertaken (section 1.5).

1.4 AUTOLOGOUS BONE MARROW TRANSPLANTATION

The use of ABMT to support very high doses of chemo- and/or radiotherapy for the treatment of haematological malignancy was first reported by McGovern et al in 1959. It was more extensively studied from the mid-1970s onwards (e.g. Dicke et al, 1979), and has been widely practised in the 1980's (Goldstone, 1986). This technique has now been applied to the treatment of the acute leukaemias, chronic granulocytic leukaemia, non-Hodgkin's lymphoma, Hodgkin's disease and solid tumours, (Goldstone, 1986). Early reports of its use in multiple myeloma have now appeared (Barlogie et al, 1986). The rationale for using high dose chemotherapy, with or without total body irradiation, with autologous bone marrow transplantation (ABMT) in the treatment of various malignancies is based on three suppositions:

(1) that increasing the dose of the cytoreductive therapy, above that normally possible without ABMT, increases the tumour cell kill; i.e. that the dose-response curve continues to rise above conventional dose levels;
(2) that the main limitation on the dose used is bone marrow toxicity; i.e. that the doses are not limited by other toxicities, especially pulmonary, gastrointestinal, and cardiac; and
(3) that the reinfusion of autologous bone marrow, harvested in 'remission', but theoretically or demonstrably contaminated with 'minimal' numbers of residual malignant cells, does not matter significantly; alternatively, that these residual cells can be effectively and selectively removed ('purged') prior to the reinfusion of the marrow.

1.4.1 Cytoreductive schedules

Phase I and II studies of various cytoreductive regimes in the setting of allogeneic BMT have helped define the most effective and least toxic regimes of chemotherapy for a number of

malignancies. The most commonly used regimes for haematological malignancies are listed in Table 1.1:

Name	Drug Combination	Reference
Cy+TBI	Cyclophosphamide + Total Body Irradiation 10 Gray	Kaizer et al (1982)
TACC	Thioguanine 200mg/m² x8 (days) Cytosine Arabinoside 200mg/m² x8 Cyclophosphamide 45mg/kg x4 CCNU (Lomustine) 400mg/m²	Herve (1985)
HDM	Melphalan 140mg/m²	Maraninchi et al (1983)
UCH	Cyclophosphamide 1.5g/m² x3 BCNU (Carmustine) 300mg/m² Adriamycin 50 mg/m² Cytosine Arabinoside 100mg/m² x8 Thioguanine 100mg/m² x8	Gribben et al (1987)
BACT	BCNU (Carmustine) 200mg/m² Cytosine Arabinoside 100mg/m² x8 Cyclophosphamide 1600mg/m² x4 Thioguanine 100mg/m² x8	Appelbaum et al (1978) Gorin (1983)
BuCy	Busulphan 4mg/kg x4 Cyclophosphamide 50mg/kg x4	Santos & Colvin (1986)

Table 1.1 Cytoreductive schedules used in bone marrow transplantation.

It will be seen that most regimes have utilised alkylating agents and a variety of other drugs, with or without TBI. Serious toxicity to tissues other than bone marrow, e.g. liver or lung, often limits the maximum tolerated doses to two or three times what can be given without bone marrow rescue.

Whether these dose increases can overcome the innate or acquired resistance of a malignant clone to these drugs is a moot point. The best evidence for this comes from ABMT results in lymphoma. Long term disease-free survival has been reported in patients with advanced large cell lymphoma, Burkitt's lymphoma and Hodgkin's disease receiving high-dose therapy and ABMT in circumstances in which cures are very rare with conventional doses (Appelbaum et al, 1978; Phillips et al, 1984; Nadler et al, 1984 (updated by Takvorian et al, 1987); Jagannath et al., 1986; Philip et al, 1987). For details of some of these studies see 1.5.4.5 below. In myeloma, high doses of melphalan can achieve a CR in about 30% of patients (Selby et al, 1987), whereas this is rarely achieved with conventional doses (MRC working party, 1985).

However, in the acute leukaemias it is still controversial whether high-dose therapy and ABMT results in fewer relapses and more cures than conventional intensive therapy without ABMT. For example, in AML, 25-35% of adults are long-term survivors after intensive chemotherapy (Cassileth et al, 1984; Vogler et al, 1984; Mayer et al, 1985; Rees et al, 1986; Preijers et al, 1987), compared with 40% after high-dose chemotherapy and syngeneic transplantation (Fefer 1986; Butturini et al, 1987;) and 39% after ABMT (Gorin et al, 1990). A dose-response relationship in the chemotherapy of ALL is suggested by the study of Gaynon et al (1988a): whereas 49% of poor prognostic children treated with standard therapy survive 3 years, 78% may survive if given very intensive treatment. It is very difficult to compare chemotherapy and ABMT studies when their patient populations differ so much. The elucidation of the benefit of ABMT compared to chemotherapy alone in these various settings requires a randomised comparison, as is currently being carried out by the Medical Research Council, in its AML X trial.

1.4.2 The rationale for 'purging': the evidence for minimal residual disease

A large number of patients who enter complete remission from leukaemia after chemotherapy will subsequently relapse. Thus, although their blood counts and their bone marrow morphology return to normal, with fewer than 5% blasts in the marrow, 'minimal residual disease ' (MRD) is frequently present.

This disease can be detected immunologically, especially when the tumour cells express rare or aberrant combinations of surface/nuclear/cytoplasmic markers, or a monoclonal marker such as kappa/lambda restriction (Smith et al, 1984). In patients with Burkitt's and non-Burkitt's lymphoma, disease-specific cell-lines can sometimes be grown from morphologically normal remission samples (Philip et al, 1984; Benjamin et al, 1983; Joshi et al, 1987).

The demonstration of a clonally specific cytogenetic marker is proof of residual disease (Benjamin et al, 1983), but only has a sensitivity of 5% (Nowell, 1987). Specific gene rearrangements can be detected in the majority of B- and T-lineage malignancies with a sensitivity of approximately 1% (Arnold et al, 1983; Zehnbauer et al, 1986, Wright et al, 1987). These techniques may be refined to look for tumour-specific DNA, or sequences of chimaeric genes created by a tumour-specific translocation, or their RNA transcripts. These sequences can be detected with exquisite sensitivity ($1/10^6$ cells) by the polymerase chain reaction (PCR; Lee et al, 1987, 1988). Recently the PCR has been used to detect tumour-specific gene-rearrangement in the absence of a gross translocation (Hansen-Hagge et al, 1989).

The exact significance of the detection of MRD is uncertain. For example, reappearance of the Philadelphia chromosome after alloBMT for chronic myeloid leukaemia is usually, but not always, followed by frank leukaemic relapse (Arthur et al,

1988). Persistence of a del (16q) did not herald relapse in two children with ALL (Holt et al, 1989). Southern blot analysis was only able to predict one out of seven relapses amongst 95 surveillance samples from 45 patients, although the technique was able to correctly exclude relapse in a number of cases with suspicious bone marrow morphology (Gaynon, 1990). The emergence of unrelated rearrangements may complicate the picture (Bunin et al, 1986). The long term prognostic significance of the detection of minute amounts of leukaemic DNA by PCR has not yet been reported.

1.4.3 Methods available for selective elimination of malignant cells from bone marrow

Table 1.2 shows the methods that have been used to remove residual neoplastic cells from bone marrow (see over). The optimal method for purging marrow should have the following characteristics:

(1) Lack of toxicity to normal haemopoietic stem cells;
(2) Greater sensitivity of clonogenic neoplastic cells compared to haemopoietic stem cells;
(3) Ability to kill the neoplastic cells independent of cell cycle;
(4) Ease of use, and the ability to inactivate, eliminate or dilute to non-toxic levels the purging agent, before the infusion of the treated marrow;
(5) Absence of mutagenicity to the normal or any neoplastic cells not eliminated by the procedure.

Unfortunately it is difficult to be certain that any of these requirements have been fulfilled. It has been usual to measure colony forming units-granulocyte/macrophage (CFU-GM) before and after purging as a measure of toxicity to normal stem cells. These are relatively differentiated progenitor cells which may not reflect the number of pluripotential, self-renewing, cells. There is conflicting evidence as to whether

CFU-GM numbers do (Rowley *et al*, 1987), or do not (Kaizer *et al*, 1985) correlate with time to reappearance of peripheral blood neutrophils and platelets. There is also a lack of reliable clonogenic assays for leukaemic cells (see below 1.4.4), and it is not certain how these correlate with *in vivo* clonogenicity.

Immunological	
Antithymocyte globulin	Kaizer *et al*, 1980
MoAb and complement	Nadler *et al*, 1984
	Janossy *et al*, 1987
and toxins	Stong *et al*, 1985
and magnetic beads	Dicke *et al*, 1985
	Reading *et al*, 1987
Pharmacological	
4-Hydroperoxy-cyclophosphamide	Yeager *et al*, 1986
Asta-Z (Mafosfamide)	Gorin *et al*, 1986
Methyl Prednisolone	Santos & Colvin 1986
Physical Separation	
Albumin density gradients	Dicke *et al*, 1979
Elutriation	Figdor *et al*, 1983
Lectins	Rhodes *et al*, 1989
Floating beads	Hirn-Scavanec *et al*, 1988
Photodynamic	
Merocyanine 540	Sieber *et al*, 1984a,b, 1987
Sulphonated Aluminium-Phthalocyanine	Singer *et al*, 1988
Long term bone marrow culture	
	Chang *et al*, 1986
Hyperthermia	
	Da *et al*, 1989

Table 1.2 Methods for the selective elimination of malignant cells from bone marrow

Thus the tumour cell kill/ CFU-GM kill therapeutic ratio is, at best, only a guide to the selectivity of the process. In general where drugs have been used, non-cycle specific ones such as alkylating agents have been used for purging, and several washes are performed prior to marrow reinfusion, which appears to prevent systemic toxicity. There is no evidence thus far for mutagenicity for the procedures used. However it would be very difficult to prove that a new cytogenetic aberration was caused by the purging procedure *in vitro* rather than the conditioning regime *in vivo*, or the inherent instability of the leukaemic clone.

In studies reported to the European BMT registry, the majority of ABMTs for acute lymphoblastic leukaemia (ALL) have been performed using purged bone marrow, whereas most ABMTs for acute myeloid leukaemia (AML) have been performed using unpurged bone marrow (Gorin and Aegeter, 1987). This is because MoAb are available which are reactive with antigens expressed by ALL cells, and which do not appear to react with haemopoietic stem cells. Until recently it was thought that such antibodies were not available for AML cells and thus purging for AML has usually been performed using chemotherapeutic agents rather than monoclonal antibodies (Linch and Burnett, 1986). Recently, reports of the use of MoAb (e.g. CD14,15,33) for purging AML autografts have appeared (Ball *et al*, 1988).

1.4.3.1 Chemotherapy methods of purging

The active cyclophosphamide metabolites, 4-Hydroperoxycyclophosphamide and Asta-Z (Mafosamide), have been most widely used for AML ABMT. However, no attempt has been made to study the killing of AML clonogenic cells. Although an assay for AML-CFU has been described (Lowenberg *et al*, 1980), it has not been used in this setting, except in one preclinical study (Singer *et al*, 1988). In the study of Yeager *et al*. (1986), doses were based on phase I studies showing that marrow treatment with

100μg/ml 4HC did not prevent engraftment in humans, but would kill cells from an AML cell line in a rat model (Sharkis et al, 1980). However it was clear from Yeager's study that engraftment could take place even without detectable CFU-GM in the marrow inoculum. Correlation of haemopoietic recovery (speed and quality) with assays of more primitive progenitor cells would be interesting. However, in one recent report, recovery of cell counts correlated better with CFU-GM and BFU-E numbers, and even with total numbers infused per kilogram of body weight than with CFU-Mix (Ball et al, 1990). Clearly a better assay of the repopulating ability of the autograft is required: the assays of Nakahata and Ogawa (1982) and Gordon et al (1985) are potential candidates.

1.4.3.2 Immunological methods of purging

If an antibody is found that reacts with a large proportion of the neoplastic cells, then it may be used to remove these cells from the marrow autograft. While initial studies used polyclonal antibodies (Kaizer et al, 1980), all current studies now report using MoAb, usually of murine origin. Often combinations of MoAb are used, each antibody reacting with different antigens in order to try to cover all the malignant cells (Janossy et al, 1987; Ball et al, 1990). Although the phenotype of the leukaemic progenitor cell is not known, it seems logical to try to include in the 'cocktail' MoAb reactive with cells from earlier stages in the differentiation of that cell lineage, e.g. CD19 as well as CD10 for common ALL. The specificity of MoAb is very high, but it is still necessary to show, in vitro at least, that the antigen with which it reacts is not expressed on haemopoietic progenitors. Ultimately the proof that they do not so react is their safe use in a clinical setting.

In order to achieve lysis or removal of the cells reactive with the antibody, three main techniques have been used: complement-mediated lysis; conjugation of the MoAb to a toxin; or

conjugation to ferrous beads for magnetic removal. Unfortunately most murine MoAb do not bind human complement and have variable reactivity with rabbit complement: IgM MoAb and to a lesser extent IgG_{2a}, IgG_{2b} and IgG_3, being better than IgG_1. Another problem has been variability between lots of rabbit complement which therefore must be carefully screened for lytic activity before use. Bast et al (1985) recommended three 30-minute cycles of MoAb plus complement, although Le Bien et al (1985) found two 35-minute periods sufficient if using more than one MoAb. High cell concentrations may inhibit cell lysis (Bast et al, 1985), and it is therefore necessary to use large volumes of rabbit serum when treating a clinical marrow harvest sample which is both cumbersome and expensive. If the amount of complement in the mixture is too high, this can be toxic to normal stem cells. It is usually found that lysis in large volumes proves less effective than pilot studies in small test tubes. The best available technique using rabbit complement and rabbit complement can induce a 4-log reduction in leukaemic cells in 80% of cases (Janossy et al, 1987).

To circumvent some of the problems of murine MoAb and rabbit complement, one approach is to use rat MoAb which fix human complement such as Campath-1 (Hale et al, 1983). Another possibility is to 'humanize' the MoAb. By creating a human Fc fragment of the Ig molecule by genetic manipulation, the molecule may also fix human complement (Hale et al, 1988; Riechmann et al, 1988). Another approach has been the use of MoAb-toxin conjugates, especially the use of ricin A chain. When combined with its B chain this is an extremely toxic substance, but when deprived of its B chain, it cannot enter cells to inflict harm. When the A chain is coupled to an antibody it can fix to cells carrying the relevant antigen. It is then internalised, thus causing selective cell death, with up to 6-log reductions in cells in preclinical studies (Preijers et al., 1989a). This has now been successfully used in the clinical setting (Stong et al, 1985; Preijers et al,

1989b). Preijers found that cell killing was highly dependent on the density of the antigen on the cell surface.

1.4.4 Monitoring the elimination of residual neoplastic cells from bone marrow

(a) The removal of the malignant progenitor cells.

It is now widely accepted that malignant tumours comprise stem cells with proliferative and self-renewing capacity, which give rise to more differentiated progeny with limited or absent proliferative capacity only (Mendelsohn & Dethlefen, 1968; Steel, 1977; Goldie & Coldman, 1983; and see Drewinko et al, 1981 for evidence for this hypothesis in myeloma). Obviously any purging procedure must not only remove mature malignant cells but also the self-renewing malignant stem cells. The stem cell compartment is difficult to measure but is probably small: cells capable of forming colonies usually form ≤0.1% of cells (Touw et al, 1985), and even these may represent a more differentiated stage than the self-renewing stem cell (Buick et al, 1981). There is evidence for phenotypic differences between the majority of blasts present in AML and the leukaemic colony-forming cells (Griffin et al, 1983; Sabbath et al, 1985), and also for heterogeneity of CD10 (= common acute lymphoblastic leukaemia antigen, CALLA) expression on colony-forming cells in common ALL (Touw et al, 1985). Thus if purging is to be employed it is preferable to have a method for monitoring the removal of the clonogenic as well as the non-proliferating malignant cells. However most studies have used only sensitive immunological analysis before and after purging (Janossy et al, 1987). Those that have tried to assess purging efficacy using a clonogenic assay have used cell lines rather than fresh material from patients (Bast et al, 1985; Falkenberg et al, 1986), mainly because clonogenic stem cell assays on fresh malignant cells are still rather unreliable and difficult to perform.

Another possible method of monitoring the removal of residual disease is the detection of specific gene rearrangements. It is likely that the gene rearrangements present in mature malignant cells are also present in the progenitor cells, and specific abnormalities can be found in most cases of T- or B-cell lineage disease. However, the sensitivity of current methods is the detection of 0.5-5% residual cells (Zehnbauer et al, 1986; Arnold et al, 1983; MacIntyre et al, 1987). It is likely that use of the polymerase chain reaction will improve this considerably.

Alternatively, if the malignant cells are aneuploid, residual cells may be detected by flow cytometrical analysis of DNA ± RNA content. The sensitivity of this method is increased by examining separate fractions after centrifugal elutriation (Barlogie et al, 1980).

(b) Preservation of normal haemopoietic stem cells. The purging procedure must aim not only to remove all the malignant cells, but must also leave sufficient normal stem cells to allow full haemopoietic and immunological reconstitution of the patients. Unfortunately no assay for the postulated pluri-potential self-renewing stem cell exists for human marrow. Most investigators have relied on leaving 'enough' colony forming units granulocyte/macrophage (CFU-GM) or the more immature mixed colony forming units (CFU-Mix), i.e. containing granulocytic, erythroid, macrophage and megakaryocytic cells. However, the correlation between these assays and the time to engraftment in patients is poor (Douay et al, 1985), and in fact some patients have engrafted from marrow grafts with no residual CFU-GM measurable in vitro (Santos & Colvin, 1986). Better methods might be the ability of long term bone marrow cultures to generate CFU-GM over 4-6 weeks or possibly the blast cell colony assay (Gordon et al, 1985). However, clinical correlations of these assays with the speed or quality of engraftment in patients have not been published. Thus it is

not possible for anyone to claim that a particular purging procedure is more toxic to the neoplastic cells (+/or progenitors) than it is to normal haemopoietic stem cells. All that can be said is that the purging procedures have been undertaken and the patients survived (or not). With some (?most) conditioning regimens that are not truly ablative it is not even possible to say whether haemopoietic recovery resulted from the infused marrow or recovery of the cells left in the patient.

1.5.4 Clinical trials of 'purging' in acute leukaemia and lymphoma

The results of clinical trials in acute myeloid and lymphoblastic leukaemia (AML and ALL), in first and later remission (CR1 and CR≥2) will now be presented.

1.5.4.1 AML in CR1

The leukaemic cells may be more susceptible to cytoreduction in patients in CR1 than in relapse or CR≥2 when cells may be resistant to chemo- and/or radio-therapy. Thus the removal of contaminating leukaemic cells in the autograft should be more easily demonstrable than in later disease where the predominant problem is the elimination of resistant clonogenic cells within the patient.

The European Bone Marrow Transport Group (EBMTG) data was analysed by Gorin *et al* (1990), and showed that *ex vivo* purging had a favourable effect on disease-free survival (DFS) (34% for unpurged ABMT, 63% for purged), and the probability of relapse was also much lower in the purged (23%) than the unpurged group (55%). This was a retrospective study of a heterogeneous group of patients studied in a non-randomised fashion; different centres selected patients according to different criteria and each centre tended either to 'purge' *all* autografts or *none* of them. However the authors seek to exclude, as far as possible. such confounding factors in their statistical analysis. The survival advantage was even more marked in those whose marrow

was harvested within three months of achieving CR1, and this may be a more homogeneous group. It is claimed by opponents of 'purging' that equivalent results (58% DFS) can be obtained with 'unpurged' ABMT (Burnett et al, 1984). Gribben et al (1987) claim 69% DFS for the University College Hospital double unpurged autograft protocol. Dicke and Spinolo (1989) argue that a pre-BMT intensification can act as an 'in vivo purge' before an unpurged ABMT with similar results (56% DFS, Spinolo et al, 1988).

Thus purging may be helpful in ABMT in CR1, but it is possible that similar results can be obtained with adequate pre-ABMT chemotherapy.

1.5.4.2 AML in CR≥2

The best results for ABMT for AML in CR≥2 are those using the BAVC combination (BCNU, Amsacrine, etoposide and Cytarabine) and an unpurged autograft from Rome (Meloni et al, 1989). They claim a 67% DFS and a 55% inversion rate (i.e. post-ABMT remission longer than previous CR). Results of Busulphan-Cyclophosphamide conditioned ABMT with 4HC purged marrow show only 30% DFS (Yeager et al, 1987). Thus in CR≥2, the conditioning regime appears to be paramount and the purging is probably of secondary importance. There will probably be no randomised trial of purging in this setting until the most effective conditioning regime is established.

1.5.4.3 ALL in CR1

Modern intensive chemotherapy regimes can produce long-term survival rates of 40% in adult ALL (Hoelzer et al, 1988; Schauer et al, 1983), and 70% in childhood ALL (Clavell et al, 1986; Gaynon et al, 1988a,b; Gaynon, 1990). ABMT has only been applied in those patients considered to be at high risk of relapse - i.e. most adults, and children with high presenting WBC. Purging of bone marrow has often been used, usually with MoAb. This is because anti-lymphoblastic MoAb do not appear to

react with haemopoietic stem cells and thus it is argued the use of purging is unlikely to be detrimental and may be useful. UcKun et al (1987) have shown the in vitro clonogenic ALL cells in CR1 have the same or similar phenotype as the original leukaemic blasts.

Gorin et al (1986) reviewed the collective but heterogeneous experience of the EBMTG of 32 patients with ALL in CR1 treated by high-dose therapy and ABMT, and found a DFS of 56%, regardless of whether purging had been applied or not. Simonsson et al (1989) report a more homogeneously treated group of high risk patients (mostly adults) undergoing ABMT for ALL in CR1. Twenty-one patients had a DFS of 65% after a TBI (± cytotoxic drug) conditioning and a marrow autograft purged with either a CD10/CD19 combination (B-lineage malignancies) or CD7 MoAb (T-lineage), and rabbit serum as the source of complement. However median follow-up was only 16 months. These results are encouraging, but longer follow-up and larger numbers of patients will be required to confirm them.

Proctor et al (1988) compared the results of three therapies for adults with ALL in CR1, although the treatments were not allocated randomly. Maintenance therapy alone produced a 3-year DFS of 12% (overall survival 12%). An ABMT without purge produced comparable figures of 30% DFS (overall survival 65%), and alloBMT gave 30% DFS (overall survival 38%). They argue that if non-purged ABMT can produce results as good as alloBMT then purging would not have improved their ABMT results.

1.5.4.4 ALL in CR≥2

The results of ABMT in this group have been more discouraging. In the above-mentioned report by Simonsson et al, 32 patients who were treated in CR≥2 had a DFS of only 31% with a median follow-up of 18½ months. However 12 patients had inversions,

and the results of conventional treatment in these situations are very poor (see below).

Kersey et al (1987) in Minnesota performed MoAb-purged ABMT on 45 patients with 'high-risk' ALL, 87% of whom were in CR≥2 having had a relapse on therapy or within 6 months off therapy, and compared them with 46 similar patients treated by allogeneic BMT. This was not a randomised study and treatment selection depended on the availability of an allogeneic donor, the immunophenotype of the leukaemic cells, and the referral pattern to this tertiary centre. There was a significantly higher relapse rate in the patients treated by ABMT (79% versus 50%), but the DFS was not significantly different (20% versus 27%), with follow-up ranging from 1.4 to 5 years. These results compare well with the very poor outcome from chemotherapy in patients relapsing on or shortly after therapy: e.g. of those relapsing less than 18 months from diagnosis and on treatment there were no survivors in the study of Rivera et al (1986), and a similar poor result was seen by Chessels et al (1986). These results are compatible with a beneficial effect of purging, but it must be remembered that the chemotherapy trials included all relapsing patients, whereas ABMT reports only include those re-entering and remaining in remission long enough to receive the ABMT. Thus the worst prognosis patients are censored from the ABMT data. The very high relapse rates suggest the need for improved conditioning for both allo- and auto-BMT before a true assessment of the value of purging can be achieved.

It is even more difficult to assess the role of purging in lower risk or unselected groups of ALL patients in CR≥2. Thus in the EBMTG, Gorin reports 32-44% DFS for ALL in CR≥2 (Gorin et al, 1986). Sallan et al (1989) report 44 patients receiving MoAb-purged ABMT after TBI and chemotherapy with a DFS of 29% after 5 years follow-up. Plouvier reports a 60% DFS in this

situation, but his cases were all children with varying lengths of first CR (Plouvier et al, 1987).

1.5.4.5 Non-Hodgkin's Lymphoma (NHL)

Since Appelbaum et al (1978) reported the first long-term disease-free survival in children with relapsed lymphoma, the use of high dose chemotherapy and ABMT has been investigated in more than 1000 patients with lymphoma (International Cooperative Study group, 1986). Some studies are under way to identify whether some patients with poor prognostic indicators might benefit from this treatment in CR1. However, most ABMTs in NHL have been performed in patients who had either relapsed at least once after previous systemic chemotherapy, or had primary resistant disease. Such patients would be expected to have a very poor outlook with 'cure' rates of 0-10% (Cabanillas et al, 1982; Cabanillas et al, 1987; O'Donnell et al, 1987). A proportion of these patients can become long-term survivors after ABMT. The main factor influencing outcome after ABMT is the responsiveness of the disease to conventional dose (re-) induction chemotherapy. In a study of 100 patients, 36% of patients with sensitive relapses were long-term survivors, 14% of those with resistant relapses, and 0% of those with primary resistant disease (Philip et al, 1987). This was a multicentre retrospective analysis, where a number of different regimes had been used, but none included marrow purging before reinfusion. Seventy-six percent of post-ABMT episodes of disease progression were isolated and involved primarily the site(s) of initial lymphoma involvement, indicating that local disease control is the main limiting factor. Patients with overt marrow involvement were excluded from consideration and marrow relapses were not a major problem post-ABMT. The implication to the authors was that purging the bone marrow before re-infusion would not have improved the results.

The opposite view was taken by Takvorian et al (1987), who studied 49 patients with 'poor prognosis' NHL according to a

single protocol involving cyclophosphamide and TBI and reinfusion of bone marrow purged with B1 (a CD20 MoAb). Although all the patients had responsive disease they had poor prognostic features: multiple relapses, previous short disease-free interval, bone marrow involvement (33 patients, but ≤5% malignant cells on histology), multiple extra-nodal involvement, or conversion to a more aggressive histology at the time of BMT (19 patients). Seventeen had never had a CR, and 8 were being treated as primary consolidation therapy of CR1. Comparison with the study of Philip et al (1987) is made particularly difficult because 6/49 patients had low-grade disease according to the Working Formulation. Disease-free survival was 67% with a median follow-up of 11 months. If only high-grade cases were considered, the DFS was 47%. Involvement of the bone marrow, which is usually considered to be a bad prognostic factor, did not have an adverse effect on DFS, which argues that these good results were due in part to the marrow purging. Again the lack of a true control group makes interpretation difficult.

1.5.4.6 Conclusion

There are no published randomised studies on which to base an assessment of the efficacy of purging bone marrow. The evidence from uncontrolled studies suggests that ABMT may be a useful therapy in certain circumstances. There are indications that purging is of benefit in AML, and possibly ALL, in CR1, and also in NHL particularly if there is involvement of the bone marrow. Purging will continue to be used and investigated on the following bases:

(1) It has been safely carried out by immunological and chemotherapeutic means.
(2) There are indications that purging may be beneficial, and at least in ALL and lymphoma, where the procedure is highly specific, it is unlikely to be harmful.

(3) Whereas the effect of purging may be difficult to discern now, it may be much more important in the future if better conditioning regimes can be found.

(4) Clinical and preclinical studies of purging provide one means of studying the physiology and immunology of these malignant processes.

1.5 THE IMMEDIATE BACKGROUND TO THE STUDIES UNDERTAKEN

If ABMT is to be applied to the treatment of MM then a means of removing neoplastic progenitor cells from the marrow prior to its reinfusion might be required. A study of the physiology of the progenitor cell might also lead to the development of novel cytotoxic therapies that could be useful in treating the disease either in the context of ABMT or otherwise. To this end:

(1) the phenotypic spectrum of myeloma cells has been studied to look for antibodies that might be useful for purging marrow, and new monoclonal antibodies have been made using various sources of plasma cell antigens as immunogens, and

(2) the clonogenic fraction of myeloma has been studied using cellular DNA content analysis, and short- and long-term cell culture.

1.5.1 The Phenotype of MM cells.

The MM plasma cell represents the terminal phase of the B-cell differentiation pathway, which was summarised in Figures 1.1 and 1.2 (pages 4 and 8). It was noted that most of the antigens associated with peripheral B cells such as clusters CD 19, 20, 21, 22, 24, 37 are absent at the plasma cell stage. A few antibodies are reactive with plasma cells, in particular CD38 (OKT10 etc). Some reports also suggest reactivity with CD9 antibodies (San Miguel et al, 1986). A number of unclustered antibodies with anti-plasma cell reactivity have been reported (Table 1.2):

Antibody	Other Reactivity	Reference
PCA-1	Monocyte, Myeloid & T cell	Anderson et al (1983)
PCA-2	Monocyte, Myeloid & T cell	Anderson et al (1983)
PC-1	? None	Anderson et al (1984)
R1-3	B cells	Gonchoroff et al(1986)
9.3 (CD28)	Activated T cells	Kozbor et al (1987)
HAN PC-1	Thymocytes, ALL, AML	Mertens et al (1985)
MM4	Activated T cells	Tong et al (1987)
YM5/7,9,21	Follicular Dendritic cells	Delmastro-Galfré et al (1987)
8A	B cells, Monocytes, Granulocytes, AML	Tazzari et al (1987)
8F6	Mature B cells, AML	Tazzari et al (1987)
62B1	Hairy Cell Leukaemia	Tazzari et al (1987)

Table 1.2 Monoclonal antibodies reported to have anti-plasma cell activity.

However, most of these MoAb are not plasma cell specific, and have not been subjected to rigorous assessment at an international workshop. Our phenotyping studies in myeloma have aimed to use antibodies from defined clusters, but other antibodies (e.g. PCA-1, 8A) have been used when available, and new antibodies have been made.

The terminally-differentiated myeloma plasma cells may not be clonogenic or, if they are, may not be the only clonogenic cell. The cell phenotypes that have been claimed to be part of the neoplastic clone also include pre-B cells and B cells:

Pre-B cells. Kubagawa et al (1979) reported the presence of idiotype-specific cytoplasmic immunoglobulin (cId) in bone marrow pre-B cells from two patients with MM. However these results still await confirmation. More recently, Caligaris-Cappio et al (1985) have described common acute lymphoblastic leukaemia antigen (CALLA = CD10)$^+$, HLA-DR$^+$, terminal deoxy-

nucleotidyl transferase $(TdT)^-$, cIg^- and sIg^- cells in myeloma bone marrow that when stimulated with phorbol ester (TPA), transform into plasma cells that synthesize the same light and heavy chains as the patient's paraprotein. While this is evidence for these cells having a role in the neoplastic process, they are not typical pre-B cells in that they lack $c\mu$ chains and are $CD19^-$, TdT^-. They suggest that these $CD10^+$ cells may lead directly to myeloma plasma cells in the bone marrow without the need to pass through secondary lymphoid organs. However, they could also be described as resembling centroblasts which are $CD10^+$, $CD38^+$ with a low expression of other B cell markers such as CD23, 24, 39, sIg and cIg (Ling et al, 1987). Centroblasts may give rise to plasma cells after passing through a small cell stage and being reactivated by antigen (Maclennan & Gray, 1986). $CD10^+$ cells have also been detected in the blood and/or bone marrow of patients with MM by Ruiz-Argüelles et al (1984), Durie and Grogan (1985) and Epstein et al (1988). Recently, Grogan et al (1987) have been able to derive 'pre-B' like cell-lines from the bone marrow of MM patients. These lines were CALLA $(CD10)^+$, TdT^+, $CD19^+$, $CD20^+$ but also expressed some 'plasma cell' antigens such as PCA-1. They expressed monoclonal cytoplasmic μ chains, but the final proof of idiotype (Id) identity with the myeloma paraprotein was lacking. We have studied CD10 expression in MM bone marrow and also found small numbers (usually <5%) of $CD10^+$ cells in about half the cases (see chapter 4).

B cells. The existence of B lymphocytes bearing sId determinants was reported some time ago (Mellstedt et al, 1974). Kubagawa et al (1979) have also reported cId^+, sIg^+ cells in bone marrow from patients with MM. However Pilarski et al (1984) feel that the high concentrations of Id^+ Ig in which these cells are bathed, make physiological pre-B cells with adsorbed Id^+ Ig the likely explanation for these findings. Walker et al (1988) have shown that peripheral blood mononuclear cells from some patients with MM do include some cells

of the neoplastic clone, since when stimulated by polyclonal B cell mitogens 4 of 24 specimens showed an excess of cells containing cytoplasmic light chain of the same type as the patient's paraprotein. However, it is not clear what cells were stimulated in their experiments; they were not necessarily B lymphocytes but could have been circulating plasma cells or plasmacytoid cells.

In conclusion, the mature myeloma plasma cell expresses few well-documented antigens; there is a lack of plasma-cell specific antibodies; and it remains uncertain how far back along the B cell differentiation pathway the neoplastic clone extends.

1.5.2 The Clonogenic Fraction in Myeloma

(a) The DNA-Synthesizing fraction. The fraction of human myeloma cells synthesizing DNA ('the growth fraction') has been measured in two ways: in vitro and in vivo. The plasma cell labelling index (LI) is the fraction of cells incorporating ^{3}H-thymidine in short term culture (1 hour), as demonstrated by autoradiography (Durie & Salmon, 1975). In MGUS and smouldering MM the LI is $\leq$0.1% (Greipp & Kyle, 1983; Kyle & Greipp, 1980). In MM, it is usually $\geq$ 0.4% with a mean of 1%, and occasionally up to 20% (Greipp & Kyle, 1983; Durie et al, 1983; Karp et al, 1984). The LI is an important biological prognostic indicator (Durie et al, 1980), but whether all cells that synthesize DNA actually complete their cell cycle and divide has not been determined. Some cells evidently undergo mitosis without proceeding to cell division, giving rise to multinucleate plasma cells. However there is a rough correlation between LI and the ability of a sample of MM marrow to form colonies in soft agar culture (Durie et al, 1983; Karp et al, 1984). The growth fraction has also been measured in vivo, by measuring the plateau number of plasma cells labelled in patients' bone marrows during a continuous intravenous infusion of ^{3}H-thymidine over 8-10 days (Drewinko et al, 1981). The

technical and ethical difficulties of this experiment were severe, including alternate day bone marrow aspirations, and very long (4-6 month) exposure times for the autoradiography because of the low concentrations of ^{3}H-thymidine that could be used *in vivo*. Plateau labelling was achieved after about 8 days and was <4% in almost all untreated or initially unresponsive MM patients. Relapsing patients had values ranging from 14% to 83%.

(b) *The proliferating ($Ki67^{+}$) fraction*

The MoAb Ki67 reacts with the nuclei of proliferating cells (Gerdes *et al*, 1983). Lokhorst *et al* (1987) have used it to study the morphology and phenotype of proliferating cells in MM. They found that Id^{+} $Ki67^{+}$ cells represented <5% of cells in MM BM. The majority of these cells were not mature PC but were 'spotted cells' with a spot of Ig in the rough endoplasmic reticulum. The immunophenotype of this cell was negative with PC markers (PCA-1, PC-1, HAN PC-1) and with most B cell antigens including HLA-DR, sIg, CD38 and CD24, but positive with HB4 and HB6, two unclustered B-cell associated MoAb.

(c) *Short-term cell culture (clonogenic assays)*. The fraction of cells able to form colonies in suspension culture (*the cloning efficiency*) gives a measure of the number of cells with proliferative capacity. Large colonies are thought to arise from cells with extensive proliferative capacity, small colonies from cells with limited proliferative capacity. A number of different methods for the short term culture and 'clonogenic assay' of myeloma cells have been reported, and a few have attempted to harvest the myeloma colonies and replate the cells to test the 'self-renewal' capacity of the progenitor cells. In general, the cloning efficiencies have been 10 to 100 times less than the LI.

Hamburger and Salmon (1977) used a double layer agar technique with feeder layers containing either human group O erythrocytes

or medium conditioned by the adherent spleen cells of mineral oil-primed BALB/c mice. Colonies were counted after 18-21 days, and were observed in 75% of cases. Cloning efficiency was very low (0.001-0.1%) but limited self-renewal was demonstrated by replating experiments. The linear relationship between number of cells plated and colonies formed was not tested below 10^5cells/ml, and thus stem cell 'killing' by drugs *in vitro* (Salmon *et al*, 1978) is difficult to interpret. There is no proof that each colony originated from a single cell, and it is possible that at least some of the 'colonies' seen represent homotypic adhesion, i.e. clumping, or even clumps present in the original 'single-cell' suspension. Although studies of the resistance to irradiation of myeloma stem cells have not been performed using this type of assay, Selby *et al* (1983) point out inconsistencies when this assay is used with other neoplastic cell types. Normal and neoplastic human cells have radiation-dose survival curves with very similar exponential components (Hall, 1978), but this assay shows tumours having a remarkable degree of radioresistance which may be artefactual. Selby *et al* also point out the 'untenable observation' that higher doses of cytotoxic drugs sometimes lead to *increased* survival of clonogenic cells, again suggesting the artefactual measurement of clumps as colonies. However this assay has been adopted by a number of other investigators. The colony-forming cell has been described as near to the plasma cell stage by phenotypic examination of cells within colonies (Bast *et al*, 1982). They found that in one case of biclonal gammopathy, single colonies contained either IgG- or IgA-positive cells, which argues against all colonies being merely cellular clumps. The assay has been used to test the effects of chemotherapeutic agents (including α-interferon) on myeloma progenitor cells *in vitro* (Salmon *et al*, 1978; Brenning, 1986), and to correlate *in vitro* and *in vivo* drug sensitivities (Durie *et al*, 1983).

Izaguirre et al (1980), from the Ontario Cancer Institute, reported a method which used a Phytohaemagglutinin (PHA)-T cell conditioned medium as a stimulator of colony growth by various normal and malignant B cells. The viscous support used was methylcellulose rather than agar and most B cell colonies required the addition of irradiated T-cells to obtain colony formation, although myeloma cells did not. Colonies, i.e. collections of more than 20 cells, were formed in only 5 days with a plating efficiency of 0.25-1%, and surprisingly no contaminating granulocyte-macrophage growth occurred. Shimuzu et al (1982) used this system to investigate the proliferative state of myeloma colony-forming units (My-CFU), and found that high specific activity ^{3}H-thymidine killed 21-45% of the My-CFU. They also showed that the radiosensitivity of My-CFU in this assay agreed with the known radiosensitivity of myeloma colony forming cells (Hamburger & Salmon 1985).

Takahashi et al (1985), also working at Ontario Cancer Institute, describe a similar technique using the same conditions used for multi-lineage colony (CFU-Mix) growth. They used PHA-leucocyte conditioned medium and 30% human plasma from a patient with aplastic anaemia, which produced growth of colonies of myeloma cells in only 14 out of 61 cases of myeloma. However, it also supported growth of CFU-Mix; Burst-forming units - erythroid; granulocyte-macrophage colonies; and megakaryocyte colonies. A high degree of expertise was evidently required to distinguish these different colonies and count them. However, they were able to serially replate the myeloma colonies and establish long-term cell-lines in several patients.

Ludwig et al (1984) have described a plasma clot culture system for growing myeloma stem cells. Cells were suspended in autologous citrated plasma supplemented with 2-mercaptoethanol, insulin, nucleotides and amino acids, and then the plasma was clotted by the addition of calcium chloride. Colonies of ≥32

cells were scored between days 6-8. Plating efficiency was a median of 0.34%, and significant myeloma colony growth occurred in >90% of cases. Interestingly the cells in the colonies were mostly of lymphoid, lymphoblastoid or lymphoplasmacytoid morphology rather than the mature plasma cells seen with the other culture systems. Linearity of plating efficiency was demonstrated down to 10^4cells/ml. This technique appears promising, but the work has not yet been confirmed by others.

In our studies of myeloma culture, we started by adapting the method of Izaguirre et al (1980), as this appeared the simplest method, and also had a high success rate and plating efficiency. The use of methylcellulose also makes relatively easy the harvesting of individual or pooled colonies for further study. However, as described in chapter 5, the method could not be validated, as cellular clumping occurred and in some cases only CFU-GM growth could be obtained. A reliable myeloma culture method must demonstrate a clear increase in cell numbers to be convincing, and many of the published methods may be measuring cell clumping, at least in part, and therefore may not be true clonogenic assays.

Recently a new method has been described for the clonogenic assay of myeloma progenitors using a double layer agar technique with an HL60 feeder layer (Millar et al, 1988). However, they were unable to obtain growth in 0.3% agar, but only in 0.23%. Phenotypic and morphological analysis of the cultured cells was carried out, but apparently only on cells washed from concurrent liquid-over-agar cultures. This assay requires independent verification before it can be widely adopted for assay of myeloma progenitors.

(d) Long-term culture. The difficulty in establishing short-term colony growth in myeloma is mirrored by the paucity of myeloma cell lines available (Matsuoka et al, 1967; Nilsson et al, 1970; Jobin et al, 1974; Burk et al, 1978; Karpas et al,

1982; Durie et al, 1985; Jenberg et al, 1987; Lohmeyer et al, 1988). Well-differentiated (i.e. true plasma cell) lines would be useful for the study of the biology of the disease and possibly the terminal stages of normal B cell differentiation. They might also serve as potential fusion partners for the production of human monoclonal antibodies. If lines could be established from myeloma cells, it is likely that they would represent the predominant clonogenic cell and their phenotype might be useful in designing combinations of antibodies for the purging of MM bone marrow prior to ABMT. They could also be used - as we have - for making new anti-plasma cell monoclonal antibodies (Delmastro-Galfré et al, 1987). However, just as the most reliable growth of myeloma colonies comes from patients with 'acute phase', relapsing disease (Takahashi et al, 1985), so also cell lines have usually been obtained from patients with aggressive neoplasms. Our cell line, described in chapter 8, came from a patient who died within one month of presentation. The biology and phenotype of such lines may not be representative of the clonogenic cells of the more usual slowly-progressive cases of MM.

CHAPTER 2

MATERIALS, PATIENTS AND METHODS

2.1. MATERIALS

2.1.1. Media and supplements

(a) For myeloma culture

Human cell lines and in vitro cultures of bone marrow were suspended in a 1:1 mixture of Iscove's modified Dulbecco's medium and Ham's nutrient mixture F-12 both with L-Glutamine (Gibco Europe Ltd, Paisley, UK) and added antibiotics (Penicillin 100units/ml and Streptomycin 0.1mg/ml, Dista products Ltd, Basingstoke, UK). In the rest of the thesis, 'culture medium' or 'Isc/Ham' refers to this combination of media.

Fetal calf serum (FCS) was obtained from various sources (Gibco, Flow Laboratories). The FCS selected was tested for its growth-promoting potential using a limiting dilution growth assay with a mouse plasmacytoma. After testing it was used at concentrations varying from 15-30% by volume.

2-Mercaptoethanol (Sigma Chemical company Ltd, Poole, UK). A stock solution of 10^{-3} molar was made up and kept at -20°C in foil-wrapped bijou (5ml) plastic bottles.

Methylcellulose, 1500 centipoise (Sigma) was made up as a 2% (w/v) mixture by weighing 2.0g into a sterile glass bottle containing a magnetic stirrer and autoclaving at 120°C for 20 minutes. Then 100ml of sterile Isc/Ham was added and stirred in the cold room for approximately 3 days. Aliquots of 20 ml were stored at -20°C.

The following tissue culture plates were used :

(i) 96-well microtitre plates (Northumbrian Biologicals Ltd, Cramlington, UK), and

(ii) 24-well Linbro plates (Flow Laboratories, Rickmansworth, UK).

(b) For granulocyte-macrophage colony culture

The medium used was McCoy's 5A tissue culture medium, Iwakata and Grace modification (Neumann & McCoy, 1958; Iwakata & Grace, 1964) from Flow Laboratories, with the following additives per 100ml of medium (Table 2.1):

Additive	Stock solution	Kept(°C)	Vol added
L-Asparagine	1%	-20°C	0.2ml
L-Glutamine	29.4g/l	-20°C	0.75ml
MEM essential amino acids(as supplied)		4°C	1.0ml
MEM non-essential amino-acids (-/-)		4°C	0.5ml
MEM vitamins (as supplied)		-20°C	0.5ml
Penicillin	125mg/ml	-20°C	0.05ml
Streptomycin	0.2g/ml	-20°C	0.05ml
L-Serine	21mg/ml	-20°C	0.05ml
$NaHCO_3$	7.5%	+20°C	0.75ml
Sodium Pyruvate	100mM	-20°C	1.25ml
Fetal Calf Serum		-20°C	15.76ml

Table 2.1 . Additives for granulocyte-macrophage culture. MEM = Minimal Essential Medium. All products from Flow Laboratories, except L-Serine (Sigma), and antibiotics (Dista Products).

The pH was always between 7.0-7.3, and because of its instability in solution, fresh glutamine was added every 2 weeks. Additives were sterilized by filtering through a 0.2μm micropore filter (Flowpore D, Flow Laboratories).

Agar stock solution was made up at 5% (w/v, i.e. 5g/100ml distilled water) for feeder layers and 3% for overlayers. These concentrations were diluted 1-in-10 in medium for final use.

Tissue culture plates used were 35mm in diameter (Nunc, Rosskilde, Denmark; via Appleton Woods, Birmingham, UK).

2.1.2. Separation of mononuclear cells from bone marrow samples

Bone marrow samples were collected into preservative-free Heparin (Monoparin, CP Pharmaceuticals, Wrexham, UK) 500units in 2ml Isc/Ham medium.

Dextran (Lomodex 70, Fisons PLC, Loughborough, UK): 0.5ml of a 3.6% solution was added to samples of blood or marrow to accelerate red cell sedimentation.

Sterile Ficoll-Paque (Pharmacia Ltd, Milton Keynes, UK), density 1.077g/ml, was used for separation of a mononuclear cell fraction. Occasionally 70% Percoll (Pharmacia), density 1.085g/ml was used instead (see section 2.3.1(d)).

2.1.3 Sheep red blood cells

Sheep blood was provided from the Birmingham university farm mixed with an equal volume of Alsever's solution. 2 litres of Alsever's was made up as follows:

> D-Glucose (BDH Chemicals Ltd, Poole, UK) 41g, NaCl 8.4g, Trisodium citrate (BDH) 16g, citric acid 1.1g, made up to 2L with distilled water. Penicillin and Streptomycin were also added.

Sheep red blood cells (SRBC) were treated with a 2% solution of 5.2 Aminoethylisothiouronium Bromide Hydrobromide (AET) (Sigma) in distilled water, adjusted to pH 8.0 with 5N NaOH (Saxon et al, 1976). Nystatin (Mycostatin, E.R. Squibb Ltd, Hounslow, UK) 50,000units in 10ml of cells, was added to the prepared cells to prevent fungal contamination.

2.1.4. Cell activators/growth factors

Phytohaemagglutinin (M form) (PHA), Gibco, made up in Isc/Ham medium.

12.0 tetradecanoylphorbol-13-acetate (TPA) (Sigma).

Calcium ionophore: Ionomycin from streptomyces (Cambridge Biosciences Ltd, Cambridge, UK).

Staphylococcus Aureus Cowan I (SAC) (Cambridge Biosciences Ltd) diluted to 1/200,000 for use.

Purified interleukin 1 (IL1) (Genzyme Corporation, Boston, USA; via Koch-Light Ltd, Haverhill, UK).

Recombinant interleukin 2 (rIL2) (Genzyme Corporation).

Hybridoma growth factor (contains B cell stimulatory Factor 2 = BSF-2 = Interleukin 6), from Ewing's sarcoma cell line supernatant (ESG) (Northumbrian Biologicals Ltd).

Purified BSF-2, gift from A.Billiau, Leuven, Belgium.

2.1.5. Buffers

Phosphate buffered saline (PBS), pH 7.4 :

Solution A	23.4g/L	NaH_2PO_4	180ml
Solution B	21.3g/L	Na_2HPO_4	820ml
+	0.9g/L	NaCl	1000ml
+	Distilled	H_2O	100ml

sterilized, if necessary, by autoclaving at 120°C for 20 minutes.

Tris-buffered ammonium chloride was used for red cell lysis: 9 volumes NH_4Cl (8.3g/L in H_2O) + 1 volume Tris Base (20.59g/L in H_2O), adjusted to pH 7.65 with 1M HCl, and filter sterilized.

2.1.6. Autoradiography

Fixative: methanol (BDH Chemicals Ltd).

Tritiated(^3H-)thymidine (Amersham International Ltd, Amersham, UK) 2.0 Ci/mmol.

Ilford K2 emulsion (Ilford Ltd, London, UK).

Kodak D19 developer (Kodak Ltd, Hemel Hempstead, UK).

Sodium thiosulphate (Sigma).

2.1.7. Cell fusion

Mouse plasmacytoma cell line X63-Ag8.653.

Polyethylene glycol (PEG) (Sigma) 50% (v/v). 10g PEG was autoclaved in a glass bottle and allowed to cool slightly. Then 10ml sterile alkaline Dulbecco's modified Eagle's medium (Flow Laboratories) was added and thoroughly mixed.

2.1.8. Immunophenotyping (immunofluorescence)

Hepes (N-2-Hydroxyethyl piperazine-N'-2 ethanesulfonic acid)-buffered RPMI-1640 medium (Gibco), pH=7.3 (Gibco) with 2% FCS (Hepes/2%) was used for washing live cells in immunophenotyping tests.

Mouse monoclonal antibodies: ascitic fluid from various sources (as noted in the various chapters), and purified antibodies from Becton Dickinson (U.K.) Ltd.

Rhodamine (TRITC)- or fluorescein (FITC)-conjugated sheep anti-mouse immunoglobulin prepared by Dr.G.D.Johnson, department of Immunology, Birmingham.

Fixative for cytospin preparations: acetone (BDH chemicals Ltd).

Propidium iodide (Sigma) was used as a nuclear counterstain at a concentration of 1μg/ml.

For double-immunofluorescence experiments the nuclear counter-stain, Hoechst dye 33342 (Sigma) was used, with a stock solution of 1mg/ml.

'Anti-fade' reagent: 'DABCO' (Aldrich Chemical Co. Ltd, Gillingham, Dorset, UK), 2.5g Diazobicyclo-octane in 90% glycerol, 10% PBS pH 8.5.

Mountant: 20g polyvinyl alcohol type II (Sigma) was added to 100ml of PBS with 0.1% sodium azide(NaN_3) (Sigma) and stirred overnight at 4°C. 50ml glycerol was then added, stirred for 16-24 hours and centrifuged at 20,000g for 15 minutes. 2.5ml DABCO was added and the pH adjusted to 8.6, and the mixture then aliquoted and stored frozen until required (Johnson et al, 1982).

2.1.9. Immune-rosetting

For coating sheep red blood cells (SRBC) a diethylaminoethyl cellulose (DEAE)-purified preparation of Sheep anti-mouse Ig (G.A.M.) (The Binding Site Ltd, Birmingham, UK) was used.

Acridine orange (Sigma) was used as a nuclear counterstain: working solution 18.75μg/ml.

Multispot polytetrafluoroethylene (PTFE) - coated slides (CA Hendley Ltd, Essex).

2.1.10 Miscellaneous

Fixative for DNA analysis on the fluorescence-activated cell sorter: 0.07% paraformaldehyde, 0.1% Triton in PBS.

2.2 PATIENTS

Between October 1985 and September 1987 bone marrow samples were collected from patients with known or suspected myeloma, treated or untreated. Cases of known myeloma would normally have received treatment and be having follow-up bone marrow examinations, in plateau phase or at the time of relapse. Cases of 'suspected myeloma' were usually being investigated because a paraprotein had been found, with or without other features of myeloma. If the patient had only a paraprotein with no other features (e.g. suppression of other immunoglobulins, bone lesions, hypercalcaemia, renal failure, cytopenias) then the bone marrow virtually never revealed myelomatosis. In all, approximately 160 bone marrows were performed, of which 79 came from 63 patients with myeloma (either previously diagnosed, or diagnosed by the sample being taken). The criteria for the diagnosis of myeloma were those of the Medical Research Council trials (MRC Working Party, 1985), which were detailed in the introduction (section 1.2.2).

The bone marrows were not all subjected to the same investigations, and each individual chapter will detail the characteristics of the patients whose bone marrows underwent the investigations outlined in that chapter. Overall the patients were as detailed in Table 2.2:

Patients	63	(79 samples)
Paraprotein Type IgG	34	
IgA	19	
Light Chain	8	(2K, 6L)
Unknown	2	
Plasma cells in marrow	2-100%	(treated)*
	14-100%	(untreated)**

Table 2.2 Details of patients studied.

* Lower % in patients on 'plateau', Higher % in relapse
** If <20%, patients had lytic lesions + paraprotein as diagnostic criteria of myeloma.

2.3 METHODS

2.3.1. Cells: collection and preparation

(a) Collection

Between 0.5 and 3 ml of bone marrow was aspirated in the normal way and the majority was placed in a sterile universal container to which 2ml of culture medium and 500 units of preservative-free heparin had been added. A small amount of marrow was spread on glass slides and stained with Leishman's stain for morphological examination and counting the proportion of plasma cells. The sample was transported within 1½ hours, usually within ½ hour, to the laboratory for processing.

(b) Mononuclear cell preparation

An attempt was made to break up any large fatty particles contained in the marrow, and/or release cells from them by serially passing the sample through 21 gauge, 23 gauge, and then, sometimes, 25 gauge needles. The marrow was diluted to a total volume of 15ml with culture medium and layered onto the top of 6ml Ficoll-Paque in the bottom of a sterile universal container. After centrifugation at 1800rpm (=400g at the interface), the interface cells were collected and washed twice in culture medium containing 2% FCS. Often particles from the marrow were still present in this 'mononuclear' cell preparation, and if large numbers were present they were partially removed by allowing them to sediment out at unit gravity. The elimination of polymorphonuclear myeloid cells was not always complete, but a second centrifugation through Ficoll-Paque often resulted in substantial losses of plasma cells, which are very sensitive to any form of manipulation. Therefore only one centrifugation was carried out.

(c) Cell counting and viability

A diluent of 0.34ml 2% acetic acid and 0.04ml 1% trypan blue was prepared and 0.02ml of cells to be counted was added (i.e. 1-in-20 dilution). The number of cells was counted using an

haemocytometer, and those excluding trypan blue were considered viable.

(d) T cell depletion and/or collection

Treatment of SRBC with AET. 1ml of sheep blood mixed with 1ml of Alsever's was kept for less than 7 days before use. The cells were first washed 5 times in PBS and then incubated with 10ml of 2% AET for 15 minutes at 37°C, mixing well. This was followed by 5 further washes in PBS. The cells were resuspended in 10ml of Hepes/2% containing 50,000u of nystatin. The cells were usable for up to 3 months if kept at 4°C.

T-cell removal. AET-treated SRBC were diluted 1-in-5 in culture medium and added to an equal volume of mononuclear cells at a concentration of 4-8x10^6 cells/ml, and the mixture was centrifuged at 150g for 5 minutes. After incubation at 4°C for 60 minutes, the pellet was gently resuspended and the suspension centrifuged over Ficoll-Paque at 400g for 30 minutes. No T cells were detectable in the interface cells by immune-rosetting, but there were large non-specific cell losses and the white cells in the pellet contained large numbers of non-rosetted (i.e. non-T) cells.

T-cell collection. In those cases where a purer T-cell population was required, the suspension was centrifuged over 70% Percoll (density = 1.085g/ml). This resulted in a higher proportion of cells in the pellet being T-cells (usually > 50%), but sometimes there was a less complete T-cell depletion of the interface (approximately 1-2% detectable T cells). To remove the SRBC from the T cells, the pellet was resuspended in NH_4Cl-Tris buffer, and the cells spun down at 150g for 5 minutes. This was repeated 2 or 3 times, as necessary, to remove all the SRBC.

(e) Adherent cell removal/collection

To separate adherent cells (predominantly monocyte/macrophages) from the mononuclear cell fractions, cells were incubated in 25cm² plastic tissue culture flasks at approximately $4x10^6$/ml in culture medium with 20% FCS at 37°C. The incubation period varied from 1 hour to overnight, after which the non-adherent cell population was removed by aspiration of the supernatant and gentle washing of the culture surface. An adherent cell population could be collected by vigorous washing down and scraping of the flask surface.

(f) Irradiation of cells

Cells were irradiated using a ^{60}Cobalt source in the Birmingham university radiation physics department, at a dose rate of approximately 9 centiGray/second.

(g) Cytospin preparation

Initial attempts at spinning cells onto slides produced highly variable results, sometimes resulting in good quality morphology, but sometimes resulting in too few or too many cells on the slide, or cells of very poor morphology. The best results were obtained as follows: (a) a fresh and viable cell preparation at $4x10^6$ cells/ml in medium with 2% FCS; (b) methanol-washed slides; (c) clean cytospin chambers; (d) 50µl of cells, followed by 50µl of medium without cells into each cytospin chamber; (e) without delay, spinning at 300rpm for 3 minutes on a Shandon Cytospin II; (f) after allowing the slide to dry thoroughly, fixing - e.g. in acetone for immunofluorescence; (g) slides not being used for immunofluorescence immediately were stored in air-tight plastic bags at -30°C; (h) before testing, the bags were allowed to reach room temperature before being opened.

(h) Freezing cells

Approximately $10-100x10^6$ cells were centrifuged into a 2ml Cryotube (Nunc) and the pellet was resuspended in 0.25ml ice-

cold FCS with 4% Dimethylsulphoxide (DMSO, Sigma). The tube was transferred to a glycerol bath at -40°C for 45 minutes, and then into a liquid nitrogen freezer. To recover the cells: after removal from liquid nitrogen the cells were placed in a 56°C water bath until just thawed, before being diluted in 10-20ml of culture medium. They were then spun down, resuspended in culture medium and the cells counted and viability assessed.

2.3.2. Immunological phenotyping methods.

(a) Surface antigen detection: indirect immune-rosetting

Because very little notice was given of a bone marrow sample being available, it was not possible to use the FACS for routine work because it had to be booked one week in advance. It was decided therefore to use indirect immune-rosetting as the basic method for surface antigen detection, a technique well-established in this laboratory (Ling et al., 1977; Kalland, 1977).

First, SRBC were coated with a DEAE-purified sheep anti-mouse immunoglobulin (IgG,IgA,IgM) polyclonal antisera (using the chromic chloride method of Ling et al (1977):

1. Each coating required 0.1ml of packed SRBC. These were washed 6 times in sterile saline.
2. 0.3mg of antibody was added to each tube. The required amount of aged chromic chloride (at least 5 months old, 1/10 dilution of a 1.0mg/ml stock solution, pH=5.0) was added to each tube. The amount used was determined by selecting the amount giving the highest titres with good negative controls.
3. The walls of the tube were washed down with 1ml saline and left overnight at 4°C.
4. 2ml of Hepes-buffered RPMI-1640 was used to wash the cells which were then resuspended in 4ml of Hepes/2%.

The monoclonal antibodies (MoAb), were diluted 1μl in 50μl of PBS containing 10% FCS and 0.1% sodium azide. An anti-idiotype MoAb generated against an unrelated myeloma paraprotein was used as a negative control. Then, $2.5x10^5$ cells to be analysed were added, in 5-20μl of culture medium, and incubated at 4°C for 30 minutes. After washing twice in Hepes/2% the cells were mixed with approximately $10x10^6$ SRBC coated with a sheep anti-mouse immunoglobulin in 150μl of Hepes/2%. This mixture was left to stand at room temperature for 10 minutes, centrifuged at 100g for 30 seconds, and left to stand for a further 10 minutes.

One drop of acridine orange (18.75μg/ml) was added and after gently resuspending the cells, one drop was spread under a coverslip on a multispot PTFE-coated slide. The percentage of rosetted non-myeloid cells was enumerated by counting the number of white cells with at least 4 SRBC attached out of a total of 100 or 200 cells. A fluorescence microscope (Leitz dialux, Leitz Instruments, Bedford UK) was used, set for fluorescein excitation by epi-illumination to visualise the fluorescing nuclei, with slight background illumination with ordinary light to visualise the erythrocytes. As acridine orange causes myeloid cell cytoplasm to fluoresce red under these circumstances, they were eliminated from the count in order to concentrate on the plasma cells, which were also recognized by their characteristic morphology. However, some other non-myeloid cells could not be excluded especially lymphocytes.

To allow for the possibility that some lymphoid cells might be included in the rosetted cells, small percentage values of rosetted cells (<10%) were regarded as **negative**; counts between 10-30% as **possible weak positivity** ; counts ≥ 30% as **definite positivity**, of plasma cells with that antibody. On a few occasions, 50μl of the suspension of rosettes was cytospun

onto slides and stained using Leishman's stain (Figure 2.1). As this always confirmed the plasma cells were the rosetted cells, this was not performed in every case.

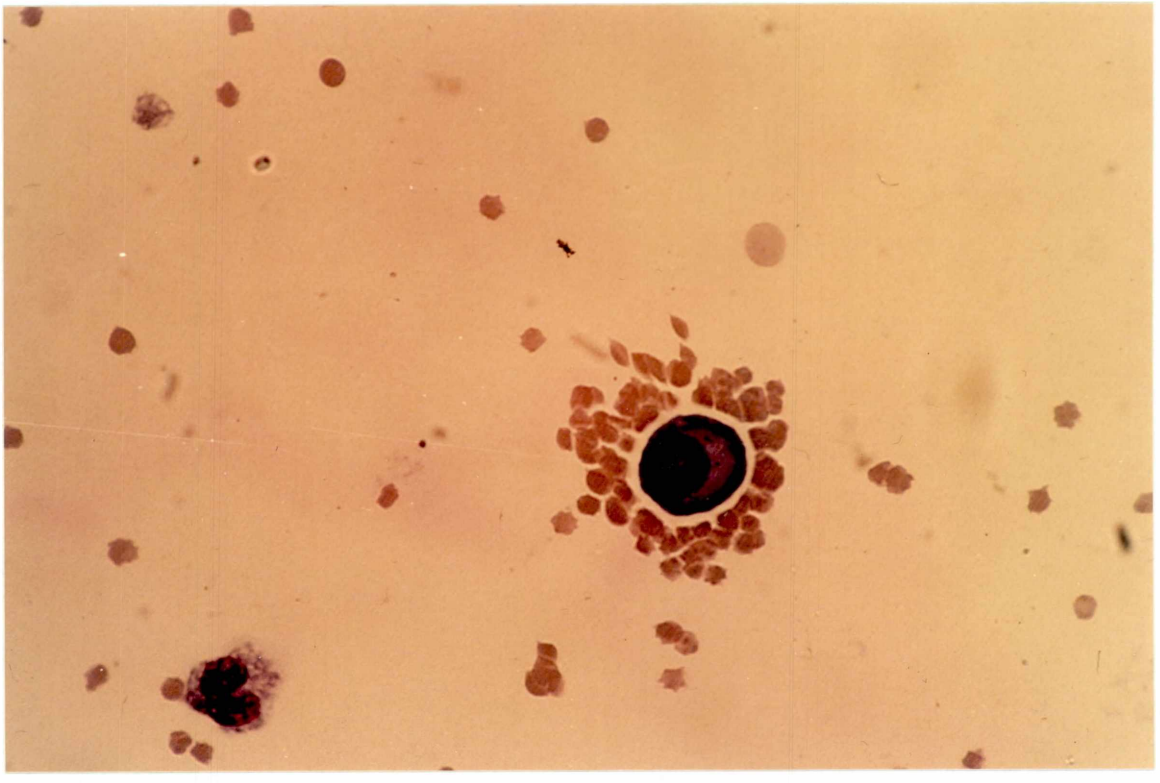


Figure 2.1 A cytospin of a myeloma plasma cell rosetted by indirect immune-rosetting with a CD38 MoAb as the first layer antibody.

(b) Double immunofluorescence

To confirm that the surface-labelled cells detected by immune-rosetting were plasma cells, in a few cases cells were double-labelled for surface antigen expression and cytoplasmic Ig using two colour immunofluorescence. The surface antigens were detected by incubating the cells first with a 1-in-50 dilution

of MoAb and then after washing twice, with a 1-in-100 dilution of a fluorescein-conjugated sheep anti-mouse Ig. After 2 further washes, the cells were cytospun onto methanol-washed slides, air-dried and fixed for 10 minutes in acetone. The slides were then stained with a rhodamine-labelled sheep anti-human Ig (G,A,M, Kappa & Lambda), at a dilution of 1-in-20, with Hoechst dye 33342 nuclear counterstain (1-in-100), for 30 minutes at room temperature. After washing for 30 minutes in PBS the slides were mounted under a coverslip in PVA-DABCO (see reagents) to prevent fading. The slides were then examined using Ploemopak filters (Leitz, Luton, UK) set for fluorescein (Block I2/3) and rhodamine (Block N2) alternately to find cells labelled for both. The nuclear counterstain is revealed by filter block A, and was used to count all the cells and as a guide to whether the cells were viable when first stained.

(c) Cytoplasmic antigen detection: immunofluorescence

Approximately 1-2x10^5 cells were cytospun onto slides (see section 2.3.1.(g) above). A small rim was created around the spot of cells by spraying the slide (with the spot covered with a drawing pin head) with PTFE spray (Klingerfon, Marshall-Howlett Ltd, Gravesend, UK). The slide was first incubated with 1μl of MoAb in 50μl PBS/10% FCS/NaN_3, at room temperature for 30 minutes, and then washed for at least 30 minutes in PBS. The slide was then incubated with a 1-in-100 dilution of fluorescein-conjugated sheep anti-mouse Ig for 30 minutes and washed in PBS for 30 minutes. The slides were then dipped in propidium iodide (PI) (1μg/ml) for 5 minutes for nuclear counterstaining, washed, and then mounted using PVA-Dabco. The slides were examined using a fluorescence microscope set for fluorescein excitation (fluorescein: green, propidium iodide: red), and results graded as negative, weak, moderate, or strong.

2.3.3. Cytochemical staining methods

(a) Chloroacetate esterase

1. Smears were fixed in formol acetone for 1 minute, and then washed in water for 10 minutes.
2. 50μl of new Fuchsin was mixed with 50μl of freshly prepared 4% $NaNO_2$. After 1 minute, 19ml of M/15 phosphate buffer (pH 7.6) was added. To this was added 3mg naphthol AS-D chloroacetate dissolved in 1ml of dimethyl formamide.
3. The slide was incubated with this solution for 15 minutes, washed in water for 10 minutes, and counterstained with methyl green for 30 seconds.

(b) Butyrate esterase

1. Smears were fixed for 1 minute in buffered formol acetone.
2. 25μl of para-rosaniline hydrochloride was mixed with 25μl of freshly prepared $NaNO_2$ for 1 minute, before 9.5ml of M/15 phosphate buffer (pH 7.3) was added. 10mg of α-naphthyl butyrate in 0.5ml 2-methoxyethanol was then added to the mixture.
3. The slide was then stained with this mixture at 37°C for 45 minutes, before being washed in water for 10 minutes, and counterstained with methyl green.

2.3.4. DNA analysis using the fluorescence-activated cell sorter

$5x10^5$ cells were centrifuged and the pellet resuspended in 0.5ml of fixing buffer (see materials). Immediately prior to analysis by the FACS, PI was added to give a final concentration of PI of 330μg/ml. This concentration is known to saturate the DNA intercalation sites. Then, $20\text{-}50x10^3$ cells

were analysed using a Becton Dickinson 440 FACS. (see chapter 3, section 3.2.1 for details of calibration).

2.3.5. Radio-isotope methods for detection of ^{3}H-thymidine incorporation

(a) Autoradiography

Cells at 2-4x10^5/ml were incubated with ^{3}H-thymidine (2μCi/mmol) at a final concentration of 3μCi/ml for 4-16 hours, after which they were washed 5 times in medium. They were then cytospun onto slides, dried and fixed in methanol for 10 minutes. In a darkroom, the slides were dipped in Ilford K2 emulsion at 56°C and allowed to drip dry. They were exposed in the dark at 4°C for 1-2 weeks, and then developed by serial passage through the following solutions:

1. Kodak D19 developer	18°C	4 minutes
2. Distilled water		quick rinse
3. 25% sodium thiosulphate	18°C	5 minutes
4. Running cold tap water		15 minutes.

The slides were counterstained using Leishman's stain.

(b) Quantitative assay of ^{3}H-thymidine incorporation

In functional studies to assess the proliferative effect of various growth factors on cell lines, ^{3}H-thymidine incorporation was measured. The protocol was as follows:

1. Cells were set up in microtitre wells on a 96-well plate at 2x10^4 cells/well (in 100μl medium + 20% FCS). The various factors under investigation were added, each set of factors being set up in triplicate or quadruplicate.
2. After 3 days, 0.3μCi ^{3}H-thymidine in 20 μl of medium was added to each well, and incubation continued for 16-

36 hours depending on the growth rate of the cell line. Then the cells were lysed and the nuclei harvested onto filtermats (Skatron AS, Norway) using the MCHI Cell harvester (Skatron AS).

3. The filtermats were placed in counting vials, and after allowing them to dry, 4ml of scintillation fluid (Optiphase X, LKB Scintillation Products, Bromma, Sweden) was added.
4. Counting was performed in various scintillation counters.

2.3.6. Cell culture: myeloma

(a) Phytohaemagglutinin-T-cell conditioned medium culture

Initial attempts at short-term culture of myeloma cells were based on the method of Izaguirre et al (1980), using phytohaemagglutinin-T-cell conditioned medium (PHA-TCM). PHA-TCM was prepared by separating T-lymphocytes from the peripheral blood of a normal subject using the SRBC method (section 2.3.1(d) above) and incubating them at a concentration of $1x10^6$/ml with 1% PHA in Ham's/Iscove medium at 37°C for 3 days in a moist atmosphere enriched with 5% CO_2. The culture supernatant was collected, filtered through 0.22μm filters and stored at -20°C.

For culture of myeloma marrow, mononuclear cells (or occasionally T-cell depleted and/or adherent cell depleted mononuclear cells) were suspended at $1\text{-}6x10^5$/ml in medium with 0.8% methylcellulose, 30% FCS, $5x10^{-4}$ molar 2-mercaptoethanol, and varying concentrations (usually 4%) of PHA-TCM. The suspension was mixed thoroughly in a vortex mixer before being plated out in 0.1ml aliquots into wells of 96-well microtitre plates. The plates were covered and inspected to check that a single cell suspension had been achieved. They were then incubated at 37°C in humidified air with 5% CO_2. Colonies (collections of

≥20 cells) were counted at 5-7 days using a Leitz Diavert inverted microscope. Single colonies were removed with a finely-drawn pasteur pipette and placed into a drop of distilled water, or hypotonic saline, on a multi-spot PTFE-coated slide to disperse the methylcellulose. When the methylcellulose had dispersed, the water was quickly evaporated using a hair dryer. Alternatively the total contents of several wells were aspirated, washed in medium and cytospin preparations made for examination of morphology using Leishman's stain, or immunofluorescence.

(b) Phytohaemagglutinin +/- interleukin 2 culture

Following the report of colony formation in chronic lymphocytic leukaemia (CLL) being induced by PHA and rIL2 (Touw & Lowenberg, 1985), from January 1986 cultures were also set up with phytohaemagglutinin (PHA) and/or recombinant interleukin 2 (rIL2) with or without PHA-TCM. The culture conditions were similar to the PHA-TCM culture, with PHA used at 1% final concentration, and rIL2 used at 100units/ml. The usual combination of growth factors was PHA ± rIL2, occasionally PHA + PHA-TCM, and rarely PHA + PHA-TCM + rIL2. The cells were harvested after 5-7 days of culture and cytospin preparations examined morphologically and immunologically (as above). Suspensions of cells were also tested for expression of surface antigens by indirect rosetting or immunofluorescence. Cultures of cells from the peripheral blood of patients with chronic lymphocytic leukaemia were also set up using PHA and rIL2.

(c) Liquid culture

Attempts were made to establish longer term growth of myeloma cells in liquid culture. For this, simpler culture conditions were generally used - i.e. Isc/Ham medium with 20% FCS, mononuclear cells being suspended at $2\text{-}4\text{x}10^5$ cells/ml in 1ml volumes in 24-well culture dishes. Cells were examined every few days for signs of growth, and sometimes wells were divided

or non-adherent cells were removed from a developing fibroblast/ macrophage layer. Various stimulants and feeder layers were added to different cultures in an attempt to stimulate proliferation. These included:

(1) The fibroblast line Flow 4000 (Flow Laboratories), which was sometimes irradiated, or its culture supernatant;
(2) Mouse spleen cell culture supernatant;
(3) Tetradecanoylphorbolacetate (TPA) ± Calcium Ionophore (Ionomycin) (Ca-I);
(4) Killed Staphylococcus Aureus Cowan 1 (SAC);
(5) Human serum;
(6) PHA-TCM; and
(7) Supernatant from an Ewing's sarcoma cell line (ESG, contains B cell stimulatory factor 2, Northumbrian Biologicals Ltd).

2.3.7. Assay of granulocyte-macrophage colony forming units

Normal bone marrow was assayed for colony forming units-granulocyte/macrophage (CFU-GM) according to the method of Pike and Robinson (1970). A white blood cell suspension was prepared from a normal person's peripheral blood by dextran-sedimentation of the erythrocytes. A feeder layer was made containing 10^6 of these cells in 1ml McCoy's 5A medium (+ additives, see materials) with 15% FCS and 0.5% agar, in the bottom of a 35mm diameter tissue culture plate. This was usually allowed to incubate for 1-2 days before the overlayer of bone marrow white cells to be assayed was added: $2x10^5$ cells in McCoy's 5A medium (+ additives), with 15% FCS and 0.3% agar. Colonies (≥40 cells), and clusters (<40 cells) were counted at 7 and 14 days.

2.3.8. Method for the attempted removal of myeloma progenitors from bone marrow: cytotoxic drugs

Bone marrow mononuclear cells from myeloma patients were incubated for one hour at 37°C with mafosfamide (Asta-Z, Astawerke AG, Bielefeld, FRG) at concentrations of 10-100μg/10^7 cells in 1ml of medium. 4-Hydroperoxycyclophosphamide (4HC, Astawerke AG) was used at 10-50μg/10^6 mononuclear cells in 1ml, and incubated for 30 minutes. The lower cell concentration was used for 4HC because of data suggesting a more gradual dose-response effect at this concentration (Korbling et al, 1982). After incubation, the cells were washed 3 times in medium prior to culturing for myeloma colony formation. To compare the effect of the drugs used in this way on normal bone marrow progenitors, normal bone marrow cells (from patients undergoing cardiothoracic surgery, or normal volunteers (NJ)) were treated in the same way, and then plated for CFU-GM culture (see section 2.3.7 above).

2.3.9. Protocol for fusion of mouse plasmacytoma cell line with human myeloma cells

Approximately 10^7 mononuclear cells from human myeloma bone marrow, or mouse spleen cells, were fused with 1-10x10^7 (usually 4x10^7) cells of the mouse myeloma cell line, X63-Ag8.653 using a modified technique based on Galfré and Milstein (1982). The mouse line was grown in RPMI-1640 medium, supplemented with 10% FCS, and thioguanine at 2x10^{-5} M to select cells deficient in the enzyme hypoxanthine-guanine-phosphoribosyl transferase (HGPRT).

1. The cells were mixed together and washed in RPMI-1640. All the medium was removed by suction and the pellet resuspended by agitation.
2. Five drops of alkaline RPMI-1640 were added and the cells wrapped around the tube by rotating horizontally

for 5 minutes. The tube was placed in a 37°C water bath for 7 minutes, with occasional rotation.

3. 0.7ml of warm (37°C) alkaline PEG was quickly added to the tube and rotated with the cells for 2½ minutes.
4. 1.0ml warm Dulbecco's medium was added rapidly and rotated for 1 minute. This step was then repeated once, before 20 ml of Dulbecco's medium was added slowly over several minutes.
5. The suspension was then centrifuged at 200g for 15 minutes, the supernatant removed, and the pellet resuspended in 30 ml of warm RPMI-1640 with HAT (hypoxanthine 100μM, aminopterin $4x10^{-7}$M, thymidine 16μM) and 15% FCS.
6. One drop of this suspension was added to each well of several 96-well culture plates using a pasteur pipette.
7. More HAT medium was added after 4 days and again at 7 days, during which time the plates were kept under observation with an inverted microscope. Colonies were clearly evident by 7 days.

CHAPTER 3

ANALYSIS OF DEOXYRIBONUCLEIC ACID CONTENT OF MYELOMA BONE MARROW CELLS

3.1 INTRODUCTION

Cytogenetic studies in myeloma have been limited by the low number of proliferating cells that can be demonstated in most patients. Flow cytometry can analyse the total DNA content of cells independent of cell proliferative state, and can also be used to estimate the fraction of cells in various stages of the cell cycle (G_0/G_1, S, G_2+M). Although specific chromosomal abnormalities cannot be detected, there is evidence from flow cytometry that in the lymphoproliferative malignancies, aneuploidy is associated with a relatively poor prognosis (Bunn et al, 1980; Shackney et al, 1980). In myeloma, studies have shown that 60-80% of cases show aneuploidy, and that this correlates with a poorer prognosis, especially if hypodiploidy or more than one aneuploid population is present (Barlogie et al, 1985). In this study, a cellular DNA content profile was obtained on fresh myeloma bone marrows with the aims of:

(a) confirming the incidence of ploidy abnormalities in myeloma,
(b) attempting to correlate the ploidy abnormalities and/or the percentage of S/G_2+M phase cells with the growth pattern of the myeloma cells in vitro, and
(c) using the DNA content abnormality as a marker to follow the malignant cell population through various processes, such as their selective removal from a marrow sample using monoclonal antibodies, as in 'purging' bone marrow for autologous bone marrow transplantation.

Unfortunately the second and third aims have not been achieved because of uncertainties over the validity of the in vitro culture results and the failure to develop effective means for selectively removing myeloma plasma cells from bone marrow (see chapter 5). However, the first aim has been fulfilled and the DNA content abnormalities have been correlated with prognosis. The technique of DNA analysis has also proved useful in

analysing the results of fusions of mouse and human myeloma cells (chapter 7) and the nature of a recently established human myeloma cell line (chapter 8).

3.2 METHODS

Bone marrow from 47 patients: 40 with MM at various stages (one was examined twice, and another was examined three times), 6 with benign paraproteinaemia, 1 with Waldenström's macroglobulinaemia) were examined for cellular DNA content. For details of the patients' treatment status and degree of marrow infiltration see Table 3.1 in the Results section (p78). A mononuclear cell fraction of fresh marrow was prepared and approximately $5x10^5$ cells were resuspended in 500μl of fixing solution consisting of phosphate-buffered saline with 0.07% paraformaldehyde and 0.1% Triton X-100 (a non-ionic detergent). Immediately prior to analysis by the FACS, propidium iodide (PI) was added to give a final concentration of PI of 330μg/ml, which is known to saturate the DNA intercalation sites. Then $20-50x10^3$ cells were analysed using a Becton Dickinson 440 FACS, and a frequency histogram of DNA content per cell obtained. Occasionally, two separate peaks of G_1/G_0 phase cells with only slightly differing DNA contents were revealed by using a combined plot of fluorescence and cell size. The coefficient of variation (CV) of the G_1/G_0 peak was calculated using the formula CV = HMx100/Vx2.35, where HM = width of G_1/G_0 peak at half maximum, and V = modal channel number of the normal diploid G_1/G_0 peak (Bunn et al, 1982). The CVs varied from 2.2-9.7% (mean = 5.4%). The DNA index was calculated by dividing the modal DNA content of the myeloma G_1/G_0 phase cells by DNA content of the normal G_1/G_0 phase cells.

3.2.1 Calibration

For the first 40 bone marrows analysed the FACS was calibrated using chicken erythrocytes, and for the last 10 cases, standard

PI-containing beads were used. However, for reasons that are unclear, this did not result in normal human diploid cells always having the same peak channel number for the G_1/G_0 peak. This makes comparison of DNA content profiles between different bone marrows difficult. However, almost all samples of myeloma marrow contain a proportion of residual normal haemopoietic cells which can be used as an internal standard (representing normal diploid cellular DNA content). In most cases the normal cells are in the majority, and so it was assumed that if there were two G_1/G_0 peaks the larger one was the G_1/G_0 peak of the normal diploid cells. In two cases when the marrow was very heavily infiltrated with plasma cells, it was not possible to distinguish which was the normal and which was the abnormal peak, and hence whether the myeloma cells were hyper- or hypo-diploid. Both of these cases have been recorded as hyper-diploid because the larger peak (corresponding to the larger fraction of cells) was the one with the higher DNA content. However, sometimes the percentage of aneuploid cells is lower than the percentage of plasma cells in the bone marrow (Bunn et al, 1982), and so these patients may have had hypodiploid clones. This problem was only realised after these analyses had been performed and so it was not possible to re-run these samples with normal (diploid) cells for comparison.

3.3 RESULTS

3.3.1 G_1/G_0-phase ploidy analysis

Typical DNA analyses for two MM bone marrows are shown in Figure 3.1. One shows a single G_1/G_0 peak, the other shows an additional (aneuploid) G_1/G_0^+ peak.

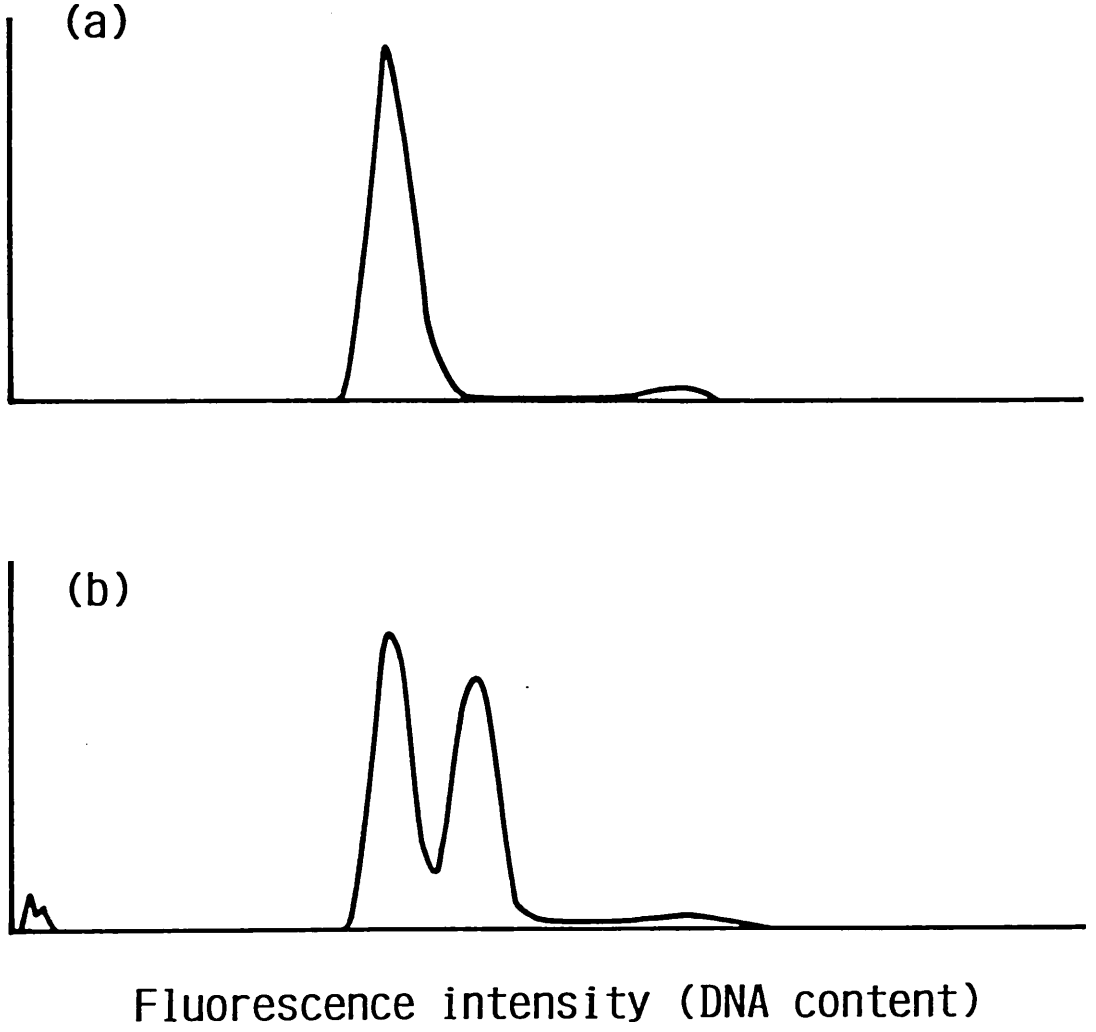


Figure 3.1 DNA content frequency histograms of two patients with MM. (a) A patient showing a single (diploid) G_1/G_0 DNA peak. (b) A patient showing an additional (aneuploid) G_1/G_0 DNA peak.

The DNA indices were calculated, and the results for the 40 MM patients are shown in Table 3.1 and Figure 3.2. If more than one aneuploid peak was present, only the largest abnormal peak was included in this analysis.

Patient	Treated?	%PC	DNA Index	Survival
GA	No	57		
DE	No	32	1.11	
SM	No	27		
ED	No	34		
HA	No	43	1.19	9
SH	No	55		34
FU	No	65	1.15	97+
HE	No	15		334+
J-SM	No	72		405+
MO	No	27		494
BI	No	35		502+
MC	No	51		513+
SP	No	85	1.14 *	544+
RI	No	20	1.10	910+
PR	Yes	4		
HU	Yes	15		
FI	Yes	31	1.23	
ME	Yes	100		54
DY	Yes	48	1.24	62+
NA	Yes	20	1.21	155
BU-2	Yes	80		156
CU	Yes	80	1.79	165
JO	Yes	45		182
LE	Yes	4		298
BR-3	Yes	1	1.35	304
SK	Yes	15		336+
BU-1	Yes	50		342
GO	Yes	95	1.15 *	343+
DI	Yes	23		367+
O-BR	Yes	42	1.22	387
BR-2	Yes	2	1.07	387
BR-1	Yes	1		393
CL	Yes	32	0.83	396
TA	Yes	30	1.33	417
SE	Yes	13		503+
HU	Yes	23	1.28	516
HI	Yes	20		541+
CA	Yes	80	1.28	551
BO	Yes	15	1.21	601
J-BU	Yes	20	1.32	642
PL	Yes	42	1.19	673+
HA	Yes	5		780
ON	Yes	8		842+

Table 3.1 The DNA index (if not 1.0), of 40 MM patients, together with the %PC on marrow smears; their treatment status and their survival in days from the date of the bone marrow examination (if known).
+: still alive at time of enquiry
*: ? hypo- or hyper-diploid (see 3.2.1, p75)

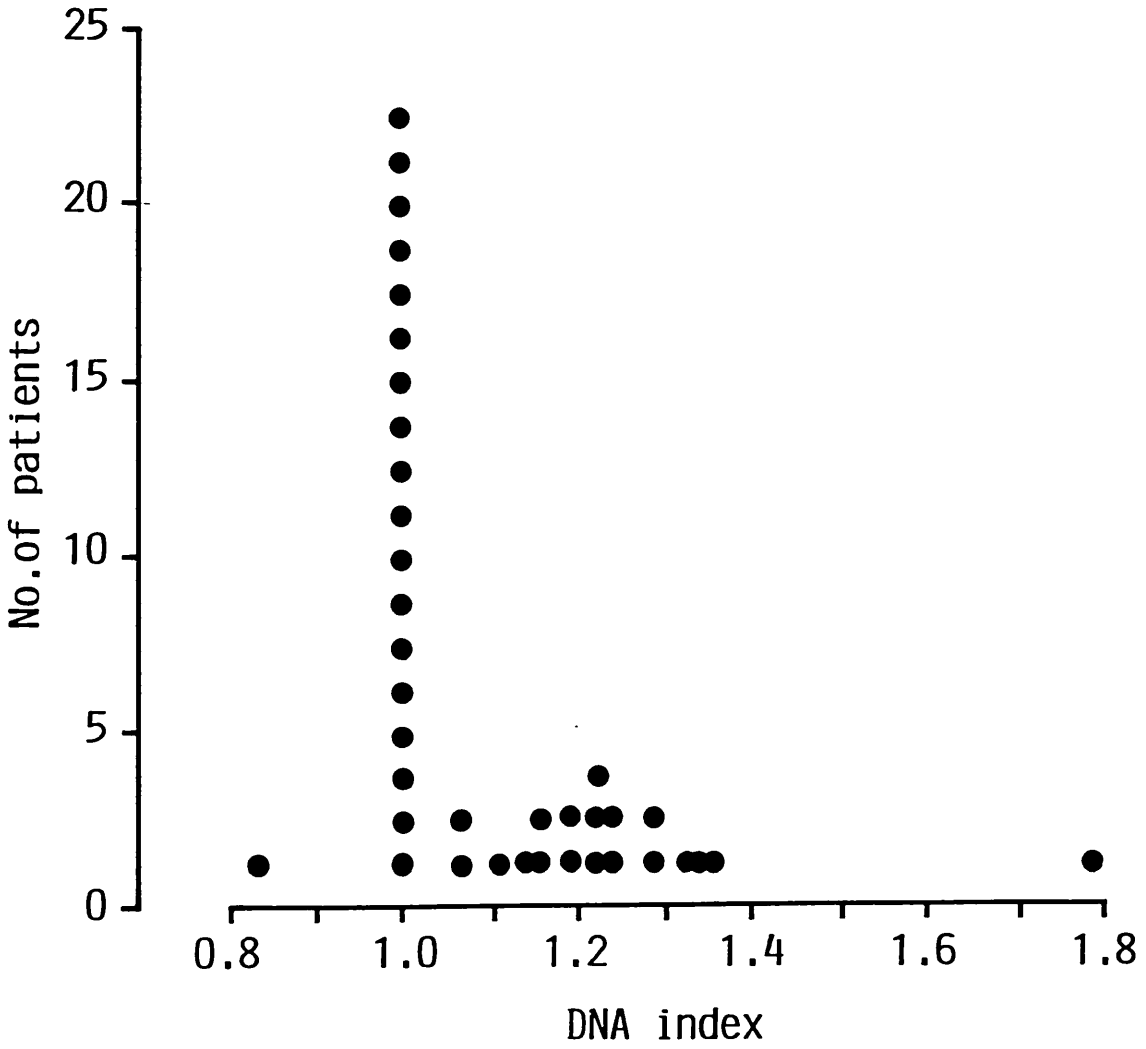


Figure 3.2 DNA Indices in 39 patients with MM.
x,y = patients with heavily infiltrated bone marrows in whom it was difficult to decide whether the abnormal clone was hypo- or hyper-diploid. If they were in fact hypodiploid then they would have had DNA indices of 0.875 and 0.86 respectively (see section 3.2.1: calibration).

Results from seven patients with benign paraproteinaemia and one with Waldenström's macroglobulinaemia who all had normal diploid DNA content, are not shown. Nineteen (47.5%) of the MM patients had detectable abnormal ploidy, 5/14 of those who had

not received treatment, and 14/26 of those who had been treated. One patient (BR) who was tested three times, had normal DNA content when first tested, later an index of 1.22, and then, later still, 1.35. The hyperdiploid clones had DNA indices ranging from 1.07-1.79, but they were predominantly grouped between 1.1-1.3. Only one sample had a definitely hypodiploid DNA content (DNA index 0.83): this patient had relapsing myeloma with 32% plasma cells in his bone marrow aspirate (Figure 3.3). He survived for just over one year from the time of the bone marrow aspirate.

Multiple clones were present in 2 patients (see Figure 3.4), and these two patients had both hypo- and hyper-diploid clones. On two occasions where the aneuploid peak was well separated from the diploid peak, the diploid and aneuploid fractions were separated using the sorting function of the FACS. The 2 fractions were cytospun onto slides for morphological examination to confirm that all the aneuploid cells were in fact plasma cells and vice versa. However with the detergent fixative used all that was seen on the cytospins was bare nuclei without any distinguishing morphological features.

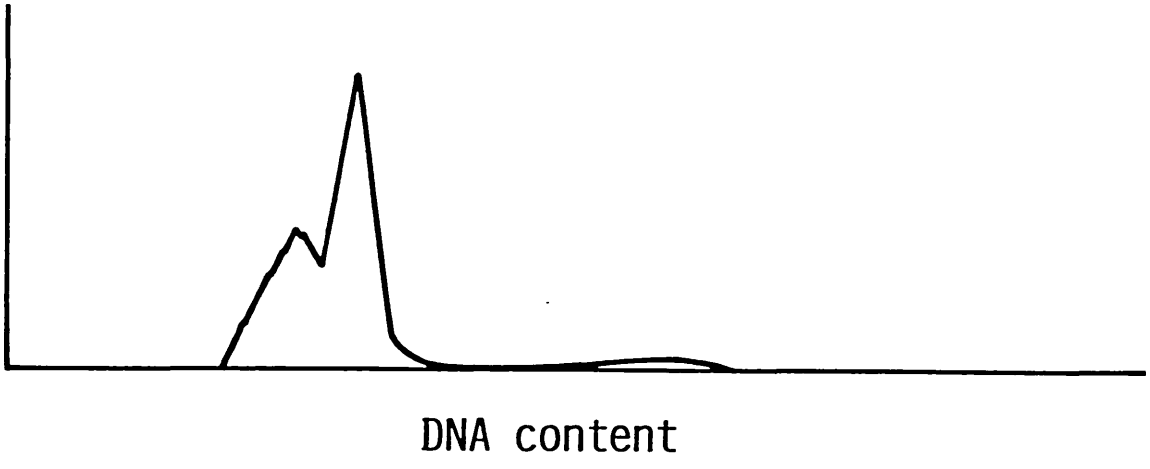


Figure 3.3 A cellular DNA histogram of a patient with an hypodiploid myeloma clone (DNA Index 0.85) with 35% plasma cells in the bone marrow.

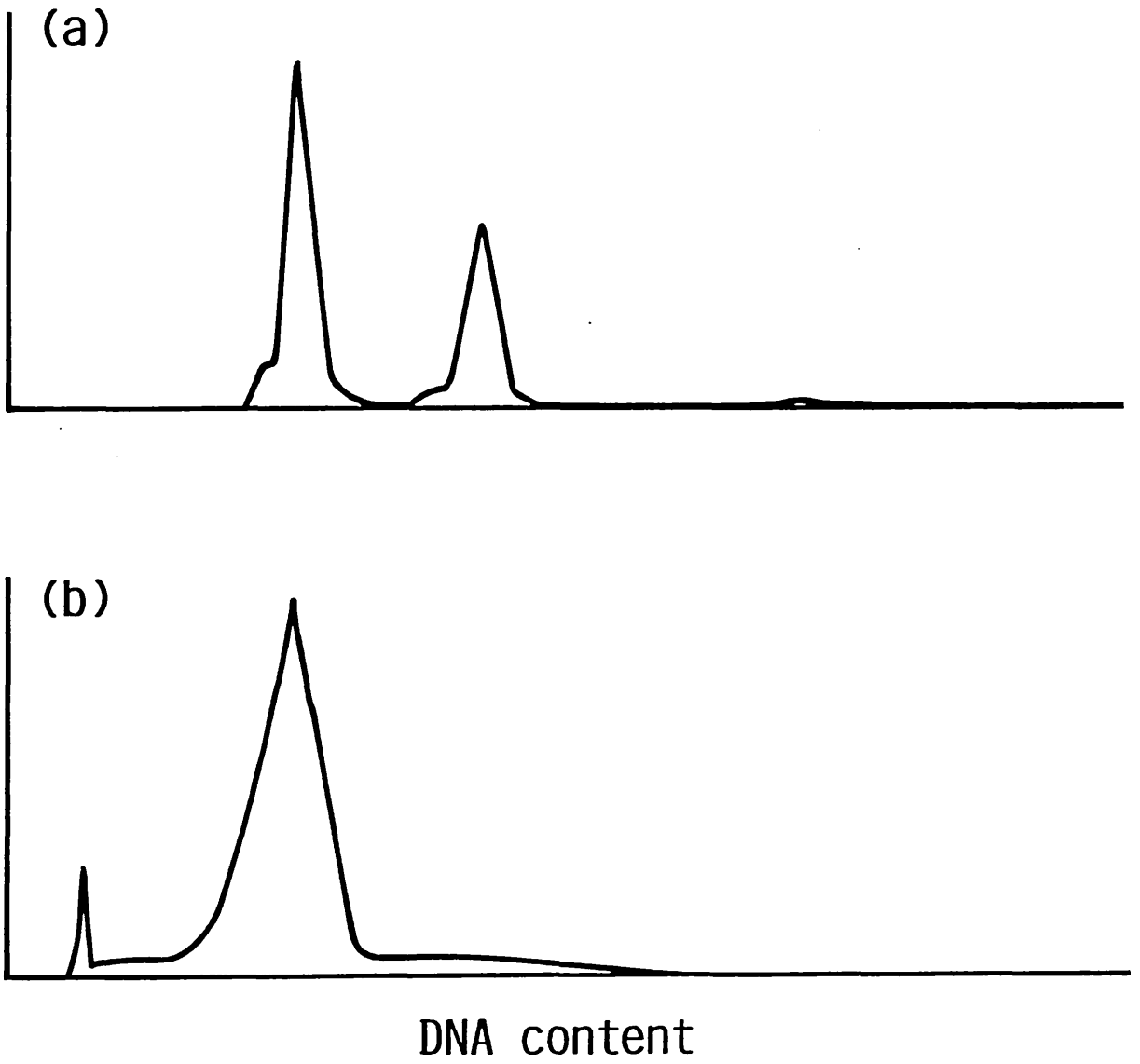


Figure 3.4 DNA histograms of two patients showing the presence of multiple aneuploid clones. (a) This patient had a heavily infiltrated BM (70% PC) and was terminally ill after multiple treatments. Her trace shows a small hypodiploid clone and a peak too large and the wrong shape to be a G_2/M peak - it probably represents a near-tetraploid clone (DNA index 1.79). (b) A very broad $G_{0/1}$ peak with at least 2 discernible shoulders - probably 1 hypo-, and 1 hyper-diploid peak.

3.3.2 S and G_2/M phase analysis

If normal bone marrow is subjected to cellular DNA content analysis, then a small number of cells will be found in the S and G_2/M phases of the cell cycle. In samples from myeloma bone marrows fewer than normal numbers of cells were found in these phases, and in heavily infiltrated bone marrows (> 80% plasma cells), often no S/G_2/M cells were detected (Figure 3.5).

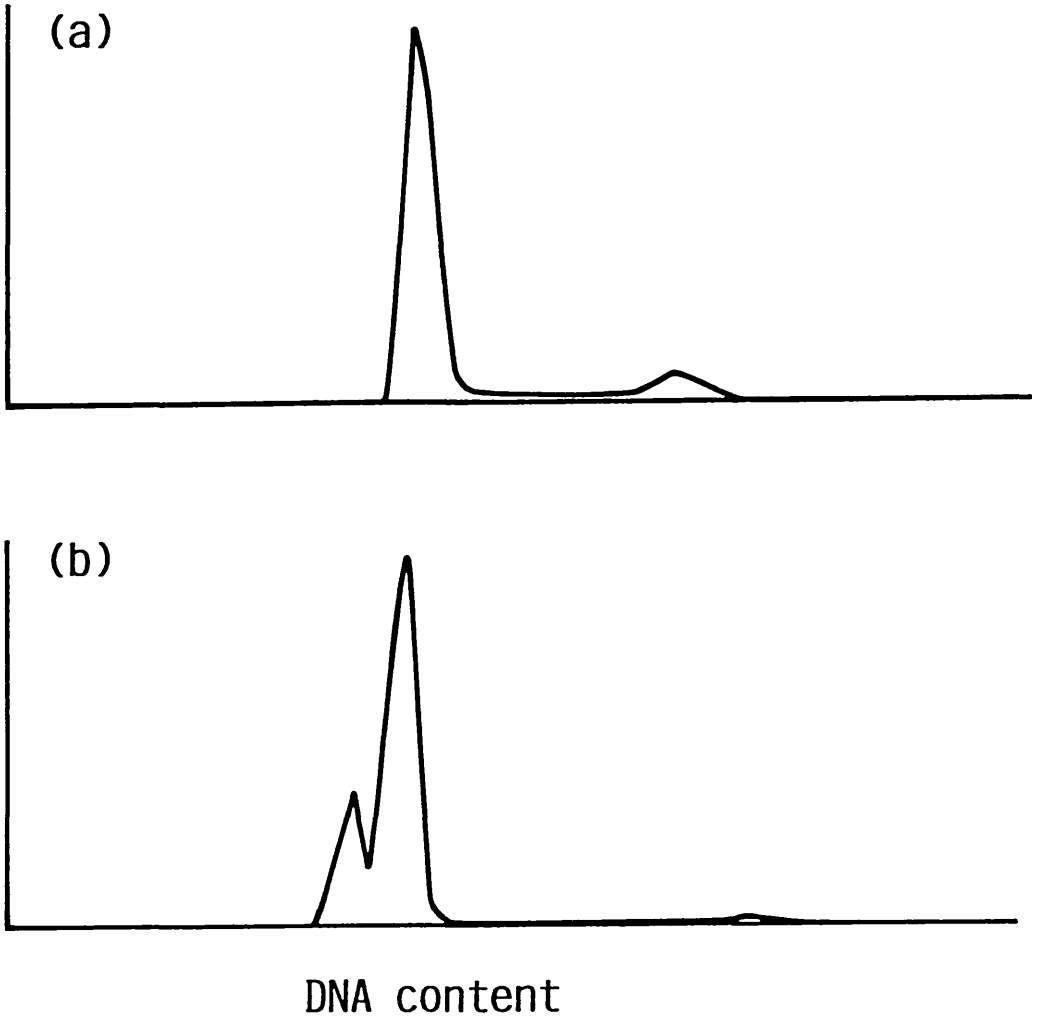


Figure 3.5 DNA histograms of a normal (a), and an heavily infiltrated MM bone marrow (b), showing the virtual absence of a S/G_2/M peak in the bone marrow containing a substantial (85%) PC infiltrate.

In 6/11 samples with a clearly demarcated aneuploid clone (DNA index > 1.2) a small corresponding (i.e. aneuploid) G_2/M peak was found, but it contained <1% of the total cells. This suggests that only a small percentage of myeloma cells are in active cell cycle.

3.3.3 Relation to prognosis

Follow up data was available for 35 of the 40 patients with MM. As these patients were sampled at varying stages in the course of their disease, the survival from the date of DNA analysis was used to compare the prognosis of patients with aneuploid clones against those with diploid clones. Actuarial survival curves for the two groups are shown in Figure 3.6.

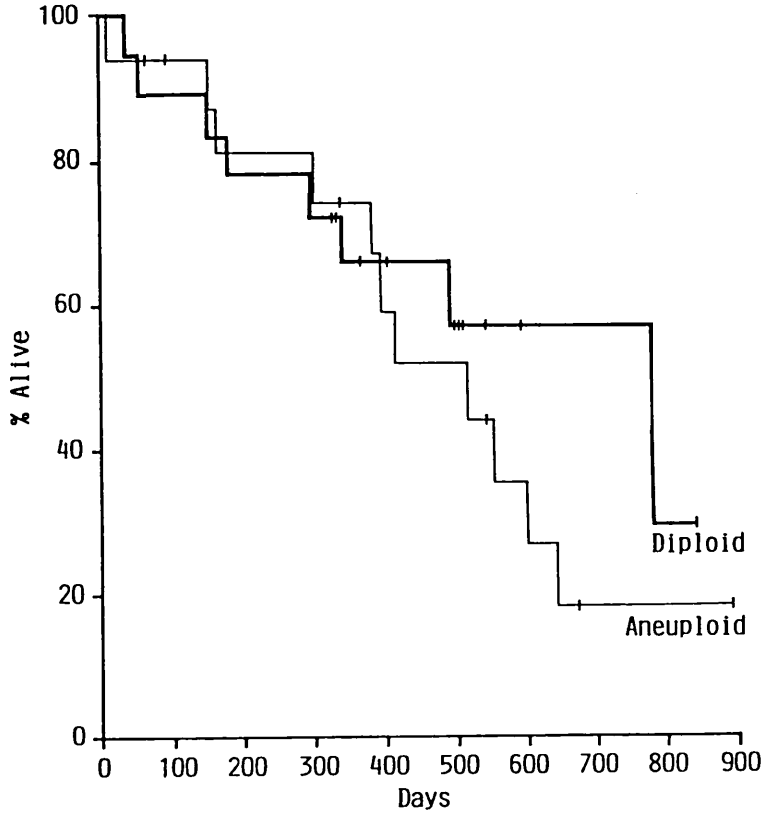


Figure 3.6 Actuarial survival curves from the date of DNA analysis for 35 patients with MM (18 diploid, 17 aneuploid).

As the number of patients studied was small and there is a lack of data on other prognostic variables in the patients studied, it was decided not to try to analyse statistically the difference between these two groups. The group with hypodiploid/multiple clones was very small, but their survival times were as follows: hypodiploid patient: 396 days; two possibly hypodiploid patients 343+ and 544+ days; two patients with multiple clones: 165 days, second patient unknown.

3.4 DISCUSSION

These results confirm that there is a high incidence (47.5%) of ploidy abnormalities in myeloma, although other workers have found even higher incidences (60-80%; Bunn et al, 1982; Barlogie et al, 1985). The lower incidence of abnormalities in our series may be due to the relatively high CVs (mean 5.4%) of our DNA analyses. Bunn's group report CVs of 4% and Barlogie's of 1-2% (Barlogie et al, 1980). This is particularly important as the majority of aneuploid MM clones have DNA indices of less than 1.15 (our data; and Latrielle et al, 1980). One important point from our series is that there is a higher incidence of abnormality in treated patients, and one patient developed an abnormal clone while on treatment. This could be due to the disease itself evolving, but it could also be due to the mutagenic effect of the drugs used. The majority of abnormalities are of the hyperdiploid type, but in this series of 40 patients at least 1, and possibly 3, were hypodiploid. In addition, two patients exhibited multiple abnormal clones, including in each case an hypodiploid one.

In at least 2 cases, it is possible that the aneuploid clone belonged to an associated myelodysplastic syndrome rather than to the myeloma itself, as the number of plasma cells in the bone marrow was very small compared with the size of the aneuploid peak. Both of these patients had associated myelo-

dysplasia, and one later died of acute myeloid leukaemia. Ploidy abnormalities are common in myelodysplasia (Peters et al, 1986), which is associated with myeloma (Copplestone et al, 1986). If DNA studies were performed using the supravital DNA stain Hoechst 33342, which does not require the use of a detergent fixative, then it might be possible to examine the morphology, immunophenotype and growth characteristics of the diploid and aneuploid cells separately. However the DNA content analyses tend to be less precise with Hoechst 33342 than with PI (Park et al, 1985).

The low number of cells found to be in the $S/G_2/M$ phases of the cell cycle confirms the work of others (Barlogie et al, 1980). This may go some way to explaining the difficulty that has been experienced in culturing myeloma cells in vitro. Although plasma cells may survive for long periods in culture, it is rare to demonstrate actual cellular division or cell number increase (see chapter 7). The only long term myeloma cell line that we have developed came from a bone marrow exhibiting prominent S and G_2/M phases, but it was not possible to tell if these $S/G_2/M$ cells were myeloma cells or normal haemopoietic cells (see chapter 8).

3.4.1 Flow cytometry analysis and survival

Barlogie's studies have shown that hypodiploid or biclonal DNA clones are associated with reduced survival (Barlogie et al, 1983; 1985; 1988). Hyperdiploidy, on the other hand, has been associated with both shorter and (non-significantly) longer survival times than diploidy in different series (Bunn et al, 1982, Barlogie et al, 1985, respectively). Our own data show a non-significant trend to poorer survival with aneuploidy but there was insufficient data on other prognostic factors to analyse this statistically. Measurement of the serum β_2M, the pre-transfusion haemoglobin and renal function at presentation are thought to provide the best indicators of survival (see Introduction, section 1.2.4, and Kelly et al, 1988).

3.4.2 Flow cytometry analysis and response to chemotherapy

The DNA index alone does not give a very good indication of the likely response to initial chemotherapy. Barlogie and co-workers have found that simultaneously measuring the RNA and DNA content of cells in MM bone marrow using acridine orange staining gives additional information about this (Barlogie et al, 1985). They analysed the relationship of the response to initial chemotherapy with vincristine, adriamycin, cyclophosphamide and glucocorticoid combinations to: the RNA index (= mean RNA content of G_1/G_0 tumour cells/ mean RNA content of normal haemopoietic cells); the DNA index; the percentage of marrow PC; the β_2M; and the tumour stage. Response was defined as a 75% reduction in paraprotein. Using multiple regression analysis, the RNA index was the most strongly associated with response to chemotherapy ($p<0.001$) followed by the DNA index ($p=0.01$). The other features were not of independent significance but the best indicator was the relative RNA index (= RNA Index/ % marrow PC). It may be that if both cellular DNA and RNA contents are measured, patients could be selected for novel or intensive treatment regimes not only on the basis of a likely poor survival outcome on conventional chemotherapy, but also on a poor chance of initial response to that chemotherapy.

CHAPTER 4

AN ANALYSIS OF MYELOMA PLASMA CELL PHENOTYPE USING ANTIBODIES FROM CLUSTERS DEFINED AT THE IIIrd INTERNATIONAL WORKSHOP ON HUMAN LEUCOCYTE DIFFERENTIATION ANTIGENS

4.1 INTRODUCTION

Cells of the B lineage cease to express many characteristic B cell antigens when they differentiate into plasma cells. Antigens lost include those detected by antibodies of clusters CD19-23 as defined at the IInd International Workshop on Human Leucocyte Differentiation Antigens (Nadler, 1986) and surface immunoglobulin (sIg) (Katagiri et al, 1984, Foon et al, 1982). Myeloma plasma cells, however, do not always lack sIg (Burns et al, 1979). The majority of plasma cells also lack major histocompatibility complex (MHC) class II antigens (Wu et al, 1976; Kuritani & Cooper, 1982; Katagiri et al, 1984), and Fc and C3 receptors (Stashenko et al, 1980). Recently, some cases of myeloma have been found to express CD10 (CALLA; common acute lymphoblastic leukaemia antigen) on some of the cells in blood and bone marrow (Ruiz-Argüelles et al, 1984; Durie & Grogan, 1985; Caligaris-Cappio et al, 1985). The IIIrd International Workshop (McMichael et al, 1987) defined 5 new B cell associated antibody clusters (CD 37-w40 and 45R), and also included studies on other antibodies which were not clustered but which were found to react with plasma cells. Plasma cell reactivity was tested mainly against plasmacytoid cell lines (JVM-3, U-266, HFB-1, RPMI-8266). Cells from only 2 cases of MM were investigated and they showed moderate reactivity with antibodies of CD38, CD39 and 2 antibodies from CD24 (HB8 and VIB-E3). Before and after the IIIrd Workshop, we carried out a phenotypic analysis of fresh MM bone marrow cells using both clustered and unclustered monoclonal antibodies (MoAb) supplied for the workshop, including one from each of the new and the previously defined B cell associated clusters. Some of these results were presented at the IIIrd Workshop (Nathan et al, 1987), and have now been published in full (Jackson et al, 1988).

4.2 METHODS AND PATIENTS

4.2.1 Patients. Forty-six patients who fulfilled the Medical Research Council criteria for entry into the IVth Myelomatosis trial (MRC working party on leukaemia in adults, 1985) were studied, including 3 with plasma cell leukaemia (i.e. peripheral blood plasma cell count $\geq 2 \times 10^9/L$). Their paraprotein type and treatment status are shown in Table 4.1.

	Myeloma		Plasma Cell Leukaemia
Paraprotein	Untreated	Treated	Untreated
IgG	15	11	1
IgA	4	7	2
Light Chain Only	1	5	

Table 4.1 Patient characteristics with their paraprotein types.

4.2.2 Antigen detection: indirect immune-rosetting and immunofluorescence.

See Methods (section 2.3.2(a) and (c)). The MoAb used, and their international cluster of differentiation (CD) number, are shown in Table 4.2 (over).

Antibody	CD	Source
control		An anti-idiotype Moab produced in the Immunology Dep, Birmingham University.
BU16	9	Immunology Dep, Birmingham University.
Anti-CALLA	10	Becton-Dickinson*.
BU12	19	Immunology Dep, Birmingham University.
BC1	20	Gallart, Barcelona.
BA5	21	Le Bien, Minnesota.
29-110	22	Kraft, Melbourne.
MHM6	23	McMichael, Oxford.
ALB9	24	Boucheix, Villejuif, France
HB8	24	Cooper, Alabama.
HB9	24	Cooper, Alabama.
VIB C5	24	Knapp, Vienna.
VIB E3	24	Knapp, Vienna.
ALla	24	Ravoet, Brussels.
LC66	24	Ravoet, Brussels.
CLB/granBlyl	24	Tetteroo, Amsterdam.
Anti-IL2 receptor	25	Becton-Dickinson*.
WR-17	37	Moore, Southampton.
OKT10	38	American Type Culture Collection.
HB7	38	Tedder, Boston.
T16	38	Bourel, Rennes. France.
T168	38	Bourel, Rennes, France.
AC2	39	Rowe, Cancer Studies, Birmingham.
G28.5	w40	Ledbetter, Seattle.
4KB5	45R	Pulford, Oxford.
PCA-1		Nadler, Boston.
BU25 (Jo5)	Anti-MHC class II	Immunology Dep, Birmingham University.
KB61		Pulford, Oxford.
7F7		Schulz, Innsbruck.
BU 11,18	Anti-RER	Immunology Dep, Birmingham University.
RFD6	Anti-RER	Janossy, London.

Table 4.2 Antibodies used, their cluster of differentiation (CD) where applicable,* and the sources from which they were obtained (*=purified antibody; others were ascitic fluid).

4.3 RESULTS

4.3.1 Reactivity of myeloma plasma cells with clustered antibodies

Bone marrow mononuclear cells from 46 patients have been analysed with various combinations of antibodies. The last 16 samples were tested against all of the B cell antibody clusters, as defined by the IIIrd Workshop. The results for antibodies from these clusters are shown in Table 4.3. (cytoplasmic typing), and Figure 4.1. (surface typing).

	Cytoplasmic Reactivity			
CD No.	Negative	Weak	Moderate	Strong
9	1	8	1	
10	8	2		
19	6	4		
20	8			
21	6			
22	9	1		
23	4	2	1	
24(HB8)	0	6	6	2
24(Other)	6			
25	12			
37	7	1	1	
38	0	6	11	13
39	5	3		
40	3	3	1	

Table 4.3 Cytoplasmic reactivity of myeloma plasma cells with clustered antibodies. (Numbers of cases tested expressing negative, weak, moderate or strong reactivity). CD = cluster of differentiation.

In the majority of cases the plasma cells were $HB8^+$, but most other antibodies from CD24 gave negative results. Most cases (26/36) were CD38 $(OKT10)^+$, but some were negative by indirect rosetting. In 8 out of the 10 $CD38^-$ cases, the pattern of

positivity of the immunofluorescence on the cytospins suggested that the cells were in fact surface $CD38^+$. One case was $CD10^+$, $CD19^+$, $CD20^+$, and $CD23^+$, and occasional weak surface reactivity was also obtained with CD9 (3 cases), CD25 (2 cases), CDw40 (2 cases), CD45R (5 cases). Reactivity on cytospin preparations was found with CD38 and HB8 (strong), and CD9, 23, 37, 39 and w40 (weak to moderate).

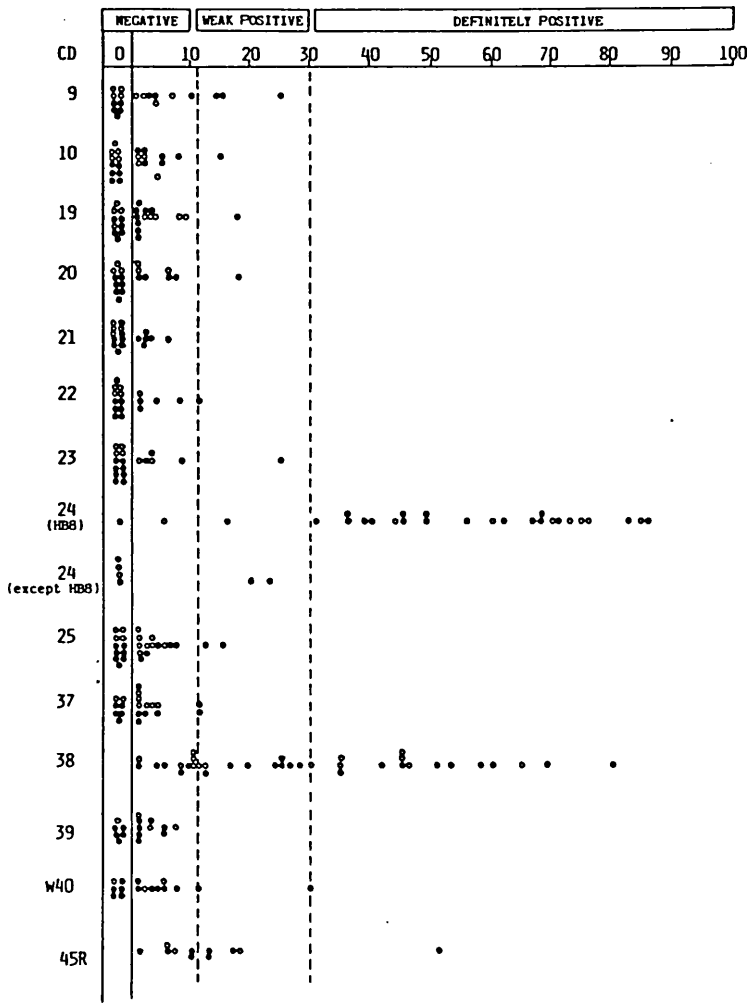


Figure 4.1 Surface reactivity of myeloma plasma cells with clustered antibodies: percentage of positive cells by indirect immune-rosetting (o=untreated, o=treated).

4.3.2 Reactivity of myeloma plasma cells with unclustered antibodies

The results using unclustered antibodies are shown in Table 4.4 and Figure 4.2. Antibody KB61 showed definite surface reactivity in 6/18 cases, and weak surface reactivity in a further 5 cases. There was very weak MHC Class II expression in a few cases. Antibodies reactive with determinants on rough endoplasmic reticulum (RER) (BU11, BU18, RFD6) were strongly reactive with the cytoplasm but negative with the cell surface. Antibody 7F7, which reacted with plasmacytoid lines in the workshop was negative on myeloma cell surface and cytoplasm. Antibody PCA-1 was found unreactive with myeloma cells, but there was known to be a problem with this antibody as supplied to the Workshop. Other workers found it unreactive in most tests (Johnson et al, 1987). Three cases of true plasma cell leukaemia are included in this study : their phenotype was not different from the other cases, i.e. $CD38^{+}$, $HB8^{+}$, $CD10^{-}$, $KB61^{+/-}$.

	Cytoplasmic Reactivity			
MoAb	Negative	Weak	Moderate	Strong
BU25 (Jo5)	11			
7F7	6	2		
KB61	4	2	2	3
BU 11,18	2	6	3	5
RFD6	0	3	6	3
PCA-1	6			

Table 4.4 Cytoplasmic reactivity of myeloma plasma cells with unclustered antibodies. BU25 is an anti-MHC class II MoAb. BU11, BU18 and RFD6 are directed against determinants on RER.

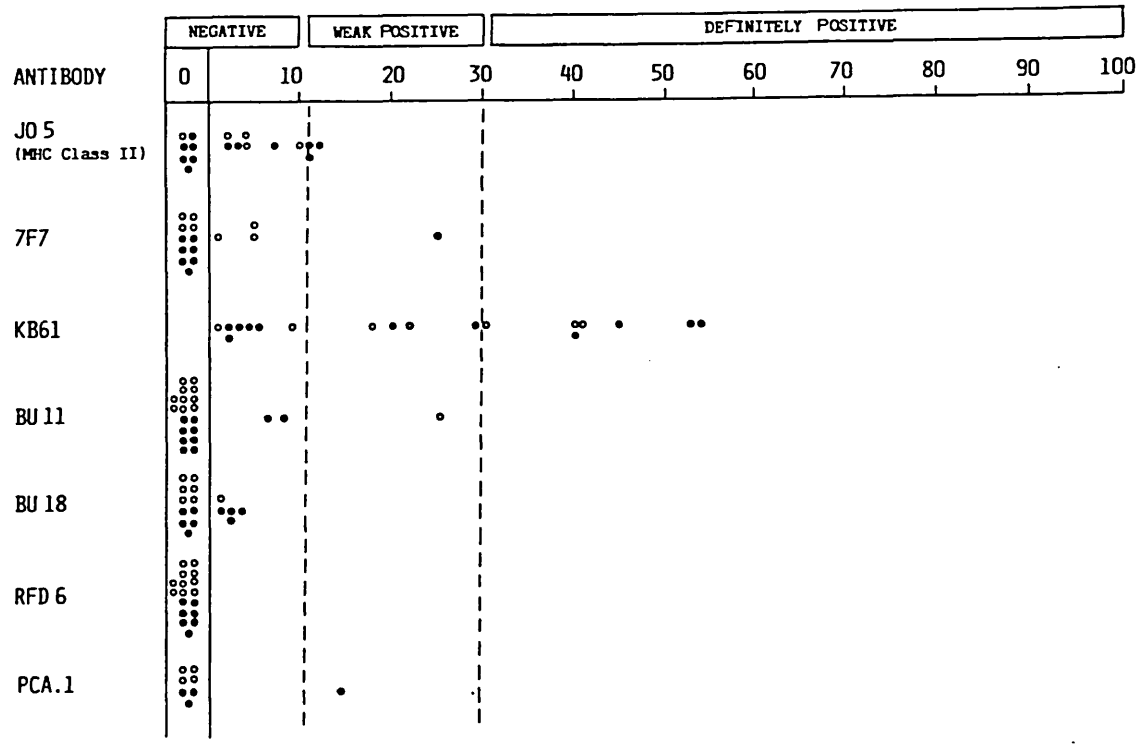


Figure 4.2 Surface reactivity of myeloma plasma cells with unclustered antibodies: percentage of positive cells by indirect immune-rosetting. Jo5 (BU25) is an anti-MHC class II MoAb; BU11, BU18 and RFD6 are directed against determinants on RER. (o=untreated, o=treated).

4.4 DISCUSSION

These results confirm that most plasma cells from the majority of cases of myeloma are $CD38^+$, $HB8(CD24)^+$, and $CD10,19\text{-}23^-$ (Nadler, 1986; Katagiri et al, 1984). Antigens of the newly designated clusters CD37, 39, w40, and 45R are also not usually present. However there is some heterogeneity of phenotype, with a few cases showing weak surface reactivity with CD9, CD10, CD19, CD20, CD23, CD25 and CD45R. The unclustered antibody KB61 (Pulford et al, 1987), which is B-cell associated, binds to the neoplastic cells of about half the cases of myeloma.

4.4.1 CD9 This antigen is expressed on a number of different haemopoietic cells including platelets, neutrophil and eosinophil polymorphonuclear leucocytes, B cells (weak), common acute lymphoblastic and non-lymphoblastic leukaemia blasts and a range of other tissue cells (Horton & Hogg, 1987). Its previously reported presence on monocytes is now disputed (Ohto et al, 1987). San Miguel et al. (1986) found surface expression of FMC8 (CD9) in 32 out of 37 cases of myeloma. In contrast, we found only 3/19 cases with a weak positive surface reaction. Katagiri et al. (1984) also demonstrated the reactivity of myeloma cells with BA2 (CD9) in 3 patients, but Caligaris-Cappio et al. (1985) failed to demonstrate any $BA2^+$ myeloma cells out of 25 cases, even though 11 of these cases expressed CD10. The technique we use for demonstrating cytoplasmic antigens will also pick up surface antigens if they are not destroyed by acetone fixation, and weak CD9 reactivity was found on immunofluorescence of cytospin preparations. It seems, therefore, that myeloma cells express CD9 weakly. The function of the CD9 molecule, being found on such a wide variety of cell types, is unknown: CD9 antibodies will aggregate platelets (Boucheix et al, 1987), and do not inhibit polymorph chemotaxis (Ohto et al, 1987). It is one of a

number of antigens shared between plasma cells and myeloid and/or monocytic cells.

4.4.2 CD10 In this series, 1/16 cases contained significant numbers of cells expressing CD10: this case also weakly expressed CD19, CD20 and CD23, and was in a terminal relapse after a very prolonged clinical course with multiple treatments. Another 9 patients had between 1 and 10% non-myeloid $CD10^+$ cells detectable: it is unlikely that these are all residual normal pre-B cells but they might be myeloma progenitors, as others have postulated. Ruiz-Argüelles et al. (1984) reported 2/14 myeloma cases having ≥15% $CD10^+$ cells in the bone marrow, and these patients' myeloma cells also expressed other B cell antigens [HLA-Class II, sIg, B1 (CD20), and B2 (CD21)]. Durie and Grogan (1985) found 4/21 cases of myeloma were CALLA$(CD10)^+$ and showed that these patients had a worse prognosis. Caligaris-Cappio et al. (1985) found 9-27% $CD10^+$ cells in bone marrows from 11/25 cases of myeloma and plasma cell leukaemia using indirect immunofluorescence on acetone-fixed cytocentrifuge specimens (the other cases contained <1% $CD10^+$ cells). These $CD10^+$ cells were of lymphoid morphology, but did not express surface or cytoplasmic Ig, CD9, 22, 24, or TdT, but they were $HLA\text{-}DR^+$ and $CD38^+$. These cells do not correspond to any currently recognized stage of normal B cell differentiation. Their phenotype is not typical of pre-B cells, but more closely resembles that of centroblasts, which may be precursors of bone marrow plasma cells (Maclennan & Gray, 1986). Centroblasts are $CD10^+$, $CD38^+$ with low expression of other B cell markers such as CD23, 24, 39, sIg (Ling, Maclennan & Mason, 1987). Caligaris-Cappio et al (1985) found that the $CD10^+$, Ig^- cells could be induced to differentiate into plasma cells with tetradecanoyl phorbol acetate, and they suggested that the clonogenic precursor cell in myeloma could be $CD10^+$. The terminal aggressive phase of myeloma, sometimes associated with cells of more immature morphology and reduced

Ig secretion, could represent the outgrowth of this $CD10^+$ cell.

4.4.3 CD24 Strong surface expression of the pan-B and granulocyte antigen CD24, as recognized by HB8, was shown by 24/27 samples. This reactivity was not shown by other CD24 antibodies. There is known to be heterogeneity of CD24 antibodies, e.g. in their reactivity with vascular endothelium (Ling et al, 1987), or with neuroblastoma cells, and in their cross-blocking activity (Stockinger et al, 1987). It could be that it is only the HB8 epitope that is expressed on myeloma cells, perhaps being incorporated into another molecule (Ling et al, 1987). Alternatively, this pattern of reactivity could be due to HB8 having the highest affinity of the cluster. In immunofluorescent staining, HB8 always stained myeloid cells more brightly than plasma cells. PCA-1 is also reported to react with both plasma cells and myeloid cells (Anderson et al, 1983), so this is further evidence of shared antigens between these two cell lineages.

4.4.4 CD38 The CD38 antigen occurs on both T and B cells. It was first described as present on thymocytes and activated T cells (Reinherz et al, 1980; Reinherz & Schlossman, 1981), but it is also present on B cell progenitors (e.g. on common acute lymphoblastic leukaemia and B progenitor lines) and germinal centre B cells (Ling et al, 1987). OKT10 is the prototype antibody, but all the antibodies reacted with the majority of myeloma cells. However, it was again evident that these antibodies did not always perform well in the indirect rosetting assay. Eight out of 10 cases negative by indirect immune--rosetting were found to be $CD38^+$ by indirect immunofluorescence. Little is known of the function of the CD38 molecule, but there are other antigens shared between T cells and plasma cells - e.g. Tp44 (CD28) (Kozbor et al, 1987). CD28 antibodies can induce antigen-independent T cell activation, and although this antigen is present on plasma cells, CD28 anti-

bodies do not appear to induce proliferation of plasma cells (Kozbor et al, 1987).

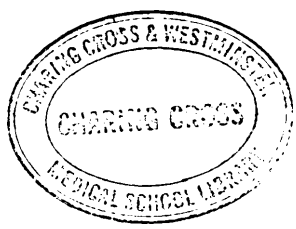
4.4.5 Unclustered Antibodies Not surprisingly, antibodies reactive with (RER) (BU 11, BU18 and RFD6) show strong reactivity with plasma cell cytoplasm. We were surprised to find a complete absence of these antigens on the cell surface, as one might expect some carry-over of the RER antigens onto the cell surface membrane during Ig secretion. These antibodies have also been shown to react with other secretory cells; e.g. thyroid acinar epithelium and exocrine pancreatic cells (Johnson et al, 1987). We have confirmed very weak or absent MHC-Class II expression of myeloma cells, as reported by other workers (Halper et al, 1978; Katagiri et al, 1984; San Miguel et al, 1986). PCA-1 (Anderson et al, 1983) as supplied to the workshop, appeared to have lost activity and was found unreactive in most tests undertaken, and no myeloma plasma cells reacted with PCA-1 in our experience. Antibody 7F7 has restricted B cell reactivity and was shown in the workshop to bind to 3 of the 4 plasmacytoid cell lines, but not to plasma cells in tissue sections (Ling et al, 1987). In our studies it reacted with neither the surface nor cytoplasm of myeloma cells. KB61 recognizes mature B cells but not germinal centre B cells, and certain macrophages (Pulford et al, 1987). It reacts with some plasma cells in tissue sections and in our studies it reacted with the surface of myeloma plasma cells in 50% of cases. An unclustered MoAb, 8A, has been reported to react with virtually all myeloma plasma cells (Tazzari et al, 1987). We have found 8A and a number of MoAb we have made ourselves to be strongly reactive with plasma cells, but they are broadly reactive with a number of other cell types, especially on tissue sections (Johnson GD and our unpublished observations).

4.5 CONCLUSION

In conclusion, the majority of myeloma plasma cells have been shown to lack expression of the B cell antigens defined by CD19-23, 37, 39, and w40. They express strongly the CD38 antigen and the HB8 epitope of CD24. Weak expression of CD9 and CD10 is also occasionally seen. The existence of small and sometimes large numbers of $CD10^+$ cells, especially in aggressive or late disease is compatible with the myeloma progenitor cell being $CD10^+$. However this does not mean that the progenitor cell is at the pre-B cell stage: it could be more closely related to the centroblast, which is $CD10^+$, $CD38^+$. That the progenitor cell is at a late B cell stage is supported by studies on a plasma cell leukaemia cell line that we have recently developed (JJN-1). The phenotype of JJN-1 is similar to that of the mature plasma cell - i.e. $CD38^+$, $HB8^+$, $CD10^-$, $CD19\text{-}23^-$ (see section 8.4.3).

CHAPTER 5

SHORT-TERM MYELOMA CULTURE USING PHYTOHAEMAGGLUTININ-T-CELL CONDITIONED MEDIA



5.1 INTRODUCTION

Izaguirre et al (1980) reported colony formation by myeloma cells using phytohaemagglutinin stimulated T-lymphocyte conditioned medium (PHA-TCM) and fetal calf serum (FCS) with methylcellulose for viscous support. We modified their technique slightly by increasing the percentage of fetal calf serum (from 20% to 30%), and adding 2-mercaptoethanol, which has been reported to increase the plating efficiency in the clonogenic assay described by Hamburger & Salmon (1977 & 1986). The aim was to develop a sensitive method that produced myeloma cell colonies from all cases of myeloma. It was hoped that the method would not support the growth of normal haemopoietic colony-forming units (e.g CFU-GM), so that the myeloma colonies could be accurately quantitated. Alternatively, if normal haemopoietic colonies were grown, an easy method for distinguishing the myeloma colonies from the others was required. As will be shown, we found that this particular culture system is unsatisfactory, as the predominant growth occurring is CFU-GM and when myeloma cell 'colonies' occur, they appear to be the result of homotypic adhesion ('clumping') rather than true clonogenic growth. As we now believe that the aggregates that were counted were not in fact colonies, they will be called 'cellular collections' for the purposes of this discussion.

5.2 PATIENTS AND METHODS

5.2.1 Patients

Thirty-seven samples of bone marrow from 34 patients were cultured with the aim of growing myeloma colonies using PHA-TCM and FCS as the growth factors. Samples were taken from 18 patients before any treatment had been given. Nineteen were from treated patients, although bone marrow was always taken at least 3 weeks after the last course of chemotherapy. See Table 5.3 in Results (p106) for details of the percentage marrow

infiltration by plasma cells, and treatment status of each patient. Most of the patients were in the MRC Myeloma Vth trial and so received ABCM or melphalan alone but exact details, e.g. which drugs and for how long, are not available.

5.2.2 Production of PHA-TCM

Over the time these experiments were being done, 4 different batches of PHA-TCM were used, made up as described in the materials and methods chapter (section 2.3.6(a)). The different batches of PHA-TCM produced similar results, even though the first two batches were produced with the PHA concentration being 0.025% instead of 1%.

5.2.3 Culture conditions

A mononuclear cell preparation was cultured for myeloma colony growth in 96-well microtitre plates as outlined in section 2.3.6(a). The culture conditions are summarised in Table 5.1.

Fetal Calf Serum	30% by volume
PHA-TCM	4% by volume
2-Mercaptoethanol	$5x10^{-4}$Molar
Methylcellulose (1500 centipoise)	0.8% by volume
Cells	$2-4x10^{5}$/ml
Iscove's/Ham's medium	To final volume

Table 5.1 Conditions for culture with PHA-TCM.

After culture for 5-7 days, single cellular collections could be removed, using a drawn-out glass pasteur pipette and spread on a slide. Alternatively, material from several microtitre wells was pooled, washed in medium and cytospun onto slides for morphological and immunological examination. It was found that some cellular collections remained on the cytospin preparations and these were assumed to represent at least part of the original collections seen in the culture wells.

5.2.4 Incubation with cytotoxic drugs

Populations of cells were incubated with cytotoxic drugs in some experiments as described in section 2.3.8.

5.2.5 Irradiation of cells

In some experiments, some cells were irradiated using the ^{60}Cobalt source of the Radiation Physics department of Birmingham university. Doses from 250-4000centiGray (cGy) were given by exposing the cells for varying lengths of time at 9cGy/second.

5.3 RESULTS

5.3.1 Culture appearance

The culture plates were examined daily using an inverted microscope under low power. After 1-2 days, in all but 3 cases, cellular collections appeared. Usually the number of collections did not increase after this; in fact, often the number decreased as their size increased. After 5-7 days the collections showed signs of deterioration, with a tendency to disperse. A representative series of counts is shown in Table 5.2:

Table 5.2 Day	0	1	2	3	4	5	6	7
Number of cellular collections	0	172	140	132	132		117	111
Appearance	small (20-30 cells)			medium (30-50 cells)			dispersing & deteriorating	

5.3.2 Numbers of cellular collections formed

The percentage of plasma cells in the bone marrow samples varied widely. The number of cellular collections per 10^5 cells plated (x 100%) was in the range 0-0.56% and showed no correlation at all with the number of plasma cells in the original marrow, nor with the patient's treatment status (See Table 5.3; Figure removed from revised thesis). However, in 3 cases, the number of collections formed was linearly related to the number of cells plated (Figure removed from revised thesis).

5.3.3 The influence of various growth factors

In some preliminary experiments 2-mercaptoethanol was excluded, but no effect on the number of cellular collections was demonstrated. Iscove's medium contains 1 mg/L human transferrin. There was no difference in the number of cellular collections with or without supplementary transferrin.

Patient	PC% in Bone	Number of 'collections' Formed/10^5 cells
	Untreated Patients	
OD	100	0
SP	85	96*
SM	72	?
FA	72	288
FU	65	?
DI	60	447
DY	57	253
SH	55	?
HE	53	451
MC	51	150
WA	50	128
CL	35	44
BI	35	?
HA	33	?
MO	27	212
PA	19	182
TH	18	0
CH	17	55
	Treated Patients	
ME	97	120*
GO	95	212*
BU-1	80	246
MO	79	?
ST	74	25
JO-1	68	229
BU-2	56	280
JO-2	45	118
CL	32	190
SI	20	320
HO	19	324
SK	17	0
MC	16	226
HU	15	211
HI	12	0
ON	8	103
SU	6	87
BU-3	5	260
VA	1	560

Table 5.3 Numbers of cellular collections formed in PHA-TCM culture of MM bone marrow, related to the %PC in the marrow smear.

*: many pure PC collections seen on cytospins.
?: cellular collections formed but were not counted.

However, transferrin and 2-mercaptoethanol were added to the medium for all other experiments. In experiments to assess the requirement for PHA-TCM, only 2 of 10 bone marrow samples failed to develop cellular collections in the absence of PHA-TCM and one other marrow showed progressive increases in the numbers of these collections up to 20% (vol/vol in the final mixture). PHA-TCM at 4% was used in most experiments.

5.3.4 Morphology of cultured cells

The cytospins generally showed a very mixed population of cells. In the first 2-3 days, the cell proportions were similar to the original mononuclear cell fraction, but thereafter the number of plasma cells usually reduced, and the number of myeloid cells and macrophages increased. The cellular collections seen on cytospins were composed of either granulocytes and macrophages or mixed collections of myeloid cells/macrophages and plasma cells in the same collection (Figure 5.1). The morphology of these collections when they had been individually picked off the plates was the same. Cytochemistry confirmed that the majority of the cells in the collections were of myeloid (chloroacetate esterase positive) and macrophage (butyrate esterase positive) origin (Figure 5.2). A representative series of differential cell counts on cytospins is shown in Table 5.4.

Days in culture	0	1	2	3	4	6	7
(%) Plasma cells+ Lymphoplasmacytoid	94	90	90	72	74	60	50
Myeloid	3			23	23	32	45
Macrophage	0			4	2	5	5
Erythroid	3			1	1	3	0
Cellular Collections	0	172	140	132	132	117	111

Table 5.4 A representative series of differential cell counts on cytospins from a culture of MM bone marrow in PHA-TCM.

When bone marrows with very high plasma cell counts were plated out some, but by no means all, of the cellular collections were composed purely of plasma cells (Figure 5.3). In some cases plasma cells were seen palisaded around a central macrophage (Figure 5.4). In two experiments wells were plated out without PHA-TCM, i.e. medium + FCS only: although the number of colonies formed was less than with PHA-TCM, the cytospins showed a higher proportion of pure plasma cell collections.

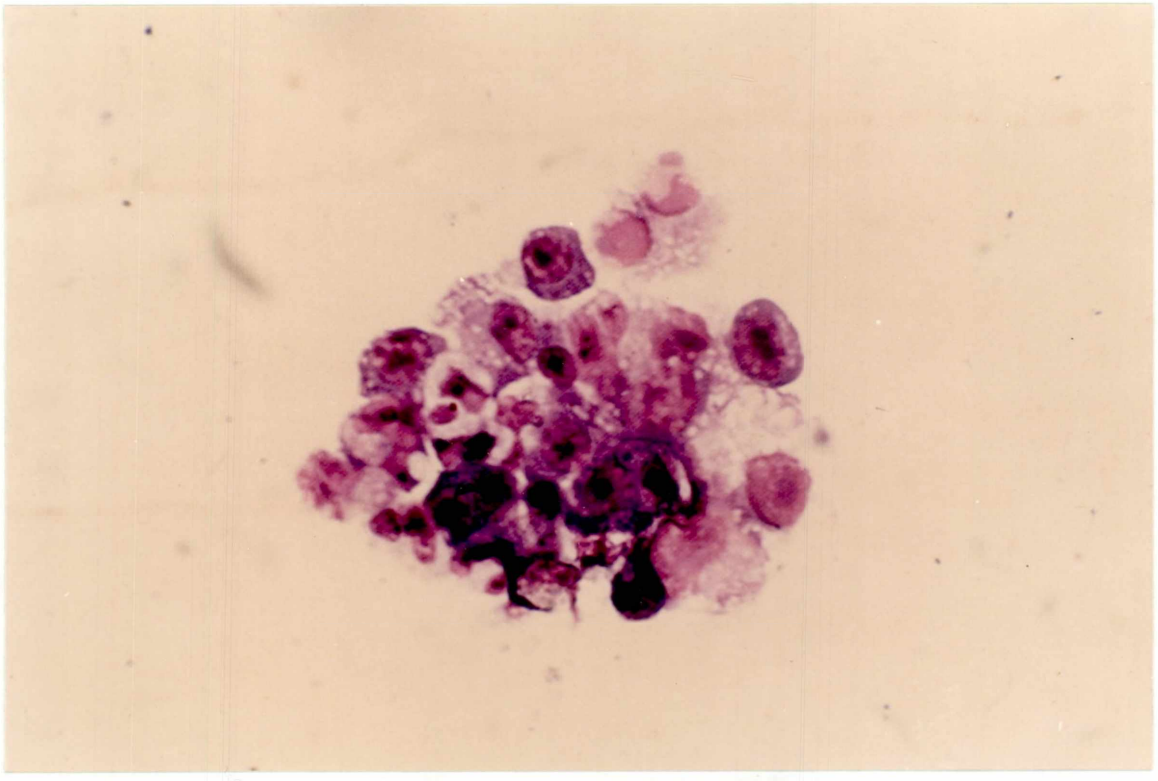


Figure 5.1 A cellular collection from a PHA-TCM culture of MM bone marrow, showing the presence of plasma cells, myeloid cells and macrophages.

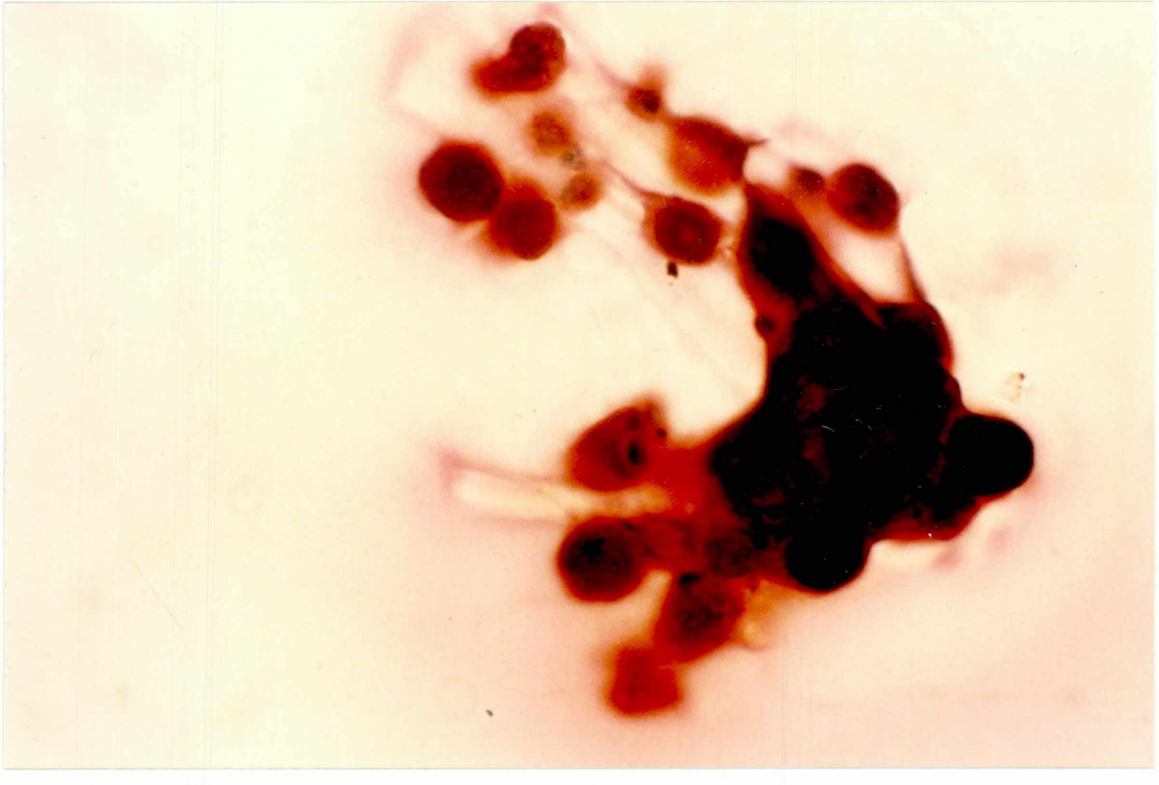


Figure 5.2 A cellular collection from a PHA-TCM culture stained for butyrate esterase, showing typical monocyte/macrophage positivity.

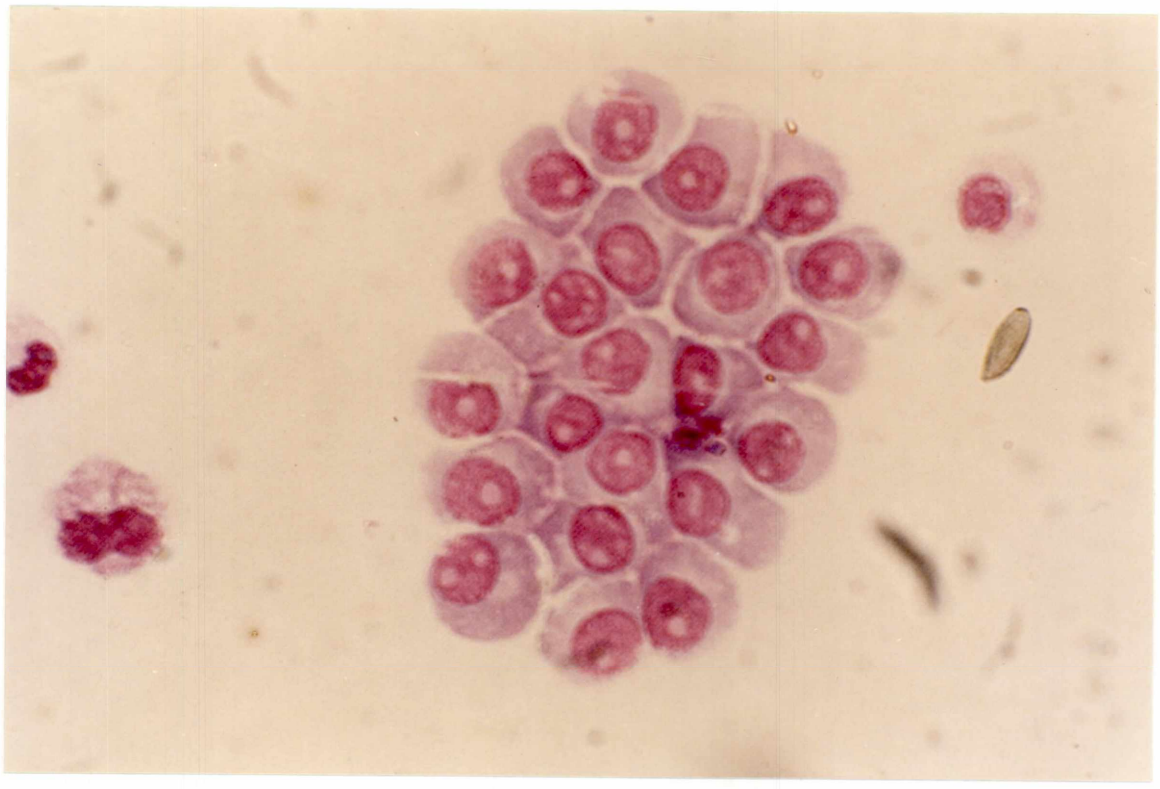


Figure 5.3 A cellular collection from a culture of a MM bone marrow containing a high percentage of plasma cells. A pure plasma cell "colony" is seen.

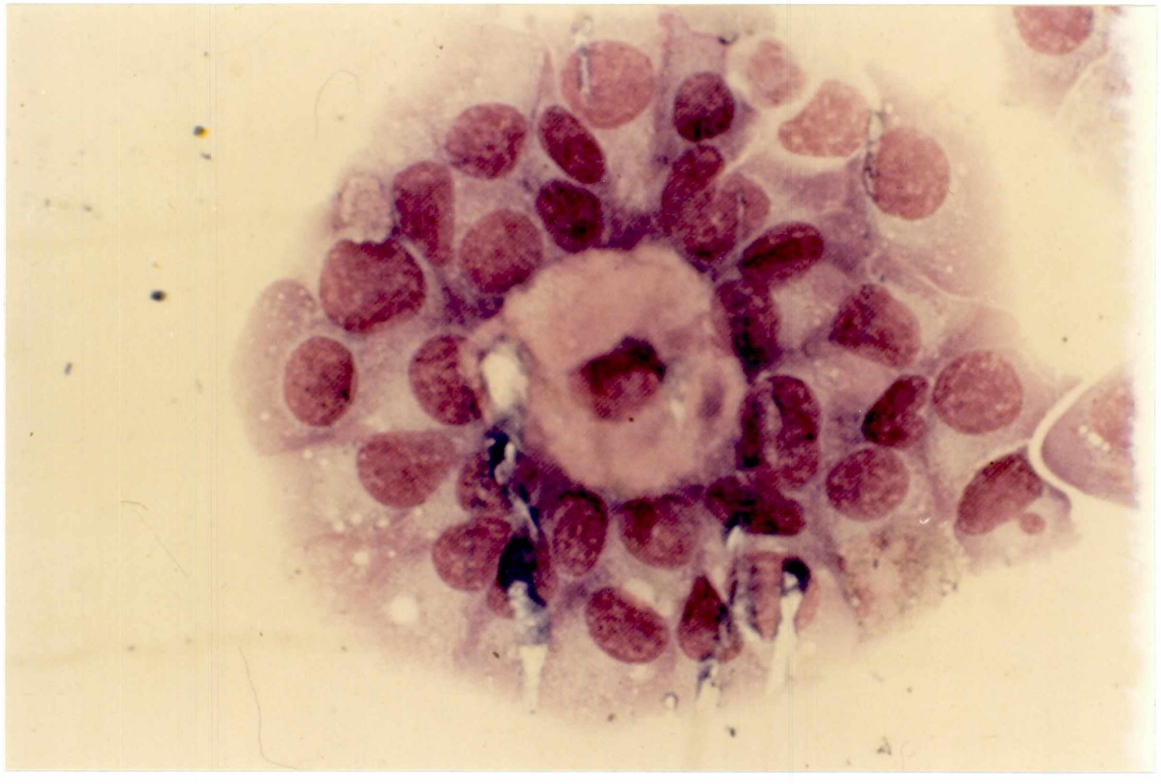


Figure 5.4 A cellular collection showing plasma cells palisaded around a central macrophage.

5.3.5 Immunofluorescence

This was performed on cytospin preparations from most of the cultures. This demonstrated definite monoclonality, by kappa/ lambda restriction of the plasma cells present, corresponding to the light chain isotype of the patient's paraprotein (Figure 5.5). However, most cellular collections were mixed in that they contained both cIg positive and cIg negative cells. In a few cases cytospins were tested with an anti-myeloid MoAb, 4.48 (gift from Dr G Brown, Immunology, Birmingham university): this reacted with some cells in the collections, even when the original marrow was very heavily infiltrated with plasma cells.

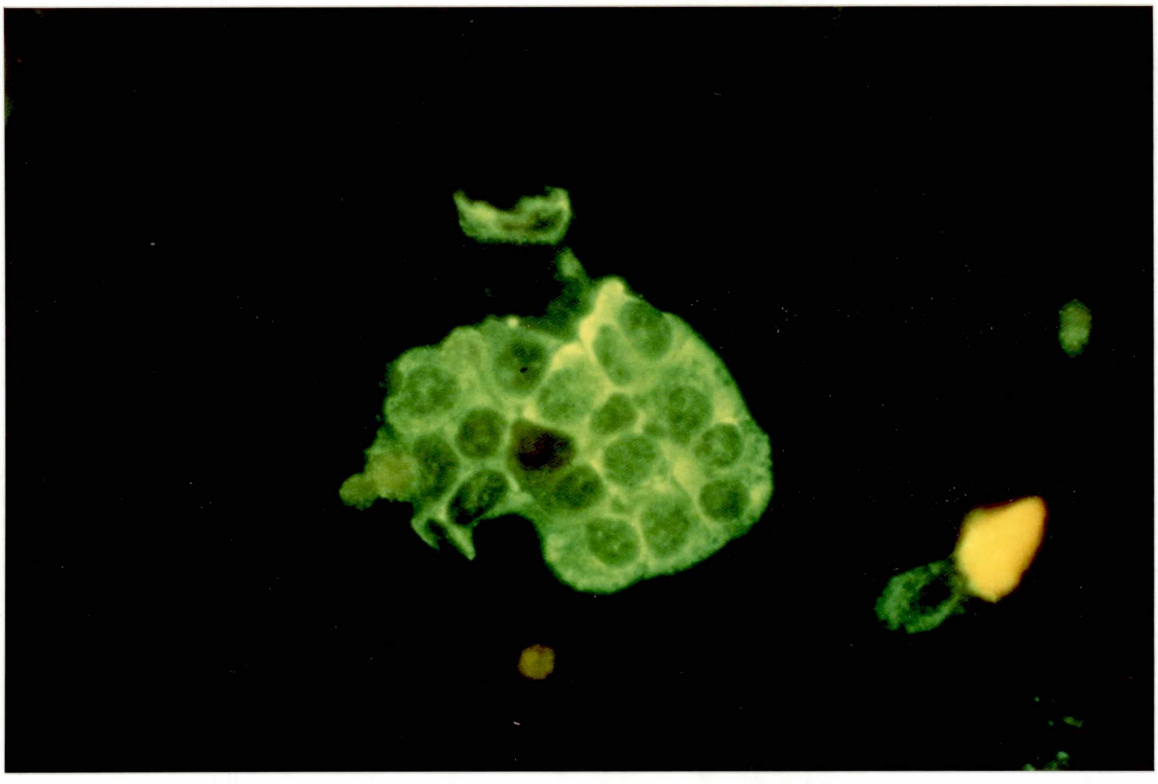


Figure 5.5 Immunofluorescence of a cellular collection from MM BM cultured in PHA-TCM, showing expression of lambda light chain in the cytoplasm of the cells (kappa was negative).

5.3.6 Effect of Irradiation

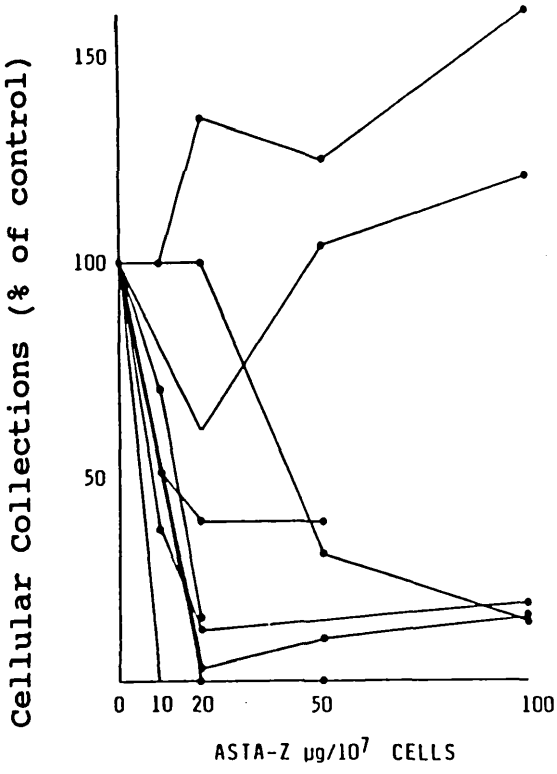
The effect of irradiating the cells before plating was examined in 5 cases. In 4 cases a single dose of 2500cGy (at 9cGy/second) was investigated. After 1-2 days in culture the

irradiated cells formed identical cellular collections to unirradiated cells in both number and size. However by 5-7 days the irradiated cells were degenerating and the collections dispersing to a much greater extent than in the unirradiated cell cultures. In one case, doses of irradiation from 500-4000 cGy were investigated. No apparent dose-response curve was observed, with similar numbers of cellular collections counted in the first few days after plating (Figure removed from revised thesis).

5.3.7 The effect of incubation with cytotoxic drugs

Myeloma bone marrow cells from 10 patients were incubated with varying doses of either Mafosfamide (Asta-Z) (9 samples) or 4-Hydroperoxycyclophosphamide (4HC) (5 samples). These drugs appeared to inhibit cellular collection formation in myeloma cultures (Figure 5.6). However this was quite unlike the progressive inhibition of growth of CFU-GM after incubation of normal bone marrow with Asta-Z or 4HC (Figure 5.7). Even at concentrations of each drug that produced 95% killing of normal CFU-GM, consistent effects on cellular collection formation in myeloma culture were not seen.

(a)



(b)

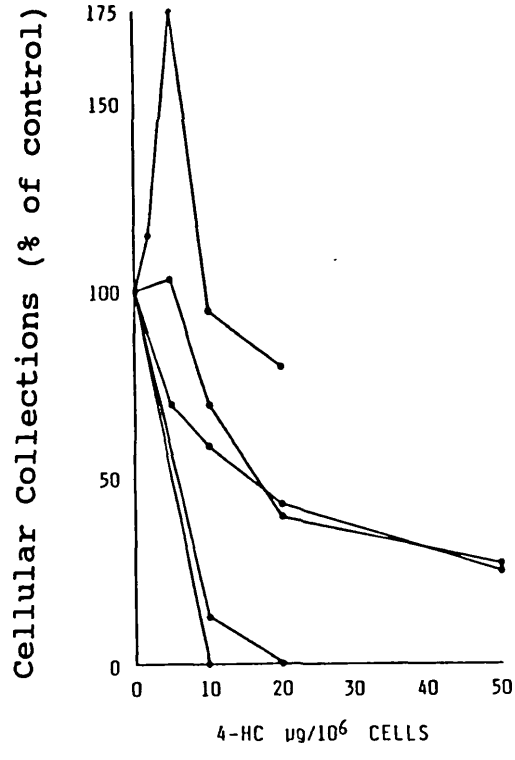
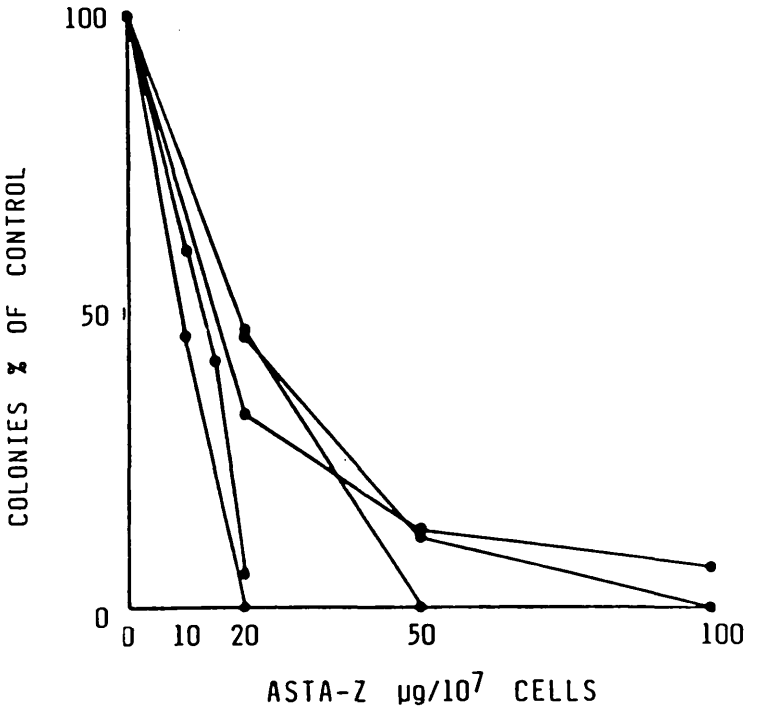


Figure 5.6 The effect of Asta-Z (a) and 4-HC (b) on formation of cellular collections in PHA-TCM culture of MM marrow.

(a)



(b)

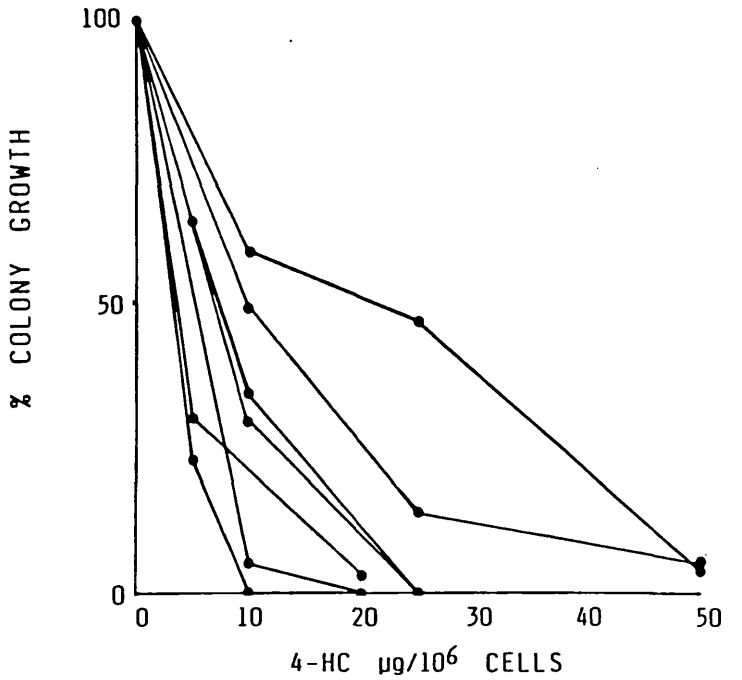


Figure 5.7 The effect of Asta-Z (a) and 4-HC (b) on growth of CFU-GM from normal bone marrow.

5.3.8 Effect of T cell and/or adherent cell depletion

In a few cases sufficient cells were available to investigate the effect of removal of T cells and/or adherent cells. The effect of recombining the various fractions (i.e. non-T, non-adherent cells, with or without irradiated T cells or adherent cells) was also examined. The predominant cell type seen in the cultures was still granulocyte-macrophage, but the number of cellular collections formed varied. The results of one experiment are shown graphically in Figure 5.8.

In this experiment it can be seen:

1. Without adherent cells the number of cellular collections formed is greatly reduced, and it is reduced still further by the removal of T cells.
2. The addition of an irradiated 'T cell' fraction 1:1 with the 'B cell' fraction results in enhanced formation of cellular collections.
3. The greatest enhancement of cellular collection formation is seen with the addition of an irradiated 'adherent' cell population.

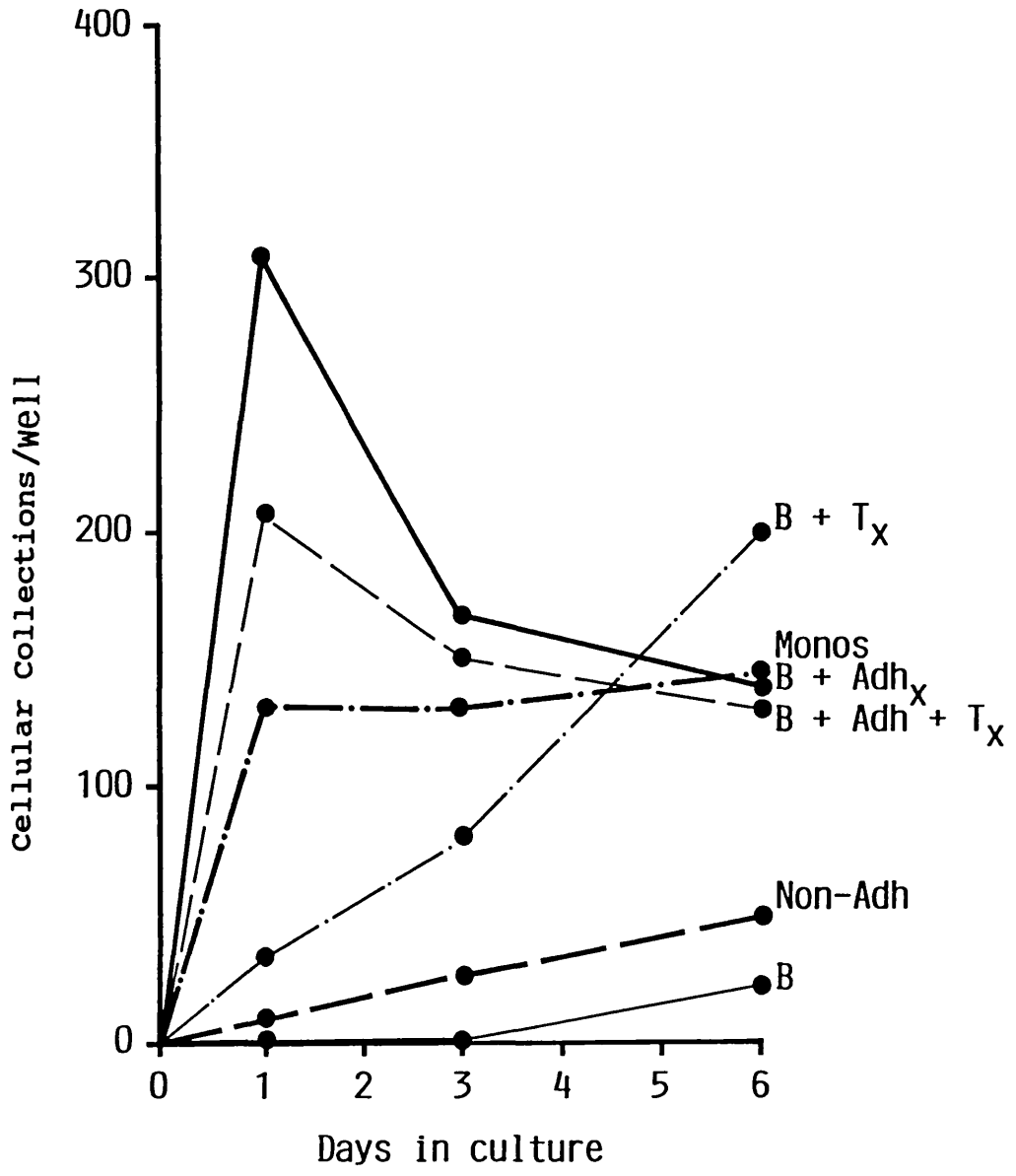


Figure 5.8 The effect on cellular collection formation of removing and/or replacing T cells and/or adherent cells before culturing.

Monos = unmanipulated mononuclear cells @ 4.5×10^4 cells/well
Non-adh = non-adherent cells @ 3×10^4 cells/well
B = Non-adherent cells, T-depleted by one round of E-rosetting @ 2×10^4 cells/well
T_X = T cells obtained by E rosetting, irradiated (20 Gray) @ 3×10^4 cells/well
Adh_X = Adherent cells removed from the surface of the flask after the adherent cells were taken off, irradiated (20 Gray) @ 3×10^4 cells/well.

5.4 DISCUSSION

In order to prove that this type of culture system was a true clonogenic assay, (i.e. that each colony arose from a single precursor cell) ideally the following criteria should have been fulfilled:

1. Immobility. Cells should not migrate within the culture system. Mixed collectons - i.e. containing normal haemopoietic cells and myeloma cells - would not be expected to occur.
2. Linearity . 'Colony' numbers should be linearly related to the number of cells plated over a useful range of cell concentrations.
3. Replication. At least some cells within the cellular collections should be shown to be synthesising DNA.
4. Drug and radiation sensitivity. In agreement with other human cell systems, clonogenic cells would be expected to show a dose-responsive reduction in colony growth with increasing amount of cytotoxic drug treatment or irradiation.
5. Self-renewal. Ideally, harvesting of 'colonies' and replating should be done to demonstrate the self-renewing capacity of the cells.
6. Reliability and sensitivity. Ideally most, if not all, cases of the disease being studied should grow reliably in the culture system. Enough colonies should grow, i.e. at least 10-100 per 10^5 cells plated, to permit meaningful experiments, e.g. testing drug sensitivity.

This PHA-TCM-based myeloma culture method will now be discussed with reference to these criteria.

5.4.1 Immobility

Assuming that cells are plated out as a true single cell suspension (see below under 'linearity'), collections of cells

that form during culture can arise either by proliferation or aggregation. It is necessary to show that cells do not migrate together, e.g. because of a survival advantage of being in contact with each other. It is apparent from these studies that methylcellulose does not inhibit the movement of cells within it, for the following reasons:

(a) Settling of cells. After plating, cells in methylcellulose all settle onto the bottom of the culture dish, as compared to agar where the cells stay fixed in position at all levels.

(b) Mixed colonies. On analysis of the cellular collectons, it was frequently found that they contained both monoclonal plasma cells and granulocyte/macrophage cells. It seems very unlikely that these cells all arose from one clonogenic precursor. In general, the collections were composed of cells in roughly the same proportion as in the mononuclear cell fraction of bone marrow as plated out. In cultures from bone marrows highly infiltrated with plasma cells, some pure plasma cell collections were seen, but none were seen if plasma cells were <40% of nucleated cells. In some marrows which were cultured with and without PHA-TCM, purer PC collections were seen without PHA-TCM. This suggests that factors within PHA-TCM (possibly the small amounts of PHA itself) may be responsible for drawing these aggregates of heterogeneous cells together. A useful experiment would be to mix cells from a kappa and a lambda myeloma together and plate them out to see if collections expressed just one, or both, of the light chain types, (cf Bast et al, 1982). We did not do this because two suitable samples were never available on the same day.

(c) Timing of formation of the cellular collections. The cellular collections formed very rapidly in culture with large numbers of collections being seen on day 1, after just overnight incubation. Thereafter the collections increased in size but, if anything, their numbers reduced. In retrospect, it is

our impression that the collections were surrounded by a 'halo', i.e. an area without cells. This suggests that cells were being drawn into aggregates and then these aggregates were coalescing. The formation of a true colony, even of 20 cells, would have implied 4-5 cell divisions within 24-48 hours. This would mean a very short intermitotic interval of 6-12 hours, in what is otherwise regarded as a slow-growing tumour.

(d) Agar cultures. In a few cases myeloma marrows were cultured in both methylcellulose- and agar-based cultures with otherwise identical conditions. Whereas cellular collections formed in methylcellulose, no myeloma sample ever formed a PC collection in 0.3% agar, though occasional granulocyte-macrophage colonies were seen. It could be argued that agar inhibits myeloma colony-forming units, but it is more likely that agar is better at immobilizing the cells, and thus stops aggregates forming.

5.4.2 Linearity

Our experiments showed a linear relationship between cellular collections formed and cells plated for cell concentrations between 1 and $5x10^5$/ml. Linearity is quoted as one 'proof' of a true clonogenic assay. While it is clearly necessary to show linearity, it is not a sufficient proof of clonogenic growth; it is likely that cellular aggregation will be related to cell density at least over a limited range. This will apply if the number of aggregates formed is, say, proportional to the number of accessory cells, e.g. T cells or macrophages, that act as central cells onto which the other cells attach.

Even if the relation is non-linear, e.g. if it is proportional to the distance between cells on the bottom of a culture dish (i.e. related to the square root of cell density), it might appear pseudo-linear over a small range especially if compounded by other variables. Most culture systems, including those used in our experiments, have only been tested for linearity

over a small range of cell concentration, 1-2 logs. In particular, linearity at very low cell concentrations is not usually reported. If in fact the relation is non-linear at low cell densities, this would imply: (a) the presence of clumps in the original 'single cell suspension'; or (b) cellular aggregation; or (c) the need for a baseline number of accessory cells to support growth. In any case it nullifies the technique as a clonogenic assay, especially for the detection of small numbers of malignant precursor cells after a bone marrow 'purging' procedure.

5.4.3 Replication

Studies of cellular DNA synthesis and replication were not performed on our PHA-TCM cultures. However it should be noted that even to prove that cells within collections are synthesising DNA, e.g. by using autoradiography or bromodeoxyuridine incorporation, does not prove that they are actually dividing or that they all proliferated from one cell. Certainly B cells are known to be able to enter S phase of the cell cycle, i.e. DNA synthesis, but not proceed to mitosis (Gordon & Guy, 1987). Only an increase in plasma cell numbers would prove that real plasma cell growth had occurred. However it is virtually impossible to show this in this sort of assay where the plating efficiencies are <1% and many colonies contain only 20-50 cells.

5.4.4 Sensitivity to cytotoxic drugs and radiation

We found that formation of cellular collections within 1-2 days was not prevented by doses of radiation that normally completely inhibit cell division, e.g. 2000-4000cGy. However, after 5-7 days the collections had deteriorated and dispersed, presumably because the cells were not viable. There was also no clear-cut dose-response curve as is normal in radiation-treated cells (Hall, 1978). This implies that the collections cannot have formed by cell division but must have been cellular aggregates. As Selby et al (1983) comment in general about

assays of human tumour stem cells, some of our cases of myeloma marrow showed the 'untenable observation' of increased cell 'survival' with increasing drug dosage (Figure 5.6). This suggests we were mistaking clumps for colonies.

5.4.5 Self-renewal

We did not study the self-renewal capacity of the cells within cellular collections.

5.4.6 Reliability and sensitivity

Cellular collections were reliably observed, in 35/38 cases, and the 'plating efficiencies' (up to 0.5%) were similar to those of Izaguirre et al, 1980. This is considerably higher than the 0.001-0.1% described by Hamburger and Salmon (1977). In view of the above comments about the invalidity of the method, this is irrelevant.

5.5 CONCLUSIONS

Unfortunately we were unable to validate this method of culture as a clonogenic assay for myeloma progenitors. It now appears that what we observed and counted as colonies were in fact artefacts. They were clumps of cells which had aggregated in the culture, in part drawn together by the action of the lectin PHA. Accessory cells such as macrophages appeared to play a role and it may be significant that the myeloma cells were sometimes seen lined up around macrophages. The nature of this cell-cell interaction and adhesion is unknown, but may involve the production of growth factors by the macrophages. With the difficulties of eliminating contaminating granulocyte-macrophage growth, it is likely to prove very difficult to validate these types of clonogenic assays. Hence our attention was transferred to the long-term culture of myeloma cells where increases in cell numbers can be more readily identified. These experiments are described in chapter 7.

CHAPTER 6

THE EXPRESSION OF INTERLEUKIN-2 RECEPTORS ON MYELOMA BONE MARROW CELLS BEFORE AND AFTER SHORT-TERM CULTURE WITH PHYTOHAEMAGGLUTININ AND INTERLEUKIN-2

6.1 INTRODUCTION

Interleukin-2 (IL2) is a glycoprotein lymphokine of 15kilo-Daltons originally called T-cell growth factor, because of its proliferative effect on T cells through a specific membrane receptor. On stimulation of T cells with antigen or mitogen, IL2 receptors (IL2R) are expressed on their surface (Uchiyama et al, 1981). IL2, which is then secreted, acts in an autocrine fashion on the T cells to stimulate further proliferation and IL2R expression (Malek & Ashwell, 1985; Smith & Cantrell, 1985). IL2R are lost from the surface of the T cells and can be found in culture supernatant (Rubin et al, 1985; Reske-Kunz et al, 1987) or, in vivo, in serum (Wagner et al, 1987). The IL2R, which contains 2 subunits of 55 and 75 kDa (Waldmann et al, 1987), is also found on activated B cells (Waldmann et al, 1984) and monocytes (Herrmann et al, 1985). There is controversy over the role of IL2 in B-cell growth and differentiation, although undoubtedly at high concentrations it can stimulate activated B cells to divide (Mingari et al, 1984). Whether it also induces differentiation of B cells into antibody-secreting cells is uncertain (Miedama & Melief, 1985).

Interleukin-2 receptors (IL2R) have been demonstrated on the cells of a number of different leukaemias, especially T-cell acute lymphoblastic leukaemia/lymphoma associated with HTLV-1 infection (Wong-Staal & Gallo, 1985), where they almost certainly have a pathogenic role. Indeed anti-IL2R MoAb have been used to treat this leukaemia. IL2R are expressed on cells from some cases of B-CLL (Touw & Löwenberg, 1985; our own unpublished observations), and hairy cell leukaemia (Korsmeyer et al, 1983). Their cell surface expression can be induced in other cases of these diseases using inducing agents such as phorbol esters, phytohaemagglutinin (PHA) and IL2, or gamma-interferon (Touw & Löwenberg, 1985). As hairy cell leukaemia may be a malignancy of activated B cells at a pre-plasma cell

stage we investigated whether MM cells expressed IL2R before and after exposure to PHA and IL2.

6.2 METHODS

6.2.1 Patients and cell preparation

Bone marrow was aspirated from 23 patients with MM, and a mononuclear cell preparation made as described in chapter 2. For further details of the patients' treatment status and PC infiltration in the bone marrow, see Table 6.2 (p127).

6.2.3 Cell culture

The mononuclear cells were suspended in 96-well microtitre plates in the following culture medium (Table 6.1):

Phytohaemagglutinin	1% by volume
Interleukin-2	100units/ml
Fetal calf serum	30% by volume
Iscove/Ham's media (1:1)	

Table 6.1 Conditions for culture with PHA and IL2

After culture for 5 days the cells were harvested from the wells and phenotyped as above. Some were also cytospun onto slides and stained for morphological examination by Leishman's stain.

6.2.3 Phenotyping

In 14 cases, the mononuclear fraction was phenotyped by indirect immune rosetting for the expression of CD3 (Leu4), CD24 (HB8) and CD25 (anti-IL2R), before and after culture. In a few cases, an attempt was made to study the cells by double immunofluorescence looking for co-expression of cytoplasmic Ig and surface IL2R: see section 2.3.2 for more details.

6.3 RESULTS

6.3.1 Culture

Cells clumped within the first 24 hours, and this was not different in cultures of cells given between 10 and 40 Gray irradiation. Over the next few days the clumps enlarged slightly. Cytospin preparations showed these clumps to contain both mature plasma cells and less mature cells with open chromatin and nucleoli, i.e. blasts (Figure 6.1). Sometimes the clumps also contained other cell types - e.g. myeloid cells or macrophage cells, as in the PHA-TCM cultures described in chapter 5.

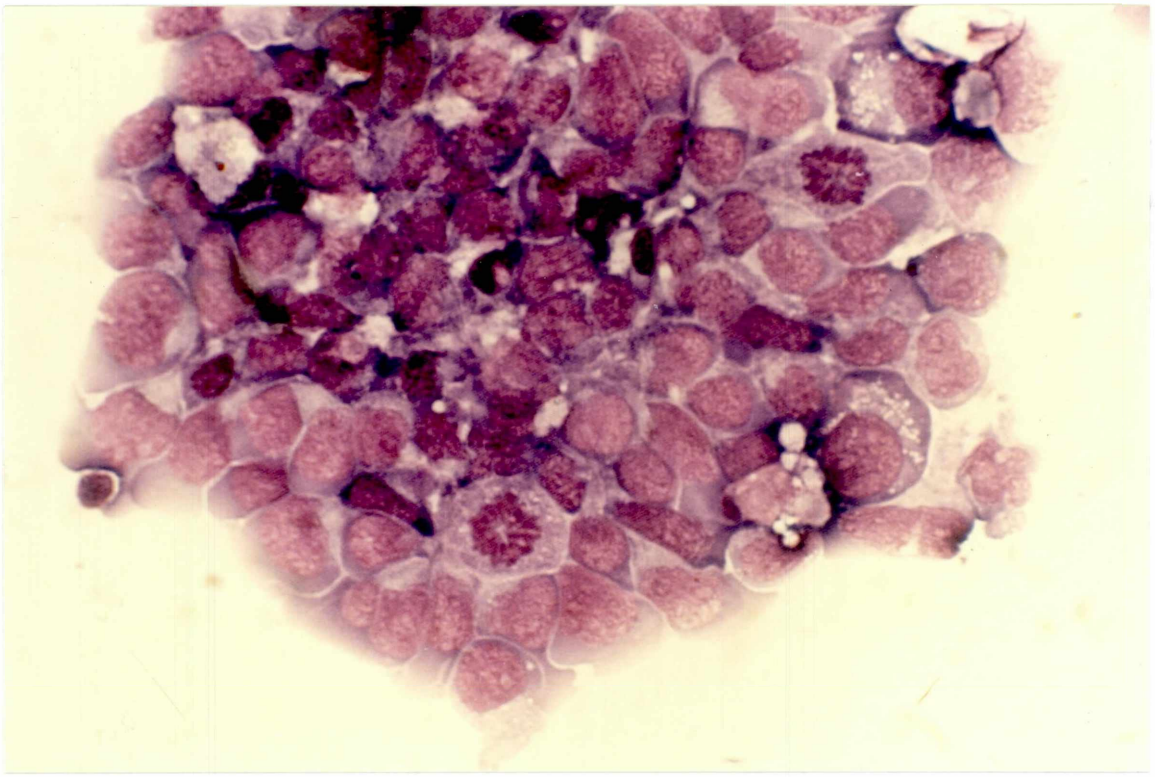


Figure 6.1 A cytospin preparation of a MM bone marrow after 5 days in culture with PHA and IL2.

6.3.2 Phenotyping

Fourteen bone marrow samples were phenotyped both before and after culture with PHA and IL2. All the samples were tested for IL2R expression (anti-IL2R, CD25), and T cell numbers (Leu4, CD3). Most were also tested for HB8 (CD24) expression, as our best available marker for plasma cells (see chapter 3). The results are presented in Table 6.2, together with the treatment status, bone marrow PC infiltration and the cytospin appearance of cultured cells for each patient. The results showed 3 different patterns:

(a) Plasma cells expressing IL2R. Figure 6.2 shows the results for 7 cases of MM marrow, which demonstrate greatly increased numbers of cells expressing IL2R after culture. There was either no, or only slight, increase in the number of T cells, and in the 4 cases tested the number of HB8 positive cells stayed at about the same level. In some cases there was considerable overlap in the numbers of cells expressing HB8 and IL2R (i.e. with percentages adding up to >100%), implying co-expression of the 2 markers on the same cells.

(b) No expression of IL2R after culture. A second group of 4 samples showed no increase in cells expressing IL2R (Figure 6.3).

(c) Contaminating T cells expressing IL2R. A third group of 3 samples started with higher numbers of T cells (4-17%), and showed significant increases in both the number of T cells, and the number of IL2R-expressing cells (Figure 6.4). It seems likely that most of the $IL2R^+$ cells were activated T cells in these cases.

Patient	Treated?	%PC	Cytospin Appearance	Pre-culture HB8	IL2R	Leu4	Post-culture HB8	IL2R	Leu4
EV	No	73	L/PC		0	0		42	14
OA	No	100	L	60	1	0	48	16	0
WA	No	50	PC?	35	0		38	1	
M-HE	No	53	PC/GM	57	0	2	64	54	2
DY	No	57	PC/L	70	5	7	63	55	5
MO	Yes	79	PC/L	80	4	8	25	0	0
SM	No	72	L/PC/GM		0	2		26	5
HI	Yes	20	L/PC/GM	67	6	16	57	30	22
FU	No	65	L/PC	76	1	2	79	41	25
BI	No	35	L/PC/GM		0	10		47	53
SH	No	55	L/PC/GM		0	9		7	4
DI	No	60	L/PC		0	4		53	6
ST	Yes	74	L/PC		2	0		20	14
UF	No	65	PC		0	0		5	1
LA	No	23	GM						
HI	Yes	25	GM						
CL	Yes	32	PC/GM						
GO	Yes	95	PC						
R-HE	Yes	52	PC/L						
OD	No	100	L/PC						
SW	No	30	*						
ED	Yes	46	PC/GM						
CO	No	34	PC/GM						

Table 6.2 The treatment status, and % plasma cell infiltration (%PC) in the bone marrow smears of the 23 patients studied.

The cytospin appearance of the cultured cells:
GM: Granulocytes and macrophages
L: Lymphocytes/lymphoblasts
PC: Plasma cells
*: A few small aggregates only

The expression of HB8 (CD24); IL2R (CD25); and Leu4 (CD3) by indirect immune-rosetting before and after culture in PHA and IL2: % of positive cells. Fourteen cases only.

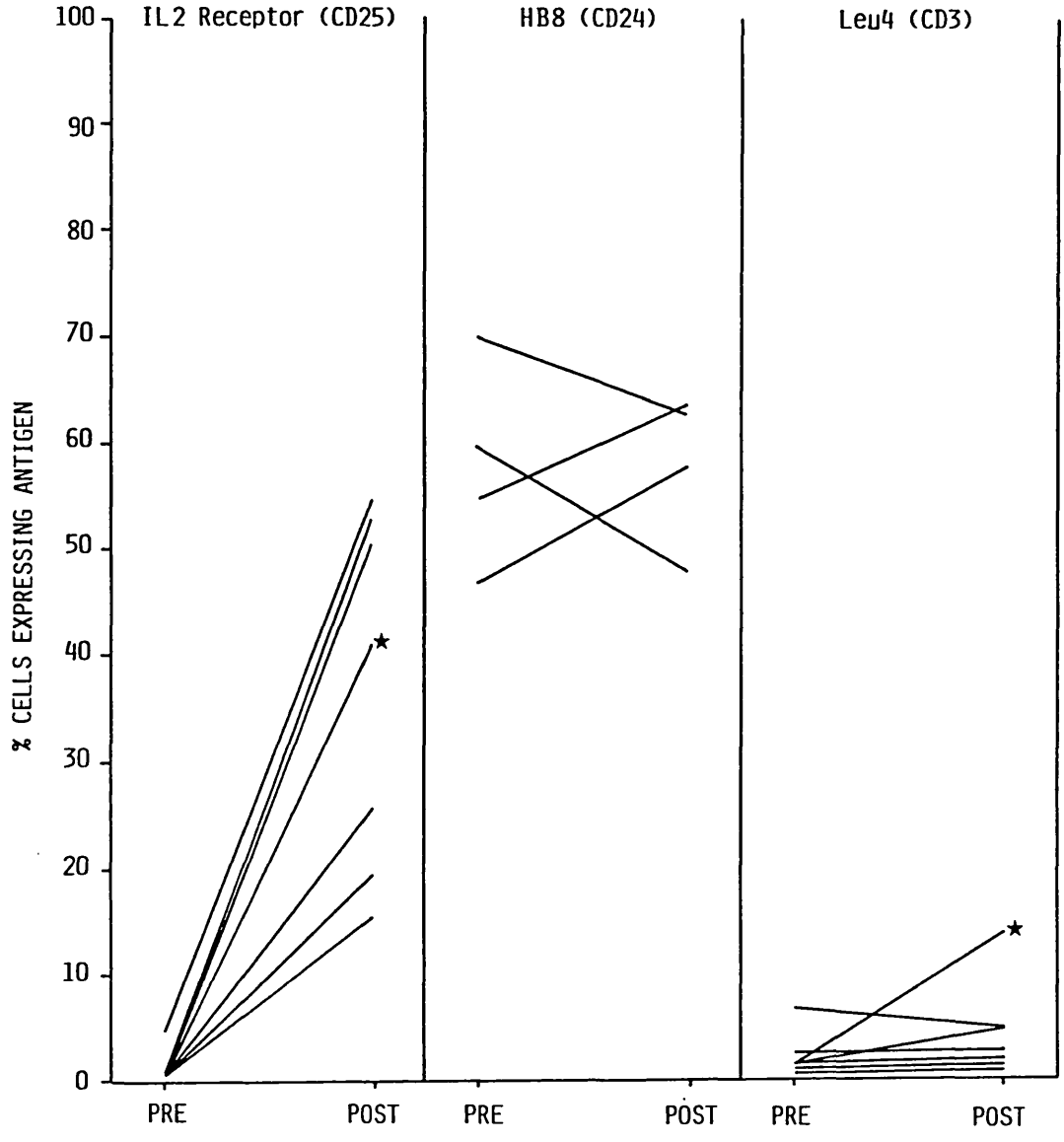


Figure 6.2 The percentage of MM bone marrow cells expressing IL2R (CD25), HB8 (CD24), and Leu4 (CD3) before and after culture with PHA and IL2. Seven cases showing an increase in IL2R expression not accounted for by T cells.
* = In this case, in which CD3 expression increased to 14%, the CD25 (IL2R) expression increased to 41%.

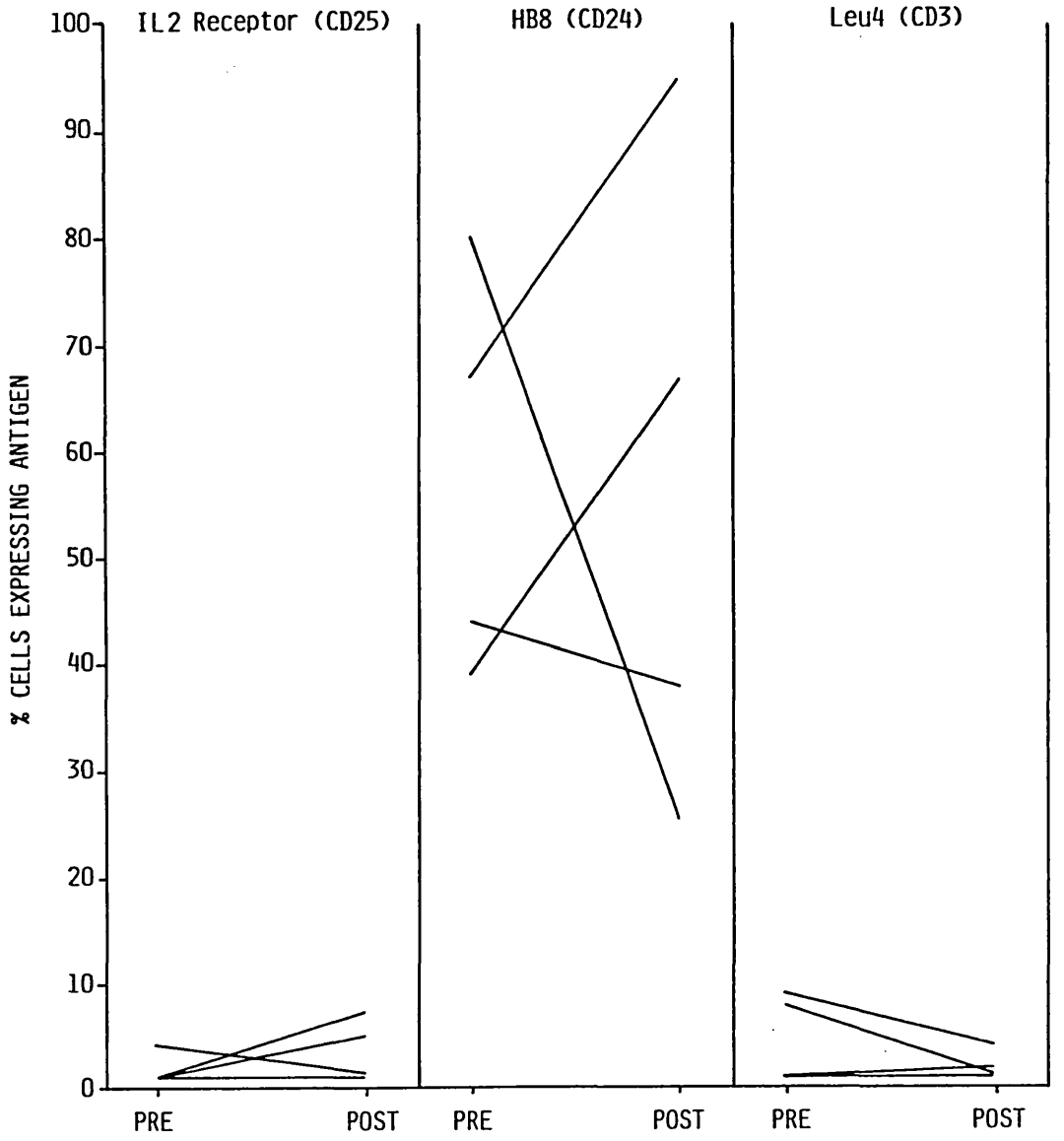


Figure 6.3 The percentage of MM bone marrow cells expressing IL2R (CD25), HB8 (CD24), and Leu4 (CD3) before and after culture with PHA and IL2. Four cases showing no increase in IL2R expression.

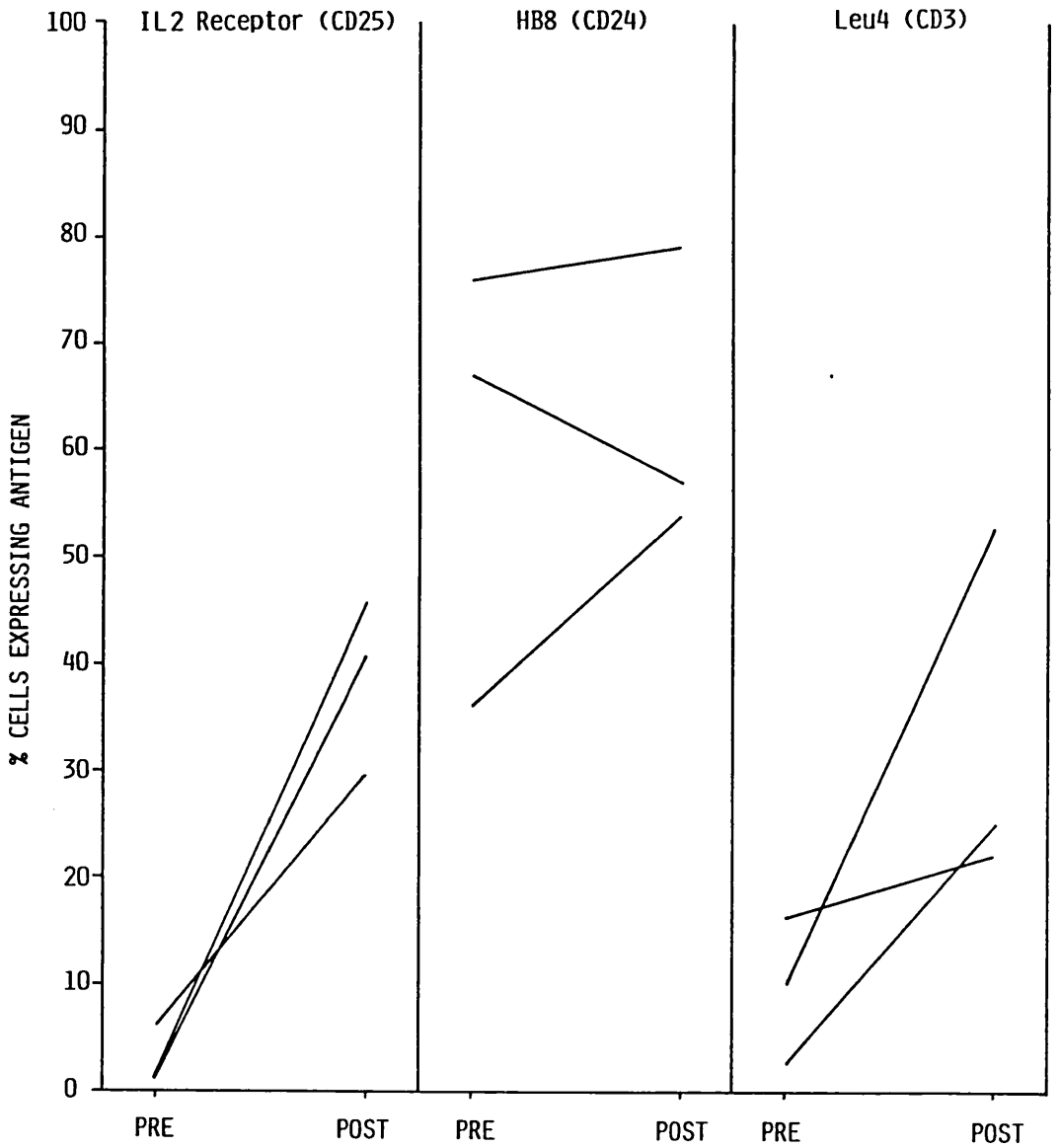


Figure 6.4 The percentage of MM bone marrow cells expressing IL2R (CD25), HB8 (CD24), and Leu4 (CD3) before and after culture with PHA and IL2. Three cases where T cell activation appears to be the reason for increased IL2R expression.

6.3.3 Double immunofluorescence

An attempt was made in a few cases to demonstrate that the IL2R were being expressed on the plasma cells by combining surface staining using an anti-IL2R MoAb with staining for cytoplasmic Ig. After reacting the cells with the anti-IL2R MoAb and the second layer conjugated antibody, the cells were cytospun onto slides and fixed in acetone. This fixation appeared to destroy the surface fluorescence as even in cases showing surface IL2R positivity by immune-rosetting, it was not possible to detect surface IL2R by immunofluorescence. Thus it was not possible to find cells co-expressing IL2R and cytoplasmic Ig.

6.3.4 The effect of T-cell depletion

The effect of removing T cells from the mononuclear cell fraction before culture with PHA and IL2 was examined in 7 cases. T cells were reduced to <1% by one 1 round of sheep erythrocyte rosetting and centrifugation over Ficoll-Paque. T-depleted mononuclear cells formed fewer and smaller aggregates after culture in PHA and IL2. When aggregates did form they were seen to be mixed on morphological examination - i.e. including both plasma cells and myeloid cells. Large blast-like cells were still occasionally seen in these cultures. Immune-rosetting (in 5 cases) showed that there were no or very few (0%, 0%, 0%, 5%, 7%) $IL2R^+$ cells after culture. T-cells remained <1% after culture, although an occasional $CD3^+$ cell was seen (possibly the large blasts seen ?).

6.4 DISCUSSION

These experiments have shown that in some cases of MM (half in this series), cells can be induced to express IL2R on their surface when incubated with PHA and IL2. However it was not possible to confirm that the cells expressing IL2R were also cIg^+ because of methodological problems. This expression was dependent on the presence of T cells in the culture, as removal of T cells prevents this induction. It may be that the PHA and IL2 stimulated T cells to secrete a factor which caused IL2R expression on the MM cells. However, it is possible that what was observed was the expression of surface IL2R, followed by the release of soluble IL2R by small numbers of T cells (Rubin et al, 1985). The soluble IL2R might then have been taken up passively by the plasma cells. To show that this was not the case it would be desirable to remove the IL2R from the cell surface and show that IL2R would reappear even if the T cells had been removed from the culture. This was not attempted.

6.4.1 The role of interleukin 2 in the proliferation of neoplastic B cells

Löwenberg and Touw (1986) have investigated the role of IL2 in the proliferation of neoplastic T and B cells. They studied IL2R expression on these cells and their capacity to proliferate or to form colonies in response to IL2. In relation to B lineage malignancies they studied 6 cases of B-cell chronic lymphocytic leukaemia (CLL) and 8 cases of pre-B/common acute lymphoblastic leukaemia (ALL). In CLL, they found 4/6 cases spontaneously expressed IL2R, and expression in the other 2 cases could be induced by exposure to PHA (0.75% vol/vol) and IL2 (25 units/ml) (Touw & Löwenberg, 1985). In liquid cultures (over an agar underlayer) CLL cells formed 'colonies' in the presence of PHA and IL2 or TPA and IL2, but not in most cases with PHA or TPA alone. In view of our experience with pseudo-colony formation in MM with PHA-TCM based cultures (chapter 5), some doubt must remain over the validity of these colony

cultures until they can be confirmed by others under more cell-immobilising conditions. This is especially so as the speed of colony formation (50 cells - i.e. 6 divisions - in 7 days) seems extremely rapid for an indolent neoplasm such as CLL. In our experience of CLL, one round of E-rosetting may remove all detectable T cells, but after culture with PHA with or without IL2, T cells can be found in small numbers, usually at the centre of a tight aggregate ('colony') of CLL cells (data not shown). Their data (from only one patient) on ^{3}H-thymidine incorporation as a measure of the proliferative response of CLL cells to IL2 are more convincing: PHA alone induced virtually no ^{3}H-thymidine uptake, suggesting a lack of contaminating T cells, whereas PHA and IL2 induced marked ^{3}H-thymidine incorporation (Löwenberg & Touw, 1986). Their data are consistent with CLL cells being responsive to IL2, though an effect via another cell type cannot be excluded.

The results with pre-B/common ALL cells were similar although none of the cells expressed IL2R before culture, and only 6/8 expressed IL2R after culture with either PHA or TPA (Touw et al, 1985). Colony formation, with PHA and IL2 occurred in 5/8 cases and in most cases required an irradiated feeder layer suggesting that the PHA and IL2 may have acted indirectly via the feeder cells, or that another 'permissive factor' was required. A direct proliferative role for IL2 in pre-B/common ALL seems unlikely on present data.

Our data are consistent with MM cells having the capacity to express IL2R. It is possible that the cells that survived in culture might more closely represent the progenitor cell fraction than fresh MM bone marrow cells. Thus the progenitor cells in MM may express or have the capacity to express IL2R. However, doubts remain because of the presence of contaminating T cells in our experiments and the possibility of them shedding soluble IL2R onto the PC. Whether IL2 plays a growth-modulating role in MM requires further investigation.

CHAPTER 7

CELL FUSION EXPERIMENTS AND LONG-TERM CULTURES IN LIQUID MEDIA

7.1 INTRODUCTION

The short term 'clonogenic' assays were found unsatisfactory for the study of progenitor cells in myeloma (chapter 5), so attention was turned to long-term culture. The objective was to allow the neoplastic plasma cells to grow in liquid culture in the hope that immortal cell lines would emerge that would be representative of the progenitor cell(s). In addition, fresh MM bone marrow cells were fused with a mouse plasmacytoma cell line, in the hope that dividing (progenitor) MM cells were more likely to fuse than the terminally-differentiated non-dividing cells.

It was intended to use the products of both techniques to provide a means of studying the phenotype and physiology of the (or a) myeloma clonogenic cell. Liquid culture of MM cells failed to produce a long term myeloma cell line, although an unfused line was established from a failed fusion protocol (see chapter 8). The fusion technique succeeded in one case in establishing a number of hybridomas which, at least for a short time, expressed both murine and human characteristics and secreted immunoglobulin (Ig) of the same light and heavy chain types as the patient. These lines were studied phenotypically and were also used as immunogens for the production of anti-plasma cell MoAb.

7.2 METHODS

7.2.1 Cell preparation

Fresh bone marrow samples from 15 cases of MM, and 2 cases of PCL were obtained. Four were from untreated cases, and 13 from treated patients who had clinical evidence of relapse at the time of the bone marrow aspiration. A mononuclear cell fraction was prepared using Ficoll-Paque and then washed twice in Iscove's/Ham's (1:1) medium (Isc/Ham) with FCS. The percentage of plasma cells in this fraction varied from 15% to 95% (median 50%). In 2 cases the effect of removing non-adherent cells from the mononuclear fraction was investigated, and in 4 cases the effect of removing T cells by E-rosetting and density-centrifugation was studied (see section 2.3.1(d) and (e)) (E^-cells).

7.2.2 Liquid Culture

The washed mononuclear cells were suspended in Isc/Ham with 20% serum, usually FCS, but in 5 experiments the effect of using fresh human serum instead was investigated. This suspension was plated out in 1ml aliquots into wells of 24-well 'Linbro' multiwell culture plates (Flow Laboratories). To this a variety of growth factors was added to try to stimulate plasma cell growth, as listed in Table 7.1. These were either added individually, or occasionally in combination (see results (section 7.3.1), and Table 7.2). In most cases some cells were cultured without any additional growth factors.

The plates were observed every few days using an inverted microscope. As the cells settled to the bottom of the wells, half the medium could easily be aspirated and replaced as necessary without disturbing the cells. This was done every 1-2 weeks in most cases. When the cells in a well appeared to be increasing, the non-adherent cells were aspirated after gentle stirring, and half of them moved on to an empty well. In a few

cases, some cells had DNA incorporation analysed by autoradiography (see methods, section 2.3.5(a)).

	%/concentration
Phytohaemagglutinin (PHA)	1%
PHA-T cell-conditioned medium	4%
Staphylococcus Aureus Cowan (SAC)	0.0005%
{ Tetradecanoyl Phorbol Acetate { Calcium Ionophore (Ionomycin)	0.1ng/ml 1μg/ml
Mouse spleen cell 3-day culture supernatant	10%
JJN-1 (plasma cell line) culture supernatant	50%
ESG (Ewing's sarcoma cell line supernatant	0.5-5%
Fibroblast feeder layer (Flow 4000 cell line)	

Table 7.1 Growth factors added, usually individually, sometimes in combination, to the cultures.

7.2.3 Cell Fusion protocol

See section 2.3.9. A fusion between fresh MM/PCL mononuclear cells and the mouse plasmacytoma cell line X63-Ag8.653 was attempted in 4 cases (3 MM, 1 PCL). One was successful and the hybrid clones were grown up in RPMI-1640 with 10% FCS and analysed in the following ways:

(a) Morphologically, on cytospin preparations stained by Leishman's stain.

(b) DNA content, by propidium iodide (PI) staining and analysis using the Becton-Dickinson 440 fluorescence-activated cell sorter (FACS). The mouse plasmacytoma cell line used in the fusion (X63-Ag8.653) was used as a control for each run. The FACS was calibrated to give a modal fluorescence intensity of X63-Ag8.653 at channel 38 with standard voltage settings and PI concentration.

(c) The culture supernatants were tested for the presence of Ig heavy (alpha, gamma, and mu) and light (kappa and lambda) chains, using a direct haemagglutination assay.

Doubling dilutions of the supernatants were made and the titre which just agglutinated sheep erythrocytes coated with MoAbs against kappa, lambda, alpha or mu chains was noted. These were repeated several times over the first few weeks of culture.

(d) Cell surface phenotype by direct immune rosetting.

(e) Cell cytoplasmic phenotype using indirect immunofluorescence on acetone-fixed cytospins.

Table 7.2 (next two pages)

The culture conditions and results of liquid culture of fresh MM bone marrow mononuclear cells from 14 cases of MM and of fresh peripheral blood mononuclear cells of one case of PCL.

Abbreviations

%PC = Percentage of plasma cells in the mononuclear fraction of freshly aspirated MM bone marrow.

Cells used:
Monos = Mononuclear fraction. Non-adh = Non-adherent cells.
E^- = T-cell depleted fraction by sheep erythrocyte rosetting.

Serum used:
FCS = Fetal calf serum. HuS = Human serum.

Growth factors:
PHATCM = PHA-T-cell conditioned medium.
SAC = Killed Staphylococcus aureus Cowan.
CaI + TPA = Calcium Ionophore + Tetradecanoyl Phorbol Acetate.
JJN-1 = plasma cell line (see chapter 8). S/N = Supernatant.
Flow 4000 = Fibroblast cell line (Flow Laboratories).
ESG = Ewing's sarcoma cell line S/N (Northumbrian Biologicals).

Results of culture:
Fibro = Fibroblasts. Macro = Macrophages.
Myeloid = Granulocytic cells. PC = Plasma cells.

TABLE 7.2 RESULTS OF LIQUID CULTURE OF FRESH MM BONE MARROW

Patient	% PC	Cells Used	Serum (20%)	Growth Factor(s)	Results of Culture: First 2 weeks	Second 2 weeks	> 4 weeks Termination
HM	95	Monos	FCS	—	Loose clumping: FCS > HuS; Cytospin: PC clustered around Macros.	Fibroblasts++	Fibroblasts++ curling up Terminated on day 25
		Monos	HuS	—			
		Monos	FCS	PHATCM	Tighter clumping, cells less viable than with serum alone	Stromal layer developed	
		Monos	HuS	PHATCM			
AM	93	Monos	FCS	Flow 4000 (cell line)	PC attach around Fibroblasts	Overtaken by fibroblasts	
JS	40	Monos	FCS	—	Fibroblasts/Macrophages form adherent layer. PC++ in non-adherent layer	PC++ with occasional mitotic figures*. Some PC with 4-8 nuclei	Infected at day 19
			HuS	—			
KF	25	Monos	FCS	—	Clumping of cells	Day 21: cytospin shows Macrophages+++. PC: single cells, healthy. No mitotic activity	Terminated day 21
			HuS	—			
			FCS	S.A.C.	(Macrophages contain SAC)		
DH	50	Monos			(Day 13 cytospins:)		
			FCS	—	PC+	Fibroblasts++	Terminated Day 20
			HuS	—	PC+, Macro++	Fibro ++, Macro++	
			FCS	S.A.C.	PC+	Fibroblasts++	
			FCS	PHATCM	PC+	Fibroblasts++	
			HuS	PHATCM	PC+, Macro+, some failed mitoses	Fibro ++, Macro++	
MF	65	Monos	FCS	—	Fibro+	Fibroblasts++ Few other cells	Fibro++, therefore terminated Day 35
		Monos	HuS	—	Fibro+, Macro+		
		Monos	FCS	PHATCM	Clumping of cells		
		Monos	HuS	PHATCM	Clumping of cells, Macro++		
		E^-	FCS	—	Fibro++		
		E^-	FCS	CaI + TPA	Clumps. Day 9:dead		
KE	62	Monos	FCS	—	Early clumping (Day 4), followed by Fibro++ (Day 11)	PC difficult to separate from Fibro	Fibro++. Terminated day 24
		E^-	FCS	—			
AC	53	Monos	FCS	—	Day 5: clumped. Day 11: Fibro+, attempt to move non-adherent cells to new wells	Day 22: apparent increase in cells. Cytospin: Macro/Fibro/Myeloid, occasional PC	Terminated day 22
		E^-	FCS	—			

TABLE 7.2 RESULTS OF LIQUID CULTURE OF FRESH MM BONE MARROW (CONT'D)

Patient	% PC	Cells Used	Serum (20%)	Growth Factor(s)	Results of Culture: First 2 weeks	Second 2 weeks	> 4 weeks Termination
PG	48	Monos	FCS	—	Day 4: clumped Day 11: Fibro+	Non-adh cytospin (day 14): PC++, Macro++ Myeloid++	Day 38: Fibro++ Terminated Day 38
		E^-	FCS	—			
		Non-adh	FCS	—	No fibro ? Slight increase in cell number	Day 21: Cells stuck down; cannot move on. ESG: no difference	Stayed static until day 104. No fibro but terminated day 104
		Non-adh	FCS	ESG			
JD	45	Monos	FCS	Mouse spleen cell S/N	Early clumping	Day 22: Fibro++ with a few clusters of cells stuck to Fibro or free-floating	Day 48 : Very few non-adh cells left. Therefore terminated day 48.
		Monos	FCS	JJN-1 S/N.	Early clumping, cells increasing		
		Monos	FCS	—			
		E^-/non-adh	FCS	—	Early clumping		
		E^-/non-adh	FCS	Mouse spleen cell S/N	Early clumping		
		E^-/non-adh	FCS	JJN-1 S/N	Early clumping		
FS	24	Monos	FCS	—	Day 13: Fibro++, Myeloid+, Macro+		Terminated day 19
JG	15	Monos	FCS	—	Day 7: Fibro++. Attempt to move on non-adherent cells	Very few cells left	Terminated day 18
		Monos	FCS	ESG			
JC (PCL- peripheral blood used)	95	Monos	FCS	ESG	Small clumps of cells	Day 14: all healthy	Day 35: very static, ? dead. Terminated day 35
		Monos	FCS	—			
		Non-adh	FCS	ESG	Cells tending to centralise in wells		
		Non-adh	FCS	--			
PW	25	Monos	FCS	ESG	Day 10: Fibro++		Day 29: Fibro++, myeloid++ Terminated day 29
		Monos	FCS	—			
		Non-adh	FCS	ESG	Day 10: Fibro+/- Other cells: not very active		
		Non-adh	FCS	—			
HV	30	Monos	FCS	ESG	Day 8: wells with ESG contain more cells than those without	Day 20: Fibro++	A few wells survived until day 42
		Monos	FCS	—			
MS	97	Monos	FCS	ESG	Day 12: appear healthy. No difference between wells with and without ESG	Day 18 Fibro+	Day 39: ?colonies on Fibro layer Day 52: no growth Day 75: no growth Terminated day 75
		Monos	FCS	—			
WE	78	Monos	FCS	ESG	Clumps+; Day 6: Fibro+	Fibro++, few other cells	Terminated day 15
		Monos	FCS	--			

7.3 RESULTS AND DISCUSSION

7.3.1 Liquid Culture of Myeloma Bone Marrow

None of the cultures from the 15 cases of MM/PCL resulted in long-term human myeloma cell lines. No beneficial effect in this respect was observed from using different sera and growth factors (Table 7.2) The cultures consisted of healthy plasma cells, which were confirmed as viable by trypan blue exclusion. These remained for 6-8 weeks, but no significant increase in plasma cell numbers occurred. During this time a fibroblast/ macrophage layer developed on the bottom of the wells, which gradually overtook the culture. The plasma cells became tightly stuck to, or wrapped up in, the fibroblasts. The cultures became more and more predominantly fibroblasts and so were thrown out. Occasionally cultures were terminated by infection. There was evidence for some proliferative activity in the plasma cells in culture, e.g. occasional mitotic figures in plasma cells on cytospins (Figure 7.1), and DNA incorporation shown by autoradiography (Figure 7.2).

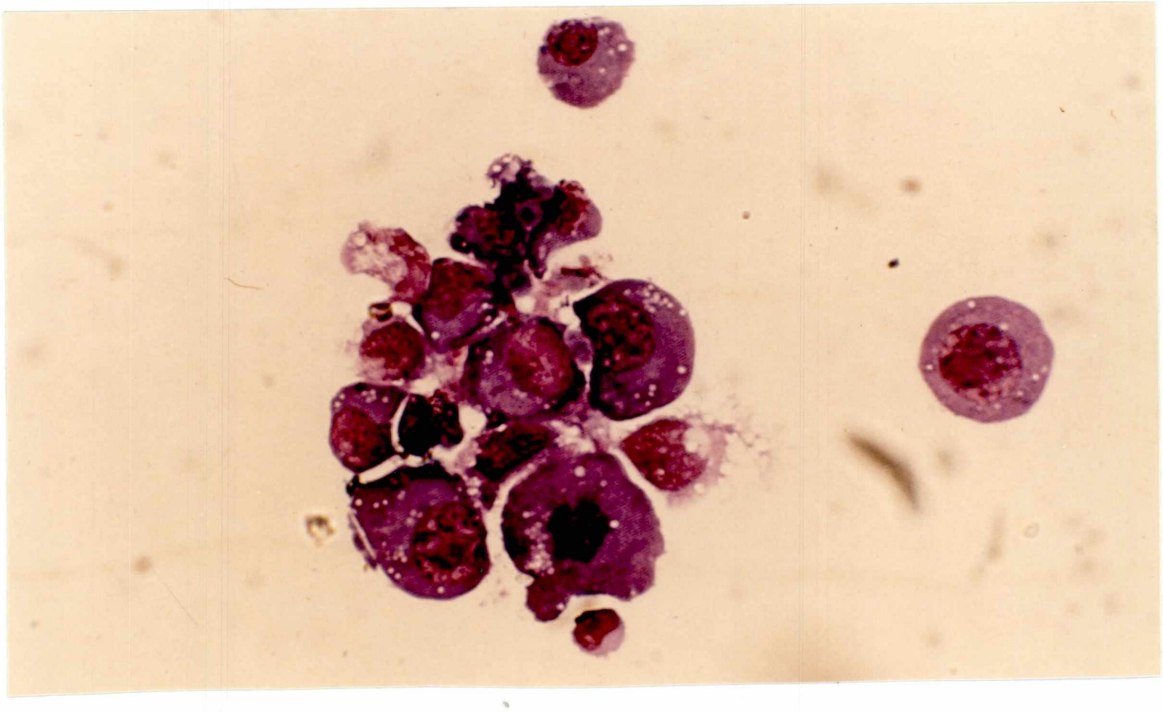


Figure 7.1 A cytospin preparation of MM bone marrow after 7 days in liquid culture, showing the presence of mitotic figures in plasma cells (Leishman's stain).

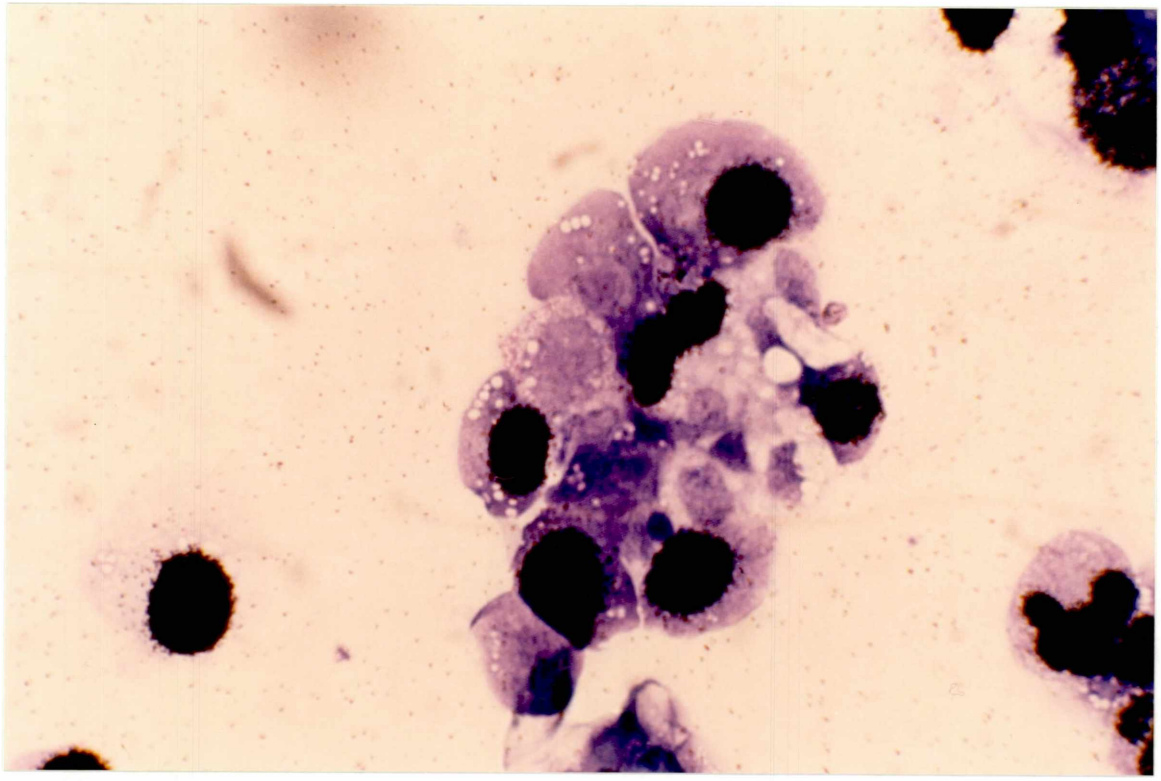


Figure 7.2 Autoradiography of a MM bone marrow after 4 weeks in liquid culture (with superimposed Leishman's stain).

The variations on the culture technique had the following effects:

(a) Human serum tended to encourage the growth of macrophages rather than fibroblasts.
(b) PHA-TCM caused early clumping of cells, but no increase in plasma cells. If anything, more myeloid/macrophage growth was encouraged.
(c) CaI + TPA clumped the cells but killed them after a week.
(d) Staphylococcus Aureus Cowan had no effect.
(e) Mouse spleen cell + JJN-1 supernatants had no effect.
(f) ESG gave a possible slight increase in plasma cells.
(g) Use of E^- cells made no difference, except in one case, in which CaI + TPA was used. In this case less

clumping was noted, presumably because this clumping was in part mediated via T-cells.

(h) Use of non-adherent cells resulted in less or slower fibroblast 'take-over' of the cultures. In one case (PG), the cultures were maintained to day 104, without fibroblasts taking over. However, even in this case no PC line was achieved, perhaps because the plasma cells require stromal cell contact or a stromal cell factor. It was evident in some of the earlier cultures that PC in culture tend to cluster round macrophages or fibroblasts, presumably deriving some benefit.

In summary, of the culture conditions examined the best for maintaining viability of MM cells was Isc/Ham medium with 20% FCS without any other additives. It was best to have cells taken from a patient with a high percentage of plasma cells and thoroughly depleted of adherent cells. Under these conditions, myeloma PC remained viable for prolonged periods, but did not grow out into cell lines. Durie et al (1985) and Garrett et al (1987) have found that, using a complex tissue culture medium known as M3, a small percentage of myeloma bone marrows will grow into cell lines. However we have not been able to confirm the benefit of M3 medium in recent experiments (J. Ball, personal communication).

7.3.2 Fusion with Mouse Plasmacytoma Cell line

Three attempted fusions of MM bone marrow with X63-Ag8.653 were unsuccessful. However, BM cells from one PCL patient with 65% PC in the bone marrow and an IgA-kappa paraprotein did fuse with X63-Ag8.653, producing 12 clones from the 576 microtitre wells plated. Unfused X63-Ag8.653 cells were prevented from growing by culturing the cells initially in HAT medium before transferring them to RPM1-1640 with 10% FCS. It was assumed, based on the liquid culture experiments, that unfused human myeloma cells would not grow, and would be overgrown by a rapidly growing hybridoma.

(a) Morphology and cell culture. The cells (Figure 7.3) resembled large lymphoblasts, with some plasmablastic differentiation: there was relatively abundant blue cytoplasm with occasional golgi areas apparent. The cells grew rapidly in RPM1-1640 + 10% FCS, with a doubling time of 2-3 days, and could be frozen in liquid N_2 and thawed.

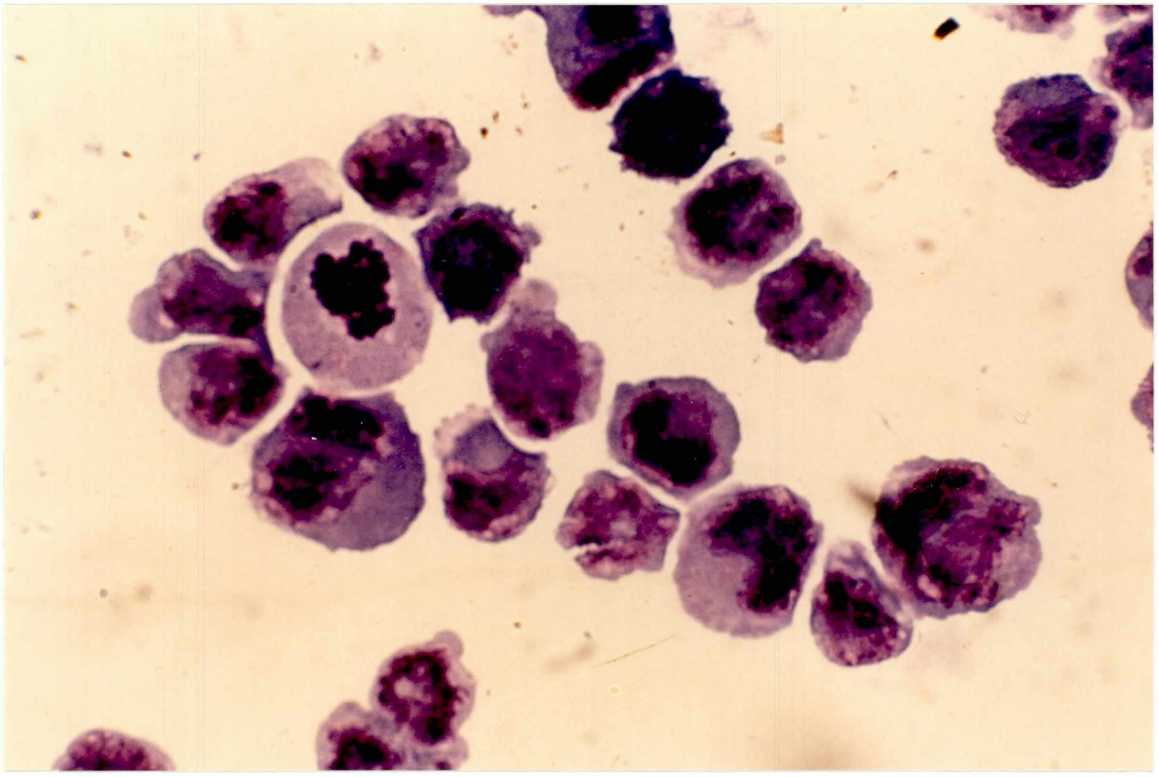


Figure 7.3 The morphology of hybrid cells formed by the fusion of a human plasma cell leukaemia and the murine plasmacytoma line X63-Ag8.653. The cells resemble large lymphoblasts, with some plasmablastic differentiation evident in some cells.

(b) DNA content, by propidium Iodide staining and FACS analysis is shown in Table 7.3 (page 131). The peak DNA content of X63-Ag8.653 was arbitrarily set to channel 38 (because that was the reading on the day of the first experiment), and at this setting the plasma cells of the original bone marrow had a DNA

content of 33 (slightly hyperdiploid with respect to normal human cells; DNA Index 1.15).

Although the DNA content scale may not be absolutely linear, most clones appeared to contain approximately the sum of the DNA content of X63-Ag8.653 and the original patient PC. This sum is strictly 71, with most of the clones within the range 68-85. These data are compatible with these clones representing one-to-one fusions between X63-Ag8.653 and the human plasma cells or their precursors from the patient's bone marrow. Three clones (marked * in Table 7.3, and including the second clone within clone A) showed much lower values - around 40. Evidently these could not represent full fusions between the two cells of interest. As can be seen from the results 12 weeks after the fusion, these clones were quite unstable showing a high degree of DNA loss after this time. This was paralleled by loss of Ig secretion (see below).

Clone	6 weeks after fusion	12 weeks after fusion
X63-Ag8.653	38	38
Patient BM PC	33	38
A	69,39* (Double)	
B	77	54
C	76	
D	68	58
E	69	
F	85	55
G	83	
H	48*	
V	70	57
W	41*	
Y	83	
Z	81	

Table 7.3 The DNA content of Human PC/X63-Ag8.653 fused clones, by PI-induced fluorescence, as measured by FACS. The DNA content of X63-Ag8.653 was arbitrarily set at channel 38.

* = clones with a DNA content around channel 40, which would not represent a full fusion (see text).

(c) Culture supernatant analysis. The clones all appeared to secrete both alpha and kappa chains (the patient's paraprotein was IgA-kappa) but this was gradually lost in most cases, over a 12 week period, as shown in Table 7.4.

	Immunoglobulin Titre (Doubling dilutions)						
Ig Type	L	G	μ	k/α	k/α	k/α	k/α
Week of culture	2	2	2	2	4	8	12
Clone							
A	0	0	0	1/3			
B	0	0	0	4/3	4/3	1/1	0/0
C	0	0	0	5/4	½/½	0/0	
D	0	0	0	6/5	3/3	0/2	0/0
E	0	0	0	6/5	2/2	0/2	0/0
F	0	0	0	2/2	0/1		
G	0	0	0	4/5			
H	0	0	0	1/3			
V	0	0	0	4/4	2/3	0/0	
W	0	0	0	7/6	3/3	0/0	
Y	0	0	0	?/?	0/1	0/0	0/0
Z	0	0	0	6/5	4/4	0/2	0/1

Table 7.4 Immunoglobulin present in supernatants from human PC/X63-Ag8.653 fused clones. There was never any lambda (L), gamma (G) or mu (μ) detectable (left hand panel). Both kappa (k) and alpha (α) chains were detectable in all the clones tested, except possibly clone Z (? = not done), after 2 weeks in culture. Over the next 10 weeks Ig secretion was progressively lost.

(d) Cell surface phenotype. The cells expressed both murine and human antigens. Both X63-Ag8.653 and the clones reacted with a rat anti-mouse Ia MoAb, called C57BL/6. The reactivity of the clones with MoAb to human antigens is displayed in Table 7.5, and compared with the phenotypes of RPM1-8226, a well-described human 'PC' line; JJN-1, our own PC line described in chapter 8; the original BM. The mouse plasmacytoma X63-Ag8.653 did not react with any of the MoAb mentioned in Table 7.5.

MoAb	CD/Ag		B	C	E	V	Y	Z	Orig. BM	JJN-1	RPMI 8226
6e1	k	c	-	-/+	-	-/+	+	-	++		
		s	+	+	+	+	+	+		++	-
12F	l	c	-	-	-	-	-	-	-		
		s	-	-	-	-	-	-		-	+
N2C1	9	c	-		-	+/-		+/-	+		
		s	-	+	-	++	+	-	-	-	++
CALLA	10	c	-		-	-	-	+/-	-		
		s	-	-	-	-	-	+/-	-	-	+/-
BU12	19	c	-/+		-/+	+/-	++	+/-	+/-		
		s	-	-	-	-	-	-	-	-	+/-
MHM6	23	c		-/+	-/+	+/-	+	-	+/-		
		s	-	-	-	-	-	-	-	-	-
HB8	24	c	+		-/+	-/+	-	+/-	+		
		s							+	+	
OKT10	38	c	+/-	-	+/-	+	++	+	+		
		s	+	-	-	+	++	++	++	++	++
AC2	39	c	+	++	++	++	+	++	+/-		
G28-5	40	c			-	-	+/-		-		
BU18	RER	c	+/-	-/+	-	-/+	+/-	+/-	++	+	++
BU25	MHC II	c			-	-	-	-	-		
		s	-	-	-	-	-	-	-	-	+++
KB61		c	-		-		-	-	-	+	
7F7		c	-		-	-		-	-		

Table 7.5 The cell surface (s) and cytoplasmic (c) phenotype of some of the human PC/X63-Ag8.653 hybridomas (B, C, E, V, Y, Z) compared with the original bone marrow (Orig. BM), the established plasma cell line RPMI-8226, and our new plasma cell line JJN-1 (see chapter 8).

k = kappa, l = lambda, RER = rough endoplasmic reticulum, MHC II = major histocompatibility complex class II antigen. KB61 and 7F7 are MoAb from the IIIrd International Workshop on Human Leucocyte Differentiation Antigens with putative anti-plasma cell activity.

All the clones and lines tested here were also negative for surface expression of OKT3 (CD3), OKT4 (CD4), B941 (CD8), NA1/34 (CD1), OKT1 (CD5), and BU15 (CD11c).

Most clones were $CD38^+$, and some were also $CD9^+$. They probably expressed weak surface kappa chains, although this could have been passively adsorbed Ig as the cells were actively secreting kappa-chains. They were negative for the human antigens detected by CD3,4,5,9,10,11c,19,23. This is similar to the original marrow plasma cells which expressed CD38, CD45 and CD24(HB8), but no other PC antigens. Unfortunately the clones were not tested for surface CD24 or CD45 expression.

(e) Cell Cytoplasmic Phenotype. The phenotype of the cells as detected by indirect immunofluorescence on acetone-fixed cytospin preparations is also displayed in Table 7.5.

There are some variations between the surface phenotype and the cytoplasmic phenotype possibly because the cytospin analysis was done on cells from a later passage than the surface phenotyping, when Ig synthesis +/or secretion, and perhaps other antigen expression, may have been lost. However, it confirms that the clones were mostly $CD38^+$. The most striking positivity was with the CD39 MoAb, AC2, which unfortunately was not used in the surface phenotyping. This antigen was only very weakly expressed on the original marrow PC. Interestingly JJN-1 and JJN-2, described in chapter 8, also expressed CD39, in contrast to the original marrow cells producing those lines.

7.4 CONCLUSIONS

Overall these results suggest that these clones represent true fusions of the murine plasmacytoma line X63-Ag8.653 with cells from the IgA-kappa secreting clone from a patient with PCL. The hybrid cells contained approximately the sum of the DNA of the individual fusion partners, and expressed both mouse Ia antigens and a number of human antigens. They secreted both alpha and kappa Ig chains, but not gamma, mu, or lambda. CD38 and CD39 were expressed on, or in, all the lines. Over a period of 12 weeks or so, DNA loss occurred and Ig synthesis and secretion stopped. No chromosome analysis was undertaken.

Fusion between mouse plasmacytoma cell lines and human MM cells has not been reported. Hybridomas between mouse plasmacytomas and human lymphocytes have been derived by similar techniques to that described here, with a view to producing specific human MoAb. Specific human MoAb have been produced against the Forssmann antigen (Nominske et al, 1980), Keyhole Limpet haemocyanin (Lane et al, 1982), Tetanus toxoid (Kozbor et al, 1982, Butler et al, 1983), and multiple endocrine organs (Satoh et al, 1982), These hybridomas were also unstable, with chromosome loss and failure of Ig secretion. More stable clones have recently been made which secrete monoclonal anti-Rhesus D antibodies by fusing Epstein-Barr virus transformed human B lymphocytes with cells of mouse plasmacytoma lines (Thompson et al, 1986). Careful and repeated cloning has been shown to be essential for prolonged stability of these types of hybridoma (Thompson et al, 1986; James & Bell, 1987). Such handling might have improved the durability of these human/X63-Ag8.653 hybridomas.

These clones were used in an attempt to make murine MoAb against human PC using them as immunogens in Balb/c mice. X63-Ag8.653, having originated from a Balb/c plasmacytoma, is not antigenic to Balb/c mice and thus antibodies produced should

have been directed against any human antigens on the surface of the infected cells. It was thus hoped that specific anti-human plasma cell MoAb would be produced. However, although a large number of murine MoAb directed against human PC were derived, none were truly PC specific: most reacted with a broad range of cell types (J. Lowe, personal communication). Analysis of these MoAb is still in progress.

CHAPTER 8

TWO NEW IgA_1-KAPPA PLASMA CELL LEUKAEMIA CELL LINES

8.1 INTRODUCTION

Only a few myeloma cell lines exist (Matsuoka et al, 1967; Nilsson et al, 1970; Jobin et al, 1974; Burk et al, 1978; Karpas et al, 1982; Jenberg et al, 1987) and some of these are not truly representative of the most mature stage of the B lineage (Goldstein et al, 1985). These lines have been useful for the study of late B-cell differentiation, and for studying the biology of the tumour; e.g. its secretion of factors such as the osteoclast activating factor, "Lymphotoxin" (Garrett et al, 1987). They have also been used for the production of human monoclonal antibody-producing hybridomas (Kozbor & Croce, 1985), and as immunogens for the production of new murine anti-human plasma cell monoclonal antibodies.

B-cell stimulatory factor 2 (BSF-2; Interleukin 6) is a 26 kDa protein that acts on activated B cells to induce proliferation and immunoglobulin secretion (Billiau, 1987). The presence of receptors for BSF-2 on human myeloma cell lines has recently been described (Taga et al, 1987). Kawano et al. (1988) have recently demonstrated that fresh isolates of myeloma plasma cells show increased ^{3}H-thymidine incorporation in response to BSF-2.

This chapter concerns the derivation and characterisation of a new plasma cell line, JJN-1, and its sub-line, JJN-2, which proliferate in response to BSF-2.

8.2 CASE HISTORY

The patient, aged 57, presented in February 1987 with weight loss and a bleeding tendency. She was found to be anaemic and the peripheral blood showed an IgA_1-kappa paraprotein (110g/l) and the presence of circulating plasma cells ($40x10^9$/l). Bone marrow examination showed 55% plasma cells. Chemotherapy with vincristine, adriamycin and dexamethasone (Barlogie et al, 1984) was commenced, but she died one month after presentation.

8.3 METHODS

8.3.1 Cell culture

The diagnostic bone marrow was aspirated into 2ml of culture medium containing 500 units of preservative-free heparin, and placed over Ficoll-Paque for centrifugation. The mononuclear cells obtained were used for an attempted fusion with the mouse plasmacytoma cell line, X63-Ag8.653, using a polyethylene glycol based protocol (Galfré & Milstein, 1982). The cells were then plated out in 96-well microtitre plates in medium containing hypoxanthine, aminopterin, and thymidine (HAT). There was no apparent growth until six weeks later, when all unfused murine plasmacytoma cells would have died in the HAT medium. Small collections of live cells were seen in the wells, the culture medium not having been changed since the initial plating. The contents of several wells were pooled and replated at approximately $2x10^5$ cells/ml in 1ml volumes of a Isc/Ham medium with 20% FCS. Cell growth was monitored by inspection under an inverted microscope and cultures in wells were divided as necessary (approximately every 10-14 days). Twelve weeks after the initial sample was taken, an Ewing's sarcoma cell line supernatant (ESG), containing hybridoma growth factor (Northumbria Biologicals Ltd, Cramlington, UK) was added to some of the culture wells at 2.5%, by volume. Hybridoma growth factor is also known as B cell stimulatory

factor 2 and interleukin(IL)6 (Billiau, 1987; Wong & Clark, 1988).

8.3.2 Surface antigen detection: indirect immunofluorescence

The monoclonal antibodies (MoAb) used were nearly all of a specificity defined at the IIIrd International Workshop on Human Leucocyte Differentiation Antigens (McMichael et al, 1987), as listed in Table 8.1 (in 8.4.3). The first layer MoAb was diluted 1-in-50 in PBS containing 10% FCS and 0.1% sodium azide. An anti-idiotype MoAb generated against an unrelated myeloma paraprotein was used as a control. The second layer antibody was a fluorescein-conjugated sheep anti-mouse immunoglobulin (prepared in the department of Immunology, Birmingham university by Dr G.D. Johnson) used at a 1-in-100 dilution. The cells were analysed using a Becton Dickinson 440 FACS.

8.3.3 Cytoplasmic antigen detection: indirect immunofluorescence

See methods chapter, section 2.3.2(c).

8.3.4 Assay of secreted immunoglobulin

Supernatants from the cell lines, cultured for 3 days, were analysed for alpha and kappa chains by an antibody-coated erythrocyte agglutination assay. Doubling dilutions of the supernatant were mixed with a drop of sheep erythrocytes coated with either an anti-α or anti-k MoAb, and the titre noted at which agglutination still just occurred.

8.3.5 DNA content analysis

$5x10^5$ cells were centrifuged at 400g and the pellet resuspended in 0.5ml of fixing buffer (0.07% paraformaldehyde, 0.01% triton in PBS). Immediately prior to analysis by the FACS, PI was added to give a final concentration of PI of 330μg/ml. This concentration is known to saturate the DNA intercalation sites. Then, $20-50x10^3$ cells were analysed and a plot of DNA content per cell derived.

8.3.6 Cytogenetic Analysis

Two cultures were established in RPMI 1640 medium supplemented with 10% FCS. These were incubated for 2 and 4 days respectively at 37°C. The 4 day cultures also had TPA added at a final concentration of 50ng/ml (Callen & Ford, 1983). Both cultures were exposed to 1ml of 10mg/ml colchicine for the final 30 minutes before harvesting. The cell suspensions were treated with 0.075M KCL as an hypotonic agent for 10 minutes, and then fixed with 3:1 methanol:acetic acid. The preparations were air dried and Giemsa-banded after trypsinisation (Seabright, 1971). These studies were done with Dr M.J. Griffiths of the department of cytogenetics, Birmingham Maternity Hospital.

8.3.7 Response to growth factors

^{3}H-thymidine incorporation was measured to assess the proliferative effect of various growth factors on the cell lines. Cells were set up in microtitre wells on a 96-well plate at $2x10^{4}$ cells/well (in 100μl medium + 20% FCS). The factors under investigation were added, each set of factors being set up in triplicate or quadruplicate. After 3 days, 0.3μCi ^{3}H-thymidine in 20 μl of medium was added to each well and incubation continued for 36 hours. Then the cells were lysed and the nuclei harvested onto filtermats (Skatron AS, Norway). The filtermats were placed in counting vials and 4ml of scintillation fluid (Optiphase X, LKB Scientific Products) was added. Beta emissions were counted in a liquid scintillation counter. The factors investigated included:

1. ESG (Northumbria Biologicals Ltd);
2. A purified preparation of BSF-2;
3. A MoAb to BSF-2 (=747) diluted 1/10,000;

(2. and 3. were gifts from A. Billiau, Leuven, Belgium)

4. Recombinant Interleukin 2 (Genzyme);

5. 3-day supernatant from Flow laboratories' fibroblast line 'Flow 4000'; and
6. 3-day supernatant from mouse spleen cells grown in RPMI-1640 medium with 15% FCS.

8.4 RESULTS

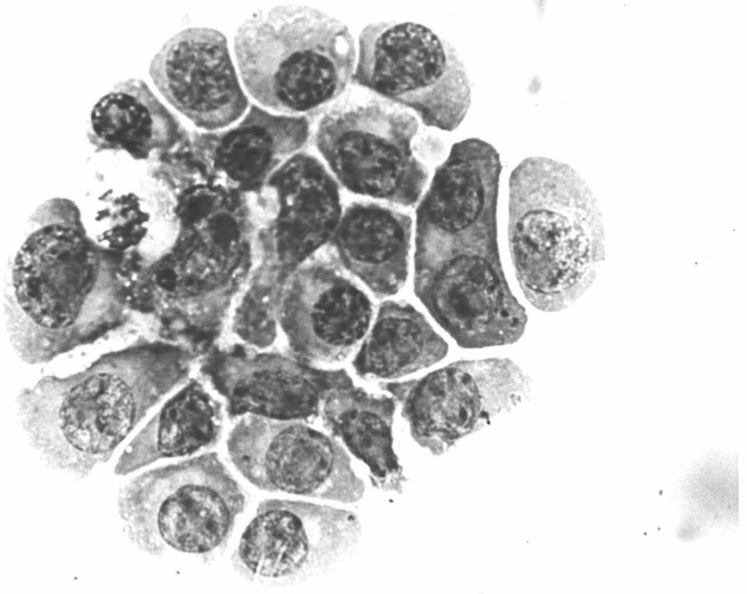
8.4.1 Cell culture

The attempt to fuse the bone marrow plasma cells from this patient with cells of a mouse plasmacytoma line was a failure as judged by the lack of any growth in the microtitre wells for 6 weeks after the fusion protocol. However the plates were not discarded and after 6 weeks live cells were seen within the microtitre wells, which on cytospin examination turned out to be of mature plasma cell morphology, with occasional mitoses. Considerable cell death occurred initially, but gradually the cell line became established with a doubling time of 10 days. This line is designated JJN-1 and is maintained in either Isc/Ham with 20% FCS or RPMI-1640 medium with 15% FCS. Within a week of adding ESG, the cells started to divide much more rapidly (doubling time 3-4 days). This line is designated JJN-2, and can be maintained in Isc/Ham with 20% FCS with 0.5% ESG. In the absence of ESG it dies off completely within 10 days.

8.4.2 Morphology

JJN-1 originally had the morphology of a mature plasma cell (Figure 8.1(a)), but in more recent passages it has shown a greater morphological range with cells resembling mature plasma cells, plasmablasts and multinucleate cells (Figure 8.1(b)). Electron microscopy (Figure 8.1(c)) shows the presence of a variable amount of rough-endoplasmic reticulum. JJN-2 contains mononuclear cells and only occasional binucleate cells, with a plasmablastic morphology (Figure 8.1(d)).

(a)



(b)

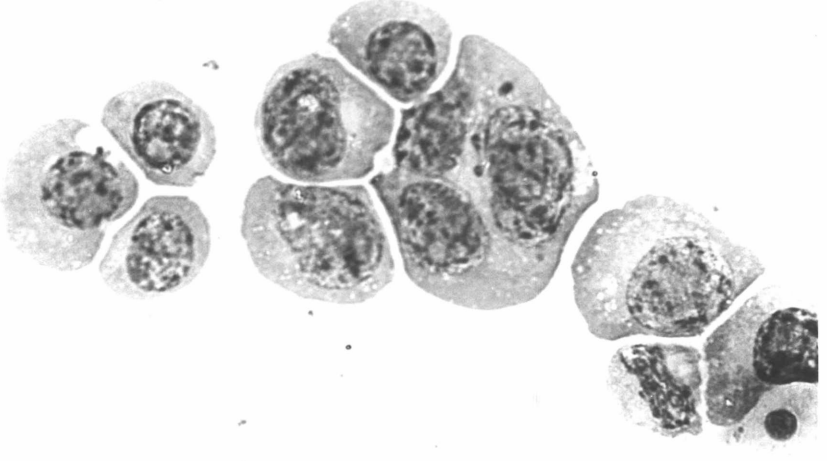
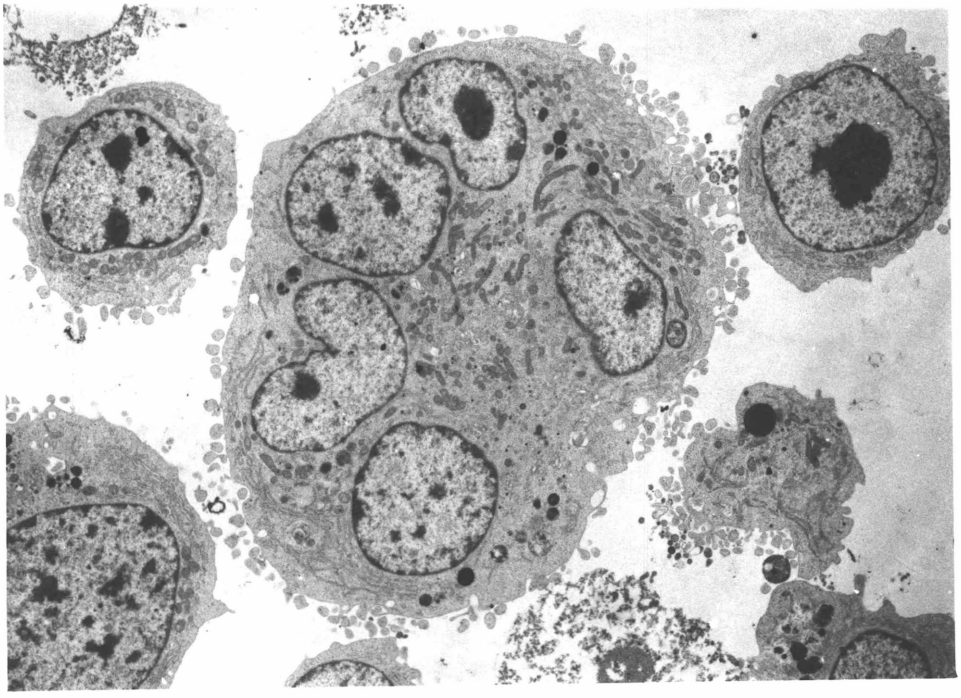


Figure 8.1. (a) JJN-1 in an early passage, having a mature plasma cell morphology (x570); (b) JJN-1 in a later passage demonstrating a more heterogeneous appearance, with mature plasma cells, plasmablasts and multinucleate cells (x570).

(c)



(d)

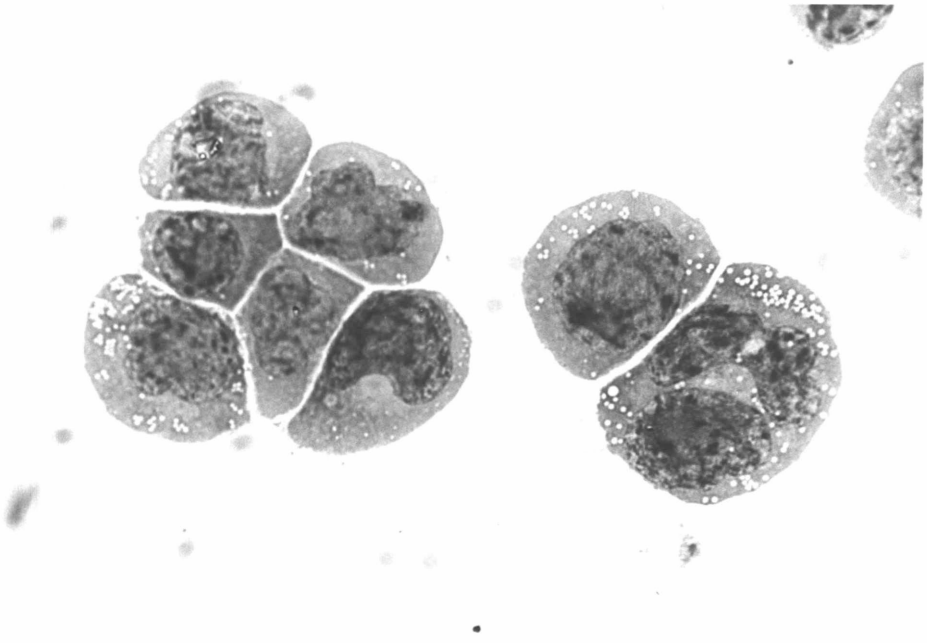


Figure 8.1 (c) electron microscopy of JJN-1 (x2800); (d) JJN-2 with a plasmablastic morphology (x570).

8.4.3 Immunophenotype

Table 8.1 shows a comparison of the phenotype of the original bone marrow and the JJN-1 and JJN-2 cell lines, from their later passages. Neither line reacted with a rat anti-mouse Ia antibody (C57BL/6) which does react with the original mouse cell line X63-Ag8.653, suggesting that these cell lines are not mouse-human hybrids. This antibody did show reactivity with the mouse-human hybridomas in chapter 7. Neither line expressed Epstein-Barr virus nuclear antigen.

MoAb	Source	CD Number/ Antigen	Original Marrow	JJN-1	JJN-2
BU16	B'ham Univ.	9	-	-	-
Anti-CALLA	Becton Dickinson	10	-	-	-
BU12	B'ham Univ.	19	-	-	-
BC1	Gallart, Barcelona	20	-	-	-
BA5	Le Bien, Minnesota	21	-	-	-
29-110	Kraft, Melbourne	22	-	-	-
MHM6	McMichael, Oxford	23	-	-	-
VIB E3	Knapp, Vienna	24	n/d	-	-
HB8	Cooper, Alabama	24	++	+	+
Anti-IL2R	Becton Dickinson	25	-	-	-
WR-17	Moore, Southampton	37	-	-	-
OKT10	American Type Culture Collection	38	++	++	++
AC2	Rowe, Birmingham	39	-	++	++
G28.5	Ledbetter, Seattle	w40	-	-	-
4KB5	Pulford, Oxford	45R	-	-	-
BU25	B'ham Univ.	MHC Class II	-	+	-
8A	Gobbi, Bologna		n/d	++	++
KB61	Pulford, Oxford		-	+	+
6e1	B'ham Univ.	Surface k	n/d	++	++
C4	B'ham Univ.	Surface l	n/d	-	-
2D7	B'ham Univ.	Surface α	n/d	+	+
N1F2	B'ham Univ.	Surface α_1	n/d	+	-
2E2	B'ham Univ.	Surface α_2	n/d	-	-
8a4	B'ham Univ.	Surface G	n/d	-	-
AF6	B'ham Univ.	Surface μ	n/d	-	-
6e1	B'ham Univ.	Cytoplasmic k	+++	+++	+++
C4	B'ham Univ.	Cytoplasmic l	-	-	-
2D7	B'ham Univ.	Cytoplasmic α	++	+	+
N1F2	B'ham Univ.	Cytoplasmic α_1	+	++	+
2E2	B'ham Univ.	Cytoplasmic α_2	-	-	-
8a4	B'ham Univ.	Cytoplasmic G	-	-	-
AF6	B'ham Univ.	Cytoplasmic μ	-	-	-

Table 8.1. Phenotype of JJN-1 and JJN-2, and the original bone marrow of the patient.

The phenotypes of JJN-1 and JJN-2 are very similar, being negative for CD9, 10, 19-23, 37, 40, 45R, but positive for HB8 (CD24), CD38, CD39 and surface kappa. JJN-1 cells show weak reactivity with BU25, an anti-MHC class II MoAb, and weak expression of surface α_1. They strongly express the antigen detected by antibody 8A, and react with KB61. No interleukin 2 (IL2) receptor expression was observed, and even after 4 days incubation with 1% phytohaemagglutinin and 100u/ml recombinant IL2, no IL2 receptors could be detected on the cells of either JJN-1 or JJN-2. The original bone marrow plasma cells were similar except for being $CD39^-$, $KB61^-$.

8.4.4 Secreted immunoglobulin

JJN-1 secreted both alpha (titre 1024) and kappa (titre 32,768) Ig chains. Although kappa chains appeared to be in excess, the sensitivity of the test is much greater for kappa than for alpha chains. JJN-2 secretes only kappa chains (titre 16,384), and when taken out of ESG, it does not regain alpha-secretion.

8.4.5 DNA content

Figure 8.2 shows the DNA content profile of the JJN-1 and JJN-2 cell lines compared with a normal diploid cell control. JJN-1 is hypodiploid, but the height and shape of the G_2/M peak suggests that it may also contain an hypotetraploid population, and a corresponding small hypotetraploid G_2/M peak can be seen. JJN-2 is predominantly hypotetraploid with substantial S and G_2/M phase peaks. There was no evidence for either cell line containing the sum of the DNA contents of a human plus a murine cell (X63-Ag.653 contains approximately 1½ times the DNA content of a diploid human cell).

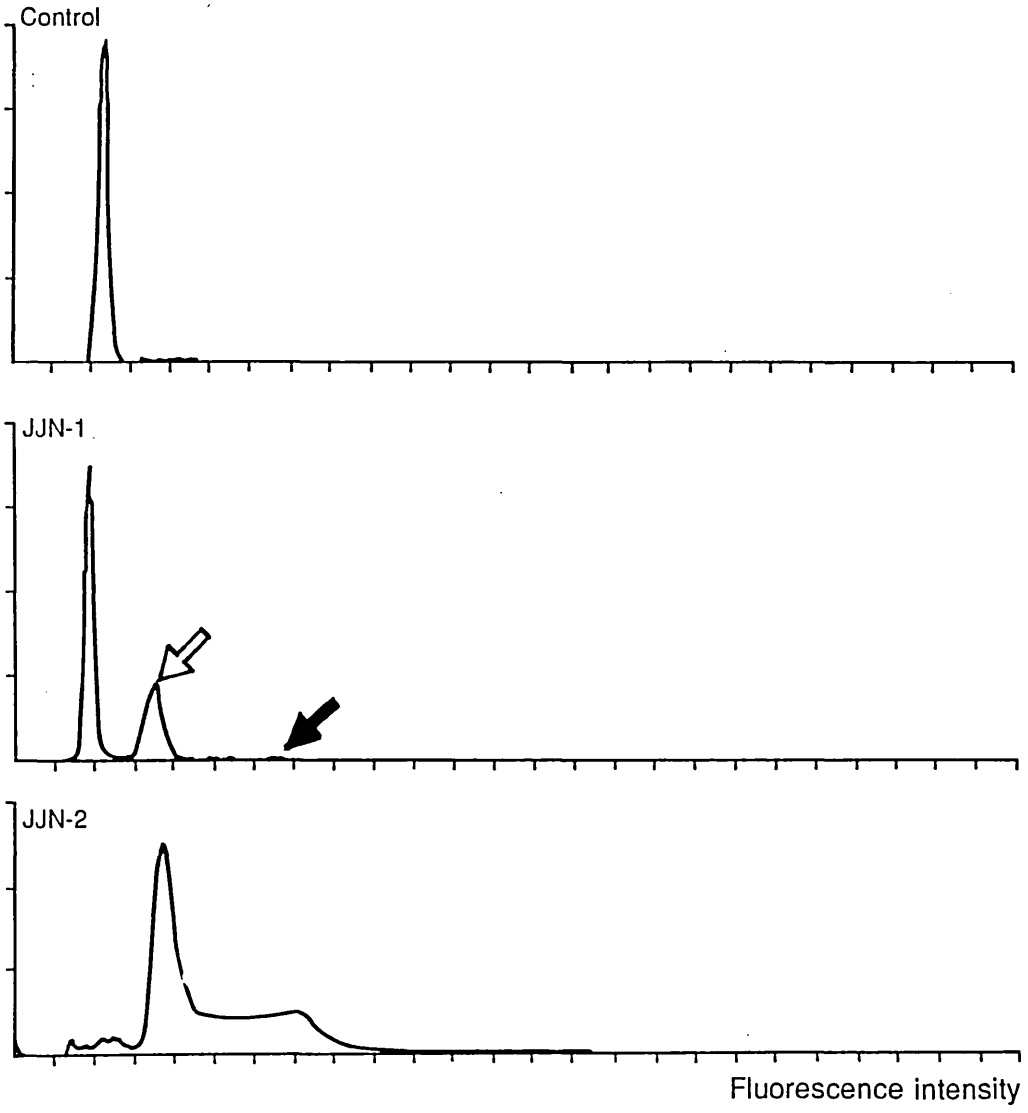


Figure 8.2. The DNA profile of JJN-1 and JJN-2, compared with a normal diploid control. Note the prominent G_2/M population in the JJN-1 trace which may conceal an hypotetraploid population (open arrow), and the possible corresponding G_2/M peak (solid arrow).

8.4.6 Cytogenetics

Metaphase cells were only obtained from the 4 day cultures with TPA. The cytogenetics of the cell lines are complex. JJN-1 was hypodiploid with 40 chromosomes:

40,XX, -7,+der(7)t(7;11)(q32;q13),-9,-10,-11,-12,-13,-14, -16,-20,-20,+mar,del(6)(q15),del(8)(p21),14q+.

JJN-2 was hypotetraploid with between 61 and 82 chromosomes. The karyotypes showed several consistent abnormalities and some marker chromosomes, e.g the following karyotype (as shown in Figure 8.3):

75,XXX, del(Xq),-1,1q+,1q+,-2,-3,del(3)(q23),5p+,5p+, del(6)(q15),del(6)(q15),-7,-7, +der(7)t(7;11)(q32;q12),del(8)(p21),del(8)(p21), -9,-9,-10,-10,-11,-11,-12,-12,-13,-13,-14,-14, 14q+,14q+,-15,-16,-16,-17,-20,del(20)(p11),+4mar.

Of the consistent abnormal chromosomes observed, 4 were frequent in both hypodiploid and hypotetraploid cells: del(6)(q15); der(7)t(7;11)(q32;q13); del(8)(p21); and 14q+. Other abnormalities were found in the hypotetraploid cells as double copies, and included a 5p+ and a del(20)(p11). These may have been present in JJN-1, although they were apparently lost in the cells analysed. Culture of thawed cryopreserved cells from the original bone marrow failed to produce any analyzable metaphases.

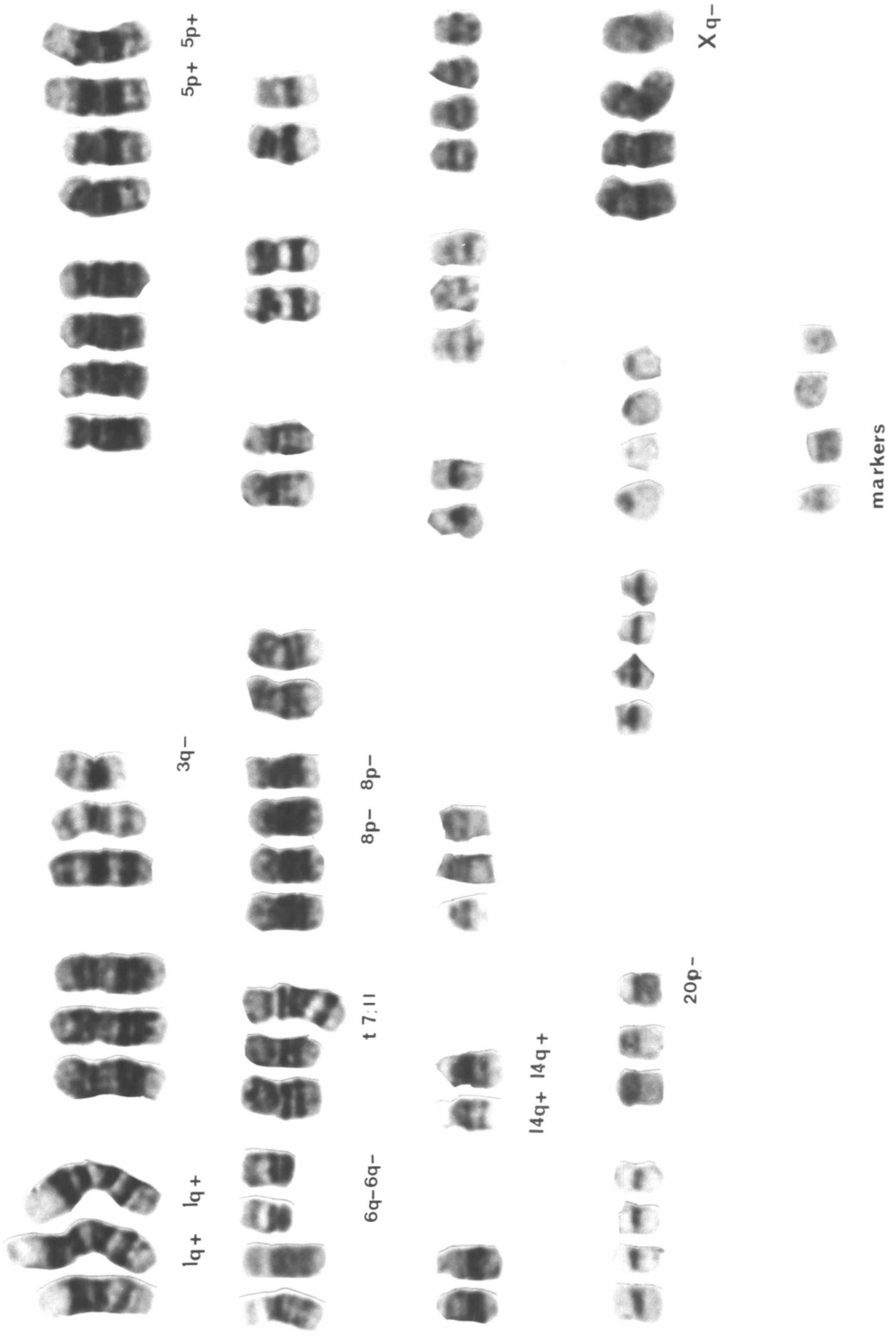


Figure 8.3. The karyotype of a representative cell from JJN-2, showing 75,XXX and the abnormalities as listed in the text.

8.4.7 Response to B-cell stimulatory factor 2

The results of one experiment are shown in Table 8.2. It can be seen that both JJN-1 and JJN-2 showed a graded response to increasing concentrations of ESG, although JJN-2 is clearly more dependent on it than JJN-1.

		Cell line			
		JJN-1		JJN-2	
		—	Anti-BSF-2	—	Anti-BSF-2
Control		100±8	111±6	100±12	110±5
ESG (%)	0.1	122±8	105±8	151±7	98±6
	0.3	115±6	115±8	158±7	86±11
	1.0	162±6	109±11	242±4	89±11
BSF-2 (%)	0.5	121±8	130±12	111±2	99±5
	1.0	120±14	128±8	108±17	85±4
	5.0	158±11	104±3	143±12	73±12

Table 8.2 Response of JJN-1 and JJN-2 to ESG and BSF-2. ^{3}H-thymidine incorporation (as a percentage of control), in the presence of varying concentrations of ESG (Ewing's sarcoma cell line supernatant), and purified BSF-2; with and without anti-BSF-2 present. Numbers are mean±S.D. for experiments done in triplicate.

Table 8.2 also shows that the stimulation by ESG was completely inhibited by the addition of a MoAb specific for BSF-2. This MoAb did not inhibit the resting ^{3}H-thymidine incorporation of JJN-1 and JJN-2 nor of a number of other cell lines (e.g. EB4- a B cell line; MOLT-4 - a T cell line). Purified BSF-2 on its own was not able to produce as great a stimulation of the lines (especially JJN-2) as the ESG supernatant. Results of other stimulation/inhibition experiments are shown in Figures 8.4 and 8.5 (over).

Recombinant interleukin 2 (IL2) had no effect at normal concentrations (10units/ml and 100units/ml), and added nothing to the stimulatory effect of ESG. Recombinant IL2 was actually inhibitory at 1000units/ml. Of the other growth factors tested: fibroblast-conditioned medium gave minor stimulation at 5% or 10%; mouse spleen-conditioned medium had no effect.

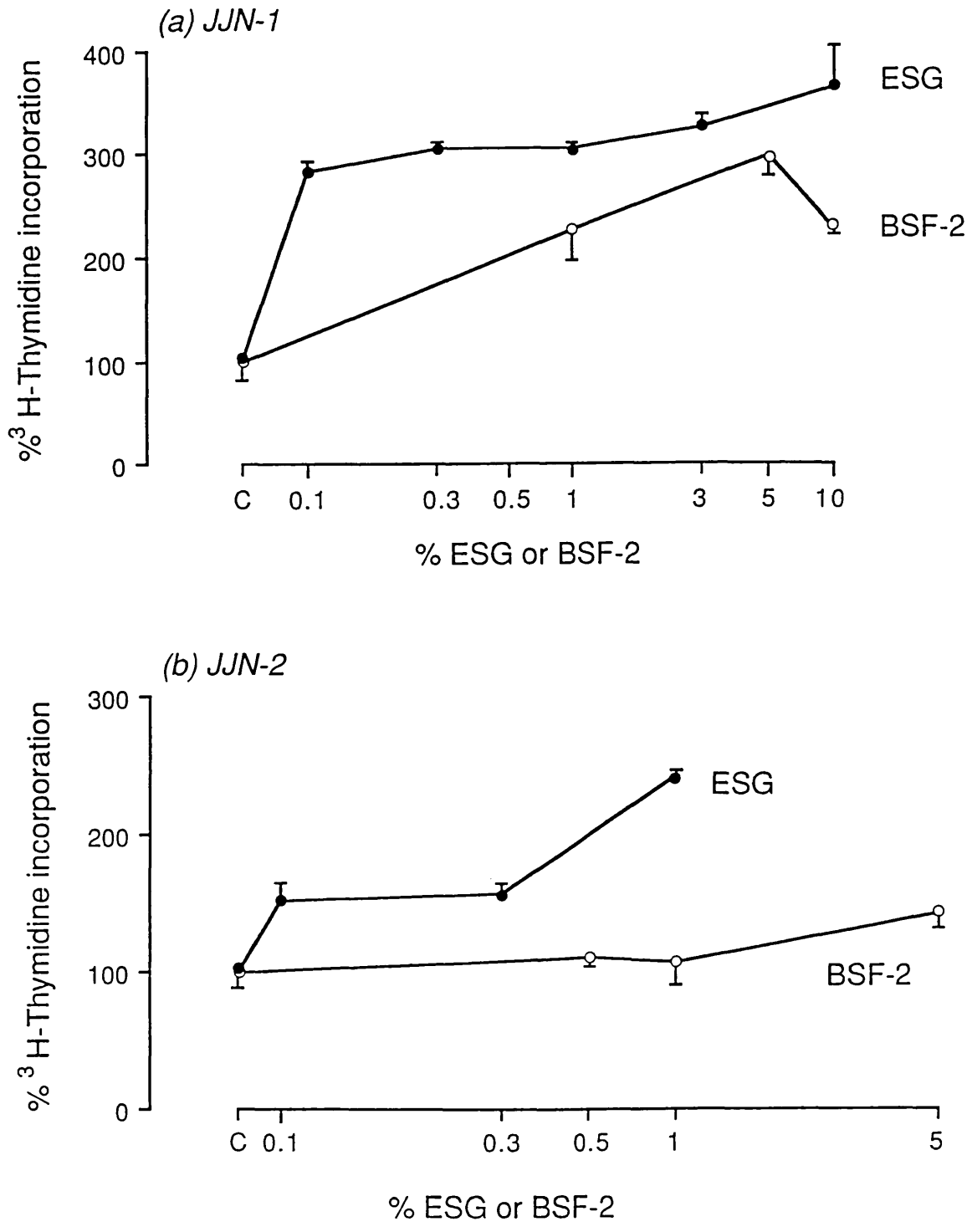


Figure 8.4 (a) the response of JJN-1 to ESG and BSF-2. A plateau of response was seen at a very low concentration in this experiment (0.1%). (b) the response of JJN-2 to ESG and BSF-2. The response to ESG was much more marked in this experiment than to BSF-2.

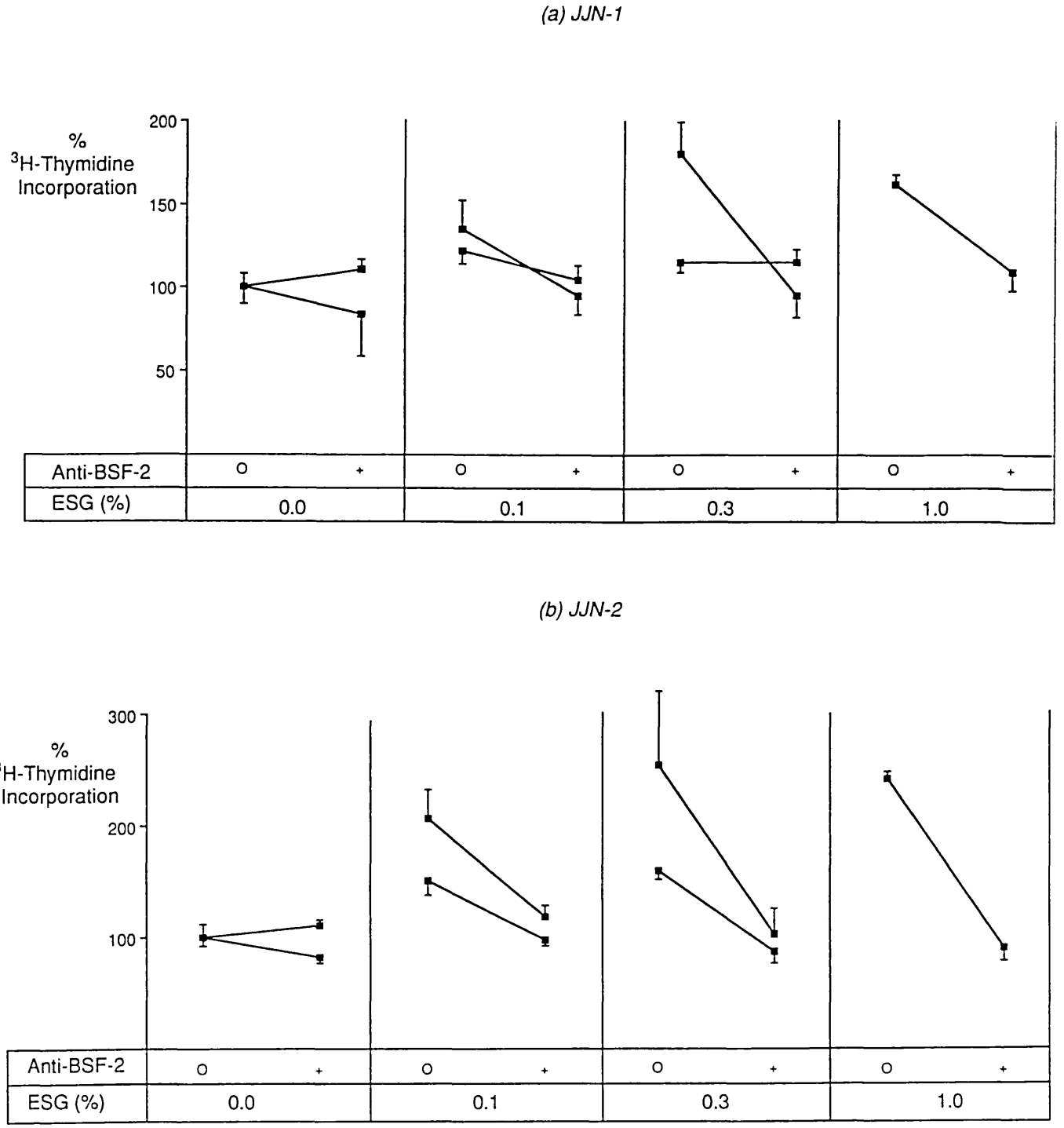


Figure 8.5 The response of JJN-1 (a) and JJN-2 (b) to ESG and BSF-2, with (+) and without (O) a MoAb to BSF-2. The results of two experiments are shown for lower concentrations of the factors. There is a dose responsive increase in ^{3}H-thymidine incorporation, which is inhibited by anti-BSF-2.

8.5 DISCUSSION

It has proved difficult to establish cell lines from plasma cell dyscrasias. Most reported myeloma cell lines have been derived from patients with advanced disease, from extramedullary sites, or from the more unusual forms of myeloma, such as IgD or IgE (Kozbor & Croce, 1985). Recently, several new lines have been derived, some using an enriched medium called M3 (Durie et al, 1985; Garrett et al, 1987). JJN-1 and JJN-2 were derived from the bone marrow of a patient with plasma cell leukaemia at diagnosis, whose paraprotein was IgA_1-kappa. The derivation of these lines from a failed fusion protocol may have been incidental, but it is possible that the mouse spleen cells acted as a feeder layer for a few days before they died. JJN-1 was derived without the addition of exogenous growth factors except FCS, whereas JJN-2 is dependent on added IL6.

8.5.1 Cytogenetics and DNA analysis

JJN-1 and JJN-2 expressed no murine antigens and contained no mouse chromosomes, so there is no evidence that they represent successful fusions between human and mouse plasma cells. It is, however, very difficult to exclude a balanced translocation with exchange of genetic information between mouse and human chromosomes. This seems an unlikely, although possible, explanation for the development of these lines. Murine cells, and in particular plasmacytomas and hybridomas, are known to be responsive to IL6, but this cannot have been responsible for the selection of JJN-1 which is IL6-independent. The near-tetraploidy of JJN-2 suggests that it may be the result of the fusion of two near-diploid (JJN-1) cells at the time of the attempted fusion of the original bone marrow with the mouse myeloma cell line. However, this could have resulted from the failure of a JJN-1 cell to complete mitosis. In fact one near octaploid cell was also observed in the cytogenetic analysis. The presence of these cells in the original bone marrow cannot be excluded, as there was a significant peak on the cellular

DNA content profile of the original marrow corresponding to a hypotetraploid G_0/G_1 population (Figure 8.2). However this peak might just be a prominent hypodiploid G_2/M peak. Unfortunately, cytogenetics on the original bone marrow are not available. The karyotypes of the two lines are complex but show many similarities. Both lines demonstrated a $14q^+$ and a del(6q), which are commonly found in myeloma and plasma cell leukaemia (Dewald et al, 1985). Abnormalities of 14q32, the location of the Ig heavy chain genes, are associated with a number of B cell malignancies (Gahrton et al, 1980; Schröder et al, 1981; Van den Berghe et al, 1979). Abnormalities of 6q are correlated with the production of tumour necrosis factor-β, a potent osteoclast activating factor (Durie et al, 1986). The derived chromosome 7 is also of interest as it may share the 11q13 breakpoint with the t(11;14)(q13;q32) seen in some cases of multiple myeloma, B-cell chronic lymphocytic leukaemia and plasma cell leukaemia (Berger et al,1985). A 5p+ was seen in many of the hypotetraploid cells, and translocations involving 5p have been reported in both B-cell and T-cell malignancies (Berger et al,1985).

8.5.2 The phenotype of the new cell lines

Previously established lines have been reported to express HLA Class II antigens, unlike normal and myeloma plasma cells (Jackson et al, 1988), although one recently described (Lohmeyer et al, 1988) does not. JJN-1 expressed MHC Class II weakly, whereas JJN-2 did not. The lack of antigens recognized by MoAb of the B-cell associated clusters CD 19-23, 37, and w40 demonstrates that JJN-1 and JJN-2 truly represent the terminal stage of B-cell differentiation. However these lines differed from the original bone marrow and the typical myeloma cell phenotype (Jackson et al, 1988) in expressing CD39. The lines also showed relatively weak expression of the HB8 epitope of CD24.

8.5.3 BSF-2 and multiple myeloma

BSF-2 is a 26kDa human protein, produced by many cell types, particularly fibroblasts and T cells (Billiau, 1987), which is a potent growth factor for mouse plasmacytomas and mouse-rat hybridomas (Van Damme et al, 1987). It is capable of inducing Ig synthesis by Epstein-Barr virus transformed human B cells i.e. it possesses B-cell differentiating activity (Hirano et al, 1986).

Kawano et al (1988) have recently described how freshly purified myeloma cells both secrete and proliferate in response to B cell stimulatory factor 2 (BSF-2; IL-6), and that polyclonal antibodies to BSF-2 can reduce the spontaneous ^{3}H-thymidine uptake of these cells. They interpreted these findings as evidence that BSF-2 may function as an autocrine growth factor for human myelomas. This interpretation appears possible although the cells were not 100% pure myeloma cells ('more than 95% pure'), and their reference on this point (Kawano et al, 1986) does not make it clear how this purity was achieved or measured. As they state themselves many other cells are now known to secrete and/or respond to BSF-2, e.g. T cells, fibroblasts, haemopoietic stem cells, all of which might have been present in their preparations. Fresh preparations of myeloma plasma cells are known to have a very low number of cells in DNA synthesis at presentation (<5%, usually <1%) (Durie et al, 1980; Durie et al, 1983) and it might be that cells from other lineages, with higher baseline proliferation rates, might have contributed to the changes in ^{3}H-thymidine incorporation seen. Our autoradiographic studies of myeloma bone marrow have shown that non-myeloma cells show a much higher ^{3}H-thymidine labelling index than do the myeloma plasma cells (data not shown).

While it can be argued that JJN-1 and JJN-2 are not truly representative of the disease in the average patient, they may represent the growth fraction and they are a pure sample of the

disease clone. It is also possible to measure true cell number increase as opposed to just ^{3}H-thymidine incorporation, which can occur in the absence of mitosis in at least some B cell systems (Gordon & Guy, 1987).

JJN-1 and JJN-2 both show increased proliferation in response to ESG, which contains BSF-2. An anti-BSF-2 MoAb is able to reduce this stimulation to control levels, but purified BSF-2 alone does not induce as large a response as ESG. The lines were also stimulated by supernatant from Flow 4000, which being a fibroblast line may also secrete BSF-2. Thus, BSF-2 causes proliferation of these cell lines, but maximal stimulation may require other factors contained in ESG. JJN-2 dies within a week in the absence of BSF-2. The MoAb against BSF-2 did not inhibit the spontaneous ^{3}H-thymidine incorporation of JJN-1 or JJN-2. The data from these cell lines suggest that BSF-2 may play a regulatory role on the growth fraction in myeloma in some cases. They do not, however, support the theory that it is an autocrine growth regulator.

8.5.4 Interleukin 2 and multiple myeloma

Although Interleukin 2 is reported to stimulate the growth of activated B cells (Mingari *et al*, 1984), these cell lines neither expressed IL2 receptors, nor proliferated in response to recombinant IL2. This does not support a regulatory role for IL2 at this late stage of B-cell differentiation.

8.6 CONCLUSION

In conclusion, JJN-1 and JJN-2 are two cell lines representing the terminal stage of B-cell differentiation and exhibiting some characteristic myeloma-associated cytogenetic abnormalities. They appear useful for the study of the physiology of late B-cell differentiation and may be useful as immunogens and screening cells in the production of new anti-plasma cell MoAb.

CHAPTER 9

CONCLUSIONS

The treatment of MM with conventional chemotherapy is palliative. Although recently introduced regimes such as ABCM show some improvement over previous treatments, relapse appears inevitable and long-term survival is poor ((section) 1.3). More intensive regimes such as high dose melphalan have induced complete remissions, but relapses are occurring in most cases (1.3.4). The aim of this research was to study the mature PC and their clonogenic progenitors in MM bone marrow in order to find ways of eliminating ('purging') malignant cells from a MM bone marrow autograft prior to its reinfusion after high dose chemo/radiotherapy. However it is by no means certain that purging marrow is necessary for the success of ABMT, as good results have been reported in AML without it (1.4.2, 1.4.3). The major difficulty is eliminating the disease in the patient, rather than in the 2-5% of the bone marrow which is harvested for reinfusion. It was also hoped that a study of the biology of the progenitor cell might lead to the development of new therapies for the disease (such as biological response modifiers).

9.1 The phenotype of multiple myeloma cells

MM plasma cells lacked expression of CD19-23, 37, 39 and w40, but they strongly expressed CD38 and the HB8 epitope of CD24 (4.3, 4.4). They sometimes expressed CD9 and small numbers of CD10 cells were also found. However HB8 reacted strongly with myeloid cells which would make it a difficult agent to use for purging BM. Similarly CD38 MoAb also reacted with T cells, and the lack of reactivity of CD24 and CD38 MoAb with haemopoietic stem cells has not been established. Strong intracytoplasmic reactivity with anti-RER MoAb was seen in MM PC. Of the other MoAb tested KB61 reacted with some PC in 50% case. MoAb 8A and a number we made ourselves reacted with most myeloma PC, but also reacted broadly with a number of other cell types (4.4.5). Their lack of haemopoietic stem cell toxicity needs careful evaluation before they can used as purging agents for ABMT. No completely specific anti-PC MoAb have yet been identified.

9.2 Culture studies

The aim was to use short-term in vitro cultures as clonogenic assays of MM progenitors, both for the study of their biology and to assay their elimination after a purging procedure. The system we used could not be validated because the cells evidently migrated into clumps rather than formed colonies in methylcellulose-containing media (5.4). Thus experiments to test the purging of BM with cytotoxic drugs (Asta-Z and 4HC) gave inconsistent results which served only to confirm the invalidity of the method as a clonogenic assay (5.3.7). Doses of irradiation sufficient to kill virtually all cells failed to prevent clumps forming (5.3.6). It may be that clonogenic assays can be developed in more immobilising media (e.g. agar) utilizing factors identified by long-term culture experiments as necessary for myeloma progenitor cell growth. However, short-term incubation of MM BM with PHA and IL2 did give some evidence for a possible role for IL2 in the growth regulation in MM (6.4).

It was hoped that long-term cultures of MM BM would allow immortal cell lines to emerge that would be representative of the dividing progenitor cells. Although MM PC remained viable for six or more weeks in liquid cultures, no cell lines were obtained by this method (7.3.1). However, one Ig-secreting PC line (JJN-1) was obtained from a failed fusion protocol (8.4). The surface phenotype of JJN-1 was CD24(HB8)$^+$, CD10$^-$, CD19-23$^-$, CD37$^-$, CD39$^+$. A more rapidly growing, IL6-dependent, hypo-tetraploid subclone (JJN-2) was selected by culture in IL6-containing medium. These results suggest that at least in some cases, myeloma progenitor cells can be at the plasma cell or immediately pre-plasma cell stage, and that IL6 may play a growth-modulating role in MM. CD39, which was expressed on JJN-1, JJN-2 and the hybridomas between human MM and a mouse plasmacytoma (7.3.2), but which was not expressed on mature PC in MM, may be a marker for the proliferating cell. These cell lines and hybridomas may be useful as immunogens or screening

cells in the production of specific anti-PC MoAb. However, initial attempts to do this have not been successful.

9.3 Future studies

The clonogenic cells in MM could be investigated as follows:

1. The development of further cell lines from MM BM, by liquid culture techniques.
2. These cell lines could be used to study the phenotype, karyotype, growth factor regulation and gene expression (including oncogenes, drug resistance genes, growth factor genes and their products) of a dividing MM cell population.
3. The cell lines might also be useful for testing new approaches to treatment. In fact, the effect of a number of interferons and interleukins on the growth, biochemistry, and growth factor messenger RNA expression of JJN-1 are currently being studied.
4. An alternative approach to studying the phenotype of proliferating cells in MM, which is applicable to all cases of MM would be to double or triple label cells for various surface antigens and a proliferation marker, e.g. nuclear incorporation of bromodeoxyuridine or expression of Ki67 (Gerdes *et al*, 1983), as performed by Lokhorst and co-workers (1986 & 1987).
5. When the phenotype of the MM progenitor cell(s) is clearly identified, a more rigorous search for effective purging MoAb will be justified. Their lack of toxicity on normal haemopoietic stem cells will have to be established in both short- and long-term BM cultures. Their clinical value will, however, be difficult to prove without very large clinical trials.

Ultimately a greater understanding of the biology of MM may lead to an improved outcome and even cure for patients with this currently incurable and unpleasant malignancy.

CHAPTER 10

REFERENCES

Alexanian R., Balcerzak S., Haut A., Gehan E., for the Southwest Oncology Group (1975).
Remission maintenance therapy for multiple myeloma. Southwest Oncology Group study..
Arch Intern Med 135,147-152.

Alexanian R., Gehan E., Haut A., Saiki J., Weick J., for the Southwest Oncology Group (1978).
Unmaintained remissions in multiple myeloma.
Blood 51,1005-1011.

Ambrus J.L., Fanci A.S. (1985).
Human B lymphoma cell line producing B cell growth factor.
J Clin Invest 75,732-739.

Anderson K.C., Bates M.P., Slaughenhoupt B., Schlossman S.F., Nadler L.M. (1984).
A monoclonal antibody with reactivity restricted to normal and neoplastic plasma cells.
J Immunol 132,3172-3179.

Anderson K.C., Park E.K., Bates M.P., Leonard R.C.F., Hardy R., Schlossman S.F., Nadler L.M. (1983).
Antigens on human plasma cells identified by monoclonal antibodies.
J Immunol 130,1132-1138.

Appelbaum F.R., Buckner C.D. (1986).
Overview of the clinical relevance of autologous bone marrow transplantation.
Clinics in Haematology 15,1-18.

Appelbaum F.R., Deisseroth A.B., Graw R.G.jr, Herzig G.P., Levine A.S., Magrath I.T., Pizzo P.A., Poplack D.G., Ziegler J.L. (1978).
Prolonged complete remission following high dose chemotherapy of Burkitt's lymphoma in relapse.
Cancer 41,1059-1963.

Appelbaum F.R., Herzig G.P., Ziegler J.L., Graw R.G., Levine A.S., Deisseroth A.B. (1978).
Successful engraftment of cryopreserved autologous bone marrow in patients with malignant lymphoma.
Blood 52,85-95.

Arnold A., Cossman J., Bakhshi A., Jaffe E.S., Waldmann T.A., Korsmeyer S.J. (1983).
Immunoglobulin-gene rearrangements as unique clonal markers in human lymphoid neoplasms.
N Eng J Med 309,1593-1599.

Arthur C.K., Apperley J.F., Guo A.P., Rassool F., Gao L.M., Goldman J.M. (1988).
Cytogenetic events after bone marrow transplantation for chronic myeloid leukaemia in chronic phase.
Blood 71,1179-1186.

Ball E.D. (1988).
In vitro purging of bone marrow for autologous marrow transplantation in acute myelogenous leukemia using myeloid-specific monoclonal antibodies.
Bone Marrow Transplantation 3,387-392.

Ball E.D., Mills L.E., Cornwell G.G., Davis B.H., Coughlin C.T., Howell A.L., Stukel T.A., Dain B.J., McMillan R., Spruce W., Miller W.E., Thompson L. (1990).
Autologous bone marrow transplantation for acute myeloid leukemia using monoclonal antibody-purged marrow.
Blood 75,1199-1206.

Barlogie B., Alexanian R. (1986).
Cellular aspects of myeloma: biologic and clinical implications.
In: Multiple myeloma and other paraproteinaemias (Ed. Delamore I.W.). Churchill Livingstone. London.

Barlogie B., Alexanian R., Dicke K.A., Zagers G. (1986).
High-dose melphalan (HDM) ± total body irradiation (TBI) and bone marrow transplantation (BMT) for refractory myeloma.
Blood 68 (suppl 1),235a.

Barlogie B., Alexanian R., Dixon D., Smith L., Smallwood L., Delaselle K. (1985).
Prognostic implications of tumor cell DNA and RNA content in multiple myeloma.
Blood 66,338-341.

Barlogie B., Alexanian R., Gehan E.A., Smallwood L., Smith T., Drewinko B. (1983).
Marrow cytometry and prognosis in myeloma.
J Clin Invest 72,853-861.

Barlogie B., Epstein J., Alexanian R. (1988).
Genotypic and phenotypic characteristics of multiple myeloma.
Hematol Oncol 6,99-102.

Barlogie B., Latreille J., Freireich E., Fu C., Mellard D., Meistrich M., Adreeff M. (1980).
Characterization of hematologic malignancies by flow cytometry.
Blood Cells 6,719-741.

Barlogie B., Smith L., Alexanian R. (1984).
Effective treatment of advanced multiple myeloma refractory to alkylating agents.
N Engl J Med 314,1353-1356.

Bast B.J.E.G., Boom S.E., Ballieux R.E. (1982).
Characterization of the colony-forming cell in monoclonal gammopathies.
Blood 60,608-612.

Bast R.C. Jr., De Fabritiis P., Lipton J., Gelber R., Mauer C., Nadler L., Sallan S., Ritz J. (1985).
Elimination of malignant clonogenic cells from human bone marrow using multiple monoclonal antibodies and complement.
Cancer Res 45,499-503.

Bast R.C.jr, De Fabritiitis P., Lipton J., Gelber R., Maver C., Nadler L., Sallan S., Ritz J. (1985).
Elimination of malignant clonogenic cells from human bone marrow using monoclonal antibodies and complement.
Cancer Research 45,499-503.

Bence Jones H. (1848).
On a new protein occuring in the urine of a patient with mollities ossium.
Proc Roy Soc (1848) p.673.

Benjamin D., Magrath I.T., Douglass E.C., Corash L.M. (1983).
Derivation of lymphoma cell lines from microscopically normal bone marrow in patients with undifferentiated lymphomas: evidence of occult bone marrow involvement.
Blood 61,1017-1019.

Berger R., Bloomfield C.D., Sutherland G.D. (1985).
Report of the committee on chromosome rearrangements in neoplasia and on fragile sites.
In Human gene mapping 8, Cytogenetics & Cell Genetics 40,490-535.

Bernard A., Boumsell L., Dansset J., Milstein C., Schlossman S.F. (eds) (1984).
Leucocyte typing I. Human leukocyte differentiation antigens detected by monoclonal antibodies.
Springer Verlag, Berlin.

Bernheim A., Berger R., Preud'homme J.L., Labaume S., Bussel A., Barot-Ciorbaru R. (1981).
Philadelphia chromosome positive blood B lymphocytes in chronic myeloid leukemia.
Leuk Res 5,331-339.

Billiau A. (1987).
Interferon β_2 as a promoter of growth and differentiation of B cells.
Immunology Today 8,84-87.

Blattner W.A. (1980).
Epidemiology of multiple myeloma and related plasma cell disorders.
In Progress in Myeloma (Ed. Potter M.) Elsevier, North Holland Inc. New York. pp.1-66.

Blattner W.A., Mason T.J., Blair A. (1980).
Changes in mortality rates for multiple myeloma.
N Engl J Med 302,814-815.

Boccadoro M., Gavarotti P., Fossati G., Pileri A., Marmont F,. Nerotto G., Gallamino A., Volta C., Tribalto M., Testa M.G,. Amadori S,. Mandelli F., Durie B.G.M. (1984).
Low plasma cell ^{3}H-thymidine incorporation in monoclonal gammopathy of undetermined significance, smouldering myeloma and remission phase myeloma:a reliable indicator of patients not requiring therapy.
Br J Haematol 58,689-696.

Boucheix C., Benoit P., Krief P., Billard M., Mishal Z., Azzarone B., Rendu E., Esnouf J., Hermant L., Bredoux R., Levy-Toledano S., Soria C., Perrot JY., Mirshahi M., Giannoni F., Bernadou A., Soria J. (1987).
Platelet aggregation induced by CD9 mAbs. Mechanisms and comparison with platelet aggregating properties of mAbs directed against other membrane proteins..
In Leucocyte Typing III (ed. by A.J. McMichael et al.). Oxford University Press, Oxford. pp.780-782.

Bregni M., Sienna S., Neri A., Bassan R., Barbui T., Delia D., Bonadonna G., Dalla Favera R., Gianni A.M. (1989).
Minimal residual disease in acute lymphoblastic leukemia detected by immune selection and gene rearrangement analysis.
J Clin Oncol 7,338-343.

Brenning G. (1986).
The in vitro effect of leukocyte α-interferon on human myeloma cells in a semisolid agar system.
Scand J Haematol 35,178-185.

Buick R.N., Chang L.J-A., Messner H.A., Curtis J.E., McCulloch E.A. (1981).
Self-renewal capacity of leukemic blast progenitor cells.
Cancer Res 41,4849-4852.

Bunin N., Mino J., Raimondi S. (1986).
Gene rearrangements as clonal markers at diagnosis and relapse in childhood acute leukemia.
Blood 68,196a.

Bunn P.A., Krasnow S., MaKuch R.W., Schlam M.L., Schechter G.P. (1982).
Flow cytometric analysis of DNA content of bone marrow cells in patients with plasma cell myeloma: clinical implications.
Blood 59,528-535.

Bunn P.A. Jr., Whang-Peng J., Carney D.N., Schlam M.L., Knutsen T., Gazdar A.F. (1980).
DNA content analysis by flow cytometry and cytogenetic analysis in mycosis fungoides and the Sézary syndrome: diagnostic and prognostic implications.
J Clin Invest 65,1440-1448.

Burk K.H., Drewinko B., Turjillo J.M., Ahearn M.J. (1978).
Establishment of a human plasma cell line in vitro.
Cancer Res 38,2508-2513.

Burnett A.K., Watkins R., Maharaj D., Mckinnon S., Tansey P., Alcorn M., Singer C.R.J., McDonald G.A., Robertson A.G. (1984).
Transplantation of unpurged autologous bone marrow in acute myeloid leukaemia in first remission.
Lancet ii,1068-1070.

Burns G.F., Worman C.P., Roberts B.E., Raper C.G., Barker C.R., Cawley J.C. (1979).
Terminal B cell differentiation as seen in human myelomas and related disorders.
Clin Exp Immunol 35,180-189.

Butler Y.L., Lane H.C., Fanci A.S. (1983).
Delineation of optimal conditions for producing mouse-human heterohybridomas from human peripheral B cells of immunised subjects.
J Immunol 130,165-168.

Butturini A., Bortin M.M., Gale R.P. (1987).
Graft-versus-leukemia following bone marrow transplantation.
Bone Marrow Transplantation 2,233-242.

Buzaid A.C., Durie B.G.M. (1988).
Management of refractory myeloma: a review.
J Clin Oncol 6,889-905.

Cabanillas F., Hagemeister F.B., Bodey G.P., Freireich E.J. (1982).
IMVP-16: An effective regimen for patients with lymphoma who have relapsed after initial combination chemotherapy.
Blood 60,693.

Cabanillas F., Hagemeister F.B., Mclaughlin P., Velaquez W.S., Riggs S., Fuller L., Smith T. (1987).
Results of MIME salvage regimen for recurrent or refractory lymphoma.
J Clin Oncol 5,407.

Caligaris-Cappio F., Bergui L., Tesio L., Pizzolo G., Malavasi F., Chilosi M., Campana D., van Camp B., Janossy G. (1985).
Identification of malignant plasma cell precursors in the bone marrow of multiple myeloma.
J Clin Invest 76,1243-1251.

Callen D.F., Ford J.M. (1983).
Chromosome abnormalities in chronic lymphocytic leukemia revealed by TPA as mitogen.
Cancer Genet Cytogenet 10,87-93.

Cassileth P.A., Begg C.B., Bennett J.M., Bozdech M., Kahn S.B., Weiler C., Glick J.H. (1984).
A randomized study of the efficacy of consolidation therapy in acute non-lymphocytic leukemia.
Blood 63,843-847.

Chang J., Morgernstern G., Deakin D., Testa N.G., Coutinho L., Scarffe J.H., Harrison C., Dexter T.M. (1986).
Reconstitution of haemopoietic system with autologous marrow taken during relapse of acute myeloblastic leukaemia and grown in long-term culture.
Lancet i,294-295.

Chen K-C., Bevan P.C., Matthews J.G. (1986).
Analysis of G banded karyotypes in myeloma cells.
J Clin Pathol 39,260-266.

Chessels J.M., Rogers D.W., Leiper A.D., Plowman P.N., Levinsky R., Blacklock H., Richards S., Festinstein H. (1986).
Bone marrow transplantationhas a limited role in prolonging second marrow remission in childhood lymphoblastic leukaemia.
Lancet i,1239-1241.

Child J.A., Norfolk D.R., Cooper E.H. (1986).
Serum beta$_2$-microglobulin in myelomatosis.
Br J Haematol 63,406-407 (letter).

Clark E.A., Ledbetter J.A. (1986).
Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp40.
Proc Natl Acad Sci USA 83,4494-4498.

Clavell L.A., Gelber R.D., Cohen H.J., Hitchcock-Bryan S., Cassady J.R., Tarbell N.J., Blattner S.R., Tantravahi R., Leavitt P., Sallan S.E. (1986).
Four-agent induction and intensive asparaginase therapy for acute lymphoblastic leukemia.
N Engl J Med 315,657-663.

Conklin R., Alexanian R. (1975).
Clinical classification of plasma cell myeloma.
Arch Intern Med 135,139-143.

Copplestone J.A., Mufti G.J., Hamblin T.J., Oscier D.G. (1986).
Immunological abnormalities in myelodysplastic syndromes. II. Coexistent lymphoid or plasma cell neoplasms: a report of 20 cases unrelated to chemotherapy.
Br J Haematol 63,149-159.

Cuzick J. (1986).
Epidemiology of multiple myeloma.
In: Multiple Myeloma and other Paraproteinaemias (Ed. by Delamore I.W.), Churchill Livingstone. London. pp46-55.

Cuzick J., Cooper E.H., Maclennan I.C.M. (1985).
The prognostic value of serum β_2-microglobulin compared with other presentation features in myelomatosis (a report to the MRC's working party on leukaemia in adults).
Br J Cancer 52,1-6.

Da W.M., Lin Y., Wei S.N. (1989).
Studies on sensitivity of human GM-CFU and L-CFU to hyperthermic killing in vitro.
Leuk Res 13,217-220.

Dalyrymple J. (1848).
On the microscopic character of 'mollities ossium'.
Dublin Quart J Med Sci 2,85.

Delmastro-Galfré P., Calabi F., Arno J., Karpas A., Hayhoe F.G.J. (1987).
Monoclonal antibodies specific for human myeloma/plasma cells.
In: Leucocyte Typing III. Oxford University Press. Oxford. pp510-511.

Dewald G.W., Kyle R.A., Hicks G.A., Greipp P.R. (1985).
The clinical significance of cytogenetic studies in 100 patients with multiple myeloma, plasma cell leukemia or amyloidosis.
Blood 66,380-390.

Dicke K.A., Spinolo J.A. (1989).
High-dose therapy and autologous bone marrow transplantation (ABMT) in acute leukemia: is purging necessary?.
Bone Marrow Transplant 4,Suppl 1,184-186.

Dicke K.A., Poynton C.H., Reading C.L. (1985).
Elimination of leukaemic cells from remission marrow suspensions by an immunomagnetic procedure.
In: Löwenberg B & Hagenbeck (eds). Minimal residual disease in acute leukaemia. The Hague: Martinus Nijhoff. pp209-221..

Dicke K.A., Zander A.R., Spitzer G., Verma D.S., Peters L., Vellekoop L., McCredie K.B., Hester J. (1979).
Autologous bone-marrow transplantation in relapsed adult acute leukaemia.
Lancet i,514-517.

Dorshkind K., (1987).
Bone marrow stromal cells and their factors regulate B-cell differentiation.
Immunology Today 8,191-193.

Douay L., Gorin N.C., Mary J-Y., Lemarie E., Lopez M., Najman A., Stachowiak J., Giarratana M-C., Baillou C., Salmon C., Duhamel G. (1985).
Recovery of CFUGM from cryopreserved marrow and in vivo evaluation after autologous bone marrow transplantation are predictive of engraftment.
Exp Hematol 14,358-365.

Drewinko B., Alexanian R., Boyer H., Barlogie B., Rubinow S.I. (1981).
The growth fraction of human myeloma cells.
Blood 57,333-338.

Durie B.G.M., Grogan T.M. (1985).
CALLA-positive myeloma: an aggressive subtype with poor survival.
Blood 66,229-232.

Durie B.G.M., Salmon S.E. (1975).
A clinical staging system for multiple myeloma. Correlation of measured myeloma cell mass with presenting clinical features, response to treatment, and survival.
Cancer 36,842-854.

Durie B.G.M., Salmon S.E. (1982).
The current status and future prospects of treatment for multiple myeloma.
Clin Haematol 11,181-210.

Durie B.G.M., Baum V., Vela E., Mundy G. (1986).
Abnormalities of chromosome 6q and osteoclast activating factor (TNFβ) production in multiple myeloma.
Blood 68,208a.

Durie B.G.M., Salmon S.E., Moon T.E. (1980).
Pretreatment tumor mass, cell kinetics, and prognosis in multiple myeloma.
Blood 55,364-372.

Durie B.G.M., Vela E., Baum V., Leibovitz A., Payne C.M., Richter L.C., Grogan T.M., Trent J.M. (1985).
Establishment of two new human myeloma cell lines from bilateral pleural effusions: evidence for sequential in vivo clonal change.
Blood 66,548-555.

Durie B.G.M., Young L.A., Salmon S.E. (1983).
Human myeloma in vitro colony growth: interrelationships between drug sensitivity, cell kinetics, and patient survival duration.
Blood 61,929-934.

Epstein J., Barlogie B., Katzmann J., Alexanian R. (1988).
Phenotypic heterogeneity in aneuploid multiple myeloma indicates Pre-B cell involvement.
Blood 71,861-865.

Falkenberg J.H.F., Fibbe W.E., Veenhof W.F.J., Koning F., Van Eeden G., Voogt P.J., Jansen J. (1986).
Selective removal of clonogenic neoplastic B cells from human bone marrow using anti-HLA-DQ antibodies and complement.
Exp Hematol 14,101-107.

Fefer A. (1986).
Current status of syngeneic marrow transplantation and its relevance to autografting.
Clin Haematol 15:1,49-65.

Fefer A., Greenberg P.D., Cheever M.A., Appelbaum F.R., Bluming A.Z., Storb R., Thomas E.D. (1982).
Identical twin marrow transplantation (BMT) for multiple myeloma.
Blood 60,(suppl):168a.

Feinleib M., MacMahon B. (1960).
Duration of survival in multiple myeloma.
J Nat Cancer Inst 24,1259-1269.

Fialkow P.J., Jacobson R.J., Papayannopoulou T (1977).
Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet, and monocyte/macrophage.
Am J Med 63,125-130.

Figdor C.G., Leemans J.M., Bout W.S., de Vries J.E. (1983).
Theory and practice of centrifugal elutriation (CE). Factors influencing the separation of human blood cells.
Cell Biophys 5,105-118.

Foon K.A., Schroff R.W., Gale R.P. (1982).
Surface markers on leukemia and lymphoma cells: recent advances.
Blood 60,1-19.

Gahrton G., Robert K-H., Friberg K., Zech L., Bird A.G. (1980).
Nonrandom chromosome aberrations in chronic lymphocytic leukemia revealed by polyclonal B-cell-mitogen stimulation.
Blood 56,640.

Gahrton G., Tura S., Flesch M., Gratwohl A., Gravett P., Lucarelli G., Michallet M., Reiffers J., Ringden O., van Lint M.T., Vernant J.P., Zwaan F.E. (1987).
Bone marrow transplantation in multuiple myeloma: report from the European Cooperative Group for Bone Marrow Transplantation.
Blood 69,1262-1264.

Galfré G., Milstein C. (1982).
Properties of the Monoclonal Antibodies Produced by Hybridoma Technology and their application to the Study of Diseases..
In: UNDP/World Bank/WHO, V.Houba & S.H.Chan (eds) p.1.

Garrett I.R., Durie B.G.M., Nedwin G.E., Gillespie A., Bringman T., Sabatini M., Bertolini D.R., Mundy G.R. (1987).
Production of Lymphotoxin, a bone-resorbing cytokine, by cultured human myeloma cells.
N Engl J Med 317,526-532.

Gassmann W., Pralle H., Haferlach T., Pandurevic S., Granbuer M., Schmitz N., Löffler H. (1985).
Staging systems for multiple myeloma: a comparison.
Br J Haematol 59,703-711.

Gaynon P.S. (1990).
Primary treatment of childhood acute lymphoblastic leukemia of non-T cell lineage (including infants).
Hematol Oncol Clin Nth Am 4:5,915-936.

Gaynon P.S., Blayer W.A., Steinherz P.G., Finklestein J.Z., Littman P.S., Miller D.R., Reaman G.H., Sather H.N., Hammond G.D. (1988b).
Modified BFM therapy for children with previously untreated acute lymphoblastic leukemia and unfavourable prognostic features. Report of Children's Cancer Study Group study CCG-193P.
Am J Pediatr Hematol Oncol 10,42-50.

Gaynon P.S., Steinherz P.G., Bleyer W.A., Ablin A.R., Albo V.C., Finklestein J.Z., Grossman N.J., Littman P.S., Novak L.T., Pyesmany A.F., Reaman G.H., Sather H.N., Denman-Hammond G. (1988a).
Intensive therapy for children with acute lymphoblastic leukaemia and unfavourable presenting features.
Lancet ii,921-924.

Gerdes J., Schwab U., Lemke H., Stein H. (1983).
Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation.
Int J Cancer 31,13-20.

Golay J.T., Clarke E.A., Beverley P.C.L. (1985).
The CD20 [Bp35] antigen is involved in activation of B cells from the G_0 to the G_1 phase of the cell cycle.
J Immunol 135,3795-3801.
Goldie J.H., Coldman A.J. (1983).
Quantitative model for multiple levels of drug resistance in clinical tumors.
Cancer Treat Rep 67,923-931.

Goldstein M., Hoxie J., Zembryki D., Matthews D., Levinson A.I. (1985).
Phenotypic and functional analysis of B cell lines from patients with multiple myeloma.
Blood 66,444-446.

Goldstone A.H.(Ed). (1986).
Autologous bone marrow transplantation.
Clin Haematol 15,No.1.

Gonchoroff N.J., Katzman J.A., Garton J.P., Ruiz-Argüelles G.J., Eilers C.R., Greipp P.R., Kyle R.A. (1986).
A monoclonal antibody reactive with a subset of human plasma cells.
Br J Haematol 62,619-630.

Gordon J., Guy G.R. (1987).
The molecules controlling B lymphocytes.
Immunology Today 8,339-344.

Gordon M.Y., Hibbin J.A., Kearney L.U., Gordon-Smith E.C., Goldman J.M. (1985).
Colony formation by primitive haemopoietic progenitors in cocultures of bone marrow cells and stromal cells.
Br J Haematol 60,129-136.

Gorin N.C. (1983).
Autologous bone marrow transplantation in haematological malignancies.
Eur J Cancer and Clin Oncol 20,1-9.

Gorin N.C., Aegerter P. (1987).
Autologous bone marrow transplantation for acute leukaemia in remission: 4th EBMTG survey.
Bone Marrow Transplantation 2(suppl.1),320-323.

Gorin N.C., Aegerter P., Auvert B., Meloni G., Goldstone A.H., Burnett A., Carella A., Korbling M., Herve P., Maraninchi D., Löwenberg R., Verdonck L.F., de Planque M., Hermans J., Helbig W., Porcellini A., Rizzoli V., Alesandrino E.P., Franklin I.M., Reiffers J., Colleselli P., Goldman J.M. (1990).
Autologous bone marrow transplantation for acute myelocytic leukemia in first remission: A European survey of the role of marrow purging.
Blood 75,1606-1614.

Gorin N.C., Douay L., Laporte J.P., Lopez M., Mary J.Y., Najman A., Salmon C., Aegeter P., Stachowiak J., David R., Pene F., Kantor G., Deloux J., Duhamel E., van der Akker J., Gerota J., Parlier Y., Duhamel G. (1986).
Autologous bone marrow transplantation using marrow incubated with Asta-Z 7557 in adult acute leukemia.
Blood 67,1367.

Gorin N.C., Herzig G., Bull M.I., Graw R.G. (1978).
Long-term preservation of bone marrow and stem cell pools in dogs.
Blood 51,257-265.

Greene M.H. (1982).
Non-Hodgkin's lymphoma and mycosis fungoides.
In: Cancer Epidemiology and prevention, (ed. Schottenfeld D., Fraumeni J.), Saunders. pp.754-778.

Greipp P.R., Kyle R.A. (1983).
Clinical, morphological, and cell kinetic differences among multiple myeloma, monoclonal gammopathy of undetermined significance, and smoldering multiple myeloma.
Blood 62,166-171.

Gribben J., Goldstone A.H., Linch D.C., Anderson C.C., Richards J.D.M. (1987).
Double autografting as potential curative therapy for acute leukaemia.
Bone Marrow Transplantation 2,(suppl.1),46-47.

Griffin J.D., Larcom P., Schlossman S.F. (1983).
Use of surface markers to identify a subset of acute myelomonocytic leukemia cells with progenitor cell properties.
Blood 62,1300-1303.

Griffiths G.M., Berek C., Kaartinen M., Milstein C. (1984).
Somatic mutation of the immune response to z-phenyl oxazalone.
Nature 304,320-324.

Grogan T.M., Durie B.G.M., Lomen C., Speir C., Wirt D.P., Nagle R., Wilson G.S., Richter L., Vela E., Maxey V., McDaniel K., Rangel C. (1987).
Delineation of a novel pre-B cell component in plasma cell myeloma: immunochemical, immunophenotypic, genotypic, cytologic, cell culture and kinetic features.
Blood 70,932-942.

Guy G.R., Gordon J. (1987).
Coordinated action of IgE and a B-cell-stimulatory factor on the CD23 receptor molecule up-regulates B-lymphocyte growth.
Proc Natl Acad Sci 84,6239-6243.

Hale G., Bright S., Chumbley G., Hoang T., Metcalf D., Munro A.J., Waldmann H. (1983).
Removal of T cells from bone marrow for transplantation: a monoclonal anti-lymphocyte antibody that fixes human complement.
Blood 62,873-882.

Hale G., Dyer M.J., Clark M.R., Phillips J.M., Marcus R., Riechmann L., Winter G., Waldmann H. (1988).
Remission induction in non-Hodgkin's lymphoma with reshaped human monoclonal antibody CAMPATH-1H.
Lancet ii,1394-1399.

Hall E.J. (1978).
Radiobiology for the radiologist. 2nd Edition.
Harper and Row. Hagerstown.

Halper J., Fu S.M., Wang C.Y., Winchester R., Kunkel H.G. (1978).
Patterns of expression of human 'Ia-like' antigens during the terminal stage of B cell development.
J Immunol 120,1480-1484.

Hamburger A., Salmon S.E. (1977).
Primary bioassay of human myeloma stem cells.
J Clin Invest 60,846-854.

Hamburger A.W., Salmon S.E. (1985).
Myeloma colony assays.
In: Hematopoietic stem cells (Ed. by Golde D., Takaku F.), Marcel Dekker Inc. New York. pp291-308.

Hansen O.P., Galton D.A.G. (1985).
Classification and prognostic variables in myelomatosis.
Scand J Haematol 35,10-19.

Hansen-Hagge T.E., Yokota S., Bartram C.R. (1989).
Detection of minimal residual disease in acute lymphoblastic leukemia by in vitro amplification of rearranged T-cell receptor delta chain sequences.
Blood 74,1762-1767.

Herrmann F., Cannistra S.A., Levine H., Griffin J.D. (1985).
Expression of Interleukin 2 receptors and binding of Interleukin 2 by Gamma Interferon-induced human leukemic and normal monocytic cells.
J Exp Med 162,1111-1116.

Herve P., for the French study group. (1985).
Autologous bone marrow transplantation (ABMT) in acute leukemia -a French review of 120 patients.
In: Autologous bone marrow transplantation: Proceedings of the first international symposium (Ed by Dicke K.A., Spitzer G. & Zander A.R.), The University of Texas M.D. Anderson Hospital and Tumor Institute at Houston. pp273-279.

Hirano T., Yasukawa K., Harada H., Taga T., Watanabe Y., Matsuda T., Kashiwamura S., Nakajima K., Koyama K., Iwamatsu A., Tsunasawa S., Sakiyama F., Matsui H., Takahara Y., Taniguchi T., Kishimoto T. (1986).
Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin.
Nature 324,73-76.

Hirn-Scavannec J., Brailley H., Maraninchi D., Mannoni P., Mawas C., Belaage M. (1988).
Elimination of leukemia cells from human bone marrow using floating beads.
Transplantaion 46, 558-563.

Hoelzer D., Thiel E., Loffler H., Büchner T., Gauser A., Heil G., Koch P., Freund M., Diedrich H., Rühl H., Maschmeyer G., Lipp T., Nowrousran M.R., Burkett M., Gerecke D., Pralle H., Müller U., Lunscken Ch., Fülle H., Ho A.D., Küchler R., Busch F.W., Schneider W., Görg Ch., Emmerich B., Branmann D., Vangel H.A., von Paleske A., Bartell H., Neiss A., Messer D. (1988).
Prognostic factors in a multicenter study for the treatment of acute lymphoblastic leukemia in adults.
Blood 71,123-131.

Holt C., Arensen E., Carstens B., McGavren L. (1989).
Persistence of pseudodiploidy del (16q) in remission bone marrows of two children with acute lymphoblastic leukemia (ALL).
Proc Am Soc Clin Oncol 8,218.

Horton M.A., Hogg N. (1987).
Platelet Antigens: new and previously defined clusters.
In: Leucocyte Typing III, (Ed. by A.J. McMichael et al.), Oxford University Press, Oxford. pp.733-746..

Ichimaru M., Ishimaru T., Mikami M. (1979).
Multiple myeloma among atomic bomb survivors in Hiroshima and Nagasaki by dose 1950-1976.
Radiation Effects Research Report, Technical Report 9-79.

International Cooperative Study Group. (1986).
Bone marrow autotransplantation in man: report of an International Cooperative Study.
Lancet ii,960-962.

Iwakata S., Grace J.J.H. (1964).
.
NY State J Med 64,2279.

Izaguirre C.A., Minden M.D., Howatson A.F., McCulloch E.A. (1980).
Colony formation by normal and malignant human B-lymphocytes.
Br J Cancer 42,430-437.

Jackson N., Ling N.R., Ball J., Bromidge E., Nathan P.D., Franklin I.M. (1988).
An analysis of myeloma plasma cell phenotype using antibodies defined at the IIIrd International Workshop on Human Leukocyte Differentiation Antigens.
Clin Exp Immunol 72,351-356.

Jagannath S., Dicke K.A., Armitage J.O., Cabanillas F.F., Horwitz L.J., Vellekoop L., Zander A.R., Spitzer G. (1986).
High-dose cyclophosphamide, carmustine and etoposide and autologous bone marrow transplantation for relapsed Hodgkin's disease.
Ann Intern Med 104,163-168.

James K., Bell G.T. (1987).
Human Monoclonal Antibody production. Current Status and future prospects.
J Immunol Methods 100,5-40.

Janossy G., Campana D., Galton J., Grob J-P., Burnett A., Hann I., Simonsson B., Totterman T. (1987).
Applications of monoclonal antibodies in bone marrow transplantation (BMT).
In: Leucocyte Typing III, (Ed. by McMichael A.J. et al.). Oxford University Press, Oxford. pp942-948.

Jenberg H., Nilsson K., Zech L., Lutz D., Nowotny H., Scheirer W. (1987).
Establishment and phenotypic characterization of three new human myeloma cell lines (U-1957, U-1958, U-1996).
Blood 69,1605-1612.

Jobin M.E., Fahey J.L., Price Z. (1974).
Long-term establishment of a human plasmacyte cell line derived from a patient with IgD multiple myeloma.
J Exp Med 140,494-507.

Johnson G.D., Davidson R.S., McNamee K.C., Russel G., Goodwin D., Holborrow E.J. (1982).
Fading of Immunofluorescence during microscopy: a study of the phenomenon and its remedy.
J Immunol Methods 55,231-242.

Johnson G.D., Maclennan I.C.M., Ling N.R., Nathan P.D., Hardie D.L. (1987).
Immunohistological analysis of the plasma cell Workshop antibodies on normal lymphoid tissue and secretory epithelia.
In: Leucocyte Typing III (ed. by A.J. McMichael et al.), Oxford University Press, Oxford. pp.507-508.

Joshi S.S., Kessinger A., Mann S.L. (1987).
Detection of malignant cells in histologically normal bone marrow using culture techniques.
Bone Marrow transplantation 1,303-310.

Kaizer H., Levy R., Cote (1982).
Autologous bone marrow transplantation in lymphoblastic lymphoma and T-cell leukaemia.
Blood 60,169.

Kaizer H., Stuart R.K., Brookmeyer R., Beschorner W.E., Braine H.G., Burns W.H., Fuller D.J., Korbling M., Managan K.F., Soral R., Sensenbrenner L., Shadduck R.K., Shende A.C., Tutschka P.J., Yeager A.M., Zinkham W.H., Colvin O.M., Santos G.W. (1985).
Autologous bone marrow transplantation in acute leukemia: a phase 1 study of in vitro treatment of marrow with 4-Hydroperoxycyclophosphamide to purge tumor cells.
Blood 65,1504-1510.

Kaizer H., Wharam M.D., Johnson R.J., Economou J.G., Shin H.S., Santos G.W., Elfinbein G.J., Tutscha P.J., Braine H.G., Munoz L.L., Leventhal B.G. (1980).
Requirements for the successful application of autologous bone marrow transplantation in the treatment of selected malignancies.
Haematol Bluttransfus 25,285-296.

Kalland T. (1977).
A simple and rapid method to distinguish between rosettes and non-specific aggregation of erythrocytes in the rosette assay for human T-lymphocytes.
J Immunol Methods 17,279-283.

Karp J.E., Burke P.J., Saylor P.L., Humphrey R.L. (1984).
Correlation of proliferative and clonogenic tumor cells in multiple myeloma.
Cancer Res 44,4197-4200.

Karpas A., Fischer P., Swirsky D. (1982).
Human plasmacytoma with unusual karyotype growing in vitro and producing light chain immunoglobulin.
Lancet i,931-933.

Katagiri S., Yonezawa T., Tamaki T., Kanayama Y., Kuyama J., Ohnishi M., Tsubakio T., Kurata Y., Tarui S. (1984).
Surface expression of human myeloma cells: an analysis using a panel of monoclonal antibodies.
Acta Haematol (Basel) 72,372-378.

Kawano M., Fujimura K., Iwato K., Asaoku H., Tanabe O., Kuramoto A. (1986).
Pathogenesis of myeloma and ist regulation by cytokines.
Acta Haematol Jpn 49,1740-1748.

Kawano M., Hirano T., Matsuda T., Taga T., Horii Y., Iwato K., Asaoku H., Tang B., Tanabe O., Tanaka H., Kuramoto A., Kishimoto T. (1988).
Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas.
Nature 332,83-85.

Kelly K.A., Durie B., Maclennan I.C. (1988).
Prognostic factors and staging systems for multiple myeloma: comparisons between the Medical Research Council studies in the United Kingdom and the Southwest Oncology Group in the United States.
Hematol Oncol 6,131-140.

Kersey J.H., Weisdorf D., Nesbit M.E., Le Bien T.W., Woods W.G., McGlave P.B., Kim T., Vallera D.A., Golman A.I., Bostrom VB., Hurd D., Ramsay N.K.C. (1987).
Comparison of autologous and allogeneic bone marrow transplantation for treatment of high-risk refractory acute lymphoblastic leukemia.
N Engl J Med 317,461-467.

Körbling M., Hess A.D., Tutschka P.J., Kaizer H., Colvin M.O., Santos G.W. (1982).
4-Hydroperoxycyclophosphamide: a model for eliminating residual human tumour cells and T-lymphocytes from the bone marrow graft.
Br J Haematol 52,89-96.

Korsmeyer S.J., Greene W.C., Cossman J., Hsu S-M, Jensen J.P., Neckers L.M., Marshall S.L., Bakhshi A., Depper J.M., Leonard W.J., Jaffe E.S., Waldmann T.A. (1983).
Rearrangement and expression of immunoglobulin genes and expression of Tac antigen in hairy cell leukemia.
Proc Natl Acad Sci USA 80,4522-4526.

Kozbor D., Croce C.M. (1985).
Fusion Partners for Production of Human Monoclonal antibodies.
In: Human Hybridomas and Monoclonal Antibodies (Ed. by Engleman E.G., Foung S.K.H., Larrick J., Ranbitscheck A.). Plenum Press, New York. pp21-36.

Kozbor D., Moretta A., Messner H.A., Moretta L., Croce C.M. (1987).
Tp44 molecules involved in antigen-independent T cell activation are expressed on human plasma cells.
J Immunol 138,4128-4132.

Kozbor D., Roder J.C., Chang T.H., Steplewski Z., Koprowski H. (1982).
Human anti-tetanus toxoid monoclonal antibody secreted by EBV-transformed human B cells fused with the mouse myeloma.
Hybridoma 1,323-328.

Kubagawa H., Vogler L.B., Capra J.D., Conrad M.E., Lawton A.R., Cooper M.D. (1979).
Studies on the clonal origin of multiple myeloma. Use of individually specific (idiotype) antibodies to trace the oncogenic event to its earliest point of expression in B-cell differentiation.
J Exp Med 150,792-807.

Kuritani T., Cooper M.D. (1982).
Human B cell differentiation. I. Analysis of immunoglobulin heavy chain switching using monoclonal anti-immunoglobulin M, G, and A antibodies and pokeweed mitogen induced plasma cell differentiation.
J Exp Med 155,839.

Kyle R.A. (1975).
Multiple Myeloma. Review of 869 cases.
Mayo Clinic Proceedings 50,29-40.

Kyle R.A. (1983).
Long term survival in multiple myeloma.
N Engl J Med 308,314-316.

Kyle R.A. (1984).
'Benign' monoclonal gammopathy: a misnomer?.
J A M A 251,1849-1854.

Kyle R.A. (1986).
Diagnosis and management of multiple myeloma and related disorders.
Progress in Hematology, volume XIV, p257-282.

Kyle R.A., Griepp P.R. (1980).
Smouldering multiple myeloma.
N Engl J Med 302,1347-1349.

Landreth K.S., Englehard D., Beare M.H., Kincade P.W., Kapoor N., Good R.A. (1985).
Regulation of human B lymphopoiesis: effect of a urinary activity associated with cyclic neutropenia.
J Immunol 134,2304-2309.

Lane H.C., Shelhamer J.H., Motowski H.S., Fanci A.S. (1982).
Human monoclonal anti-keyhole limpet hemocyanin antibody-secreting hybridoma produced from peripheral blood B lymphocytes of a keyhole limpet hemocyanin-immune individual.
J. Exp. Med. 155,333-337.

Latreille J., Barlogie B., Dosik G., Johnston D.A., Drewinko B., Alexanian R. (1980).
Cellular DNA content as a marker of human multiple myeloma.
Blood 55,403-408.

Le Bien T.W., Stepan D.E., Bartholomew R.M., Strong R.C., Anderson J.M. (1985).
Utilisation of colony assay to assess the variables influencing elimination of leukemic cells from human bone marrow with monoclonal antibodies and complement.
Blood 65,945-950.

Lee M.S., Chang K.S., Cabanillas F., Freireich E.J., Trujillo J.M., Stass S.A. (1987).
Detection of minimal residual cells carrying t(14;18) by DNA sequence amplification.
Science 237,175.

Lee M-S., Chang K-S., Freireich E.J., Kantarjian H.M., Talpaz M., Trujillo J.M., Stass S.A. (1988).
Detection of minimal residual bcr/abl transcripts by a modified polymerase chain reaction.
Blood 72,893-897.

Linch D.C., Burnett A.K. (1986).
Clinical Studies of ABMT in acute myeloid leukaemia.
Clin Haematol 15,167-186.

Ling N.R., Bishop S., Jefferis R. (1977).
Use of antibody-coated red cells for the sensitive detection of antigen and in rosette tests for cells bearing surface immunoglobulin.
J Immunol Methods 15,279-289.

Ling N.R., Maclennan I.C.M., Mason D.Y. (1987).
B cell and plasma cell antigens: new and previously defined clusters.
In Leucocyte Typing III (ed. by A.J. McMichael et al.) pp.302-335. Oxford University Press. Oxford.

Lohmeyer J., Hadam M., Santoso S., Förster W., Schulz A., Pralle H. (1988).
Establishment and characterization of a permanent human IgA_2/Kappa myeloma cell line.
Br J Haematol 69,335-343.

Lokhorst H., Boom S., Terpstra W., Gerdes J., Bast B. (1987).
Determination of the growth fraction in monoclonal gammopathy with the monoclonal antibody Ki67.
Br J Haematol 66,430 (abstract).

Lokhorst H.M., Boom S.E., Bast B.J.E.G., Ballieux R.E. (1986).
Determination of the plasma cell labelling index with bromodeoxyuridine in a double-fluorescence technique.
Br J Haematol 64,271-275.

Lokhorst H.M., Boom S.E., Bast B.J.E.G., Peters P.J., Tedder T.F., Gerdes J., Petersen E., Ballieux R.E. (1987).
Novel type of proliferating lymphoplasmacytoid cell with a characteristic spotted immunofluorescence pattern.
J Clin Invest 79,1401-1411.

Love E.M. (1986).
The chemotherapy of multiple myeloma.
In: Multiple myeloma and other paraproteinaemias (Ed. I W. Delamore). Churchill Livingstone. London. pp353-374.

Löwenberg B, Touw I.P. (1986).
Interleukin 2: its role in the proliferation of neoplastic T and B cells.
J Pathol 149,15-21.

Löwenberg B., Swaart K., Hagemeijer A. (1980).
PHA-induced colony formation in acute non-lymphocytic and chronic myeloid leukaemia.
Leuk Res 4,143-149.

Ludwig H., Fritz E., Peest D. (1984).
A plasma clot culture system for growing and antiproliferative drug sensitivity testing of myeloma stem cells.
Leuk Res 8,701-711.

MacIntyre E.A., Smith W., Frazer L., Gribben J., Goldstone A.H., Isaacson P.G., Linch D.C. (1987).
The use of pooled bone marrow aspirates to detect gene rearrangements in high grade NHL with morphologically uninvolved marrow.
Bone Marrow Transplantation 2(suppl.1) p.224.

MacIntyre W. (1850).
Case of mollities and fragilitas ossium accompanied with urine strongly charged with animal matter.
Med Chir Soc Tr 33,211.

Maclennan I.C.M., Gray D. (1986).
Antigen-driven selection of virgin memory B cells.
Immunol Rev 91,61-85.

Maclennan I.C.M., Kelly K., Crockson R.A., Cuzick J., Chapman C. (1988).
Results of the MRC myelomatosis trials for patients entered since 1980.
Hematol Oncol 6,145-158.

Malek T.R., Ashwell J.D. (1985).
Interleukin 2 upregulates expression of its receptor on a T cell clone.
J Exp Med 161,1575-1580.

Mandelli F., Tribalto M., Avvisati G., Cantonetti M., Petrucci M.T., Boccadoro M., Pileri A., Marmont F., Resegotti L., Lauta V., Dammacco F. (1988).
Recombinant interferon alfa-2b (Intron-A) as post-induction therapy for responding multiple myeloma patients. M84 protocol.
Cancer Treat Rev 15 suppl A,43-48.

Maraninchi D., Abecasis M., Gastaut J.A., Sebahoun G., Cahn J.Y., Herve P., Novakovitch G., Carcasonne Y. (1983).
High-dose melphalan and autologous transplant for relapsed acute leukaemia.
Cancer Chemother Pharmacol 10,109-111.

Martin P.J., Najfeld V., Hansen J.A., Penfold G.K., Jacobson R.J., Fialkow P.J. (1980).
Involvement of the B-lymphoid system in chronic myeloid leukemia.
Nature 287,49-50.

Matsuoka Y., Moore G.E., Yagi Y., Pressman D. (1967).
Production of free light chains of immunoglobulin by a haemopoietic cell line derived from a patient with multiple myeloma.
Proc Soc Exp Biol Med 125,1246.

Mayer R.J., Schiffer C.A., Peterson B.A., Silver R.T., Cornwell G.G., McIntyre O.R., Rai K.R., Budman D.R., Ellison R.R., Maguire M. (1985).
Intensive post-remission therapy in adults with acute non-lymphocytic leukemia with Ara-C by continuous infusion or bolus administration: preliminary results of a CALGB phase I study..
Sem Oncol 12(Suppl 3),84-90.

McGovern J.J., Russell P.S., Atkins L., Webster E.W. (1959).
Treatment of terminal leukemic relapse by total-body irradiation and intravenous infusion of stored autologous bone marrow obtained during remission.
N Engl J Med 260,675-683.

McMichael A.J. et al. (Eds), (1987).
Leucocyte Typing III. White Cell Differentiation Antigens. Oxford University Press. Oxford.

Medical Research Council Working Party on Leukaemia in Adults. (1980).
Prognostic features in the third myelomatosis trial.
Br J Cancer 42,831-840.

Medical Research Council Working Party On Leukaemia In Adults, prepared by Maclennan I.C.M. and Cusick J. (1985).
Objective evaluation of the role of vincristine in induction and maintenance therapy for myelomatosis.
Br J Cancer 52,153-158.

Mellstedt H., Ahre A., Bjorkholm M., Holm G., Johansson B., Strander H. (1979).
Interferon therapy in multiple myeloma.
Lancet i,245-247.

Mellstedt H., Hammarström S., Holm G. (1974).
Monoclonal lymphocyte population in human plasma cell myeloma.
Clin Exp Immunol 17,371-384.

Meloni G., De Fabritiis P., Amadori S., Petti M.C., Pulsoni A., Sandrelli A., Mandelli F. (1989).
Autologous bone marrow transplantation in patients with acute myeloid leukemia in second complete remission (CR).
Bone Marrow Transplant 4,Suppl 1,207-208.

Mendelsohn M.L., Dethlefsen L.A. (1968).
Tumor growth and cellular kinetics.
In: The proliferation and spread of neoplastic cells. William and Wilkins, Baltimore. pp197-212.

Mertens N., Dehou M.F., Vanderbruggen H., van Riet I., van Camp B. (1985).
A monoclonal antibody (HAN-PC1) reacting with a maturation antigen on plasma cells.
Protides of the biological fluids 32,891-896.

Miedema F., Melief C.J.M. (1985).
T-cell regulation of human B-cell activation: A reappraisal of the role of interleukin 2.
Immunology Today 6,258-2259.

Millar B.C., Bell J.B.G., Lakhani A., Ayliffe M.J., Selby P.J., McElwain T.J. (1988).
A simple method for culturing myeloma cells from human bone marrow aspirates and peripheral blood in vitro.
Br J Haematol 69,197-203.

Mingari M.C., Gerosa F., Carra G., Accolla R.S., Moretta A., Zulder R.H., Waldmann T.A., Moretta L. (1984).
Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells.
Nature 312,641-643.

Monconduit M., Le Loet X., Bernard J.F., Michaux J.L. (1986).
Combination chemotherapy with vincristine, doxorubicin, dexamethasone for refractory or relapsing myeloma.
Br J Haematol 63,599-601.

Nadler L.M. (1986).
B cell/leukaemia panel workshop: summary and comments.
In: Leucocyte Typing II, volume 2: Human B Lymphocytes (ed. by Reinherz EL, Haynes BF, Nadler LM, Bernstein ID). Springer Verlag, New York. pp3-43. .

Nadler L.M., Botnick L., Finberg R., Canellos G.P., Takvorian T., Bast R.C., Hellman S., Schlossman S.F. (1984).
Anti-B1 monoclonal antibody and complement treatment in autologous bone-marrow transplantation for relapsed non-Hodgkin's lymphoma.
Lancet ii,427-431.

Nakahata T., Ogawa M. (1982).
Hematopoietic colony-forming cells in umbilical cord blood with extensive capacity to generate mono- and multi-potential hematopoietic progenitors.
J Clin Invest 70,1324-1328.

Nathan P.D., Jackson N., Franklin I.M., Ball J., Johnson G.D. (1987).
Myeloma cell phenotyping with anti-plasma cell, T10, CD24 and rough endoplasmic reticulum monoclonal antibodies.
In Leucocyte Typing III (ed. by A.J. McMichael et al.). Oxford University Press, Oxford. pp506-507.

Nemerow G.R., McNaughton M.E., Cooper N.R. (1985).
Binding of monoclonal antibody to the Epstein Barr virus (EBV)/CR2 receptor induces activation and differentiation of human B lymphocytes.
J Immunol 135,3068-3073.

Neuman R.E., McCoy T.A. (1958).
Growth promoting properties of pyruvate oxaloacetate and α-keto-glutarate for isolated Walker carcinosarcoma cells.
Proc Soc Exptl Biol Med 98,303-306.

Nilsson K., Bennich H., Johansson S.G.O., Pontén J. (1970).
Established immunoglobulin-producing myeloma (IgE) and lymphoblastoid (IgG) cell lines from an IgE myeloma patient.
Clin. Exp. Immunol. 7,477-489.

Nominski R., Berglund C., Lane Y., Lostrom M., Bernstein I., Young W., Hakamori S., Hill L., Coorey M. (1980).
Human monoclonal antibody against Forssman antigen.
Science 210,537-539.

Nowell P. (1987).
Molecular monitoring of pre-B acute lymphocytic leukemia.
J Clin Oncol 5,692.

O'Donnell M.R., Forman S.J., Levine A.M., Tenito., Farbstein M.J., Faher J.L., Gill P., Lazar G., Nademanee A., Neely S., Snyder D.S. (1987).
Cytarabine, cisplatin and etoposide chemotherapy for refractory non-Hodgkin's lymphoma.
Cancer Treat Rep 71,187.

Ohto H., Hitoni Y., Kambayashi H., Sato T., Maeda H. (1987).
CD9 defined antigen (p24) is expressed on eosinophils but not on normal monocytes.
In: Leucocyte Typing III (Ed. by A.J. McMichael et al.). Oxford University Press, Oxford. pp731-732.

Osserman E.F., DiRe L.B., DiRe J., Sherman W.H., Hersman J.A., Storb R. (1982).
Identical twin marrow transplantation in multiple myeloma.
Acta Haematologica (Basel) 68,215-223.

Park C.H., Kimler B.F., Smith T.K (1985).
Comparison of the supravital DNA dyes Hoechst 33342 and DAPI for flow cytometry and clonogenicity of human leukemic marrow cells.
Exp Hematol 13,1039-1043.

Peters S.W., Clarke R.E., Hoy T.G., Jacobs A. (1986).
DNA content and cell cycle analysis of bone marrow cells in myelodysplastic syndromes (MDS).
Br J Haematol 62,239-245.

Pezzutto A., Dörken B., Moldenhauer G., Clark E.A. (1987b).
Amplification of human B cell activation by a monoclonal antibody to the B cell-specific antigen CD22, Bp130/140.
J Immunol 138,98-103.

Pezzutto A., Dorken B., Rabinovitch P.S., Ledbetter J.A., Moldenhauer G., Clark E.A. (1987a).
CD19 monoclonal antibody HD37 inhibits anti-immunoglobulin-induced B cell activation and proliferation.
J Immunol 138,2793-2799.

Philip I., Philip T., Favrot M., Vuillaume M., Fontaniere B., Chamard D., Lenoir G.M. (1984).
Establishment of lymphomatous cell lines from bone marrow samples from patients with Burkitt's lymphoma.
J N C I 73,835-840.

Philip P. (1980).
Chromosomes of monoclonal gammopathies.
Cancer Genet Cytogenet 2,79.

Philip T., Armitage J.O., Spitzer G., Chauvin F., Jagannath S., Cahn J-Y., Colombat P., Goldstone A.H., Gorin N.C., Flesh M., Laporte J-P., Maraninchi D., Pico J., Boshy A., Anderson C., Schots R., Biron P., Cabanillas F., Dicke K. (1987).
High-dose therapy and autologous bone marrow transplantation after failure of conventional chemotherapy in adults with intermediate-grade or high-grade non-Hodgkin's lymphoma).
N Engl J Med 316,1493-1498.

Phillips G.L., Herzig R.H., Lazarus H.M., Fay J.W., Wolff S.N., Mill W.B., Lin H-S., Thomas P.R.M., Glasgow G.P., Shina D.C., Herzig G.P. (1984).
Treatment of resistant malignant lymphoma with cyclophosphamide, total body irradiation, and transplantation of cryopreserved autologous marrow.
N Engl J Med 310,1557-1561.

Pike B.L., Robinson W.A. (1970).
Human bone marrow colony growth in agar-gel.
J Cell Physiol 76,77-84.

Pilarski L.M., Maut M.J., Ruether B.A., Beich A. (1984).
Severe deficiency of B lymphocytes in peripheral blood from multiple myeloma patients.
J Clin Invest 74,1301-1306.

Plouvier E., Amsallem D., Noir., Herve P. (1987).
Autologous bone marrow transplantation for the acute lymphoblastic leukemia of childhood during second remission.
In: Dicke K.A., Spitzer G., Jagannath S., eds. Autologous bone marrow transplantation: Proceedings of the IIIrd International Symposium. Houston, University of Texas. p89-92.

Preijers F.W.M.B., De Witte T., Wessells J.M.C., De Gast G.C., Van Leeuwen E., Capel P.J.A., Haanen C. (1989b).
Autologous transplantation of bone marrow purged *in vitro* with Anti-CD7-(WT1-) Ricin A immunotoxin in T-cell lymphoblastic leukemia and lymphoma.
Blood 74,1152-1158.

Preijers F.W.M.B., De Witte T., Wessels J.M.C., Meyerink J.P.P., Haanen C., Capel P.J.A. (1989a).
Cytotoxic potential of anti-CD7 immunotoxin (WT1-ricin A) to purge *ex vivo* malignant T cells in bone marrow.
Br J Haematol 71,195-201.

Preisler H., Davis R.B., Kirschner J., Dupre E., Richards F., Hoagland H.C., Kopel S., Levy R.N., Carey R., Schulman P., Gottleib A.J., McIntyre O.R., and, the Cancer and leukemia Group B (1987).
Comparison of three remission induction regimens and two postinduction strategies for the treatment of acute nonlymphocytic leukemia: A cancer and Leukemia Group B study.
Blood 69,1441-1449.

Proctor S.J., Hamilton P.J., Taylor P., Carey P., Hargrave S., Evans R.G.B., Summerfield G., Finney R., Saunders P., Goff D., Reid M.M. (1988).
A comparative study of combination chemotherapy versus marrow transplant in first remission adult lymphoblastic leukaemia.
Br J Haematol 69,35-39.

Pulford K.A.F., Ralfkiaer E., Macdonald S.M., Erber W.N., Falini B., Gatter K.C., Mason D.Y. (1987).
A unique monoclonal antibody recognozing a 40kDa antigen shared between a subpopulation of B-lymphocytes and macrophages.
In: Leucocyte Typing III (Ed. by A.J. McMichael et al.). Oxford University Press, Oxford. p358.

Quesada J.R., Alexanian R., Hawkins M., Barlogie B., Boren E., Itri L., Gutterman J.U. (1986).
Treatment of multiple myeloma with recombinant α-Interferon.
Blood 67,275-278.

Rabin E.M., Ohara J., Paul W.E. (1985).
B-cell stimulatory factor 1 activates resting B cells.
Proc Natl Acad Sci USA 82,2935-2939.

Ralph P.R., Jeong G., Welte K., Mertelsmann R., Rabin H., Henderson L.E., Sonza L.M., Boone T.C., Robb R.J. (1984).
Stimulation of immunoglobulin secretion in human B lymphocytes as a direct effect of high concentrations of IL2.
J. Immunol. 133,2442-2445.

Reading C.L., Thomas M.W., Hickey C.M., Chandran M., Tindle S., Ball E.D., Poynton C.H., Dicke K.A. (1987).
Magnetic affinity colloid (MAC) cell separation of leukaemia cells from autologous bone marrow aspirates.
Leuk Res 11,1067-1077.

Reinherz E.L., Schlossman S.F. (1981).
The characterization and function of human immunoregulatory T lymphocyte subsets.
Immunolology Today 2,69.

Reinherz E.L., Haynes B.F., Nadler L.M., Bernstein I.D. (Eds) (1986).
Human B Lymphocytes.
Leukocyte Typing II, Vol.2, Springer Verlag, New York.

Reinherz E.L., Kung P.C., Goldstein G., Levey R.H., Schlossman S.F. (1980).
Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage.
Proc Natl Acad Sci USA. 77,1588-1592.

Reske-Kunz A., Osawa H., Josimovic-Alasevic O., Rüde E., Diamantstein T. (1987).
Soluble interleukin 2 receptors are released by long term-cultured insulin-specific T cells transiently after contact with antigen.
J Immunol 138,192-196.

Rhodes E.G.H., Flynn M. (1989).
Peanut agglutinin shows specificity for bone marrow plasma cells.
Br J Haematol 71,183-187.

Riechmann L., Clark M.R., Waldmann H., Winter G. (1988).
Reshaping human antibodies for therapy.
Nature 332,323-327.

Rivera G.K., Buchanan G., Boyett J.M., Cannitta B., Ochs J., Kalwinsky D., Amylon M., Vietta T.J., Crist W.M. (1986).
Intensive retreatment of childhood acute lymphoblastic leukemia in first bone marrow relapse: a Pediatric Oncology Study Group study.
N Engl J Med 315,273-278.

Romagnani S., Gindizi G.M., Almerigogna F., Biagiotti R., Alessi A., Mingari C., Liang C-M., Moretta L., Ricci M. (1986).
Analysis of the role of interferon-gamma, interleukin 2 and a third factor distinct from interferon-gamma and interleukin 2 on human B cell proliferation. Evidence that they act at different times after B cell activation.
Eur J Immunol 16,623-629.

Rowley S.D., Zuehlsdorf M., Braine H.G., Colvin O.M., Davis J., Jones R.J., Saral R., Sensenbrenner L.L., Yeager A., Santos G.W. (1987).
CFU-GM content of bone marrow graft correlates with time to hematologic reconstitution following autologous bone marrow transplantation with 4-Hydroperoxycyclophosphamide purged bone marrow.
Blood 70,271-275.

Ruiz-Argüelles G.J., Katzmann J.A., Greipp P.R., Gonchoroff N.J., Garton J.P., Kyle R.A. (1984).
Multiple myeloma: circulating lymphocytes that express plasma cell antigens.
Blood 64,352-356.

Sabbath K.D., Ball E.D., Larcom P., Davis R.B., Griffin J.D. (1985).
Heterogeneity of clonogenic cells in acute myeloblastic leukemia.
J Clin Invest 75,746-753.

Sallan S.E., Niemeyer C.M., Billett A.L., Lipton J.M., Tarbell N.J., Gelber R.D., Murray C., Pittinger T.P., Wolfe L.C., Bast R.C.jr (1989).
Autologous bone marrow transplantation for acute lymphoblastic leukemia.
J Clin Oncol 7,1594-1601.

Salmon S.E., Hamburger A.W., Soehnlen B.J., Durie B.G.M., Alberts D.S., Moon T.E. (1978).
Quantitaion of differential sensitivity of human tumor stem-cells to anticancer drugs.
N Engl J Med 298,1321-1327.

San Miguel J.F., Caballero M.D., Gonzalez M., Zola H., Lopez Borrasca A. (1986).
Immunological phenotype of neoplasms involving the B cell in the last step of differentiation.
Br J Haematol 62,75-83.

Santos G.W., Colvin O.M. (1986).
Pharmacological purging of bone marrow with reference to autografting.
Clin Haematol 15:1,67-83.

Satoh J., Prabhakar B.S., Haspel M.V., Ginsberg-Fellner F., Notkins A.L. (1983).
Human monoclonal auto-antibodies that react with multiple endocrine organs.
N Engl J Med 309,217-220.

Scarffe J.H., Anderson H., Palmer M.K., Crowther D. (1983).
Prognostic significance of pretreatment serum β_2-microglobulin levels in multiple myeloma.
Eur J Cancer Clin Oncol. 19,1361-1364.

Schauer P., Arlin Z.A., Mertelsmann R., Cirrincione C., Friedman A., Gee T.s., Dowling M., Kempin S., Strauss D.J., Kozner B. (1983).
Treatment of acute lymphoblastic leukemia in adults: results of L-10 and L-10M protocols.
J Clin Oncol 1,462-472.

Schröder J., Vuopio P., Autio K. (1981).
Chromosome changes in human chronic lymphocytic leukemia.
Cancer Genet Cytogenet. 4,11-21.

Seabright M. (1971).
A rapid banding technique for human chromosomes.
Lancet ii,971-972.

Segi M. (1977).
Graphic presentation of cancer incidence by site, area and population compiled from the data published in 'Cancer incidence in five continents, volume III'.
Segi Institute of Cancer, Nagoya, Japan.

Selby P., Buick R.N., Tannock I. (1983).
A critical appraisal of the human tumor stem-cell assay.
N Engl J Med 308,129-134.

Selby P., Zulian G., Forgeson G., Nandi A., Milan S., Meldrum M., Viner C., Osborne R., Malpas J.S., McElwain T.J. (1988).
The development of high dose melphalan and of autologous bone marrow transplantation in the treatment of multiple myeloma: Royal Marsden and St Bartholomew's hospital studies..
Hematol Oncol 6,173-179.

Selby P.J., McElwain T.J., Nandi A.C., Perren T.J., Powles R.L., Tillyer C.R., Osborne R.J., Slevin M.L., Malpas. (1987).
Multiple myeloma treated with high dose intravenous melphalan.
Br J Haematol 66,55-62.

Shackney S.E., Skramstad K.S., Cunningham R.E., Dugas D.J., Lincoln T.L., Lukes R.J. (1980).
Dual parameter flow cytometry studies in human lymphomas.
J Clin Invest 66,1281-1294.

Sharkis S.J., Santos G.W., Colvin M. (1980).
Elimination of acute myelogenous leukemic cells from marrow and tumour suspensions in the rat with 4-hydroperoxycyclophosphamide.
Blood 55,521-523.

Sharma S., Mehta S., Morgan J., Maizel A. (1986).
Molecular cloning and expression of a human B-cell growth factor gene in Escherichia Coli.
Science 235,1489-1492.

Sheehan T., Judge M., Parker A.C. (1986).
The efficacy and toxicity of VAD in the treatment of myeloma and related disorders.
Scand J Haematol 37,425-428.

Sieber F. (1987).
Marrow purging by merocyanine 540-mediated photolysis.
Bone Marrow Transplantation 2(suppl 2),29.

Sieber F., Hilton J., Colvin O.M. (1984b).
Photodynamic therapy of drug-resistant tumor.
Exp Hematol 12.abstract 170.

Sieber F., Spivak J.L., Sutcliffe A.M. (1984a).
Selective killing of leukemic cells by merocyanine 540-mediated photosensitization.
Proc Natl Acad Sci (USA) 81,7584-7587.

Simonsson B., Burnett A.K., Prentice H.G., Hann I.H., Brenner M.K., Gibson B., Grob J.P., Lonnerholm G., Morrison A., Smedmyr B. (1989).
Autologous bone marrow transplantation with monoclonal antibody purged marrow for high risk acute lymphoblastic leukaemia.
Leukemia 3,631-636.

Singer C.R.J., Linch D.C., Bown S.G., Huehns E.R., Goldstone A.H. (1988).
Differential phthalocyanine photosensitization of acute myeloblastic leukaemia progenitor cells: a potential purging technique for autologous bone marrow transplantation.
B J Haematol 68,417-422.

Smith B.R., Weinberg D.S., Robert N.J., Towle M., Luther E., Pinkus G.S., Ault K.A. (1984).
Circulating monoclonal B lymphocytes in non-Hodgkin's lymphoma.
N Engl J Med 311,1476.

Smith K.A., Cantrell D.A. (1985).
Interleukin 2 regulates its own receptor.
Proc Natl Acad Sci USA 82,864-868.

Spinolo J.A., Dicke K.A., Horwitz L.J., Jagannath S., Spitzer G.S. (1988).
Autologous bone marrow transplantation for acute myeloid leukemia in 2CR.
Exp Hematol 16,487 (abstract).

Stashenko P., Nadler L.M., Hardy R., Schlossman S.F. (1980).
Characterization of a human B lymphocyte-specific antigen.
J Immunol 125,1678-1685.

Steel G.G. (1977).
Growth kinetics of tumors: cell population kinetics in relation to the growth and treatment of cancer.
Clarendon Press. Oxford.

Stockinger H., Majdic O., Köller U., Knapp W. (1987).
Subclustering of the CD24 cluster..
In: Leucocyte Typing III (Ed. by A.J. McMichael et al.). Oxford University Press, Oxford. pp367-368.

Stong R.C., Uckin F., Youle R.J., Kercey J.H., Vallera D.A. (1985).
The use of multiple T-cell-directed intact ricin immunotoxins for autologous bone marrow transplantation.
Blood 66,627.

Taga T., Kawanishi Y., Hardy R.R., Hirano T. (1987).
Receptors for B cell stimulatory factor 2. Quantitation, specificity, distribution and regulation of their expression.
J Exp Med 166,967-981.

Takahashi T., Lim B., Jamal N., Tritcher D., Lockwood G., McKinney S., Bergsagel D.E., Messner H.A. (1985).
Colony growth and self renewal of plasma cell precursors in multiple myeloma.
J Clin Oncol 3,1613-1623.

Takvorian T., Canellos G.P., Ritz J., Freedman A.S., Anderson K.C., Manch P., Tarbell N., Coral F., Daley H., Yeap B., Sclossman S.F., Nadler L.M. (1987).
Prolonged disease-free survival after autologous bone marrow transplantation in patients with a non-Hodgkin's lymphoma with a poor prognosis.
N Engl J Med 316,1499-1505.

Tazzari P.L., Gobbi M., Dinota A., Bontadini A., Grassi G., Cerato C., Cavo M., Pileri S., Caligaris-Cappio F., Tura S. (1987).
Normal and neoplastic plasma cell membrane phenotype. Studies with new monoclonal antibodies.
Clin Exp Immunol 70.192-200.

Thomas E.D. (1983).
Marrow Transplantation for malignant diseases.
J Clin Oncol 1,517-531.

Thompson K.M., Hough D.W., Maddison P.J., Melamed M.D., Hughes-Jones N. (1986).
The efficient production of stable, human monoclonal antibody-secreting hybridomas from EBV-transformed lymphocytes using the mouse myeloma X63-Ag8.653 as fusion partner.
J Immunol Methods 94,7-12.

Tong A.W., Lee J.C., Stone M.J. (1987).
Characterization of a monoclonal antibody having selective reactivity with normal and neoplastic plasma cells.
Blood 69,238-245.

Touw I., Löwenberg B. (1985).
Interleukin 2 stimulates chronic lymphocytic leukemia colony formation in vitro.
Blood 66,237-240.

Touw I., Delwel R., Bolhuis R., van Zauen G., Löwenberg B. (1985).
Common and pre-B acute lymphoblastic leukemia cells express interleukin 2 receptors, and interleukin 2 stimulates in vtiro colony formation.
Blood 66,556-561.

Uchiyama T., Broder S., Waldmann T.A. (1981).
A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac(+) cells.
J Immunol 126,1393-1397.

Uckun F.M., Hanson M.S., Amburs J.L., Chandon M., Ledbetter J.A. (1987).
In: Dicke K.A., Spitzer G., Jagannath S., eds. Autologous bone marrow transplantation: Proceedings of the IIIrd International Symposium. Houston, University of Texas. pp.55-58.

Van Damme J., Opdenakker G., Simpson R.J., Rubira M.R., Cayphas S., Vink A., Billiau A., Van Snick J. (1987).
Identification of the human 26-KD protein, interferon beta-2 (IFN-beta2), as a B cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor.
J Exp Med 165,914-919.

Van den Berghe H., Parloir C., David G., Michaux J.L., Sokal G. (1979).
A new characteristic karyotypic anomaly in lymphoproliferative disorders.
Cancer 44,188-195.

Van Dobbenburgh O.A., Rodenhuis S., Ockhuizen T,. Weltevreden E., Houwen B., Fidler V,. Meijer S., Marvink J. (1985).
Serum β_2-microglobulin: a real improvement in the management of multiple myeloma?.
Br J Haematol 61,611-620.

Vogler W.R., Winton E.F., Gordon D.S., Raney M.R., Go B., Meyer L. (1984).
A randomised comparison of post-remission therapy in acute myelogenous leukemia: a Southeastern Cancer Study Group trial.
Blood 63,1039-1045.

Wagner D.K., Kiwanuka J., Edwards B.K., Rubin L.A., Nelson D.L., Magrath I.T. (1987).
Soluble interleukin-2 receptor levels in patients with undifferentiated and lymphoblastic lymphomas: correlation with survival.
J Clin Oncol 5,1262-1274.

Waldmann T.A., Goldman C., Tsudo M. (1987).
The role of the multi-chain IL-2 receptor complex in the control of normal and malignant T-cell proliferation.
In: Leucocyte Typing III (Ed. by McMichael A.J. et al.) Oxford University Press, Oxford. pp927-931.

Waldmann T.A., Goldman C.K., Robb R.J., Depper J.M., Leonard W.J., Sharrow S.A., Bongiovanni K.F., Korsmeyer S.J., Greene W.C. (1984).
Expression of Interleukin 2 receptors on activated human B cells.
J Exp Med 160,1450-1466.

Walker L.A., Johnson G.D., Maclennan I.C.M. (1988).
Selective induction of light chain synthesis in cultures of blood lymphocytes from patients with IgG myelomatosis.
Clin Exp Immunol 71,193-197.

Wong G.C., Clark S.C. (1988).
Multiple actions of Interleukin 6 within a cytokine network.
Immunolology Today 9,137-139.

Wong-Staal F., Gallo R.C. (1985).
The family of human T-lymphotropic leukemia viruses: HTLV-1 as the cause of adult T-cell leukemia and HTLV-III as the cause of acquired immunodeficiency syndrome.
Blood 65,253-263.

Wright J.J., Poplack D.G., Bakshi A., Reaman G., Cole D., Jensen J.P., Korsmeyer S.J. (1987).
Gene rearrangements as markers of clonal variations and minimal residual disease in acute lymphoblastic leukemia.
J Clin Oncol 5,735.

Wu L.Y.F., Blanco A., Cooper M.D., Lawton A.R. (1976).
Ontogeny of B-lymphocyte differentiation induced by pokeweed mitogen.
Clin Immunol Immunopathol 5,208-217.

Yeager A.M., Kaizer H., Colvin M., Rowley S.O., Braine H.G., Stuart R.K. (1987).
Autologous bone marrow transplantation for acute myeloid leukemia.
In: Dicke K.A., Spitzer G., Jagannath S., eds. Autologous bone marrow transplantation: Proceedings of the IIIrd International Symposium. Houston, University of Texas. pp.59-68.

Yeager A.M., Kaizer H., Santos G.W., Saral R., Colvin O.M., Stuart R.K., Braine H.G., Burke P.J., Ambinder R.F., Burns W.H., Fuller D.J., Davis J.M., Karp J.E., May W.S., Rowley S.D. (1986).
Autologous bone marrow transplantation in patients with acute non-lymphocytic leukemia, using ex vivo marrow treatment with 4-Hydroperoxycyclophosphamide.
N Engl J Med 315,141-147.

Zehnbauer B.A., Pardoll D.M., Borke P.J., Graham M.L., Vogelstein B. (1986).
Immunoglobulin gene rearragements in remission bone marrow specimens from patients with acute lymphoblastic leukemia.
Blood 67,835-838.

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PUBLICATIONS

Franklin I M, Nathan P, Jackson N, Rhodes E G H, Ball J (1986)
Marrow purging for multiple myeloma - preliminary studies in vitro with cytotoxic drugs and monoclonal antibodies.
Bone Marrow Transplantation 1, supplement 1 p299 (Abstract).

Jackson N, Franklin I M, Nathan P, Ball J (1987)
CD24 and CD38 Monoclonal Antibodies: Suitable for purging Myeloma Bone Marrow for Autologous Bone Marrow Transplantation?
Bone Marrow Transplantation 2, supplement 1 p217 (Abstract).

Nathan P D, Jackson N, Franklin I M, Ball J, Johnson G D (1987)
Myeloma phenotyping with anti-plasma cell, T10, CD24, and rough endoplasmic reticulum monoclonal antibodies.
Paper B5.8 in McMichael et al (Eds), Leucocyte Typing III (White Cell Differentiation Antigens), Oxford University Press, Oxford, p506-507.

Jackson N, Ling N R, Ball J, Bromidge E, Nathan P D, Franklin I M (1988)
An analysis of myeloma plasma cell phenotype using antibodies defined at the IIIrd International Workshop on Human Leucocyte Differentiation Antigens.
Clin Exp Immunol 72,351-356.

Jackson N, Lowe J, Ball J, Bromidge E, Ling N R, Griffiths M J, Larkins S, Franklin I M (1989)
Two new IgA_1-kappa plasma cell leukaemia cell lines (JJN-1 & JJN-2) which proliferate in response to B-cell Stimulatory Factor 2.
Clin Exp Immunol 75,93-99.

Abstracts presented at scientific meetings

Jackson N, Ball J, Franklin I M (1987)
Myeloma cells express interleukin-2 receptors after exposure to phytohaemagglutinin and interleukin-2.
Br J Haematol.
(Oral presentation at the British Society for Haematology meeting, April 1987)

Jackson N, Franklin I M, Ball J, Lowe J (1987)
An analysis of myeloma plasma cell phenotype using antibodies from the B cell clusters defined at the IIIrd International Workshop on Human Leucocyte Differentiation Antigens.
(Presented at the combined British Societies of Haematology and Immunology, November 12th 1987).