THE DEVELOPMENT AND APPLICATION OF LECTIN/MONOCLONAL ANTIBODY PURGING IN THE TREATMENT OF MULTIPLE MYELOMA

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ABSTRACT

The aim of the work described in this thesis was to find a means of selectively removing neoplastic cells from myeloma bone marrow *in vitro* to be used in conjunction with autologous bone marrow transplantation.

The inspiration for this work derived from the demonstration by Professor McElwain that tumour clearance could be attained *in vivo* by using very high doses of alkylating agents. It was soon apparent that autologous stem cell rescue was required to counteract the morbidity of such treatment, but that this entailed reinfusion of neoplastic cells mixed with the normal haemopoietic cells.

Obstacles to successful *in vitro* purging were:

a) uncertainty as to the identity of the malignant cell in myeloma and,

b) lack of monoclonal antibodies reactive with the surface of plasma cells that could be used as purging agents.

Studies were undertaken to develop a method for growing myeloma cells *in vitro*, as a means to identify target cells for purging. After unsuccessful attempts to reproduce several previously described techniques for growing myeloma colonies, rigorous criteria were established for assessing true *in vitro* myeloma colony formation. After experimenting with many nutrient media, 17 out of 32 consecutive myeloma bone marrow samples were observed to yield true colonies using one particular serum-free medium. Immunophenotyping of these colonies revealed 2 populations of light-chain restricted B-lineage cells, ($CD19^+$ and $RFD6^+$), and these were selected as target cells for purging.

Next, studies with a panel of lectins led to the novel observation that peanut agglutinin (PNA) binds to bone marrow plasma cells but not to normal haemopoietic progenitor cells. Two different methods were then developed to separate PNA^+ cells from bone marrow, one exploiting exposed Thomsen Friedenreich [T] antigen (galactose ß1,3 N Acetyl galactosamine α-, the PNA ligand) on sialidase-treated normal red blood cells with PNA in a rosetting technique, and the other utilising magnetised microspheres for which a lectin coating technique was developed. Using the latter technique small scale experiments in normal and myeloma bone marrow samples confirmed the initial prediction that removal of PNA^+ cells did not affect the *in vitro* growth of normal haemopoietic colonies. CD19-coated immunomagnetic depletion was then combined with PNA purging and shown to achieve satisfactory depletion of target cells with conservation of normal cells.

The scaling-up procedure, well known to be the stumbling block of many successful small scale purging models, was achieved by trial and error using a variety of manual and automated techniques. Experiments were performed using normal bone marow spiked with radio-labelled tumour cell lines and myeloma bone marrow cells until a successful technique suitable for clinical use had been established.

A clinical pilot study was then initiated to assess the feasibility of autologous bone marrow transplantation in myeloma using marrow that had been purged *in vitro* with PNA- and CD19-coated magnetic microspheres. At the time of writing 5 myeloma bone marrow harvests have been successfully purged and cryopreserved. Two of these have been used to rescue patients following high-dose chemoradiotherapy. Both purged autografts engrafted rapidly and the patients are in good health 21 and 24 months later, although 1 patient still has a serum paraprotein.

This work has demonstrated the feasibility of autologous bone marrow transplantation in myeloma with combined lectin/monoclonal antibody purging of malignant cells. This will now allow further investigation of the potential benefits of high-dose chemoradiotherapy.

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3. To examine the potential of lectins as purging agents in myeloma.

4. To explore methods of *in vitro* purging of myeloma bone marrow using a combination of lectins and monoclonal antibodies.

5. To adapt *in vitro* purging systems for clinical use in autologous bone marrow transplantation for patients with myeloma.

6. To examine the role of *in vitro* purging of bone marrow in the treatment of patients with myeloma.

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CHAPTER ONE

INTRODUCTION

MULTIPLE MYELOMA

1.1 The first description of multiple myeloma

Although the first description of the clinical features of multiple myeloma is usually attributed to Henry Bence Jones (Bence Jones, 1847 and 1848), it was William MacIntyre, physician to the Metropolitan Convalescent Institution and to the Western General Dispensary, Marylebone, who had already noted many important clinical features of the disease. In 1845 he had been asked to see a patient under the care of Dr Thomas Watson: it was MacIntyre who linked the patient's severe bone pains with the pathological fractures and, in view of the history of oedema, examined the patient's urine and noted all the important characteristics of the protein in the urine that subsequently and with little justice became known as Bence Jones protein. MacIntyre noted that the precipitate formed on heating this patient's urine could redissolve with a further rise in temperature, only to reprecipitate as the urine cooled. He was also aware that this protein had a tendency to precipitate at temperatures considerably lower than than those of other proteins: thus, he noticed that the protein redissolved at the "coagulating point" of albumin, namely, 160-170°F (71-770°C) - the inference being that the protein had precipitated at a temperature well below this (MacIntyre W, 1850). Both MacIntyre and Watson independently sent a sample of this patient's urine to Bence Jones.

Henry Bence Jones, only 31 years old at the time, was physician to St George's Hospital and had established a reputation as a chemical pathologist. While

MacIntyre's paper dealt mainly with the clinical features of the disease, including significant bone pathology found at post mortem, Bence Jones' papers were concerned with the properties and analysis of the proteinuria, and for this reason he is credited with the discovery of the protein.

John Dalrymple (1846), surgeon to the Royal Ophthalmic Hospital, Moorfields, and a member of the Microscopical Society, was sent post mortem material from this same patient, taken from two lumbar vertebrae and a rib. He described the appearance of the pathological cells, the majority being round to oval with the smaller cells "one and a half to twice larger than the average blood cell or blood disc". Dalrymple also noted that the larger and more irregular cells frequently contained two and often three nuclei; these descriptions are consistent with malignant plasma cells with some multinucleated forms.

Between them William MacIntyre, Henry Bence Jones and John Dalrymple described all the essential features of the disease now known as multiple myeloma in the period 1846 to 1850.

1.2 Archaeological evidence of myeloma

The discrete "punched out" appearance and widespread distribution of myelomatous bone lesions has enabled anthropologists to report cases of multiple myeloma in archaeological specimens. With the aid of X-ray analysis, differentiation from other disorders affecting the skeleton such as osteolytic carcinomatous metastases, osteitis fibrosa cystica, histiocytosis X, leukaemia, and possibly fibrous dysplasia, has been possible.

For example, in 1930 the skeleton of an American Indian was excavated from an Indian site near Binghamton, NY, and found to be riddled with discrete destructive

lesions characteristic of myeloma. The date of occupation of this site was about 800AD (Ritchie and Warren, 1932). Several other cases of probable myeloma have been reported in skeletal finds of American Indian settlements dating back to 200AD (Morse D, 1974; Brooks ST and Melbye J, 1967).

1.3 Definition

"Plasma cell myeloma (PCM), or multiple myeloma, is an uncontrolled, malignant proliferation of plasma cells in the marrow. The neoplastic cells synthesize abnormal amounts of monoclonal immunoglobulins (IgG, IgA, IgD, or IgE) or kappa or lambda light chains; rarely, there is no detectable secretion of immunoglobulin by tumor cells. The clinical manifestations of myeloma vary but include tumor formation, osteolysis, impaired haemopoiesis, hypogammaglobulinemia, and renal disease." (Bersagel, 1990). The monoclonal cells arise as a result of malignant transformation at some stage in B lymphocyte development: progeny of this malignant clone produce a unique immunoglobulin composed (usually) of a single class of heavy and light chains and bearing an idiotypic marker determined by identical variable regions in the heavy and light chain immunoglobulin genes (Barlogie et al,1989). The uncontrolled proliferation of this clone distinguishes multiple myeloma from the benign monoclonal gammopathies (monoclonal gammopathy of undetermined significance (MGUS) (Kyle 1978; Durie 1985), and chronic cold aggglutinin syndrome) in which proliferation of the abnormal clone is stable and limited over long periods of time by unknown mechanisms.

1.4 Incidence and Epidemiology

The annual incidence of myeloma ranges from 2.6 to 3.3 per 100,000 population per year and in the UK the incidence of recognised myeloma is approximately 2 per 100,000 (Martin, 1961; Kyle et al, 1969; WHO, 1965). In all studies the incidence is higher in males and in blacks. The mean age at diagnosis is 65 years (Bergsagel et al, 1979) and the incidence increases with age being highest for men over 80 years and women over 70 years. Of the 1,830 patients entered into the IVth, Vth, and VIth MRC Myelomatosis Trials in the UK, only 18% have been below the age of 55 years at diagnosis (Janet Dunn, Statistician, MRC Trials office, personal communication) - an important point when considering aggressive treatment options.

There is evidence of a genetic predisposition to myeloma: there have been at least 75 reports of familial myeloma (Sullivan, 1972), and MGUS and macroglobulinaemia have also been reported in families (Kyle, 1975).

High-dose radiation exposure is strongly associated with the development of myeloma (Ishimaru et al, 1979; Najarian et al, 1979), as observed among 109,000 survivors of the atomic bombs at Hiroshima and Nagasaki between 1950 and 1976 (Hewell, 1976; Ichimaru et al, 1982; Cuzick 1981). These patients were relatively young (20-59 years) and the risk was greatest when the bone marrow had received greater than 50 rad, with a latency of up to 20 years. Atomic bomb survivors have had elevated serum immunoglobulin levels (Garrett et al, 1987) and B lymphocyte chromosomal abnormalities (Salmon, 1973).

Associations between myeloma and several occupations and environmental exposure have been reported: farming (Gallagher et al, 1983; Pearce et al, 1985), woodworking, leather work, commercial painting industries and cosmetology have all

been implicated in small studies, and an increased risk of developing myeloma has been postulated following exposure to pesticides, plastics, rubber, petroleum products (Rinsky et al, 1987) and asbestos (reviewed by Durie BGM, 1988).

1.5 Clinical features

The clinical and laboratory features of myeloma are attributable to a combination of bone marrow infiltration by malignant cells and to the secretory products of myeloma cells. Symptoms of anaemia are a common presenting feature (70% of patients) and in advanced disease pancytopenia occurs due to bone marrow failure: the latter may be due to replacement of normal haemopoietic tissue by malignant cells or by suppression of haemopoiesis presumably by factors secreted by the myeloma cells.

Bone pain is a common presenting complaint (and occurs in at least 70% during the course of the disease, (Bersagel, 1990)); in many patients this is the dominant feature of this distressing disease. Skeletal X-rays show either the characteristic punched- out osteolytic lesions of myeloma or diffuse osteoporosis. The causative osteoclast activating factors (OAF) have recently been identified as tumour necrosis factor (TNFβ or lymphotoxin) (Garrett et al, 1987) and interleukin-1 (IL-1β) (Cozzolino et al, 1989; Kawano et al, 1988 and 1989; Kurihara et al, 1990) both of which are produced by malignant plasma cells. The abnormal osteolytic activity leads to hypercalcaemia (Delamore 1982) and subsequent dehydration, present in about 30% of patients at diagnosis (Kyle, 1975) and this is an important contributing factor in renal failure.

Approximately 50% of patients become uraemic during the course of their

disease and renal failure is often the presenting feature (Johnson et al, 1980). Immunoglobulin light chains are secreted into the circulation, usually as monomers or dimers (MW 22,000-44,000) that can be filtered by the glomeruli. Some light chains produce AL amyloid (Solomon, 1986), while others are directly toxic to glomeruli or, more commonly to tubular cells (Cooper et al, 1984; Solomon et al, 1991), or form casts which obstruct the tubules. Infection and drugs (such as nephrotoxic antibiotics) also contribute to the renal failure.

Immunosuppression, due mainly to failure of production of normal immunoglobulins, and leading to frequent bacterial infections, is a prominent clinical feature in myeloma (Pilarski et al, 1986) and is a common cause of death (in 33% of patients during chronic phase, and 22% in the acute terminal phase (Bersagel,1990)). In advanced disease associated with bone marrow failure neutropenia is an added risk factor.

In 2% of patients a hyperviscosity syndrome may occur, with purpura, haemorrhages, visual failure, CNS symptoms and neuropathies, and heart failure. This results from polymerisation of the abnormal immunoglobulin and is more likely to occur with IgA, IgM, or IgD.

Abnormal bleeding is not uncommon and is due to interference with platelet function and coagulation factors by the paraprotein, or by thrombocytopenia. The binding of monoclonal light chains to peripheral nerve antigens has been implicated in the development of peripheral neuropathy in plasma cell dyscrasias (Latov et al, 1980).

1.6 Diagnostic criteria

At least two of the following criteria must be present for the diagnosis of myeloma (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 (Mellstedt et el, 1974).

(c) Definite lytic bone lesions (not just osteoporosis).

Equivocal myeloma is defined in the MRC VIth trial protocol in which excess bone marrow plasma cells are not accompanied by other diagnostic criteria and there is no objective evidence of progressive myelomatosis: such patients are treated expectantly, but about half progress within a short time to frank myeloma (Maclennan et al, 1991)

1.7 Prognostic features

Survival in myeloma varies widely from a few days to more than 10 years, and it is important both in conducting clinical trials and in trying out new forms of treatment to assess prognosis from the presenting features of the disease. Many series of patients with myeloma have been assessed for prognostic variables by multivariate analysis (e.g. Durie et al, 1975) (reviewed by Hansen and Galton, 1985). Renal function was 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. Light chain

only, or "Bence-Jones myeloma" has a much poorer prognosis, especially lambda-light chain disease (Durie and Salmon, 1982). The MRC working party on leukaemia in adults (1980) devised a simple three-stage system based on the blood urea and haemoglobin (after rehydration) and the performance status:

Table 1

MRC STAGING FOR MYELOMA

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

$Beta_2$-microglobulin ($beta_2M$) is an 11 kilodalton protein linked to Class I HLA molecules on the surface of most cells. It is released into the blood in proportion to cell turnover, and its serum level is widely held to be an important predictor of survival in myeloma (Child et al, 1985; Bataille et al 1984; Durie et al 1990).

1.8 Treatment of myeloma

With conventional chemotherapy the course of the disease usually progresses through three phases: induction of partial remission after which further chemotherapy appears to be ineffective: plateau phase in which there is evidence of disease but no clinical or laboratory features of tumour progression: and relapse, after which further remissions become increasingly difficult to attain.

When attempting a new form of treatment in any disease it is important constantly to review the best results attainable with current protocols, and in myeloma these can be broadly divided into chemotherapy, syngeneic or allogeneic bone marrow transplantation (BMT), and autologous BMT using either bone marrow or peripheral blood as a source of stem cells (Barlogie 1990).

Chemotherapy

Controversy still exists over whether a simple combination of melphalan (Bergsagel et al, 1962) and prednisolone (MP) (50-60% response rate, median survival approx. 3 years) is as effective as a combination of alkylating agents such as the M2 protocol (vincristine, carmustine, cyclophosphamide, melphalan and prednisone) in the USA (70-80% response, median survival 28 months). In the UK Maclennan et al (1988) found a small but significant advantage for ABCM (adriamycin, carmustine, cyclophosphamide and melphalan) over MP in a large randomised trial, but a review of 10 prospective randomised trials of MP versus combination chemotherapy failed to show a clear advantage of the more toxic combinations (Bergsagel, 1989; Boccadoro et al, 1991).

Since nearly 50% of myeloma cases are, or become, resistant to melphalan (Barlogie et al, 1984; Hall et al, 1986; Durie et al, 1988) the observation that about

40% of these patients respond to continuous infusion of vincristine and adriamycin plus high doses of dexamethasone (VAD regime) (Barlogie et al, 1984) was considered to be a major breakthrough in myeloma therapy. Unfortunately the treatment of newly-diagnosed patients with VAD does not prolong survival (Alexanian et al, 1990), and in fact high doses of dexamethasone alone may be as effective as VAD (Alexanian et al, 1986).

Alpha interferon (αIFN) (Mellstedt et al, 1979) may be of benefit in three different contexts when treating myeloma:- firstly in prolonging plateau phase (median duration of response 26 versus 14 months in 101 patients (Mandelli et al, 1990): secondly in combination with alkylating agents as initial therapy (a very high complete remission rate of 30% was obtained in a pilot study of 54 patients (Oken et al, 1990)): and finally αIFN has been used successfully to regain control of the disease when early relapse is detected following high dose therapy (Barlogie and Gahrton, 1991).

What was thought at the time to be a real breakthrough in myeloma therapy came in 1983 when McElwain reported that complete remission could be gained by means of a single, very high, dose of melphalan (HDM). 20-30% of patients attain complete remission with HDM (Gore et al, 1989). These observations led directly to exploration of the role of autologous bone marrow rescue following high-dose therapy, since the morbidity and mortality associated with prolonged bone marrow aplasia caused by these doses are unacceptably high (Barlogie et el, 1988).

Allogeneic bone marrow transplantation

The opportunity to offer allogeneic BMT (Gahrton et al, 1986, 1989, 1990; Buckner et el, 1989) to patients with myeloma is severely limited, but despite

unusually high transplant-related mortality rates (40%) in a report of 90 such transplants from the European Bone Marrow Transplant Group, there was a projected 40% long-term survival: the median duration of relapse-free survival among those with a complete response was four years (Horowitz et el, 1990). Interestingly increased age (>42) was not a predictor for poor outcome in this series (Gahrton et al, 1991). With an upper age limit for allogeneic BMT of 55 years - which excludes 75% of patients with myeloma - and with only approximately 25% of patients having an HLA-compatible donor, no more than 7% of all patients with myeloma can be offered allogeneic BMT and fewer still syngeneic BMT (Fefer et el, 1986). In contrast, about 70% of myeloma patients are candidates for autologous BMT, since up to the age of 70, treatment-related mortality does not increase with age (Jagannath et al, 1990).

Autologous bone marrow transplantation (ABMT)

Barlogie and Gahrton (1991) reviewed the results of autologous BMT from several major centres with large numbers of patients. In unpurged ABMT only the Royal Marsden group reported complete remission rates as high as 50%; other investigators found that 20-30% achieved complete remission. In all series the relapse-free survival did not exceed 18-24 months, but overall survival at best was 80% at 4 years (Jagannath et al, 1990; Barlogie et el, 1990; Harousseau et el, 1987; Gore et el, 1989) which represents a modest improvement over chemotherapy alone.

Several groups are currently investigating the efficacy of purged autografts in myeloma, but numbers are smaller. Three out of 11 patients remain disease free up to 23 months after receiving an autograft purged with Mabs against pre-B, B and plasma cells plus complement at Dana Farber Cancer Center (Anderson et al, 1989). Results are less good from the Bologna group who reported on 10 autografts purged

with the Mab 8A (against plasma cells and B cell precursors) (Gobbi et al, 1989) and Reece et al (1989) who used 4-hydroperoxycyclophosphamide for purging. In both the latter series very high transplant-related mortality rates obscure any possible benefit derived from the purging.

Increasing enthusiasm for peripheral blood stem cell autografts has been generated in the last few years (Fermand et el, 1989; Reiffers et al, 1989). This technique is particularly pertinent to myeloma firstly because complete remission is rare following conventional chemotherapy and the bone marrow is obviously contaminated with myeloma cells, and secondly it is often difficult to harvest adequate numbers of haemopoietic cells from myelomatous bone marrows. As is the case with purged autografts experience of peripheral blood stem cell transplants is limited, and it is not yet possible to assess the relative efficacy of this treatment in myeloma. However, complete remission rates of 20% (comparable to those in ABMT) have been obtained (Fermand et al, 1989), and in the series of Reiffers et al (1989) 11 out of 15 patients were in complete or partial remission at 12 months regardless of the use of blood alone or combined blood and bone marrow as a source of haemopoietic stem cells.

No comparative trials of autografting versus chemotherapy have been reported, and it is impossible to draw conclusions as to the relative efficacy of autologous bone marrow transplantation using peripheral blood or bone marrow as a source of stem cells, and whether *in vitro* purging has any effect on long-term survival.

Important conclusions to be drawn from the results of the pilot studies of autografting in myeloma are as follows:

1. High-dose chemo/radiotherapy produces remissions (at least 50% reduction in

myeloma protein) in virtually all patients with advanced or refractory myeloma, although only 20-30% achieve complete remission.

2. Better results are obtained when BMT is offered early in the course of the disease before many cycles of chemotherapy have been administered.

3. Patients under the age of 55 should be considered for allogeneic BMT, and below 70 years for autografting.

4. There is no evidence for or against the benefits of ex vivo bone marrow purging in terms of outcome: however one study indicates that prognosis is not affected by the quantity of tumour cells in marrow autografts (Gianni et al, 1989).

1.9 Aetiology and pathogenesis

The possible role of chronic antigen stimulation in the pathogenesis of myeloma (for example, the coincidence of myeloma in Finnish patients with rheumatoid arthritis is twice as high as in the general population (Spiegelberg et al, 1976)) has not been established: however, monoclonal proteins can have auto-reactivity (Davidson et al,1987), and it has been postulated that myeloma cells may arise from B cells within the idiotype (anti) idiotype network of Jerne (1987), in which case the anti-idiotypic antibodies produced may lack specificity for the original stimulating antigen (Raychaudhuri et al, 1987).

The role of oncogenes

Cytogenetic studies in myeloma have been hampered by the difficulty in obtaining adequate metaphase chromosomes, but abnormalities of chromosomes 1, 11 and 14 have been identified (Dewald et al, 1985). Although elevated expression of

c-myc RNA was found in 25% of patients with myeloma (Selvanayagam et al, 1987 and 1988; Metzer et el, 1987), this resulted only rarely from DNA rearrangement and was not associated with DNA amplification (Sumegi et al, 1985). Currently interest is focussed on the Kirsten-ras and Harvey-ras genes which are located on the short arms of chromosomes 1 and 11, the frequent sites of chromosomal breakpoints (Gould et al, 1988). The finding of increased expression of p21 protein in 17 out of 23 patients with active myeloma (Tsuchiya et al, 1988) has suggested a role for the H-ras oncogene in the pathophysiology of myeloma. However, a recent study of myeloma cell lines showed no evidence of H-ras activation (Hamilton M, 1991).

1.10 The myeloma progenitor cell

Bergsagel's definition of myeloma as a proliferation of malignant plasma cells raises two questions central to this thesis - firstly, do plasma cells have true self-renewal capacity, or do they merely accumulate as end cells? Hamburger (1977) and Bast (1982) both report evidence which suggests that plasma cells are stem cells and capable of self-renewal. These observations have been neither confirmed nor refuted since then. Secondly, is the major stem cell compartment in myeloma in mature B cells committed to the production of idiotype-specific monoclonal protein, or at a very early stage of haemopoietic development?

Malignant B lymphocytes in the peripheral blood in myeloma

For many years there have been studies showing the presence of circulating peripheral blood B lymphocytes bearing the same idiotype as the secreted myeloma immunoglobulin (Mellstedt et al,1974; Abdou et al, 1975; Warner et al, 1978; Schedel

et al, 1980; Bast et al, 1982; Ruiz-Arquelles et el, 1984), suggesting that the malignant clone comprises not only bone marrow plasma cells but also small B lymphocytes. Pre-B cells in the peripheral blood (Pilarski et al, 1985) and bone marrow (Kubagawa et al, 1979) have also been found to be idiotype positive. The difficulties of performing and interpreting immunofluorescence studies of surface immunoglobulin because of passively adsorbed antibody are well known (King et al, 1981; Gobbi et al, 1984), and it was to be hoped that the demonstration of clonal immunoglobulin gene rearrangements in peripheral blood (Berenson et al, 1987; Chiu et al, 1989) would resolve the matter. In 2 out of 10 patients clonal gene rearrangements were detected in peripheral blood B lymphocytes separated by immunomagnetic beads (van Riet et al, 1989). However, Clofent et al (1989) found no clonal rearrangements in enriched peripheral blood B lymphocytes from 21 patients with myeloma when contamination by circulating myeloma cells was rigorously excluded.

In conclusion, there is still some controversy as to whether circulating B lymphocytes really are part of the malignant clone.

Hypothesis 1.

The myeloma progenitor is a committed B lymphocyte

In 1985 Caligaris-Cappio et al reported on common acute lymphoblastic leukaemia (CALLA)-positive lymphoid-like cells in myeloma bone marrow, that failed to express terminal deoxynucleotidyl transferase (TdT) and cytoplasmic or membrane immunoglobulin. These cells (unlike CALLA+ cells from normal bone marrow) could be induced to form plasma cells after stimulation with TPA (12-O-tetradecanoyl phorbol 13-acetate). At about the same time a CALLA+ aggressive subtype of

myeloma with a poor prognosis was described (Durie et al, 1985; Wearne et al, 1986). It was suggested that the immunoglobulin-secreting myeloma stem cell could emerge just after immunoglobulin rearrangements had occurred in the pre-B phase of B cell development when CALLA, HLA-DR and B1 (CD20) are normally expressed. Further support for this concept came from Lokhorst et al (1987) who described a novel type of proliferating lymphoplasmacytoid cell with a characteristic spotted immunofluorescent pattern in myeloma bone marrow.

Following the argument that the neoplastic proliferation must occur in a cell that is already committed to the production of an idiotypic monoclonal protein and has stable heavy and light chain gene rearrangements, Warburton et al (1989) postulate that the proliferating precursor cell in myeloma arises from germinal centre B cells - the site of affinity maturation of antibody responses via somatic mutation and of isotype switching. Warburton does concede, however, that the initial molecular changes contributing to eventual malignant transformation "are possible at any stage of B-cell development" - i.e. they may occur at an early stem cell stage. Thus a later event is responsible for the deregulation of proliferation (Durie et el, 1985), and the malignant clone becomes independent of continuing antigen exposure (King et el, 1989).

Hypothesis 2.

The myeloma progenitor is an early haemopoietic stem cell

The findings of coexpression on terminally differentiated B cells of early B (Epstein et al, 1988 and 1990), myeloid and T-cell markers (Grogan et al, 1989) have been used to support the theory that the oncogenic events in myeloma occur at an

early stage of haemopoietic development (Barlogie et al, 1989). It should be noted that the apparently aberrant expression of mixed cell lineage antigens was observed on myeloma cell lines rather than on native myeloma bone marrow.

Purging myeloma bone marrow

Effective purging of marrow aims to eliminate the clonogenic fraction of the tumour: the controversies and difficulties involved with identifying the myeloma stem cells have been outlined above. One aim of this project was to attempt to establish short-term in vitro cultures of myeloma cells and identify the clonogenic fraction by means of immunophenotyping and recloning experiments. Another obstacle to overcome is the paucity of monoclonal antibodies against the cell membrane of plasma cells which could be used as purging agents.

In the following section a brief outline of previously described methods for growing myeloma colonies is given, followed by an analysis of cell membrane characteristics relevant to the search for suitable purging agents.

1.11 Methods for short-term in vitro myeloma culture

In 1977 Hamburger et al described a method for growing myeloma colonies in soft agar using as a source of putative growth factor(s) either human type O erythrocytes or medium conditioned by the adherent spleen cells of mineral oil-primed BALB/c mice. Colonies were assessed after 2-3 weeks and comprised mainly plasma cells. The technique was used extensively to study the kinetics of myeloma cell growth and also the in vitro activity of various cytotoxic compounds. However, in 1983 Durie reported that "Unfortunately, because of difficulties with reproducible

serial myeloma colony growth in recent months, possibly related to problems with the BALB/c mice used for preparation of the myeloma conditioned medium, these studies have not been further extended."

A highly reproducible method for growing myeloma colonies was devised by Izaguirre et al (1980): conditioned medium from PHA-stimulated T lymphocytes (PHA-TCM) was used as a source of colony stimulating factor and colonies were scored after 3 weeks of incubation. More detail of the method, and reasons why this may not represent a true clonogenic assay are given in the next chapter. Despite its simplicity and reproducibility, the method does not appear to have been widely adopted.

Ludwig et al (1984) described a plasma clot system for growing myeloma colonies with the aim of reproducing the patient's individual in vivo situation. Colonies were scored at 6-8 days, the cells were found to be monoclonal and consisted of lymphoid cells at various stages of maturation ranging from lymphocytic and lymphoblastoid to lymphoplasmacytic and plasma cells. The system was used to assess the effect of anti-myeloma cytotoxic drugs in vitro.

A further culture method (Takahashi et al, 1985) was devised to facilitate the simultaneous assessment of normal myeloid precursors and myeloma stem cells. Here PHA-stimulated leucocyte-conditioned medium (PHA-LCM) and plasma from a patient with aplastic anaemia were the source of growth factors. Multilineage colonies were assessed at 14 days and the cultures were incubated for a further 2 or more weeks for myeloma colonies. Patients with more aggressive disease generated more myeloma colonies and these cultures were recloned extensively, eventually giving rise to myeloma cell lines (Epstein-Barr virus negative).

A double layer agar technique described by Millar et al (1988) was used to grow myeloma colonies from human bone marrow. Irradiated HL60 cells were used as stimulants and after 2-3 weeks myeloma colonies were scored. Two cell types, plasmacytoid and lymphoid, were observed, both expressing the plasma cell antigen HAN PC1. Interestingly, these colonies could be grown from bone marrow and peripheral blood even at times of apparent disease quiescence.

In a series of elegant and rigorous experiments, Clofent et al (1988) attempted to isolate a slowly proliferating population of malignant plasma cells by depleting the samples of T lymphocytes and culturing the remaining cells by a limiting dilution technique. Positive cultures were generated only in the presence of B cells latently infected with Epstein-Barr virus (EBV) in vivo, and no cells attributable to the original myeloma cells were detected in the cell lines. This study serves to remind one of the problems of EBV superinfection.

1.12 Growth factors in myeloma

Human B cell stimulatory factor 2 (BSF-2), a T-cell derived factor that causes the terminal maturation of activated B cells to immunoglobulin-producing cells, was renamed IL-6 (Poupart et al, 1987; Kincade et el, 1989). In 1988 Kawano et al reported that purified myeloma cells express IL-6 mRNA, secrete IL-6, express IL-6 receptors,and that anti-IL-6 antibody inhibits the in vitro growth of myeloma cells. Further studies on purified fresh myeloma cells and myeloma cell lines (Horii et el, 1988; Anderson et al, 1989) demonstrated proliferation of these cells in response to IL-3, IL-5 and IL-6, but not to interleukins 1, 2 or 4. It was noted that immunoglobulin secretion did not necessarily accompany proliferative responses to

IL-6. Bataille et al, (1989) detected serum IL-6 in different plasma cell dyscrasias, and found that serum levels correlate with disease severity, the presence of serum IL-6 being detected in only 3% of the MGUS group, 35% of the overt myeloma group and in 100% of those with plasma cell leukaemia. Serial studies in 3 patients confirmed these observations. Paracrine rather than autocrine regulation of myeloma cell growth was suggested by workers in the same group (Klein et al, 1989), and support for involvement by accessory cells comes from reports of the augmentation of IL-6-induced proliferation by IL-1_{α} (Kawano et al, 1989; Kishimoto 1989), by GM-CSF (Zhang et al, 1990) and IL-3 (Bergiu et al, 1989).

Finally, Caligaris-Cappio et al (1991) have described long- term cultures of stromal cells actively producing IL-6 that were developed from the bone marrow of myeloma patients: these were used as feeder layers for autologous peripheral blood mononuclear cells and from this culture mononuclear B lymphocytes and plasma cells as well as osteoclasts, developed. Their growth was paralelled by the detection of IL-6 and IL-3 in the culture supernatant. It was concluded that bone marrow stromal cells support the growth of B lymphocytes, plasma cells and osteoclasts, and the in vivo dissemination of myeloma. Bone marrow stromal cells from normal individuals and patients with MGUS do not produce IL-6 unless activated by inflammatory mediators (Nemunaitis et al, 1989), suggesting that in myeloma bone marrow stromal cells are abnormally activated. This work also supports the hypothesis that clonogenic myeloma cells circulate in the peripheral blood: because of adhesion structures on the surface of malignant plasma cells such as H-CAM (CD44) (Picker et al, 1988), and N-CAM (CD56) (van Camp et al, 1990), circulating clonogenic cells are able to respond locally to specific growth factors secreted by bone marrow stromal cells.

1.13 Surface receptors associated with plasma cells

Most of the antigens expressed by peripheral blood B lymphocytes such as those of the clusters CD 19, 20-24, 37 are not found on plasma cells. Exceptions are CD38 (Tedder et al, 1984) which shows discontinuous expression on B cells, being present on pre-B cells and on more differentiated pre-plasma and plasma cells, and the unclustered monoclonal antibody 8A (Dinota et al,1989) which is reported to bind to all B lymphocytes and plasma cells.

The following are monoclonal antibodies which react with the surface of plasma cells: PCA-1, PCA-2 and PC-1 (Anderson et al, 1983 and 1984), R1-3 (Gonchoroff et al, 1986), HAN PC-1 (Mertens et al, 1985), MM4 (Tong et al, 1987), and 8F6 and 62B1 (Tazzari et al, 1987).

Most of the above had disadvantages when the present author was attempting to find purging agents for myeloma cells: either they were not widely available (MM4, 8A, 8F6, 62B1), or did not bind with sufficient avidity to plasma cells (PCA-1, see below), or did not bind to all plasma cells, or bound to some normal haemopoietic progenitor cells (CD38). For this reason attention was directed to examining the possible reactivity of lectins with myeloma cells.

1.14 Lectins

The term lectin was introduced by Boyd and Shapleigh in 1954 (from the latin *legere*, to pick out, to choose) to describe plant derived proteins which agglutinate cells and bind carbohydrates with a high degree of specificity. Lectins have subsequently been described as occurring in almost all types of living organism. The definition of a lectin has been refined somewhat and that adopted by the Nomenclature

Committee of the International Union of Biochemistry is "a carbohydrate-binding protein of non-immune origin that agglutinates cells or precipitates polysaccharides or glycoconjugates" (Goldstein et al, 1980).

The first experimental use of a lectin was by Paul Ehrlich in 1891, when he used ricin as the antigen for his classical experiments in immunology. Ricin had been extracted from seeds of the Euphorbiaceae family and its red cell agglutinating properties observed by Stillmark in 1888: he went on to purify similar extracts from other seeds which displayed different patterns of agglutinating activity in human and animal erythrocytes, but it was Elfstrand in 1898 who recognised the specificity of the reactions and coined the term haemagglutination.

Plant extracts became an area of intense scientific interest over the next twenty years. Landsteiner, who described human blood groups in 1900, experimented widely with a larger variety of plant products and was the first to describe a non-toxic lectin, phytohaemagglutinin. Landsteiner and Raubitschek (1909) described the first experimental inhibition of lectin effects by incubation with carbohydrate when they showed that hog gastric mucin strongly inhibited agglutination of red cells by ricin.

Although many species were found to have haemagglutinating activity, many years elapsed before useful applications were found for lectins. A truly blood group specific lectin was isolated in 1935 from *Anguilla japonica* (the Japanese eel), which is blood group O specific, and a similar agglutinin with O(H) specificity was found in 1944 (Jonsson et al) in eel serum and used for blood group typing. This was thought to be an immune phenomenon with the lectin an unusual antibody. It took 30 years for the true nature of the reaction to be elucidated (Bezkorovainy, 1971). Several more blood group-specific agglutinins followed (Reknnen et al, 1948; Boyd and

Regoera, 1949) and stimulated a further resurgence in interest in lectins. Watkins and Morgan (1952) showed that simple sugars could inhibit lectin mediated agglutination and thus a more strict definition of a lectin became possible.

The major role of lectins in cancer research was strengthened by two highly significant discoveries. Nowell in 1960 showed that *Phaseolus vulgaris* (PHA) was mitogenic for human peripheral blood lymphocytes (previously thought terminally differentiated) and Aub in 1963 demonstrated that wheat germ agglutinin (WGA) preferentially agglutinated cancer cells. Thus the relationship between cell surface carbohydrate expression, growth and differentiation was formulated and it was clear that lectins, by virtue of their specific carbohydrate reactivities, had a major role to play.

1.15 Peanut agglutinin

In 1927 Thomsen reported on a change in blood group reactivity of red cells following treatment with neuraminidase. This new blood group became known as the Thomsen-Friedenreich or T antigen. Many years later in 1964 Bird demonstrated potent anti-T activity in extracts from the seeds of *Arachis hypogea*, the peanut.

Peanut agglutinin (PNA) is unusual among the lectins in that it is unglycosylated. Its molecular weight is 110,000D (Lotan et al, 1975; Terao et al, 1975). It is a tetrameric molecule with subunits of similar molecular weight. Lotan et al (1975) found all four subunits to be identical - molecular weight 27-28,000D, although other groups have found variations, Miller (1983) showing two pairs of differing subunits. This may be a function of the different species studied as an exhaustive study of 4556 subspecies of *Arachis* showed the presence of 6-8 isolectins

(Pueppke et al, 1981), almost all having equal activity as agglutinins (Miller, 1983). Data from X-ray crystallography confirmed that PNA is a tetrameric molecule composed of two similar dimeric subunits (Salunke et al, 1985).

PNA contains a single calcium and magnesium molecule per subunit (Neurohr et al, 1980), is rich in acidic and hydroxylic amino acids and contains no cystein (Lotan et al, 1975; Miller et al, 1983). Its secondary structure is of β-pleated sheets (Hermann et al, 1978). The amino acid sequences of all legume lectins appear to have a considerable degree of homology (Higgins et al, 1983), and PNA has been partially sequenced (Lauwereys et al, 1985) and contains many homologous regions when compared to soybean or broad bean lectin.

The binding characteristics of PNA have been extensively studied by carbohydrate inhibition and spectroscopic techniques. The initial discovery of PNA by Bird in 1964 was followed by many groups investigating carbohydrate hapten-inhibition. Uhlenbruck (1969) showed strong inhibition of binding by galactose β 1,3 N acetyl galactosamine α- which has been confirmed by subsequent studies.

Table 2

Relative efficiency of carbohydrates as inhibitors of PNA-induced agglutination

CARBOHYDRATE	RELATIVE INHIBITORY POTENCY
Galactose	1.0
Galactosamine	2.22
N-Acetyl galactosamine	<0.1
Gal ß1,3 galNAc	54.5

(From Pereira et al, 1976) Galactose normalised to 1.0 (6.0 µmol required for 50% inhibition)

A cell interacts with adjacent cells and with structural components of the extracellular matrix via the external surface of its plasma membrane. Normal cell growth requires that the surface membrane should be capable of receiving and transmitting regulatory signals from the micro-environment: tumour cells are capable of indefinite proliferation, and it is likely that cell surface alterations are important in determining the characteristic features of malignant disease. Myeloma cells are largely confined to the bone marrow and their close relationship with bone marrow stromal cells has been noted above (Caligaris-Cappio et al, 1991): it seems reasonable to assume that some characteristic peculiar to malignant myeloma cell membranes is responsible for their localisation and subsequent growth advantage.

The major membrane constituents of membranes are lipids and proteins together with a small but significant amount of carbohydrate. When considering membrane-related changes in malignancy no causally related alterations in amino acid

sequence have been found in tumour membrane glycoproteins. Changes that do occur have been found only in the sugar residues and many of these are not associated exclusively with the malignant phenotype (Gallagher, 1985).

During the course of this project it was observed that normal and myelomatous plasma cells display specific, high-affinity binding of peanut agglutinin. It was hoped that this observation could be used to develop a method of clinical purging for ABMT in myeloma. This hope was duly realised and a clinical trial of lectin-purged ABMT is currently in progress. In parallel with these clinical studies, investigations of the nature and functional significance of the PNA-binding structures were commenced. To date the results of these studies reveal that in a myeloma cell line, CD44 is a major PNA-binding stucture. Since many $CD44^+$ cells are PNA^-, this implies that CD44 glycosylation is altered during terminal B-cell development.

CD44 glycoproteins have recently been identified as adhesion molecules (Jalkanen et al, 1986): further evidence for a role of CD44 in cell-to-cell adhesion comes from the work of Belitsos et al (1990) and Lewinsohn et el, (1990) who found that a Mab recognising CD44 induced the homotypic in vitro aggregation of several haemopoietic cells, and suggested that this may mimic a biological activity of CD44 as a mediator of adhesion processes in its role as a lymphocyte homing receptor.

Some recent interesting observations on the saccharide sequences of human granulocyte cell surfaces have suggested a possible relation between high-molecular-weight glycopeptides and cell migration (Van Beek et al, 1984). Mature granulocytes ready to migrate from the bone marrow into the bloodstream show a transient expression of the large glycopeptides found on many transformed cells in culture. On reaching the peripheral blood the granulocyte surface reverts to

predominant expression of lower-molecular-weight species. Immature granulocytes also have low concentrations of the larger glycopeptides and are confined to the bone marrow. Most myeloid leukaemia cells, which are immature by most cytological and immunological criteria, strongly express high-molecular-weight transformation-type glycopeptides and they migrate into the peripheral blood: it may be that in these leukaemias such large glycopeptides are synthesised precociously.

The binding of PNA to various malignant cells and in particular to malignant B cells has been extensively studied and is reviewed briefly in chapter 3.

Summary

New developments in the study of myeloma at the laboratory level comprise examination of the role of oncogenes and the search for the myeloma progenitor cell. On the clinical side there are few new chemotherapy regimens which offer improvement in survival and attention is being focussed more on the role of bone marrow transplantation, particularly autologous bone marrow transplantation because of its wide applicability to the relatively old population of patients. It is clear that new strategies in the treatment of myeloma are required and that there is a need for small pilot studies of experimental treatments. The work described in the following pages represents one such new strategy for treating myeloma.

Development of a technique for *in vitro* culture of myeloma bone marrow

2.1.Aims

To develop a reproducible technique for short-term *in vitro* myeloma colony formation and use this to identify the clonogenic fraction of myeloma cells by means of immunophenotyping and re-plating experiments.

Several culture techniques were assessed:

2.2. The B cell colony culture technique described by Izaguirre (1980)

INTRODUCTION

At the time of beginning work on this project the most widely-known method for growing myeloma colonies in vitro was that of Hamburger and Salmon (1977) which relied for colony stimulating activity (CSA) on conditioned medium from cultured spleen cells of Balbc mice which had previously been primed with mineral oil. This method was reproducible (Bast et al 1982) and had a 75% plating efficiency: however it was a difficult and time-consuming technique and more importantly there were serious problems with the availability of the particular strain of mouse necessary to generate conditioned medium (Durie et al, 1983). For these reasons the simpler method described by Izaguirre et al (1980) was assessed. Here the source of CSA is phytohaemagglutinin (PHA)-stimulated T-lymphocyte conditioned medium (PHA-TCM) and an irradiated T-cell feeder layer, although the latter was reported not to be an absolute requirement for myeloma colony formation. The method purportedly

gave rise to colonies with a higher plating efficiency than that of Hamburger, which was more suited to our requirements. The setting up of basic bone marrow in vitro culture techniques, (methods to grow colony forming units of granulocytes and macrophages (CFU-GM), erythroid precursors (BFU-E and CFU-E), mixed progenitors (CFU-GEMM) and long-term bone marrow cultures, entailed designating a special clean area of the laboratory and the use of a Class II laminar flow cabinet and humidified CO_2 incubator at 37°C which were strictly used for only known myco-plasma-free materials. Since *in vitro* cell culture experiments were not currently in progress in the laboratories used in this project (University Departments of Haematology in Birmingham and Liverpool), work was required to establish autoclaving facilities and codes of practice for sterile techniques in addition to batch-testing large numbers of animal serum samples in order to find the optimal growth-promoting product appropriate to each culture technique. Invaluable and generous advice was given by Dr G.E. Francis of the Haematology Department, Royal Free Hospital, London, and also Dr M. Gordon, Department of Immunology, Royal Marsden Hospital, Fulham Road, London. Dr N. Testa of Patterson Laboratories, Christie Hospital, Manchester, kindly taught the method of growing normal bone marrow in long term liquid cultures.

2.3.1 Bone marrow samples.

Diagnostic bone marrow aspirates were obtained from 7 patients with newly-diagnosed myeloma (aged 61-75 years; 4 female, 3 male), and 3 previously treated patients who had relapsed after a period (> 6 months) off treatment. Bone marrow plasma cell infiltrate ranged from 25-67%. One patient was receiving weekly injections of cyclophosphamide when tested. Bone marrow samples (5-10ml) were taken into medium 199 (Flow Laboratories, Rickmansworth, UK) containing preservative-free heparin 300iu and Dextran 70 0.5ml. MNC were collected from the interface after centrifugation at 400g for 25 minutes at 220°C over Ficoll- Hypaque (Pharmacia Ltd, Milton Keynes, UK) at a relative density of 1.077. The cells were then washed twice in Iscove's modified Dulbecco's medium (IMDM) and suspended in a 1:1 mixture of IMDM and Ham's medium (Gibco, Paisley, Scotland) containing human transferrin 50μg/ml, penicillin 50iu/ml and streptomycin 50iu/ml.

2.3.2 PHA-stimulated T lymphocyte conditioned medium (PHA-TCM)

This was prepared as described by Izaguirre by isolating normal peripheral blood T lymphocytes using a sheep erythrocyte rosette technique and incubating them with phytohaemagglutinin.

AET-treated sheep erythrocytes (AET-SRBC)

5.0ml SRBC 50% v/v in Alsever's medium was added to a solution of 0.806g AET (5.2 aminoethylisothiouronium bromide hydrobromide) in 20ml distilled water,

pH 9.0 and incubated at 37°C for 20 min. After washing x4 in 0.1M phosphate buffered saline (pH 7.2) the cells were resuspended in RPMI 1640 + 25% foetal calf serum (FCS).

2.3.4 T-rosetting

Peripheral blood MNC were harvested after Ficoll separation. One drop of concentrated AET-SRBC was added to $1x10^7$ peripheral blood MNC in 5ml RPMI/FCS. After centrifugation at 400g for 10 min the cells were incubated on ice for 1 hr before being resuspended and layered over Ficoll. The T cell rosettes were resuspended and the sheep red cells lysed with 12ml sterile distilled water for 15 sec followed by 4ml 3.6% NaCl. 1% v/v PHA was added to T lymphocytes at $1x10^6$ cells/ml in RPMI +10%FCS and the mixture was incubated for 3 days in 95% CO2, 5% air at 37°C. The supernatant was harvested, filter sterilized and stored in 2ml aliquots at -200°C.

2.3.5 Myeloma culture

Bone marrow MNC were suspended in concentrations ranging from $5x10^3$/ml to $6x10^4$/ml in Ham's/Iscove's medium with 0.8% w/v methylcellulose (1500 centipoise, Sigma Chemicals Ltd, Poole, Dorset), 30% FCS, $5x10^{-4}$ molar 2-mercaptoethanol and varying concentrations of PHA-TCM from 0-20% v/v. The suspensions were mixed thoroughly in a vortex mixer before being plated out in 0.1ml aliquots into wells of 96-well microtitre plates (Northumbrian Biologicals Ltd, Cramington, Northumberland), 5 replicates for each experiment. The plates were covered and incubated at 37°C in a humidified atmosphere enriched with 5% CO2 in air.

2.3.6 Assessment of cultures

Cells were scattered singly across the wells for the first 24 hours. After 2-3 days clusters (<20 cells) and colonies (>20 cells) appeared. The number of colonies did not increase significantly after that and colonies showed signs of deterioration after 7-10 days. Myeloma colonies were scored at 3-7 days and the results expressed as a mean of 5 wells. Single colonies were removed with a finely drawn Pasteur pipette, or several colonies were pooled and washed in medium. Cytocentrifuge preparations ($1x10^5$ cells per slide) were made for examination of morphology using Romanowski stains and immunofluorescence.

2.3.7 Detection of cytoplasmic immunoglobulin

Cytospin preparations of pooled colonies were fixed for 10 minutes in acetone. A double-layer technique (Walker et al, 1983) was used, slides being incubated with mouse anti-human kappa and lambda light chain monoclonal antibodies (Mabs) (gift from J Lowe, Dept of Immunology, University of Birmingham, available from Unipath, Bedford, UK) in a moist chamber for 30 minutes. After washing, the slides were further incubated for 30 minutes with fluorescein-conjugated sheep anti-mouse immunoglobulin. After washing again, a Leitz fluorescent microscope with epi-illumination was used to read the slides. Cells were also examined using Mabs against antigens present on myeloid cells (AGF 4.48; Fisher et al, 1982), monocytes/macrophages (HJ11; Dept of Immunology, University of Birmingham) and T lymphocytes (UCHT-1; Dr PC Beverley, University College, London), but in no case were these found to be expressed on more than 5% of cells examined.

2.3.8 *In vitro* testing with cyclophosphamide derivatives

Bone marrow mononuclear cells were incubated for one hour at 37°C with mafosfamide-lysine (ASTA-Z) (Asta-Werke AG, Bielefeld, FRG) at concentrations of 10-100μg/10^7 cells in 1 ml of medium.4-hydroperoxycyclophosphamide (4-HC) (Asta-Werke AG) was used at 10-50μg/10^6 mononuclear cells in 1 ml, and cells were incubated for 30 minutes. After incubation the cells were washed 3 times in medium prior to culturing. Myeloma and normal bone marrow samples were treated in the same way except that myeloma marrows were plated out in microtitre wells as described and the normal marrow cells were cultured in 35mm plates over underlayers, as described by Pike and Robinson (1970). Colonies of CFU-GM were scored at 14 days and expressed as a percentage of the colonies obtained after incubation and washing in the absence of drug.

2.4 RESULTS

Colonies appeared from 3 to 7 days and numbers ranged from 30-200 per well. In 3 samples the number of colonies was directly related to the number of cells plated between $5x10^3$ and $6x10^4$. The plating efficiency overall (colonies per cells plated) ranged from 0.05-2.8% (Figure 1).

In experiments to assess the requirement for PHA-TCM only 2 out of 10 bone marrow samples failed to grow in the absence of PHA-TCM: one other showed a progressive increase in colony numbers with increasing concentrations of PHA-TCM up to 20% v/v. The remaining 7 had maximal numbers of colonies in the presence of 4% PHA-TCM.

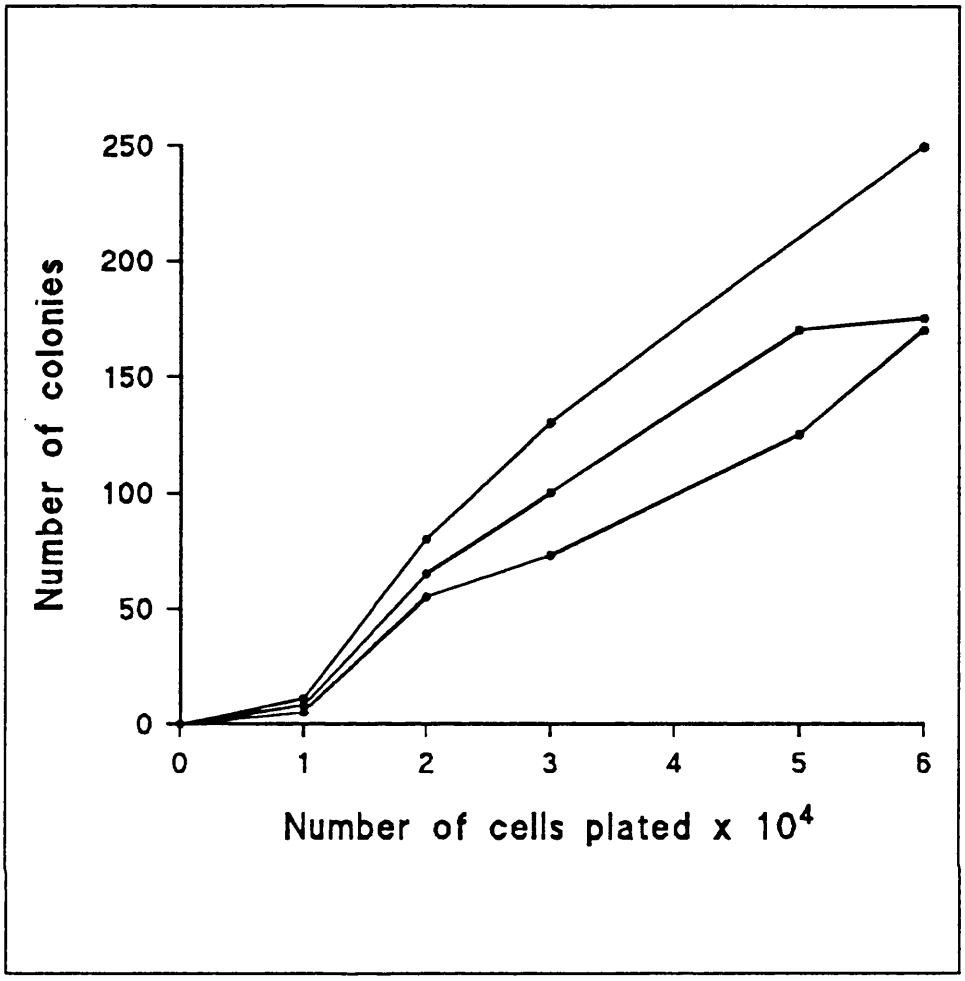
FCS at high concentration (30%v/v) was essential for the appearance of plasma cells in the colonies and at lower concentrations more mixed colonies were seen.

On Romanowski staining of cytocentrifuge preparations the majority (>80%) of cells were mature plasma cells with a small number of plasmablasts and lymphocytes. However, in 3 cases less mature forms, both plasmablasts and lymphoblastoid cells, predominated. Immunofluorescence studies confirmed that the intra- cytoplasmic immunoglobulin of the cultured cells was of the same light and heavy chain isotype as that found in the patient's serum. A small number of mixed granulocyte-macrophage-erythroid colonies was present in 3 cases. Single colonies picked from the plates and stained confirmed the lymphoplasmacytoid origin of the colonies.

2.4.1 *In vitro* drug effect

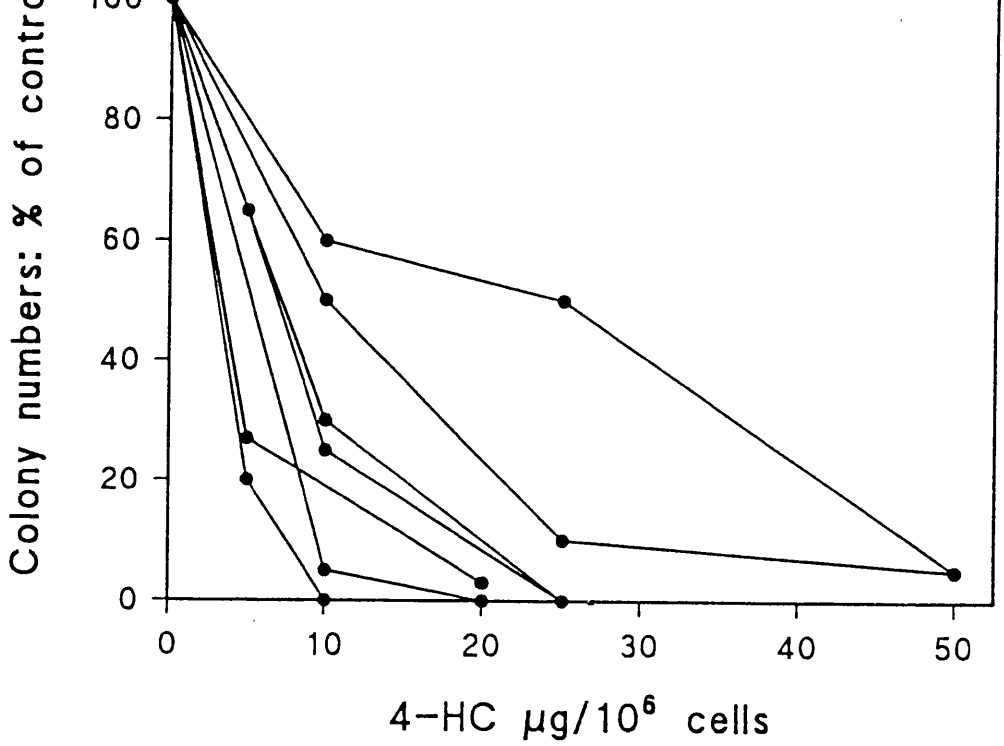
Normal bone marrow samples cultured for CFU-GM showed progressive inhibition of growth with increasing concentrations of both ASTA-Z and 4-HC, such that a concentration of 50µg/ml of each drug was inhibitory to 95-100% of CFU-GM (Figure 2). When ASTA-Z or 4-HC was incubated with myeloma bone marrows progressive inhibition of myeloma colony growth was less marked and less consistent (Figures 3 and 4). Even at concentrations of each drug that produced 95% killing of normal CFU-GM, consistent effects on the myeloma colonies were not seen.

Figure 1.



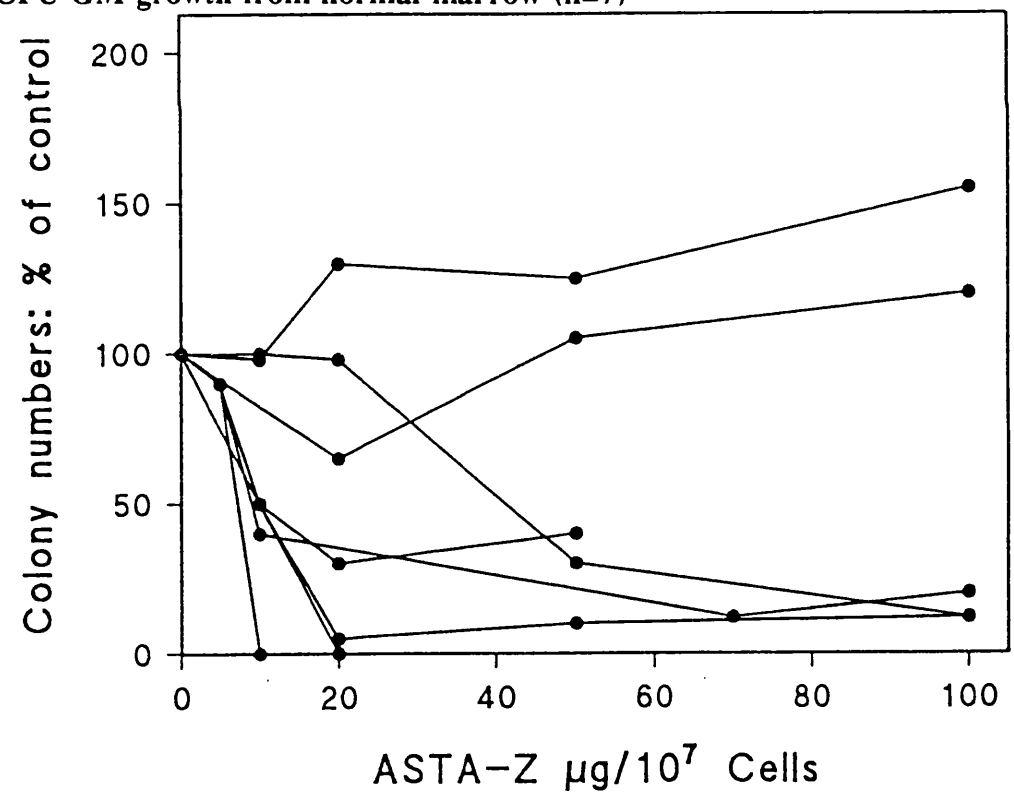
The linear relationship between the number of cells plated (in 0.1 ml in each microtitre well) and the number of myeloma colonies formed. The results for three different myeloma patients are shown.

Figure 2.



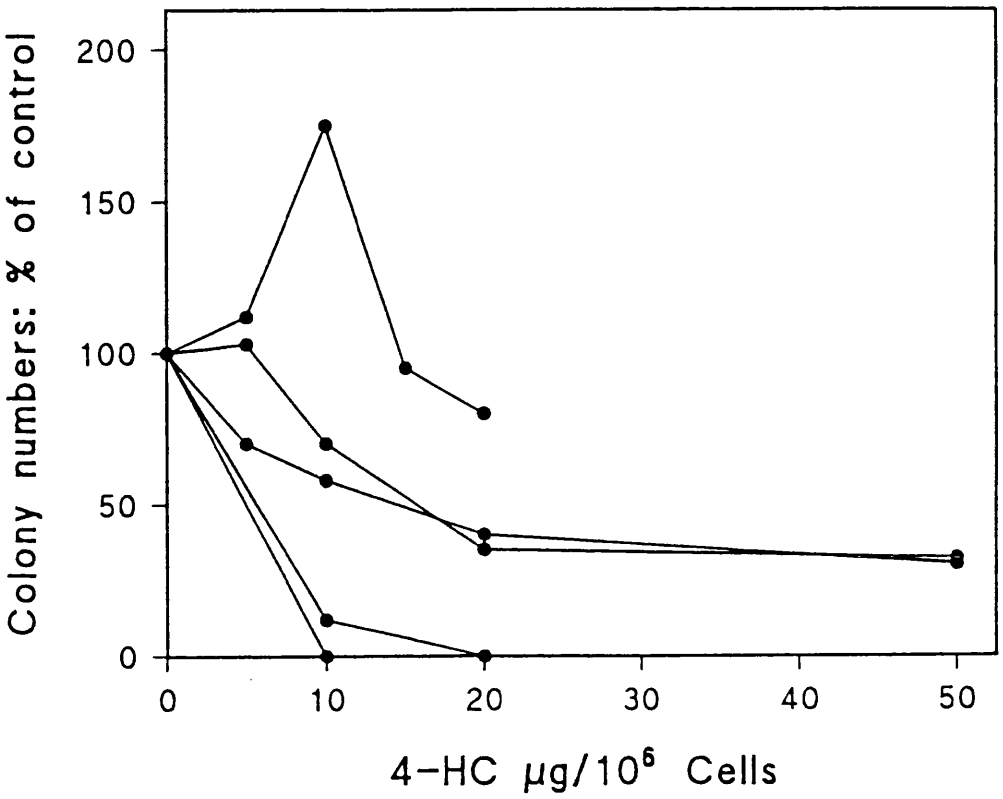
The effect of varying concentrations of 4-hydroperoxycyclophosphamide (4-HC) on CFU-GM growth from normal marrow (n=7)

Figure 3.



The effect of varying concentrations of mafosfamide-lysine (ASTA-Z) on CFU-GM growth on normal marrow (n=5)

Figure 4.



The effect of varying concentrations of ASTA-Z on myeloma colony formation (n=8).

2.5 DISCUSSION

The method for *in vitro* myeloma colony formation as described here produces a rapid accumulation of apparent colonies comprising mainly plasma cells but also lymphoblasts, plasmablasts and lymphocytes. The clonal origin of the cells was demonstrated by finding intracytoplasmic immunoglobulin in the cultured cells of the same isotype as in the patients' serum. Contaminating growth of cells of non-B origin was insignificant if FCS concentration was high, and, as reported by Izaguirre, it was not necessary to remove T cells from the bone marrow MNC preparation. Finally, most bone marrow cultures yielded colonies even in the absence of PHA-TCM.

Several factors lead one to suspect however that the culture conditions described did not yield true clonogenic myeloma colonies. First, the remarkably rapid appearance of groups of cells within 3 days raises the possibility of cell clumping rather than cell division. Secondly the linear relationship between numbers of cells plated and colonies scored would support clumping as an explanation for the rapid appearance of groups of cells. Thirdly, no clearly demonstrable growth-promoting agent could be identified in the culture medium (for example 8 out of 10 bone marrow samples grew in the absence of PHA-TCM). Fourthly no dose- response relationship could be observed with cytotoxic drugs.

A final series of experiments performed some months later compared the colony numbers of myeloma bone marrow (n=3) plated out as above with irradiated (20Gy) bone marrow MNC (n=3). The irradiated samples yielded similar numbers of colonies (68+/-25 colonies per $6x10^4$ cells plated) as unirradiated samples (78+/-18 colonies per $6x10^4$ cells plated), which led to the conclusion that this method is not

satisfactory for the study of myeloma clonogenic growth *in vitro*.

Summary

The experiments described in this chapter which aimed at growing myeloma colonies by a previously described technique failed to provide us with a reliable method for examining clonogenic cells in myeloma. This early work however, clarified the particular problems of growing myeloma cells and made it possible to define strict criteria for assessing myeloma cell growth.

CHAPTER 3

DEVELOPMENT OF A TECHNIQUE FOR IN VITRO CULTURE OF MYELOMA BONE MARROW. PART II: FURTHER BONE MARROW CULTURE EXPERIMENTS TO GROW MYELOMA COLONIES.

Aims

To experiment with different bone marrow cell growth promoters and culture conditions used with other cell types in an attempt to develop a reproducible system for growing myeloma colonies *in vitro*.

3.1 Introduction

The experience with the Izaguirre technique as just described and that of other workers (Boom et al, 1988) highlights the particular difficulties associated with myeloma cells that need to be overcome in a successful *in vitro* culture system. These can be summarised as follows:

1. Plasma cells and some lymphocytes may remain in a viable but non-proliferating state in culture medium for considerably longer periods than other haemopoietic cells.

2. Myeloma cells have a propensity to clump and form pseudo-colonies.

3. Demonstration of clonal growth of myeloma cells must include evidence of cell division including an increase in cell numbers of clonal origin and markers of cell proliferation (e.g. tritiated thymidine incorporation, expression of nuclear proliferation antigens such as Ki-67 or bromodeoxyuridine synthesis).

The identity of specific myeloma colony stimulating factor(s) is still unknown,

although several candidates have been proposed, e.g. IL-6 either alone or in combination with other factors.

The following section details a variety of bone marrow culture systems aimed at circumventing these problems. In particular a variety of culture supernatants and feeder layers was tested as a source of growth factor.

3.2 MATERIALS AND METHODS

3.2.1 Patients

Bone marrow was derived from diagnostic aspirates from patients with newly-diagnosed myeloma (n=72) and from patients with established myeloma who had relapsed following a period of at least 3 months without treatment (n=8). Further patients with monoclonal gammopathy of undetermined significance (MGUS) were studied (n=2) and one patient had a solitary plasmacytoma of bone. Normal bone marrow samples were taken at the time of sternotomy from fully informed, consenting, haematologically normal patients undergoing coronary artery bypass surgery. Ethical committee approval from Broad Green Hospital had been granted.

3.2.2 Cell preparations

Bone marrow samples (5-10ml) were taken into 2ml IMDM containing preservative-free heparin at a final concentration of 10u/ml. Density gradient centrifugation over Ficoll (as previously described) yielded MNC, which were washed twice.

Monocytes and T lymphocytes were removed by plastic adherence (incubation for 1 hour at 37°C in a 25cm^3 Falcon tissue culture flask) and double AET-treated

sheep erythrocyte (E) rosetting (B lymphocytes >95% by CD19 staining; monocytes <1% by morphology and CD14 reactivity) (Kaplan and Clark 1974).

3.2.3 Growth promoters

PHA-TCM (see Chapter 2 for method)

Helper T lymphocyte conditioned medium (H^+-TCM)

H^+-TCM was prepared from PHA stimulated $OKT4^+$ T-helper lymphocytes obtained by an indirect panning technique (Wysocki and Sato 1978). Bacteriological grade 35mm plates (Sterilin) were coated with 5µg/ml goat anti-mouse immunoglobulin (Serotec, Kidlington, UK) in 10ml of 50 mM Tris buffer, pH 9.5, for 1 hour at 22°C. The plates were washed 3 times with phosphate-buffered saline (PBS) and once with PBS supplemented with 1% heat- inactivated FCS. E^+ rosetted T lymphocytes ($3x10^6$/ml) from normal human peripheral blood were incubated at room temperature for 20 minutes with 200µg/ml Leu2 (anti- suppressor cell, Becton Dickinson). The cells were washed 3 times in ice-cold PBS and resuspended in PBS supplemented with 5% FCS. 10^7 cells of this suspension were plated in 2ml onto the antibody-coated polystyrene dishes and incubated for 1 hour at 40°C, with occasional gentle mixing. The cells were decanted by tilting the plates and gently washing and swirling these twice with 3ml PBS/5%FCS to remove all non-adherent cells, which yielded >90% $Leu3^+$ T helper cells . $Leu3^+$T-helper cells were incubated with 1% PHA as above to make H^+-TCM. This was added to various myeloma cultures at 10-20% v/v instead of PHA-TCM.

Myeloma culture supernatants

Supernatants from Ficoll-separated myeloma bone marrow cells (n=10) cultured for 48 hours in 5ml IMDM with 5% FCS were collected, filtered and added at 10-20% v/v to various myeloma cultures (n=20: T cell and adherent cell-depleted, plated at $1x10^5$ cells per ml in 35mm plates containing 0.3% methylcellulose, or in liquid cultures of 5ml RPMI/5%FCS).

Other supernatants (n=8) were collected from myeloma bm MNC which had been cultured in DCCM.1 low-serum medium (Biological Industries, Cumbernauld, Glasgow) for 9 days, and used in the same way.

Low molecular weight B-cell growth factor (LMW BCGF)

Partially purified LMW BCGF (12kD) was obtained from Cellular Products (Buffalo, USA) and added to myeloma bm in liquid cultures. For a positive control it was established that 10% BCGF induced optimal proliferation in normal tonsil purified B cells (n=2) activated with sub-optimal doses of insoluble anti-μ (10μg/ml).

Conditioned medium from C5637 and T-24 cell lines

Conditioned media from these two bladder carcinoma cell lines (kindly given by Dr Fogh, Sloan Kettering Memorial Centre, New York and Dr R Callard, Institute of Child Health, London, respectively) were added to various cultures of myeloma bm cells. The C5637 supernatant acts on primitive haemopoietic cells and contains a synergistic factor (SF-1) which, together with macrophage colony stimulating factor, is capable of stimulating high proliferative-potential colony-stimulating cells (McNiece et al,1989). The T-24 supernatant contains a B-cell differentiation factor (BCDF) that

can stimulate imunoglobulin secretion in normal human B cells without necessarily causing clonal expansion (Gallagher et al,1986).

The conditioned media were added at 10-20% v/v both singly and in combination to liquid cultures (5ml) of Ficoll-separated myeloma bm cells and to feeder layer cultures as described.

3.2.4 Liquid cultures

Ficoll-separated myeloma bone marrow cells were seeded at $5x10^5$/ml to $1x10^6$/ml in $25mm^3$ tissue culture flasks (Flow Laboratories, Rickmansworth, UK) in IMDM supplemented with various concentrations (10-20%,v/v) of serum and/or growth promoter. At intervals (2-3 weeks) the entire culture was centrifuged at 400g for 10 minutes and the cells resuspended using a fine gauge needle before counting (using Trypan blue dye exclusion to count viable cells) or re-plating as required.

3.2.5 Proliferation assays

In a variety of experiments (5 samples) rigorously T-cell depleted myeloma bone marrow MNC were seeded at $1x10^5$ cells /well in 96-well microtitre plates in RPMI +10%FCS with the following additions:- TPA 10ng/ml, 20% CM from C5637, TPA + CM from C5637, myeloma culture CM, H^+-TCM 20%, supernatant from myeloma E^+ cells cultured for 48 hours in RPMI/5%FCS, CM from the myeloma cell line RPMI 8226, CM from the T24 cell line, IL-6, and control wells with no additional factors. After 48 hours' culture at 37°C in a CO2 incubator the wells were pulsed with 5µCi ^3H- Thymidine: 18 hours later the cells were harvested and the radioactivity was measured in a scintillation counter. At the time of radioactively

pulsing the cells duplicate wells were harvested, and cytocentrifuge preparations were made for phenotypic analysis with a panel of Mabs.

3.2.6 Feeder layer techniques

Irradiated (20Gy) autologous T lymphocytes, with and without adherent cells, were immobilised in 0.5% agar containing IMDM supplemented with 10-30% FCS. Feeder layers were prepared from normal bm samples using methylprednisolone (Gordon et al,1985) and by the method of Dexter et al (1976) were prepared for over-layer with myeloma cells. Another form of feeder layer was the irradiated adherent stromal layer from myeloma bm long-term cultures.

T-lymphocyte- and adherent cell-depleted myeloma bm cells were mixed with 0.8% methylcellulose, IMDM and various growth promoters such as PHA-TCM, H^+-TCM, C5637 or T-24 conditioned medium and various sources of serum (human, bovine and horse). Cultures were incubated at 37°C and colonies forming in the methylcellulose were picked off individually using a finely-drawn pipette, and stained with a Romanowsky stain and Mabs.

A further feeder layer technique was adapted from the murine long-term bm cultures of Dorshkind (1986) in which B lymphocytes were induced to grow by transferring the cultures to Whitlock conditions after two weeks. Bone marrow aspirate cells from normal or myeloma bome marrows were cultured at $2x10^6$ cells/ml in a $25cm^3$ (Falcon) containing 5ml IMDM supplemented with 10% FCS (Biological Industries, Glasgow, UK), 10% horse serum (Imperial Laboratories, Salisbury, UK), 10^{-6}M hydrocortisone sodium succinate, $5x10^{-5}$ 2-mercaptoethanol, 50u/ml penicillin and 50µg/ml streptomycin. The flasks were cultured at 37°C in a humidified incubator

enriched with 5% CO2. After 2 weeks, or when an adherent layer was established, all culture medium was removed and the non-adherent cells were resuspended at $2x10^6$cells/ml in either RPMI 1640 supplemented with 5%FCS or DCCM.1 culture medium. Thereafter cultures were fed at weekly intervals by removing 50% of the medium and replacing it with fresh medium.

3.2.7 Low serum conditions

Ficoll-separated MNC from myeloma bm were suspended at $5x10^5$ to $2x10^6$ cells/ml in DCCM.1 medium supplemented with 2% bovine serum albumin (BSA; Sigma Chemical Co., Fraction V) and transferrin (25mg/ml) in $25cm^3$ flasks, and cultured at 37°C. Weekly cell counts were performed and cytospin preparations were made to examine morphological and phenotypic changes over time.

3.2.8 EBV studies

Cultures which yielded myeloma colonies were examined by fluorescence for the presence of Epstein-Barr nuclear antigen (EBNA). These studies were kindly performed by Dr CG Woodward at Leeds Public Health Laboratories.

3.2.9 Alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining technique

Cytospin preparations (10^5 well-separated cells per slide) were fixed in acetone for 2 minutes and stained by APAAP (Erber et al,1984) with the following first-layer monoclonal antibodies at concentrations previously determined to give optimal results: anti-kappa, anti-lambda, Leu M3 (anti-monocyte), Leu 12 (anti- CD19), Leu 3 (anti-T cell), all from Becton Dickinson, anti-CD38 was a kind gift from Dr Tedder, Dana

Farber Institute, USA, and RFD6 (anti-plasma cell) was used by kind permission of Professor Janossy, Royal Free Hospital, London. Irrelevant control first-layer antibodies of appropriate isotype were always included. The second- and third-layer antibodies consisted of rabbit anti-mouse Ig and mouse anti-alkaline phosphatase-alkaline phosphatase conjugates respectively. Alkaline phosphatase activity was then detected with naphthol AS-MX phosphatase (9.2mg/ml) substrate and fast red TR (1mg/ml) as capture agent; levamisole (0.2M) was added to inhibit endogenous alkaline phosphatase activity. Cells were formally counterstained with haematoxylin and eosin.

3.2.10 Immunoglobulin gene rearrangement studies

These were kindly performed by Miss C Olive in the Department of Haematology, Royal Liverpool Hospital. Genomic DNA was extracted from peripheral blood lymphocytes or Ficoll-separated bm cells by the method of M Jeanpierre (1987). The concentration and purity were determined by spectrophotometry.

10µg genomic DNA was digested with the restriction enzymes ECOR1 and Bam H1, and subjected to electrophoresis in 0.8% agarose slab gels according to the method of Southern (1975). Hybridization was carried out at 65°C in a solution of 5xSSPE containing 0.1% SDS, 0.1% BSA, 0.1% ficoll 400, 0.1% PVP and 100µg/ml denatured herring sperm DNA, and 0.05-1.0µg of the following nick-translated ^{32}P-labelled probes:- J_H, C_{kappa} and C_{lambda} (kind gift of Professor T Rabbits, MRC Unit, Addenbrookes Hospital, Cambridge, UK). Filters were washed at 65°C for 30 minutes each in the following solutions:- 2xSSC, 2xSSC + 0.1% SDS, and finally 0.1xSSC and 0.1% SDS.

3.3 RESULTS

3.3.1 Culture conditions unsuccessful in producing myeloma colonies

Growth promoters

No evidence of selective myeloma growth was obtained with any of the growth promoters tested. Some clumps/colonies of plasma cells were observed, but either T-cell or myeloid colonies were so numerous that accurate assessment of putative myeloma colonies was not possible.

Serum

None of the different sources and batches of serum produced evidence of myeloma growth, although markedly different numbers of myeloid colonies were observed.

Different feeder layers

Again, there was no selective growth of myeloma cells. Individual clumps/colonies containing plasma cells were observed among myeloid and T-cell colonies, but when such clumps were examined morphologically they never consisted exclusively of light-chain-restricted myeloma cells.

Under conditions similar to the Whitlock-Witte model for murine B lymphopoiesis (Dorshkind 1986), selective growth of B cell/plasma cell colonies was observed in 1 case of MGUS, 1 solitary plasmacytoma, and 1 myeloma bm. In these three cases, long-term bm cultures were initiated, and after 2 weeks, by which time a confluent stromal layer had formed, non-adherent cells were discarded, and the culture medium replaced with RPMI 1640 plus 5% FCS (MGUS) or DCCM.1 (plasmacytoma and

myeloma bone marrow cultures). After a delay of 6-11 weeks small clusters, followed by colonies of 50-100 cells appeared in the non-adherent fraction. Analysis of pooled colonies, which were negative for EBNA fluorescence in the case of the myeloma bm culture (not tested in the other two cultures), revealed that >95% of cells were of B cell origin, but were composed of both kappa-, and lambda-expressing cells. It was thought that this might represent an outgrowth of normal B cell clones, and prompted a study of low-serum conditions in myeloma.

3.3.2 Successful growth of myeloma colonies

Growth under low-serum conditions

When ficoll-separated myeloma bm cells were incubated in DCCM.1 nutrient medium containing 2% BSA and transferrin (25mg/l), 17 of 32 samples formed what was believed to be genuine myeloma colonies.

3.3.4 Demonstration of clonal growth

In APAAP preparations, the colonies grown under low-serum conditions clearly consisted of light-chain restricted B/plasma cells, with no myeloid and very few T cells. In the 3 cultures studied before and after culture, identical JH and light-chain rearrangements were observed after culture.

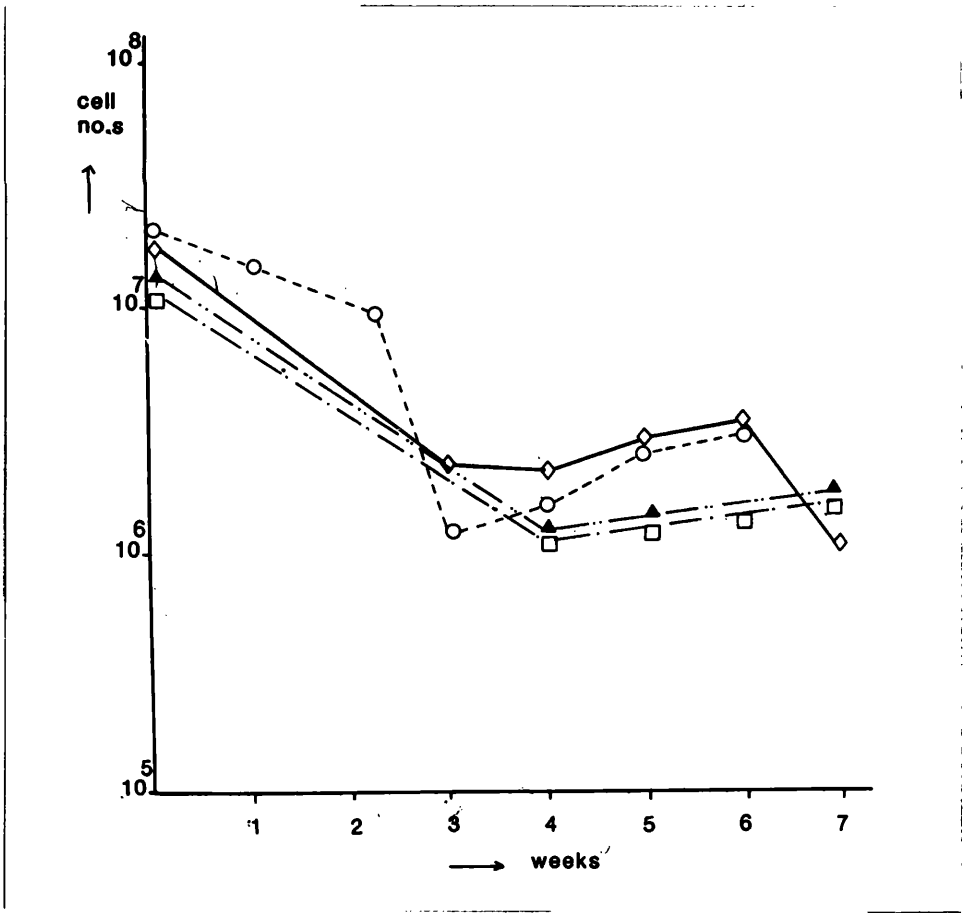
A small proportion of T lymphocytes (1.5+/-2.0%) was present in the majority (13/17) of the cultures during the proliferative phase. In no case was there evidence of T cell colony formation, and there was an average 90% reduction in relative numbers of T lymphocytes during the period of culture (up to 6 weeks). However, in 5/32 myeloma bone marrow samples, the Ficoll-separated cells were rigorously T-cell

depleted prior to culture, and none of these 5 samples subsequently showed any signs of clonal growth.

3.3.5 Characteristics of the myeloma cell growth

All 17 cultures in which growth was observed displayed similar growth patterns with an initial lag phase of 3-4 weeks during which there was a rapid diminution in cell numbers (Figure 5). During the period of active cell growth cells doubled in number in 8-10 days. During the first 3-4 weeks of culture the pH of the culture medium remained constant and the medium was not changed: thereafter the cultures became acid, and the medium was changed weekly.

Figure 5



Cell numbers during culture of myeloma bone marrow (four representative experiments). Total cell numbers fell markedly during the first 3 weeks of culture as the non-B cells died. After about 3 weeks there was a modest increase in cell numbers and these cells were clonal B/plasma cells.

3.3.6 The observed myeloma cell growth was attributable to cell proliferation and not to clumping

During the period of active cell growth (i.e. after approximately 4 weeks in culture), an overall increase in cell numbers was recorded in each case.

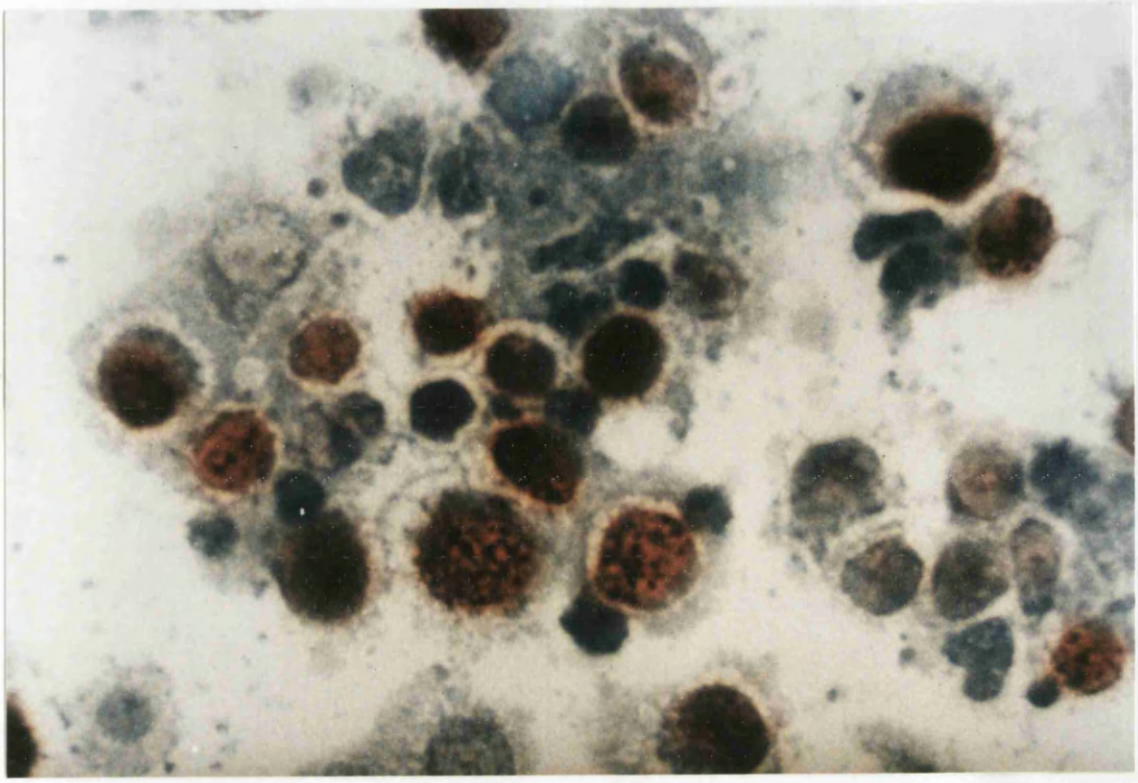
Irradiation (20Gy) of bm at the outset of culture completely abolished growth, while at the same time myeloma grew from the non-irradiated controls (3 experiments) after the usual 4-week lag phase.

3.3.7 Mitoses/Ki-67

Mitotic figures (2-5%) were consistently observed in 13/17 cultures. In APAAP preparations these mitoses were shown to be in cells of B lineage and no mitoses were observed in the few T- cells present in the culture.

There was a marked increase in the numbers of cells in growth cycle, as reflected in increased numbers of Ki-67 positive nuclei (Table III, Figure 6). Without more sophisticated staining techniques (e.g. dual staining for Ki-67 and surface membrane antigens) it was not possible to demonstrate with certainty whether Ki-67 positivity was confined to plasma cells. Some Ki- 67^{+} cells were undoubtedly plasma cells, but others could have been $CD19^{+}$ lymphocytes.

Figure 6



Nuclear staining by Ki67 Mab (by APAAP technique) in a serum-free culture of myeloma bone marrow subsampled at 2 weeks.

3.3.8 Replating experiments

In 3/17 cultures cells from developing colonies were withdrawn and pooled so that colonies were broken up: the cells were then re-cultured at $5x10^5$ in the same medium. The re-cultured cells were counted at intervals and were shown to have a doubling time of 8-10 days. 8/17 cultures were re-plated into 0.8% methylcellulose, but only two of these formed colonies after a further 3 weeks. 2 additional cultures were re-plated in limiting dilutions, but no colonies ensued. It appeared to be crucial to re- plate cultures early on in the growth phase as no culture was successfully re-plated after the first week of the growth phase.

3.3.9 EBV studies

Colony cells from 5 out of the 17 growing myeloma cultures were pooled and examined for EBNA membrane fluorescence. In the 5 cultures examined , all cells were negative for EBNA. These cultures were very similar in their growth and phenotypic characteristics to those not examined for EBNA.

In 3 of the 15 samples in which myeloma growth was not obtained colonies with very different growth characteristics emerged. Their growth rate was considerably faster (doubling time 2-4 days) and the colony size was much greater , frequently attaining >500 cells. 100% of cells from these 3 cultures were strongly positive for EBNA, and the cultures have continued to grow vigorously ever since (>6 months). The differences between the EBNA positive and negative cultures were strikingly obvious:- a shorter lag period before colonies appeared, larger colony size, greater proliferative rate, and infinite self-renewal potential. Phenotyping studies showed expression of CD19 and PNA on the same cells, whereas in myeloma colonies cells were either $PNA^{+}CD19^{-}$ or vice versa. Finally, cytoplasmic Ig light and heavy chain expression was different from that of the original myeloma samples, suggesting that EBV transformation of normal B lymphocytes had occurred.

3.3.10 Phenotype of myeloma colonies

When clusters and colonies first appeared the cultures showed a mixture of $CD19^{+}$ lymphocytes and plasma cells staining with RFD6 and CD38. After 3 weeks of proliferation >90% cells had the morphological appearance of plasma cells and were $CD19^{-}$. $CALLA^{+}$ cells were only detected in 1 culture (Tables III and IV) from a patient with plasma cell leukaemia who had 15% $CALLA^{+}$ cells in the bone marrow at diagnosis.

Table III

Phenotype of myeloma b.m. cells before and after culture in serum-free medium

Patient	Culture period (weeks)	Morphol PC* (%)	Kappa (%)	Lambda (%)	RFD6 (%)	CD38 (%)	CD19 (%)	cALLA (%)	CD3 (%)	Ki-67 (%)
1	0	67	5	65	58	61	4	0	11	0
	4	8	1.5	97	80	84	25	0	0.5	25
2	0	48	53	4	45	N/D	2	<1	20	11
	4	57	100	0	60	N/D	45	0	0	35
3	0	52	49	4	54	N/D	5	0	13	4
	4	81	100	0	80	76	17	0	0	18
4	0	35	53	3	31	N/D	10	N/D	7	2.5
	4	77	95	2	80	N/D	15	<1	4.5	15
5	0	83	1	90	87	81	2	0	5	0
	4	70	0	100	74	88	20	0	1.5	15
6	0	42	5	51	46	55	16	0	3	0
	4	32	2	77	30	N/D	68	0	0.8	30
7	0	58	65	6	N/D	70	14	0	25	19
	4	49	90	3	52	66	33	0	2	12
8	0	40	48	2	42	N/D	2	0	8	0
	4	80	95	4	78	70	32	0	3	20
9	0	59	75	3	N/D	68	32	25	9	N/D
	4	69	95	<1	71	65	25	15	0	4
10	0	29	32	6	20.5	32	11	0	5.1	N/D
	4	62	89	1.5	61	71	14	0	0.8	N/D

Patient	Culture period (weeks)	Morphol PC* (%)	Kappa (%)	Lambda (%)	RFD6 (%)	CD38 (%)	CD19 (%)	cALLA (%)	CD3 (%)	Ki-67 (%)
11	0	39	5	48	38	38	11	0.5	18	4
	4	58	2	97	62	53	25	0	1	11
12	0	40	11	53	38	40	11	0.5	12	0
	4	63	6	88	62	54	25	0	0	21
13	0	51	51	2	48	14	4	N/D	23	0.5
	4	80	96	0	85	86	18	0	<1	3
14	0	60	72	7	62	N/D	2	0	8	0
	4	50	98	3	53	62	42	0	1.5	15
15	0	44	45	5	42	45	5	0	11	0
	4	82	97	3	85	N/D	11	0.2	1	0.5
16	0	61	62	3	56	N/D	3	0	4	8
	4	69	98	0	72	65	19	0.5	0.5	5
17	0	32	38	4	26	24	10	0	9	0.5
	4	78	96	0.1	80	58	12	0	1	6
Patient group mean +/-sd	0		54+/-13[a]	61+/-17[b]						
	4		96+/-3	92+/-9						

a) Mean is for Kappa myelomas only b) Mean is for Lambda myelomas only

*Morphological plasma cells. N/D - not done

Table IV Phenotype of myeloma bone marrow cells before and after culture in serum-free medium

Phenotype of colonies in 17 samples (percent)

	Morpholog -ical plasma cells	Dominant light chain	RFD6	CD19	CALLA	CD3	Ki-67
Before culture	47+/-17	56+/-14	46+/-16	8+/-7	2+/-6	11+/-6	4+/-5
After culture (4-6 weeks)	66+/-14	94+/-6	68+/-14	26+/-14	1+/-4	1+/-1	15+/-9

3.4 DISCUSSION

This study clearly shows that non-fractionated bone marrow from approximately 50% of patients with untreated myeloma will grow in vitro under low serum conditions, without a feeder layer and without exogenous growth factors. Great care was taken to demonstrate that growth involved clonal plasma cells, that cell proliferation rather than clumping was actually taking place, and that cells were not transformed by EBV. Clonality was established by the demonstration of both light-chain restriction and clonal gene rearrangement. Actual proliferation occurred since increasing cell numbers were directly demonstrated, since cells were shown to contain mitoses and the Ki-67 nuclear proliferation antigen, and since irradiation abolished growth.

It is not clear why the present culture system succeeded in producing pure myeloma cell growth, when other systems have not clearly done so. The absence of T-cell growth factors was clearly important in preventing the outgrowth of T cells, but

the absence of inhibitory factors present in serum or in the T-cell conditioned medium used by many authors may also have contributed. It is also possible that the serum free medium contains some factor essential for myeloma cell growth. It is also perhaps worth noting that small numbers of T cells were consistently present in our cultures and it cannot be excluded that they were able to produce factor(s) contributing to growth. Clearly, though, whatever the factor(s) contributing to successful growth, the culture conditions were not ideal since growth was demonstrated in only about half the cases tested. In this regard, it would be interesting to test the effect on this culture system of recombinant cytokines such as IL-6, IL-1, IL-4, IL-7 etc.

The present work indicates that plasma cells and B cells both have proliferative potential in myeloma since both cell types containing the Ki-67 nuclear proliferation antigen were observed. Furthermore, because substantial numbers of non-plasmacytoid B cells were present at 4 weeks while after 6 weeks most cells were plasma cells, it is suggested that these B cells were myeloma precursors. Such cells, however, consistently lacked cALLA and the present study could find no evidence to support previous suggestions that the myeloma progenitor is cALLA positive (Grogan et al, 1987).

A notable feature of the present culture system was the long lag phase before colonies of B/myeloma cells emerged. The significance of this lag phase is not known, but it may indicate that production of growth factor by a minority cell type is necessary for successful growth. Also, of course, the consistent presence of this lag phase provided further evidence excluding cell clumping as a possible explanation of these findings.

The culture method described here provides a means of examining the effect

of a whole range of cytokines on myeloma cell growth and therefore of defining further the factors important in the control of B cell growth and differentiation. More relevant to the aims of the present study, it was now felt that the target cells for purging ($CD19^+$ B lymphocytes and plasma cells) had been sufficiently defined by their appearance in myeloma colonies to justify pressing on with the search for a means to remove these cells from myeloma bone marrow. It is clear that a great deal more work is required on myeloma culture studies, particularly with the use of more sophisticated techniques for detecting immunoglobin gene rearrangements in separated cell populations of growing myeloma cells (see section 8.1 on plans for future work).

Summary

After experimenting with many different cell growth promoters and culture conditions, a newly marketed serum-free culture medium was found to yield myeloma cell colonies which met our criteria for assessing clonogenic growth. As a result of the immunophenotyping studies on colonies, it was concluded that $CD19^+$ lymphocytes and plasma cells should be targetted for *in vitro* purging, and the project at this point changed direction to concentrate on finding suitable purging agents for these cells.

CHAPTER 4

Lectin reactivity of myeloma bone marrow

4.1 Aims

To examine a panel of lectins for specific reactivity with plasma cell membranes in the search for a purging agent for myeloma

4.2 Introduction

In the search for a surface-reacting agent for the selective removal of plasma cells it was soon clear that there are few monoclonal antibodies that bind all plasma cells with sufficiently high affinity, owing to the relative paucity of cell surface antigens on these cells. Table V lists some of the Mabs reported to react with the cell surface of plasma cells. The following Mabs have been used for the purposes of purging plasma cells:- anti-PCA-1 (Coulter Immunology), which recognises plasma cell- associated antigens on myeloma, plasma cell leukaemia, and plasmacytoma tumour cells, in addition to being weakly expressed on granulocytes and monocytes (Anderson et al, J Immunol, 1983).This Mab was evaluated against myeloma cell lines using an immunomagnetic bead system (Shimazaki et al, 1988) but such a high immunomagnetic bead:target cell ratio was required for effective cell separation that this Mab would not be practical for clinical use. Another plasma cell surface-reactive Mab is MM4 described by Tong et al, 1987, using rabbit complement to effect cell lysis. This Mab was not generally available at the time of this study and has not been evaluated in this study. A recently described Mab reactive against B lymphocytes and plasma cells and currently in use as a purging agent for myeloma bone marrow is 8A

(Tazzari et al, 1987, Dinota et al, 1989), but again this has not been evaluated in the current study.

Most of the surface antigens that distinguish haemopoietic cell types from each other are predominantly carbohydrate, and for this reason it was thought worthwhile examining lectins, which have specificity for carbohydrates, for their selective reactivity with plasma cells.

Table V

MONOCLONAL ANTIBODIES REPORTED TO HAVE ANTI-PLASMA CELL ACTIVITY

Antibody	Other reactivity	Reference
PCA-1	Monocyte, Myeloid and T cell	Anderson et al (1983)
PCA-2	Monocyte, Myeloid and T cell	Anderson et al (1983)
PC-1	?none	Anderson et al (1984)
RI-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-Galfre 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)
CD38	?Haemopoietic progenitors	Tedder et al (1982)

4.3 MATERIALS AND METHODS

4.3.1 Patients

Bone marrow (bm) or tonsil (n=2) samples were was obtained with informed consent and local ethical committee approval from haematologically normal patients undergoing either coronary artery bypass surgery or tonsillectomy.

The myeloma studies involved diagnostic bm aspirates from a total of 34 patients with typical multiple myeloma, one patient with plasma cell leukaemia, and 11 with MGUS. Peripheral blood or bm was obtained from normal volunteers or patients with a range of B cell and monocytic haematological malignancies of differing maturities as specified in the text.

4.3.2 Cell preparations

Peripheral blood and bone marrow.

Material was aspirated into heparin (30 u/ml blood/aspirate), diluted 1:1 with phosphate-buffered saline (PBS), and then centrifuged (400G for 25 minutes at 22°C over Ficoll. Interface cells were washed twice in PBS before staining.

Tonsil

Lymphocytes were teased from the solid tissue in PBS, washed twice in PBS and then isolated over Ficoll as above. Monocytes and T lymphocytes were removed by plastic adherence and double AET-treated sheep erythrocyte (E) rosetting (B cells >95% by CD19 staining; monocytes <1% by morphology and CD14 reactivity) (Kaplan and Clark, 1974). Methods are described in Chapter 4.

T cells

Peripheral blood and bm T cells were purified by E rosetting followed by hypotonic lysis to remove the rosetting erythrocytes.

Desialylation

Cytospin preparations of normal bm were incubated (15 minutes at 37°C) with sialidase (*Clostridium welchii* Type V; Sigma) at a final concentration of 0.8 u/ml and then washed (PBS) three times before lectin staining.

4.3.3 FITC-labelled lectins.

The five lectins employed are listed, together with their carbohydrate specificities, in Table VI. All were directly fluoresceinated and obtained from Sigma. Cells suspended in PBS/azide (0.1%) were stained at 4°C in microtitre plates ($5x10^5$ cells per well in 200µl) for 30 minutes. The optimal final concentration (Table 3) of each lectin was determined in preliminary titration experiments.

4.3.4 Immunoalkaline phosphatase detection of PNA binding.

Cytocentrifuge preparations (10^5 cells/slide) were fixed in acetone (5 min at 22°C), air dried, and washed before staining by an indirect immunoalkaline phosphatase method: the slides were exposed first to PNA (10µg/ml for 60 min at 22°C), then to rabbit anti-PNA immunoglobulin (30 min at 22°C; 1/20 final dilution) (all three reagents from Serotec, Kidlington, Oxford). Cells were washed three times between staining steps in in Tris (0.1 M)- buffered saline (pH 7.6) and the alkaline phosphatase was detected with naphthol AS-MX phosphatase substrate (0.2mg/ml) and fast red TR (1mg/ml) as capture agent; levamisole (0.05 M) was added to inhibit endogenous alkaline phosphatase. Cells were counterstained with haematoxylin and eosin.

Negative controls were performed by omitting the PNA, by prior incubaton of the lectin with 0.1 M galactose, or by substitution of the rabbit anti-PNA with a rabbit anti-*lens culinaris* antibody as an irrelevant antibody.

4.3.5 Double staining with PNA and monoclonal antibodies.

FITC-PNA (final concentration 10µg/ml) was added (30 min at 4°C) to cells ($1x10^6$/ml) suspended in PBS/azide. After washing (PBS/azide), the PNA-labelled cells were reacted in an indirect phycoerythrin technique with one of the following Mabs (previously titrated for optimal concentrations): Leu12 (CD19), Leu4(CD3), LeuM3 (CD14, anti-monocyte), anti-cALLA (CD10) and anti-CD34 (HPCA-1, anti- human progenitor cell) (all from Becton Dickinson) and HB-7 (CD38; kind gift of Dr T.F. Tedder, Dana-Farber Cancer Institute). Cells were preincubated in goat serum (1:5 v/v dilution; 30 min at 4°C) to prevent non-specific binding of Mab to the PNA-coated cells, and non-immune mouse IgG1 and 2a were included as class-specific negative first-layer controls.

After washing, Mab was detected with phycoerythrin-conjugated $F(ab')_2$ rabbit anti-mouse Ig (10µg/ml; 30 min at 4°C). After further washing, the doubly labelled cells were examined in a FACS analyser setting the Mab and PNA thresholds such that <1% of cells were respectively reactive with the class-specific first- layer control or with PNA previously incubated at 10µg/ml with an equal volume of 0.2M galactose.

4.4 RESULTS

4.4.1 Screening of bone marrow cells with a panel of fluoresceinated lectins

Only *Arachis hypogaea* (peanut agglutinin, PNA) showed any specificity for myeloma cells (Table VI). This lectin was therefore selected for further immunocytochemical study.

Table VI. Reactivity of F/P-separated bone marrow cells with fluoresceinated lectins

%positive cells (+/- 1SD)

Lectin	Carbohydrate specificity	Optimal concn (µg/ml)	Normal (n=5;PC's 1.5+/-0.7%)	MGUS (n=3;PC's11+/-4%)	MM (n=4;PC's 52+/-18%)
Ulex europaeus	αL-fucose	20	41+/-15	43+/-12	15+/-8
Tetragonolobus purpurea	αL-fucose	20	6+/-3	7+/-2	12+/-5
Bandeira simplicifolia I	αN-Acetylgalactosamine/α-galactose	12.5	2+/-2	2+/-1	ND
Bandeira simplicifolia II	αN-acetylglucosamine	20	52+/-4	48+/-18	26+/-8
Arachis hypogaea	Galactose-β1,3Nacetylgalactosamine	10	5+/-2	16+/-3	56+/-20

4.4.2 PNA-immunoalkaline phosphatase staining of fixed myeloma cells

All plasma cells in 33/34 bone marrow samples from myeloma patients were PNA positive (Figure 7, Table VII). The plasma cells in normal and MGUS bone marrows also showed strongly positive PNA binding. The one negative myeloma bone marrow expressed IgG in the plasma cell cytoplasm,and apart from being PNA negative, was indistinguishable from other myeloma samples. In addition, the plasma cells were unreactive with PNA in the single case of plasma cell leukaemia examined.

The only other cells to react consistently with PNA were monocytes and macrophages

(Table VII), although the reactivity was less strong than with plasma cells. A small proportion of granulocytes reacted in some samples. Control slides were consistently negative.

Table VII.

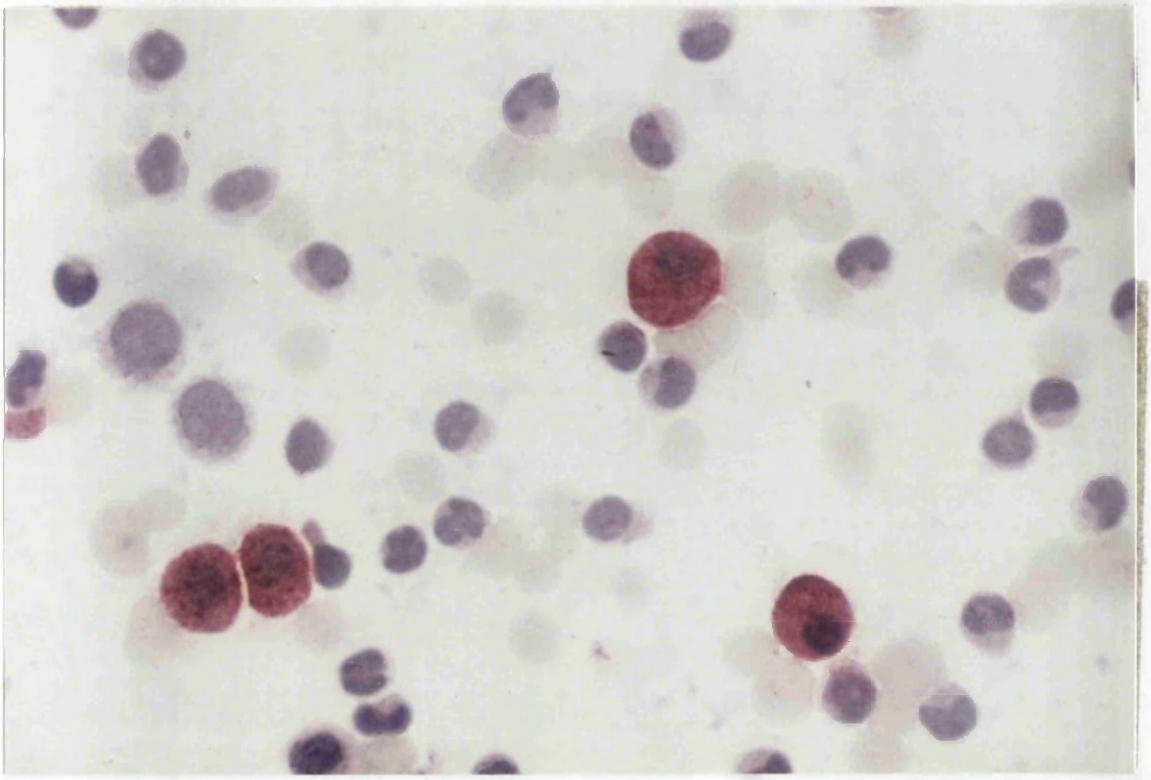
PNA immunoalkaline phosphatase staining of myeloma BM cells

%PNA-positive cells (+/- 1SD)

	Plasma cells		Monocytes		Neutrophils
Source	Pos.	Neg.	Pos.	Neg.	
MM* (n=32)	100	0	67+/-18	32+/-10	0
Normal (n=10)	100	0	67+/-10	30+/-19	2+/-1 in 3 samples
MGUS (n=11)	100	0	55+/-32	45+/-32	3+/-2 in 2 samples

* The single unreactive case of MM and the plasma-cell leukaemia excluded.

Figure 7



PNA positive plasma cells (stained by alkaline phosphatase technique) in a Ficoll-separated myeloma bone marrow.

4.4.3 PNA-immunoalkaline phosphatase staining of normal and malignant lymphocytes and monocytes, and sialidase-treated mononuclear cells

Purified tonsil B lymphocytes were PNA negative (n=2). In addition, malignant cells from bone marrows of 8/10 lymphoma patients, acute lymphoblastic leukaemia (n=1), and peripheral blood samples from hairy-cell leukaemia (n=2) and chronic lymphocytic leukaemia (n=5) were all negative. Bone marrow cells in two cases of follicular lymphoma reacted weakly with PNA. Purified normal T cells from blood and marrow were consistently unreactive.

Normal peripheral blood monocytes were weakly PNA positive (71+/-13%, n=5). In acute monocytic (M5) (94% cells positive; n=1) and chronic myelomonocytic leukaemias (62% positive; n=2), monocytes and their precursors were reactive with PNA but positivity was greater in earlier forms.

Sialidase treatment of normal bone marrow cells (n=5), prior to staining with PNA, resulted in all nucleated cells becominng PNA positive. This was expected since PNA binds to the Thomsen-Friedenreich (or T) antigen (galactose β1,3 N-acetylgalactosamine) which on most haemopoietic cells is masked by sialic acid.

4.4.4 FACS analysis of bone marrow cells doubly stained with PNA and a panel of Mab's

PNA-positive cells were unreactive with CD3, 10, 19 and 34, confirming that T cells and non-plasmacytoid B cells lack PNA receptors (Figures 8 and 9). In contrast, PNA-positive cells were reactive with anti-plasma cell (CD38) and anti-monocyte (CD14) Mab. However, as expected, some PNA- and CD38-positive cells were singly stained since not all plasma cells are CD38 positive and since not all CD38-positive cells are plasma cells. The FACS data confirmed that not all monocytoid cells react with PNA.

Figure 8

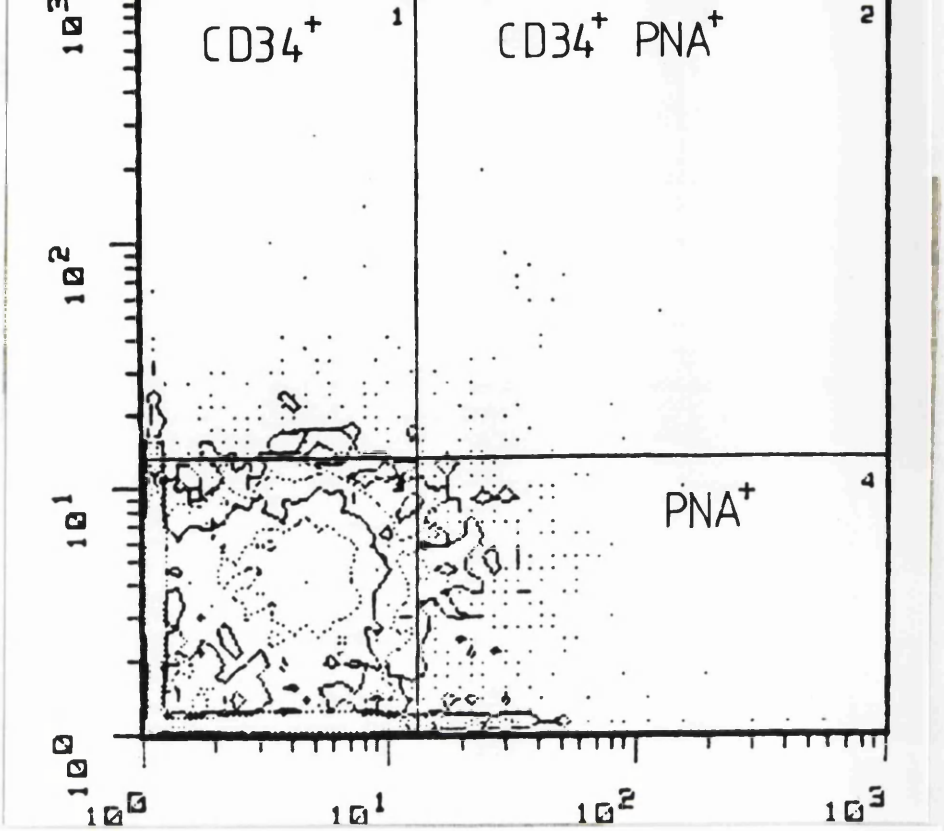
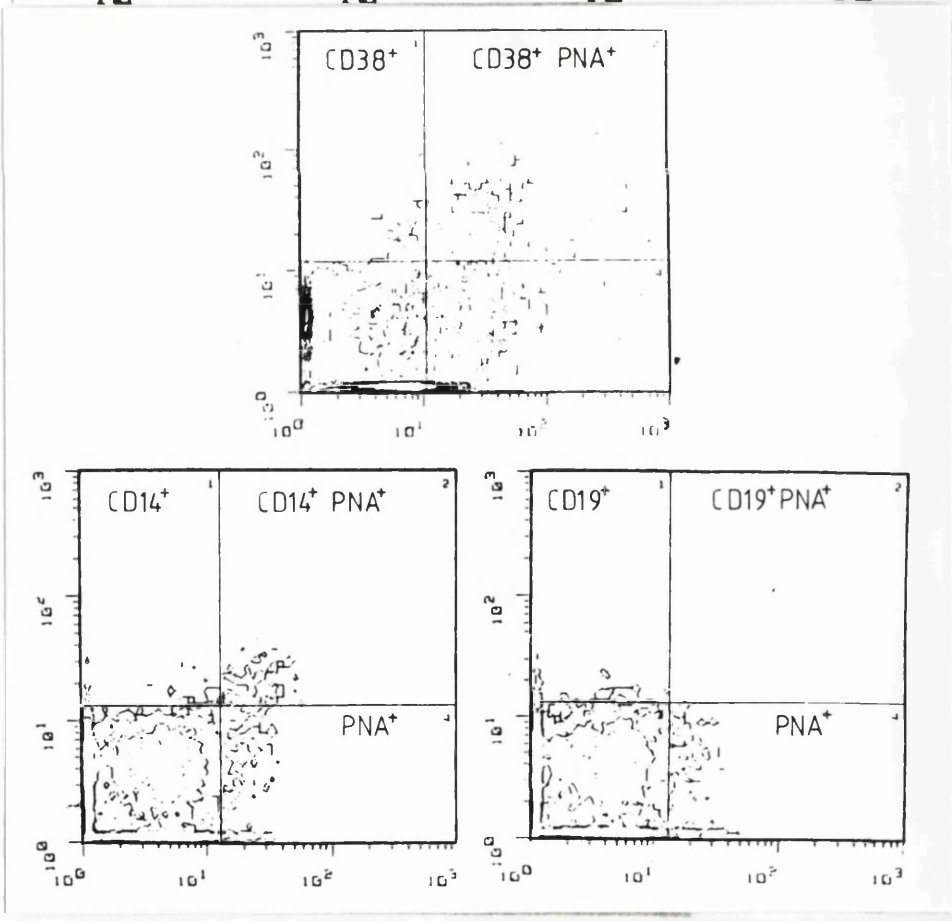


Figure 9



Two-colour FACS of FITC-PNA (horizontal axis) versus staining with CD34 (Fig 8), CD38 (Fig 9a), CD14 (Fig 9b), and CD19 (Fig 9c) on the vertical axis. The Mab staining was detected by an indirect method employing phycoerythrin-linked F9ab')$_2$ rabbit anti-mouse Ig. A significant population of CD38$^+$ PNA$^+$ is demonstrated while CD19$^+$ cells are shown to be PNA negative.

4.5 DISCUSSION

This study shows that all plasma cells from normal and myeloma bone marrows are consistently reactive with PNA. The only other bone marrow cells found to react with PNA were from the monocyte/macrophage series. However, staining was in general less strong than for plasma cells. Since immature monocytes were more strongly reactive with PNA than were mature forms, it is possible that heterogeneity of staining may reflect monocyte maturity as previously suggested by Rosenberg et al (1985). B and T lymphocytes were unreactive with PNA.

PNA has been shown in recent years to bind a wide variety of epithelial tumours, including those of gastrointestinal (Cooper and Reuter, 1983), breast (Howard et al, 1981) and urinary bladder (Lehman et al, 1984) origin.

More relevant to the studies reported here have been numerous reports of PNA binding to normal and malignant lymphoid cells. For example, immature B (from sites other than bone marrow) and T cells, both normal and malignant, have been shown to react with PNA (Levin et al, 1980; Reisner et al, 1979; Veerman et al, 1985). However, few authors have commented on the PNA reactivity of plasma cells; Rose et al (1981) note in passing that a plasmacytoma was PNA positive but do not investigate the general reactivity of plasma cells. The present findings concerning the PNA positivity of plasma cells therefore do not conflict with previous studies.

The present work also confirms the observation of Haimovitz et al (1982) that peripheral blood monocytes react with PNA. Haimovitz also reported that, upon maturation in vitro, PNA positivity disappeared from most of the cells. Our work supports this in that monoblasts from a patient with acute myeloblastic leukaemia (FAB classification M5A) reacted strongly with PNA, whereas mature monocytes in

normal marrow and CMML reacted weakly or not at all.

The demonstration that PNA specifically stains a very high proportion of myelomatous plasma cells raised the possibility that the lectin may be a useful purging agent for multiple myeloma. In addition, PNA does not appear to bind to human haemopoietic progenitor cells, as shown by the lack of reactivity with $CD34^+$ cells.

The fact that bone marrow B cells are unreactive with PNA and yet may contain at least a proportion of myeloma progenitor cells meant that PNA purging would need to be combined with some additional method for removing bone marrow B cells.

Summary

This chapter has described experiments with a panel of lectins aimed at finding an agent that reacts with the surface of plasma cells but not with normal haemopoietic cells. PNA largely fulfils these requirements and appears to bind avidly to all plasma cells in the majority of myeloma bone marrow samples. It is not entirely selective as it binds to monocytes and granulocytes but can be considered as a potential purging agent from these preliminary studies as it appears not to bind to $CD34^+$ cells. Further experiments were therefore planned to find means of using PNA to remove plasma cells selectively from myeloma cells.

CHAPTER 5

SMALL-SCALE PURGING OF MYELOMA MARROW USING PNA AND CD19 MONOCLONAL ANTIBODY

5.1 AIMS

1. To assess the use of the lectin peanut agglutin (PNA) in combination with the anti-B cell monoclonal antibody CD19 for purging of myeloma bone marrow.

2. To check the yield and viability of normal progenitor cells in purged marrow using in vitro bone marrow culture.

5.2 Introduction

Initial experiments to use PNA to remove plasma cells selectively from myeloma bone marrow explored:- a) simple agglutination as in the soybean agglutinin techniques described by Reisner et al (1982) to remove T lymphocytes from allogeneic bone marrow donor cells; b) erythrocyte rosetting techniques using either PNA-agglutinable rabbit erythrocytes (Reisner et al, 1977) or autologous desialylated erythrocytes which are also PNA-agglutinable. c) Finally bone marrow cells pre-incubated with PNA were run down a column of Sepharose labelled with anti-PNA antibody and eluted cells were assessed for depletion of target cells.

The method utilising desialylated autologous erythrocytes proved to be a

highly effective means of separating PNA-positive cells from other bone marrow cells and some preliminary purging experiments were performed on a larger scale using peripheral blood buffy coat cells mixed with a PNA-positive tumour cell line.

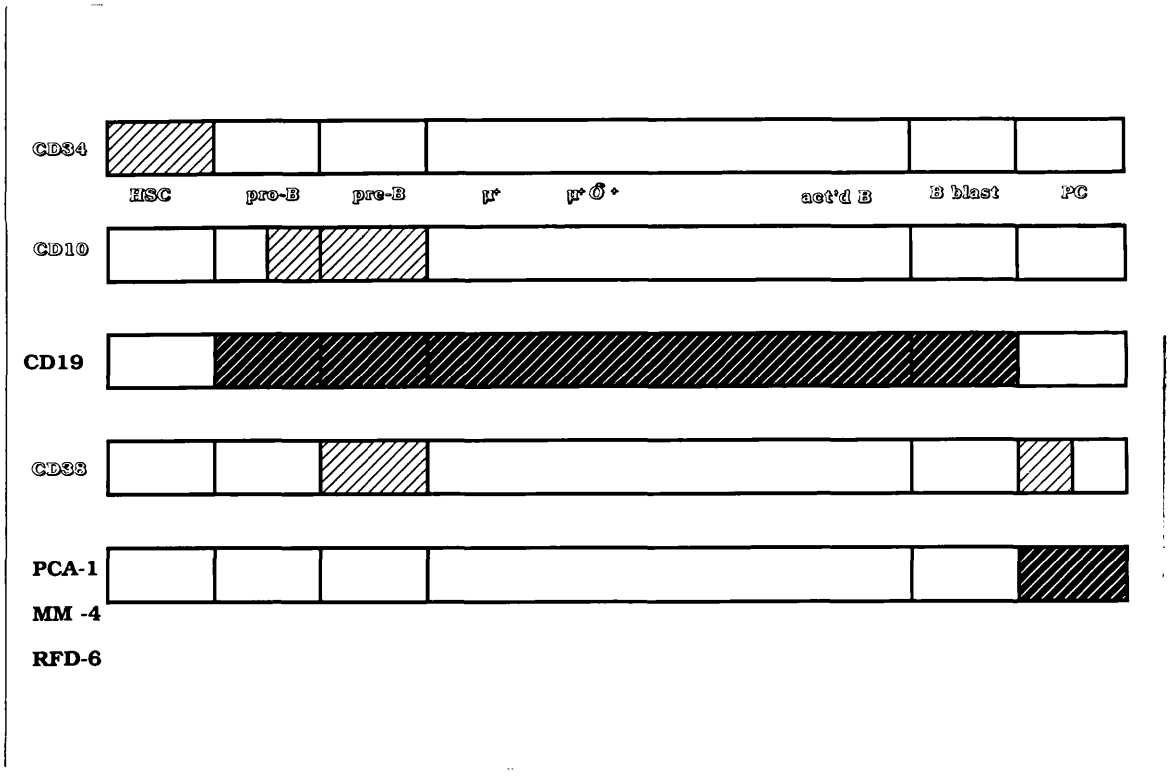
However, the method entails prior incubation of bone marrow cells with relatively high concentrations of lectin, and although no toxicity of PNA for haemopoietic colony assays could be demonstrated, it was deemed desirable to look for alternative techniques for purging which had been proven to be safe for clinical use; hence the series of experiments using PNA linked to magnetized microspheres (Kemshead et al, 1986).

In addition to removing plasma cells from myeloma bone marrow by means of PNA-coated magnetic beads, evidence implicating earlier cells in B cell development as being part of the myeloma clone, including our own myeloma colony work, suggested that effective purging of myelomatous marrow should include anti-B cell elements as well. The CD19 antigen is expressed on B lymphocytes from the early pre-B stage right up to, but not including, plasma cells (Figure 10); the IgM anti-CD19 monoclonal antibody has been chemically bound to Dynabeads (Dynal, UK) and successfully used in several purging systems such as lymphoma and acute lymphoblastic leukaemia (Kvalheim et al, 1988). This preparation was chosen to combine with PNA-coated magnetic beads in an attempt to remove all plasma cells and $CD19^+$ B lymphocytes from myeloma bone marrow. Model systems designed to ascertain optimal magnetic bead:target cell ratios were comprised of normal peripheral blood or normal bone marrow MNC mixed with a small proportion of tumour cell lines known to express receptors in high number for CD19 or PNA.

Toxicity experiments were performed on normal or myeloma bone marrow MNC by incubating them with increasing concentrations of PNA and assessing their ability to generate normal haemopoietic colonies of various lineages.

Finally, the effect on normal haemopoietic precursors of purging normal and myeloma bone marrow with PNA and anti-CD19 (each one separately then in combination) was assessed by cell numbers, phenotypic profile, and normal haemopoietic colony numbers at various stages of purging.

Figure 10



Reactivities of monoclonal antibodies with B cells at varying stages of normal development.

5.3 MATERIALS AND METHODS

Cell preparations

5.3.1 Bone marrow and peripheral blood cells

Diagnostic bone marrow aspirates (approx 10ml) were obtained from patients with newly-diagnosed myeloma in the usual way with heparin as anticoagulant (n=8 for the erythrocyte rosetting experiments) (bone marrow plasma cells 20-67%). MNC were harvested from the interface after separation over Ficoll, washed and counted.

Peripheral blood cells were obtained by venepuncture from normal adult volunteers and MNC were isolated as above.

5.3.2 Tumour cell lines

An EBV transformed B-cell line (Kirk) which was originally grown from a myeloma bone marrow in this laboratory was found to bind PNA strongly. The cells were cultured in IMDM containing 10%FCS at a cell density of $1x10^5$ cells/ml. The cell density was adjusted twice per week replacing half the supernatant fluid.

Tumour cells that strongly expressed the receptor for CD19 were obtained from the bone marrow of a patient with hairy cell leukaemia (HCL). Monocytes and T lymphocytes were removed from the HCL bone marrow by plastic adherence and double AET-rosetting respectively. The final preparation contained > 98% CD19 cells as demonstrated by APAAP staining.

5.4 Purging techniques using PNA and CD19

5.4.1 Separation of PNA^+ cells by agglutination

Myeloma bone marrow cells from 2 patients were suspended at $3x10^6$ cells/ml in PBS/1%BSA and incubated with 0.25mg PNA for 20 min at 22°C. The cells were washed 3 times in the same medium and then gently layered on top of a solution of bovine serum albumin (BSA) (5% w/v in PBS). After 20 min at room temperature, most of the agglutinated cells were sedimented, whilst the unagglutinated cells remained at the interface with the BSA solution. Top and bottom fractions were harvested separately; the agglutinated cells were incubated for 10 min in 10ml D-galactose solution (0.2M in PBS), then collected after centrifugation and washed in PBS. Samples of both fractions were assessed for CFU-GM content and for morphology (cytospin preparations stained with Romanowski and PNA-immunoalkaline phosphatase stains).

5.4.2 Separation of PNA^+ cells by rosetting with rabbit erythrocytes

Venous samples from the ears of 2 rabbits (Animal House, University of Liverpool Medical School) were taken into Li/heparin plastic tubes. The cells were washed 3 times in PBS and centrifuged for 5 min at 200G. Bone marrow MNC from 3 patients with myeloma (bone marrow plasma cells 20-67%) were incubated at $3x10^6$cells/ml in PBS/1%BSA with 0.25mg PNA for 20 min at 22°C. The PNA-labelled cells were washed 3 times in PBS and resuspended in 0.5ml PBS/1%BSA. Rabbit erythrocytes ($2x10^7$ cells in 0.3ml PBS) were added, and the mixture was incubated, with occasional gentle agitation, for 20 min at 22°C. After

agglutinates were observed the cell mixture was layered either over a solution of 5%BSA in PBS or over Ficoll. The latter was centrifuged for 40 min at 200G whereas the BSA gradient was allowed to stand for 20 min. Agglutinated and unagglutinated cells were collected from the bottom and interface respectively in both cases: the agglutinated cells were incubated with D-galactose as above to release the MNC, and all fractions were assessed for CFU-GM and morphology as before.

5.4.3 Removal of PNA^+ cells from myeloma bone marrow using desialylated autologous red blood cells.

$2x10^6$ bone marrow MNC (n=3) suspended in 0.5ml PBS/1%BSA were incubated with 0.15mg PNA (optimal concentrations having been ascertained by previous titration), for 20 min at 22°C. The cells were washed 3 times in PBS/1%BSA. 2 drops of concentrated neuraminidase-treated autologous red blood cells were added, and after centrifuging gently (5min at 200g) the cells were carefully resuspended and layered over Ficoll. The non-rosetted cells were collected from the interface, washed and assessed both for CFU-GM generation and morphology (cytocentrifuge preparations stained by Romanowski and PNA positivity).

The rosetted cells were subjected to hypotonic lysis to remove the red blood cells then examined in the same way for CFU-GM and morphology.

5.4.4 Removal of PNA^+ cells by means of PNA-coated magnetic beads

Dynabeads (M-450 uncoated; Dynal UK Ltd, Rockferry, Wirral, UK) were coated with PNA (Serotec) by a method modified from that of Morecki et al (1987).

285mg of uncoated Dynabeads were washed twice in PBS and resuspended in 10ml of PBS containing 20mg PNA at 4°C with end-over-end rotation for 24 hours. The supernatant was then removed and the PNA-coated beads were washed 3 times in PBS, the last wash incorporating 0.1% human albumin and lasting 24 hours with end-over-end rotation at 4°C. After removal of the supernatant, the PNA-coated beads were finally suspended at 28.5mg/ml in PBS/0.1% human albumin.

In order to test the efficiency of coating with the lectin, 10µl of washed PNA-coated beads was added to 40µl of a 2% suspension of neuraminidase treated human red blood cells. This was incubated for 30 min at 22°C with gentle agitation and then examined microscopically in a Neubauer counting chamber, to ensure that all the PNA-coated beads had bound to cells.

For individual purging experiments the PNA-coated beads were incubated with normal peripheral blood, normal bone marrow and myeloma bone marrow MNC at magnetic bead:target cell ratios of 10:1 to 100:1 at 4°C and 22°C. Bead coated cells were separated for 2 min on a magnetic particle concentrator (MPC) (Dynal).

5.4.5 Removal of $CD19^+$ cells by means of anti-CD19-coated immunomagnetic beads

Dynabeads commercially coated with an IgM monoclonal antibody against the CD19 antigen (M-450 Pan-B; Dynal UK) were used. CD19-coated beads were incubated with cells at bead:target cell ratios of 10:1 to 100:1 at 4°C and 22°C. Bead coated cells were separated for 2 min on a magnetic particle concentrator.

5.4.6 Combined PNA and CD19-coated magnetic bead purging

Experiments were performed to test the efficacy of sequential purging of normal bone marrow/H342-labelled tumour cells with PNA-coated beads followed by CD19-coated beads (or vice versa) both at 4°C (optimal for CD19 binding) and at 22°C (optimal for PNA binding). Further experiments were carried out in which both sets of coated beads were added simultaneously, again both at 4°C and 22°C.

5.4.7 Monoclonal antibody staining

Mab were detected by the APAAP technique in cytocentrifuge preparations ($1x10^5$ cells per slide), and the following Mab were employed:- anti- CD19 (Leu 12), anti-kappa, anti-lambda, (Becton Dickinson) and anti-plasma cell antibody (RFD6) (Department of Immunology, Royal Free Hospital, London). In order to detect intracytoplasmic immunoglobulin, cytospin preparations containing $1x10^5$ cells were fixed in acetone for 10 min and washed in PBS. The cells were incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-human kappa light chain and tetramethyl rhodamine isothiocyanate (TRITC)-labelled goat anti-human lambda light chain (Southern Biotechnology Associates, Seralab). 1µl of FITC-anti-kappa and 5µl of TRITC-anti-lambda were diluted to 50µl in PBS and the cells incubated with 5µl of this mixture for 30 min at 22°C. After washing in PBS the slides were mounted under coverslips using Apathys mounting medium and examined under a Leitz microscope fitted with UV light.

5.4.8 Detection of PNA^+ cells

Staining of cell preparations was performed by the immunoalkaline phosphatase technique outlined in the previous chapter.

5.4.9 Sialidase treatment of peripheral blood red cells

Autologous red blood cells were obtained from peripheral venous blood samples from each of the 8 myeloma patients in this series of experiments. After centrifuging the heparinised blood 2ml packed red cells were incubated with neuraminidase (*Clostridium welchii* Type V; Sigma), final concentration 1.0u/ml, at 37°C for 15 min. The cells were washed 3 times in PBS and assessed for agglutinating ability with the PNA^+ cell line Kirk.

5.4.10 Labelling of tumour cell lines with H 33342

Tumour cells were labelled by the method of Reynolds et al (1986) using the fluorescent supravital DNA dye Hoechst 33342 (H342). The H342 (Sigma) was dissolved in distilled water at a concentration of 1mg/ml. This was added at a concentration of 2µg/ml to $1x10^6$ tumour cells/ml in RPMI 1640 containing 15% v/v FCS and incubated for 1 hour at 37°C. After washing twice in RPMI/FCS the cells were incubated for a further 2 hours in RPMI/FCS at 37°C. The labelled tumour cells (either the PNA^+ Kirk cell line or the $CD19^+$ HCL cells or both) were then seeded into

normal peripheral blood or bone marrow MNC at ratios of 1:10 or 1:20. After purging the cell mixtures with PNA-coated and/or anti-CD19-coated magnetic beads, cytocentrifuge preparations of $1x10^5$ purged cells were made and examined under a Leitz fluorescent microscope using a UV excitation filter of 350nm. For each target/normal cell mixture triplicate slides were examined for residual fluorescence following purging at various bead:target cell ratios from 10:1 to 100:1.

5.5 Bone marrow culture following purging

5.5.1 Bone marrow samples

Normal bone marrow was obtained from haematologically normal patients undergoing coronary artery bypass surgery. Informed consent by the patient and approval by the Ethics Committees of Queen Elizabeth Hospital, Birmingham, and Broad Green Hospital, Liverpool, were obtained. Additional bm samples were obtained at the time of bm harvest from normal donors for allogeneic bone marrow transplant (BMT), and a further source was from informed, consenting patients undergoing inguinal hernia operations. Ethical Committee approval in the latter case was obtained from the Royal Liverpool Hospital.

Approximately 5ml bone marrow was aspirated into tissue culture medium containing 300iu preservative-free heparin. For double-layer agar CFU-GM cultures bone marrow erythrocytes were sedimented by the addition of 0.5ml Dextran 70 prior to culture. In all other techniques the bm mononuclear cell fraction (MNC) was collected from the interface after centrifugation over Ficoll-Paque (Pharmacia Ltd, Milton Keynes, UK) at a density of 1.077, and washed in tissue culture medium.

5.5.2 Foetal calf serum

15 batches of FCS from different commercial sources were tested in parallel for the best growth-promoting properties. A sample of FCS from Biological Industries, Cumbernauld, Glasgow (batch no. 0625133) gave clearly superior results. A large volume of this batch was bought and kept frozen at -40°C for use throughout the entire project.

5.5.3 Horse serum

5 batches of horse serum were tested in parallel in long- term bm culture and batch no. 116075 (Flow Laboratories Ltd, Irvine, Scotland) was chosen for use in all subsequent experiments.

5.5.4 Colony forming unit granulocyte/macrophage (CFU-GM)

In the early stages of this project a modification of Pike and Robinson (1970) was used; this is a double layer technique in which peripheral blood leucocytes act as a source of colony stimulating factor (CSF) and are embedded in a 0.5% agar under-layer. The bm cells to be studied are layered on top in 0.3% agar and incubated as described below. For the majority of experiments throughout this project CFU-GM colonies (>40 cells) were counted after 14 days.

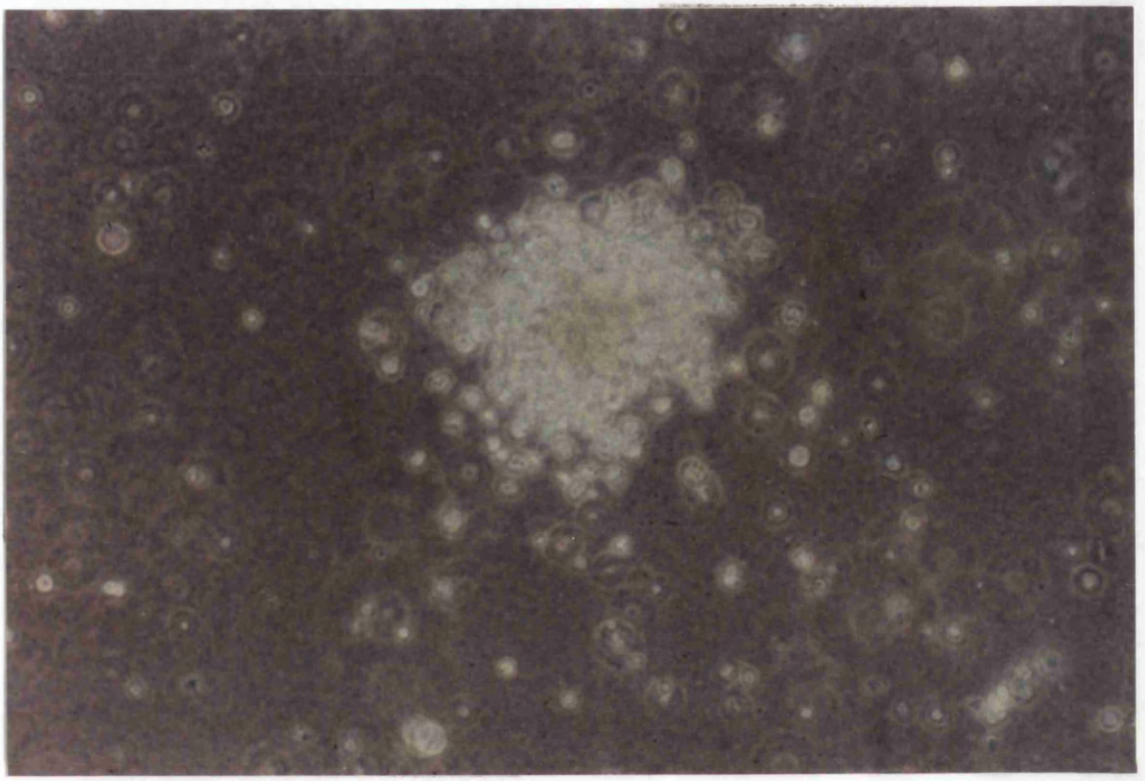
Later on a simple one-layer agar method for growing CFU-GM was adopted in which the source of CSF was in the supernatant of the cultured bladder carcinoma cell line C 5367, kindly given by the laboratory of the late Dr Fogh, Sloan Kettering, New York.

5.5.5 CFU-GM (1)

Peripheral venous blood (10ml) was collected from 3 normal adult volunteers into a plastic universal container with 0.3ml preservative-free heparin (1000u/ml) and 0.5ml Dextran 70. Bm cells were collected into the same heparin/Dextran mixture with the addition of 2ml McCoy's 5A tissue culture medium containing 15% FCS. After the erythrocytes had sedimented, the pbl were adjusted to $2x10^6$cells/ml in McCoy's/15%FCS and warmed in a 37°C water bath. A Master solution was prepared, of 5:1 McCoy's/FCS and boiled 5% Bacto agar (Difco Laboratories, Michigan, USA) and kept at 37°C. An equal volume of Master solution and cells was mixed thoroughly (final cell concentration $1x10^6$/ml) before plating out in 1ml volumes into 35mm Sterilin tissue culture wells.

The overlayer containing bone marrow cells was prepared in the same manner except that the cells were at a final concentration of $1x10^5$/ml and the agar at 0.3%. Cells were plated out in triplicate and incubated in a fully humidified incubator with 5% CO2 at 37°C, and colonies (>40 cells) were counted at 10 - 14 days (Figure 11).

Figure 11



A granulocyte/macrophage colony grown from a normal bone marrow in 0.3% agar.

5.5.6 CFU-GM (2)

Bone marrow MNC were obtained after Ficoll separation and resuspended at the desired concentration in IMDM. Agar (3%) was melted in boiling water and added to the plating mixture which comprised:- FCS 20%, C5637 conditioned medium 10%, and cells adjusted in IMDM for a final concentration of $1x10^5$cells per well in 35mm Sterilin plates. Incubation and scoring was the same as for CFU-GM (1) method.

5.5.7 Colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte CFU-GEMM

A modification of the method of Fauser and Messner (1978) was used. Ficoll-separated bone marrow MNC were plated in methylcellulose with conditioned medium from PHA-stimulated peripheral blood leucocytes (PHA-LCM), serum from a patient with severe aplastic anaemia and purified human erythropoietin as sources of colony stimulating factors. The final plating mixture was as follows:-

0.8% methylcellulose (15 centipoise), (Dow Chemical Company)

FCS 20%

PHA-LCM 5% (see method below)

2-mercaptoethanol $5x10^{5}$M

erythropoietin 2.5iu/ml (Terry Fox Laboratories, Vancouver, Canada)

serum from patient with SAA 10%

bm MNC at final concentration $1x10^{5}$/ml

IMDM

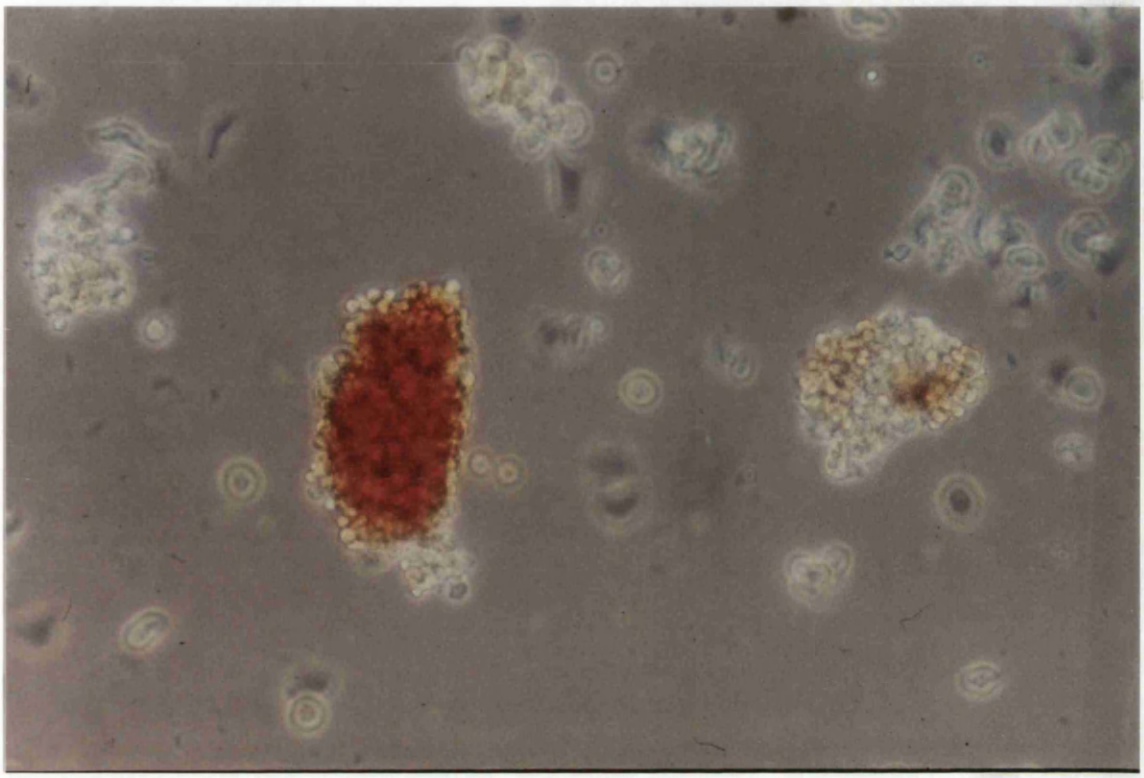
5.5.8 PHA-stimulated leucocyte conditioned medium (PHA-LCM)

Peripheral blood leucocytes from normal donors were isolated after Ficoll separation and adjusted to $1x10^{6}$cells/ml in IMDM containing 20% FCS. Phytohaemagglutinin (PHA) (Gibco) was made up from the lyophilized preparation according to the manufacturer's instructions and a 1/100 dilution made in IMDM/FCS. 50µl of diluted PHA was added to 2ml cells at $1x10^{6}$/ml (final concentration of PHA

was 1% v/v) and the mixture was incubated in a 25ml Falcon flask in a humidified CO2 incubator. After 7 days the cell suspension was centrifuged and the supernatant was filtered and stored in aliquots at -40°C.

Bone marrow MNC were added to the other reagents and mixed thoroughly using a Whirlimix before plating out in 1ml volumes into 35mm Sterilin plates and incubating in the usual way. Mixed colonies containing at least erythroid and granulocytic cells, as judged by their in situ appearance, were scored at 14 days. Erythroid bursts (BFU-E) containing >500 haemoglobinised cells were also scored at 14 days (Figure 12). In some samples the contents of an individual plate were harvested, washed in IMDM and cytocentrifuge preparations were made for special stains. The presence of megakaryocyte precursors was confirmed by Mab staining with anti-GpIB (Dako, Glostrup, Denmark).

Figure 12



One erythroid colony (CFU-E) and one mixed colony (CFU-GEMM) grown in 0.8% methyl cellulose from a normal bone marrow.

5.5.9 Long-term bone marrow cultures (LTBMC)

The method of Dexter et al (1976) was used, in which bone marrow MNC were incubated in 10ml volumes in 25ml Falcon flasks at a final cell concentration of $2x10^6$/ml with the following reagents:- IMDM, FCS 10%, horse serum 10%, and hydrocortisone sodium succinate $5x10^{-7}$M. The flasks were incubated in a humidified CO2 incubator at 33°C. After 2 weeks the flasks were inspected for the formation of a stromal layer. Half the culture fluid containing non-adherent cells was removed and relaced with fresh LTBMC culture medium. The non-adherent cells were assayed for

CFU-GM and CFU-GEMM in the usual way, and the process was repeated at weekly intervals until there was a major fall-off in CFU numbers, usually at about 5-6 weeks.

5.5.10 Toxicity of PNA for normal haemopoietic progenitors

Normal bone marrow (n=7) and myeloma bone marrow (n=3) MNC were cultured for CFU-GM in the presence and absence of increasing concentrations of PNA, between a final concentration of 25 and 100μg/ml. The cells were incubated with PNA for 20 min at 22°C and washed 3 times in IMDM before plating out with 0.3% agar, FCS and CM from C 5637 in the usual way. Colonies were scored at 14 days. In addition, 3 normal bone marrow samples were cultured in LTBMC and the generation of CFU-GM was monitored weekly for 6 weeks. In each case a control experiment was included with no prior incubation of the cells with PNA.

5.6 RESULTS

5.6.1 Separation of PNA^+ cells by agglutination

Cells collected at the interface (non-agglutinated) showed 70% and 85% reduction in plasma cells from the original myeloma bone marrow samples, as assessed by Romanowsky staining. When both the non-agglutinated and agglutinated fractions were assessed for CFU-GM content the agglutinated (PNA^+) fraction yielded 25 and 30% of the total CFU-GM colonies.

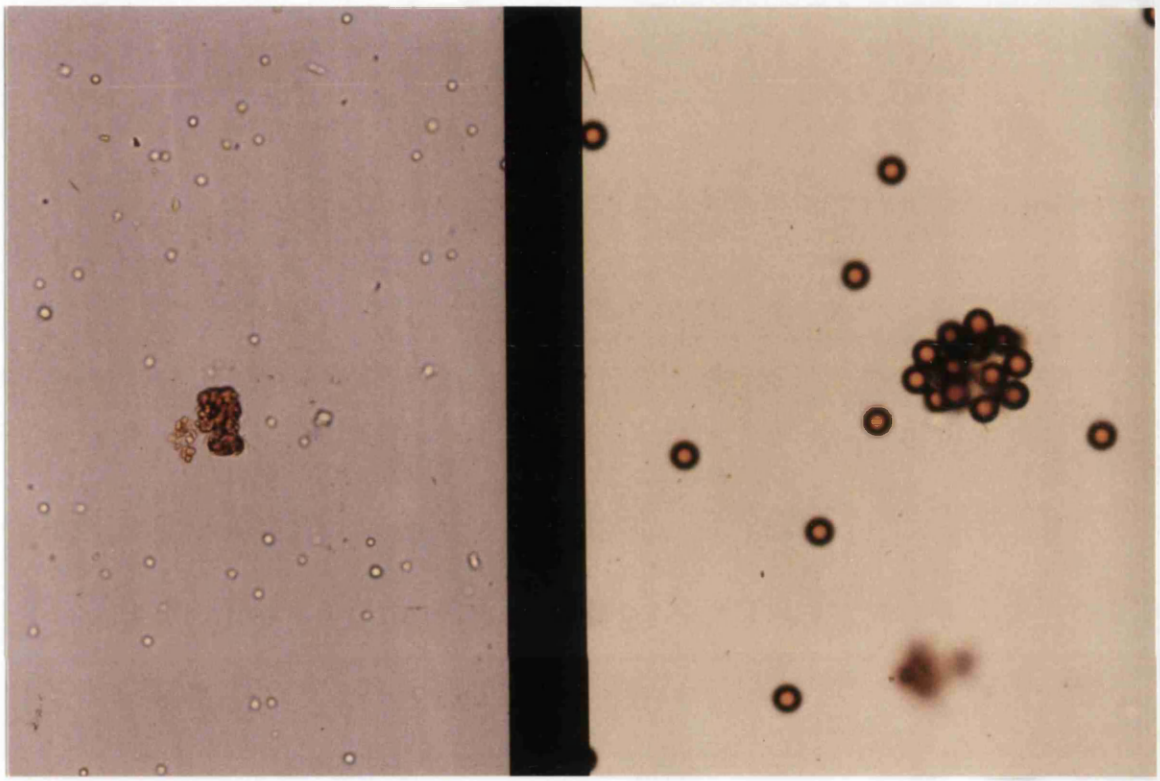
5.6.2 Separation of PNA^+ cells by rosetting with rabbit erythrocytes

In the two myeloma bone marrow samples tested with two different rabbits' red blood cells, reduction of plasma cells was from 45 to 30% in one sample, and from 25 to 20% in the second, as assessed by Romanowski staining. No further investigation of this method was carried out in view of these initial poor depletions.

5.6.3 Removal of PNA^+ cells from myeloma bone marrow using desialylated autologous erythrocytes

Dense red cell agglutinates were collected after density centrifugation (Figure 13). After hypotonic lysis of the red cells and staining cytocentrifuge preparations of cells, it was found that there was a mean 95% (+/-7,SD) removal of plasma cells from the the non-agglutinated fraction, as assessed by Romanowski stain (Figure 14,a & b). After allowing for cell loss there was a mean 52% (+/-11,SD) recovery of CFU-GM in the non-agglutinated fraction, and 5% (+/-2) recovery of total post-purge CFU-GM in the agglutinated fraction.

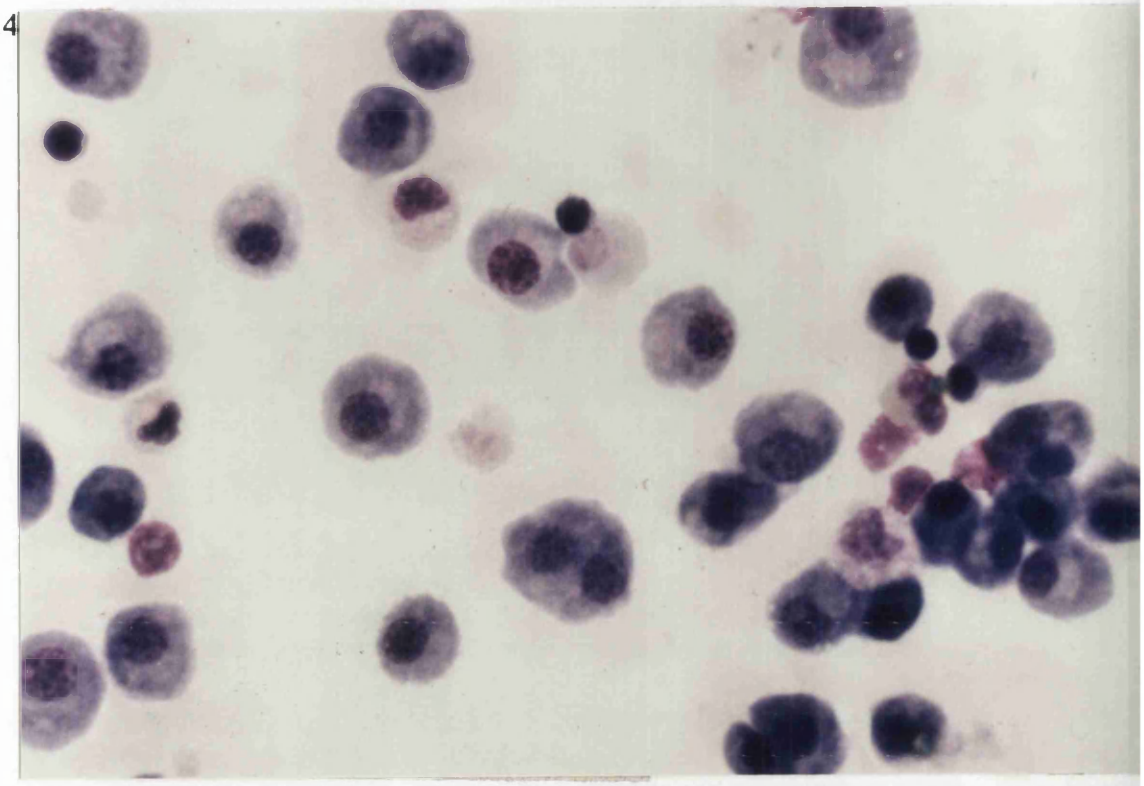
Figure 13



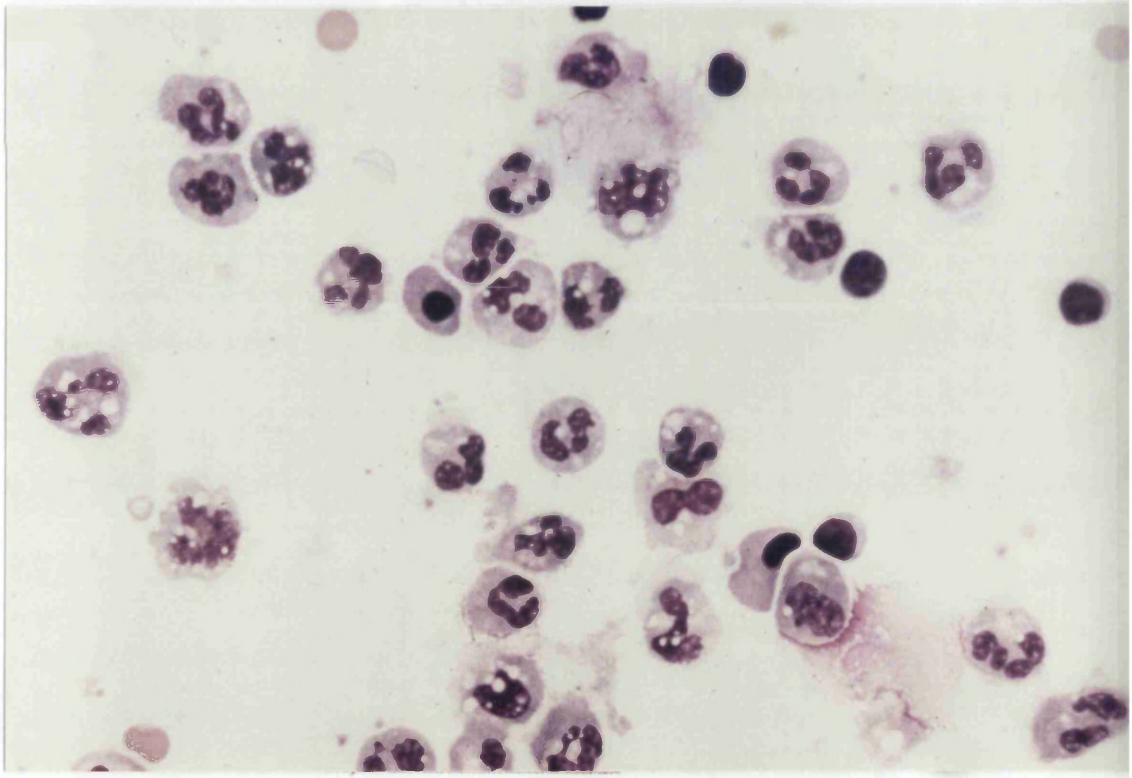
Two methods for removal of PNA^+ cells from bone marrow: on the left PNA^+ cells are agglutinated by neuraminidase-treated autologous red blood cells, on the right PNA-coated magnetised microspheres are attached to a PNA^+ target cell prior to application of the magnet.

Figure 14

a)



b)



a) and b) above show the same myeloma bone marrow stained by Romanowsky stain before (a) and after (b) purging using the PNA/neuraminidase treated red cell agglutination technique. Less than 1% plasma cells remained after purging.

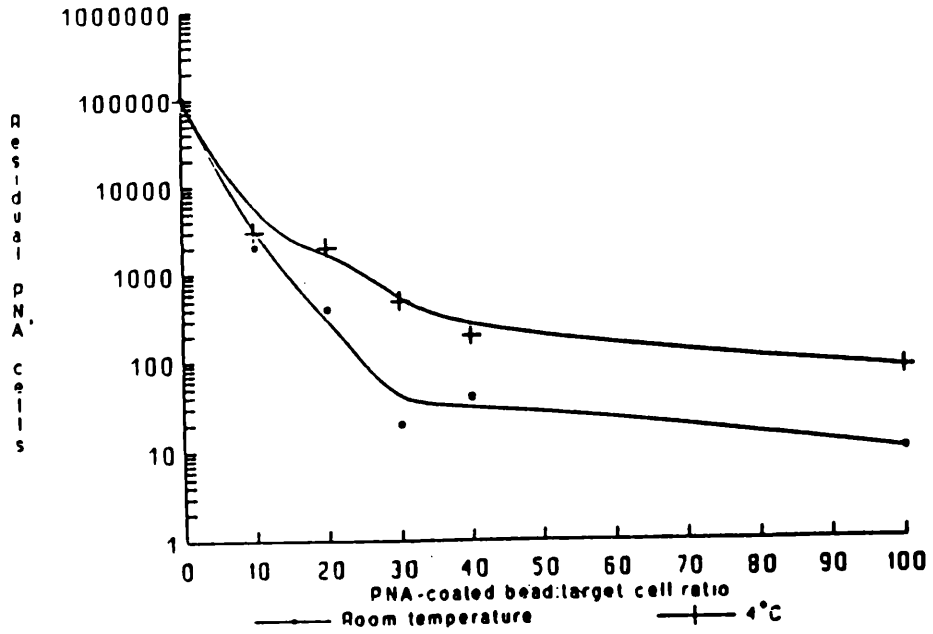
5.6.4 PNA purging with PNA linked to magnetic beads

When 10^6 normal peripheral blood cells mixed with 10^5 PNA^+Kirk cells labelled with H342 and incubated with increasing concentrations of PNA-linked magnetic beads, more efficient removal of PNA^+ cells was observed at 22°C than at 4°C (Figure 15; Table VIII). At 22°C a 3.7-log cell removal was obtained at a bead:target cell ratio of 40:1, and a 4-log removal at a ratio of 100:1. When two purging cycles were tested in 6 subsequent experiments with different cell proportions, no fluorescent cells were detectable at ratios greater than 20:1.

Table IX shows efficacy of purging myeloma bone marrow MNC (n=5) with PNA-coated magnetic beads at 22°C, assessing the removal of plasma cells morphologically in cytospin preparations stained by Romanowski, anti-PNA/alkaline phosphatase (Figure 16, a & b) and anti-CD19 APAAP. Fluorescent cytoplasmic immunoglobulin staining was performed on 2 samples and no positive cells were seen after purging. Complete removal of plasma cells was achieved at a ratio of 40:1 using one purging cycle: as expected, the numbers of $CD19^+$ cells remained unchanged.

Figure 15

Removal of PNA$^+$ target cells with PNA-coated beads at varying bead:target cell ratios



Removal of PNA$^+$ target cells with PNA-coated beads at varying bead:target cell ratios.

Table VIII.

Effect of bead:tumour cell ratio on the removal of PNA$^+$ tumour cells from normal peripheral blood MNC by PNA-MB

Temperature	PNA-MB:target cell ratio				
	10:1	20:1	30:1	40:1	100:1
	Log removal				
4°C	1.8	1.9	2.5	2.9	3.2
22°C	1.9	2.6	3.9	3.7	4.0

Table IX

Efficacy of removal of PNA$^+$ cells from myeloma BM MNC (2 x 10^7 cells, mean of five samples) by PNA-MB

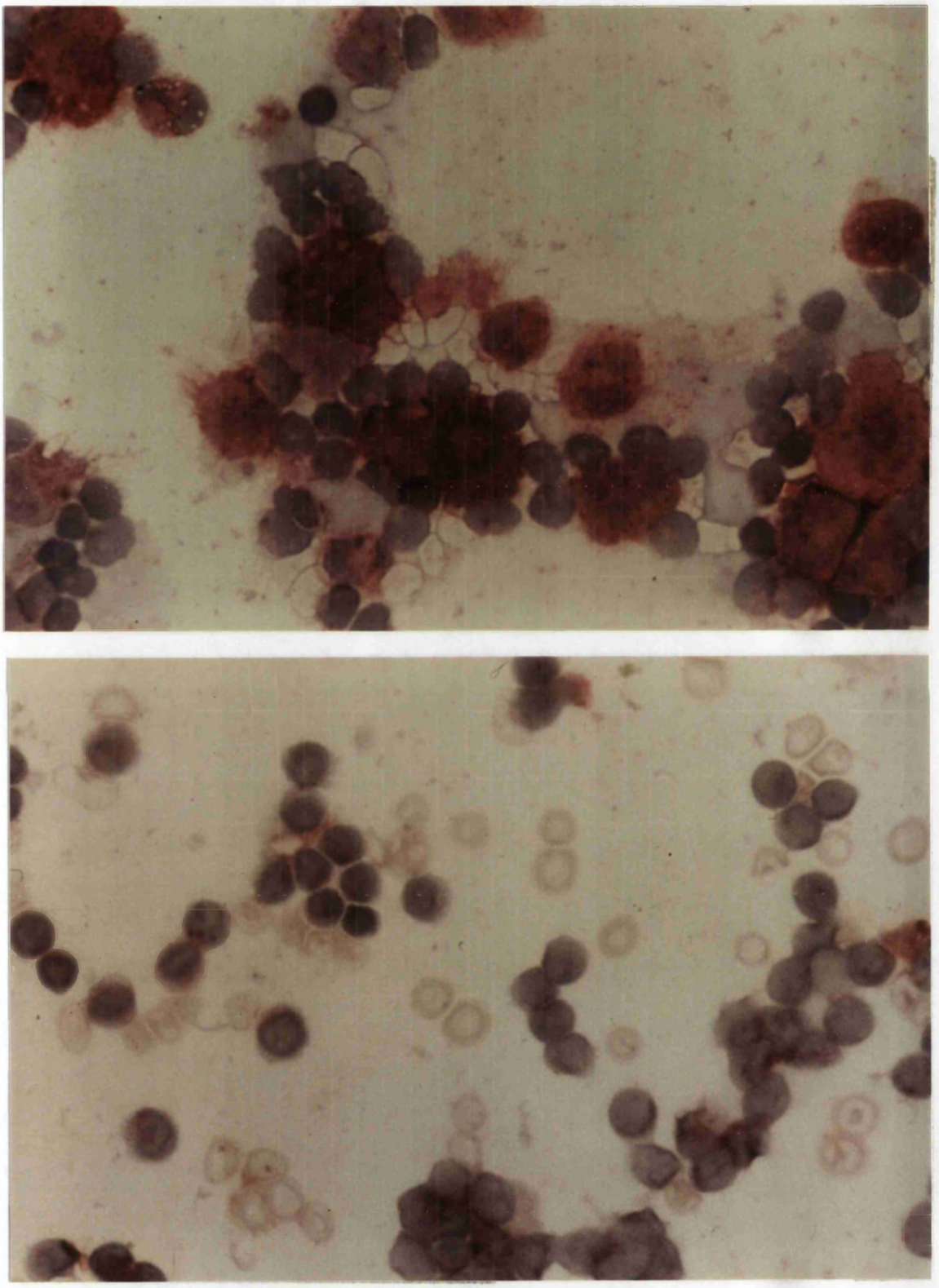
	Percent positive cells (mean +/- SD)			
PNA purging	Morphological plasma cells	PNA$^+$	RFD6$^+$	CD19$^+$
Before	20 +/-31	25 +/-26	20 +/-32	5 +/-3
After	0	0	0	5 +/-4

* Morphology in Romanowsky- or APAAP-stained cytocentrifuge preparations.

Figure 16

a)

b)



Normal bone marrow spiked with a PNA$^+$ cell line (Kirk) stained with PNA-alkaline phosphatase before (a) and after (b) purging with PNA-coated magnetic microspheres.

5.6.5 CD19 purging with anti-CD19-coated immunomagnetic beads

In experiments involving spiking of normal bone marrow MNC with H342-labelled CD19$^+$HCL cells and a range of bead:target cell ratios, a ratio of 40:1 produced a 4.2-log removal of fluorescent tumour cells when incubated at 4°C (Table X). Similar results were obtained when purging with CD19$^+$ tumour cell-spiked peripheral blood MNC preparations after incubation at 4°C.

When $2x10^6$ myeloma bone marrow MNC were treated with anti-CD19-coated beads, CD19$^+$ cells were effectively removed by one cycle of purging at a bead:target cell ratio of 40:1, (mean 4%, range 2-5% CD19$^+$ cells before purging and 0-0.1% after purging as assessed in APAAP-stained cytocentrifuge preparations (Table X)).

Table X

Removal of CD19$^+$ cells from spiked normal and myeloma BM

	Bead:target cell ratio				
Cell population	10:1	20:1	40:1	100:1	Assessed by
Normal peripheral blood MNC plus 10% HCL	2.1	4.2	5	5	APAAP
Normal BM plus 10% HCL		4	5		APAAP
Normal BM plus 10% H342-HCL	2.3		4.2	5	Fluorescence
Myeloma BM			4.5		APAAP
Myeloma BM			4.7		Cytoplasmic Ig

Shown is the log removal of CD19$^+$ cells with increasing bead:target cell ratios.

5.6.6 Combined PNA and CD19 purging on normal and myeloma bone marrow samples

Preliminary experiments involved sequential purging of H342-labelled tumour cells mixed with normal bone marrow first with anti-CD19-coated magnetic beads at

4°C followed by PNA-coated beads at room temperature. Others were treated in the reverse order with PNA purging first. Although effective depletion of target cells was achieved in both cases at bead:target cell ratios >20:1 (assessed by remaining fluorescent H342-labelled cells), non-specific cell loss was high (30-50% after target cell removal was taken into account) in 4 experiments. Further experiments were therefore performed by incubating a combination of CD19-coated beads and PNA-coated beads with bone marrow at 22°C with bead:target ratios of 40:1. This proved to be an effective compromise between successful removal of target cells and non-specific cell loss.

When this system was applied to normal bone marrow MNC preparations (n=3) spiked with 5% H342-labelled $CD19^+$ HCL and 5% H342-labelled PNA^+ Kirk cells, a mean of 4.3-log removal of fluorescent cells (range 3.5 - 4.6) was achieved (Table XI).

Myeloma bone marrow MNC (n=3) were purged in a similar way with one cycle of the combined CD19/PNA magnetic bead treatment and a >3-log removal of target cells (range 3 - 4) was demonstrated by APAAP staining for PNA^+ and $CD19^+$ cells (Table XII).

Fluorescent cytoplasmic immunoglobulin staining was performed on one myeloma sample and a 4-log removal of target cells was demonstrated, again with no identifiable immunoglobulin-containing cells remaining after purging.

Table XI

Combined PNA/CD19 purging: efficacy of removal of H-342-labelled PNA^+ and $CD19^+$ cells simultaneously from normal BM (mean of three samples)

Normal BM	Number of cells	Percent PNA^+	Percent $CD19^+$	Bead:target ratio	Log removal
plus 5% Kirk-H342 and 5% HCL-H342	5×10^7	25[a]	11[a]	40:1	4.3

[a] Number of target cells assessed by APAAP prior to addition of tumour cells.

Table XII

Removal of plasma cells and $CD19^+$ lymphocytes from myeloma BM by combined PNA-MB and CD19-MB

	Number of cells[a]	Percent plasma cells	Percent $CD19^+$ cells	Bead:target ratio	Log removal of target cells
Myeloma BM	10^7	33	7	40:1	>3

[a] Mean of three samples

5.6.7 Effect of purging on normal haemopoietic progenitor cells

Haemopoietic cell recovery after purging experiments is shown in Table XIII. Progenitor cell recovery was at least 67% after purging with PNA-coated magnetic beads alone, and at least 51% with combined PNA- and CD19-coated magnetic bead purging.

Table XIII

Normal haemopoietic progenitors remain after purging

	Cells removed	CFU-GM[a] before	CFU-GM after	Percent recovery[b]	CFU-GEMM[c] before	CFU-GEMM after	Percent recovery
Normal BM[d]	PNA^+	105 +/-28	161 +/- 19	67 +/-13	8 +/- 3	20 +/- 4	72 +/- 12
Normal BM[d]	PNA^+ $CD19^+$	93 +/- 15	148 +/- 21	51 +/- 8	9 +/- 3	14 +/- 6	56 +/- 11
Myeloma BM[e]	PNA^+ $CD19^+$	67 +/- 21	150 +/- 42	57 +/- 3	6 +/- 3	16 +/- 9	80 +/- 21

[a] CFU-GM: colonies per 2 x 10^5 cells plated

[b] Data collected for nucleated cell loss during purging and expressed as percentage of pre-purge colony numbers.

[c] CFU-GEMM: colonies per 10^5 cells plated.

[d] Ten million BM MNC spiked with 10^6 tumour cells (three experiments).

[e] Ten million myeloma BM MNC (four experiments).

5.6.8 Toxicity studies of PNA on normal haemopoietic cells

PNA had no direct inhibitory effect on CFU-GM formation when added to normal (n=7) and myeloma (n=3) bone marrow cultures over a range of final concentrations of 25 - 100µg/ml. Further experiments were performed at 100µg/ml of PNA under a range of culture conditions, and no inhibitory effect on CFU-GM, CFU-GEMM and CFU-GM generated in LTBMC was observed (Table XIV).

Table XIV

Effect of PNA (100µg/ml) on progenitor cell growth from normal and myeloma marrow (%).

		Normal marrow			Myeloma marrow	
Bone marrow	No. expts	Without PNA	With PNA	No. expts	Without PNA	With PNA
CFU-GM	5	100	123	3	100	115
CFU-GEMM	3	100	109	-	-	-
BFU-E	3	100	86	-	-	-
CFU-GM in LTBMC ....for 3 weeks	2	100	89	-	-	-
... for 5 weeks	2	100	68	-	-	-

5.7 DISCUSSION

This study has shown that combined purging of myeloma bone marrow with PNA- and CD19-coated magnetic beads can be used to achieve efficient removal of plasma cells and $CD19^+$ B lymphocytes.

This efficiency of removal of plasma cells is considerably greater than that previously reported for magnetic bead separation using the monoclonal antibody PCA-1 (Shimazaki et al, 1988). In that study Shimazaki and colleagues recommend a magnetic bead to target cell ratio of 500:1 using PCA-1 as the anti-plasma cell agent, whereas in our study comparable efficiency of purging with PNA is achieved at a magnetic bead to target cell ratio of 40:1.

This study has also shown that PNA does not have a direct toxic effect on haemopoietic progenitors (CFU-GM, -GEMM, and LTBMC) when added in concentrations in excess of those needed for purging. It was previously demonstrated (Chapter 4) that the $CD34^+$ phenotypic haemopoietic progenitors that are capable of repopulating irradiated bone marrow (Berenson et al, 1987) are not reactive with PNA. Since cells of the monocyte/macrophage series are known to be reactive with PNA special pains were taken to show that an adequate bone marrow stromal layer can be formed in LTBMC after PNA^+ cells have been removed. Phenotypic analysis of CFU-GM colonies from PNA-depleted bone marrow using a panel of Mab shows that there is no overall difference in the representation of monocytic cells between purged and non-purged colonies.

$CD19^+$ cells were removed using an established immunomagnetic bead method (Kvalheim et al, 1988), and the above experiments show that it is possible to combine the monoclonal antibody and lectin in one-stage purging procedures without undue

loss of efficiency or progenitor cells.

More sensitive techniques for detecting residual malignant cells after purging are under investigation, using DNA technology to find clonal gene rearrangements but until amplified gene products are available, the detection of cells bearing cytoplasmic immunoglobulin as in this study is probably the best indicator of the thoroughness of purging.

Summary

In conclusion, a method has been described for removing cells of B lineage from myeloma bone marrow. Since the precise stage of normal B cell development at which malignant transformation occurs in myeloma is unknown, the purging agents described here are intended to eliminate both earlier B cells and plasma cells. Whether the latter are capable of initiating tumour relapse is also unknown, and further myeloma bone marrow culture studies together with gene rearrangement analysis may be a means of answering these questions.

The present findings relate to small-scale experiments. The following chapter describes the techniques used to apply combined PNA- and CD19- purging to large volumes of bone marrow on a scale relevant to clinical use.

CHAPTER 6

LARGE-SCALE PURGING EXPERIMENTS

6.1 Aims

To repeat the small-scale purging experiments outlined in the previous chapter on large volumes of bone marrow to be used for autologous bone marrow rescue.

6.2 Introduction

In order to adapt small-scale in vitro manipulation of bone marrow to the large volumes and cell numbers required for clinical application in autologous bone marrow transplantation (ABMT) certain criteria had to be met. The first was an adequate supply of normal haemopoietic stem cells to ensure predictable bone marrow reconstitution following marrow ablative therapy. We had already shown that PNA does not bind to $CD34^+$ putative progenitors, that the lectin was not directly toxic to committed haemopoietic progenitors, and that removal of all detectable PNA^+ cells from small samples of bone marrow did not specifically remove cells capable of generating in vitro colonies. It was now necessary to repeat these experiments on a large scale. Although there is by now a sufficiently large body of experience in ABMT for guidelines to be published concerning the minimum number of nucleated bone marrow cells required for reliable reconstitution (Gorin et al, 1981), the data for minimum CFU-GM numbers necessary is not so clear-cut (Gorin et al, 1981; To et al,1984; Douay et al, 1986), and some authorities consider them to be a poor guide to predicting haemopoiesis following ABMT (Kaiser et al, 1985). However, when

dealing with purged marrow, the final nucleated cell count is inevitably going to be far below the usual marrow preparation used for ABMT. For this reason in vitro colony formation had to be the major criterion by which to assess the viability of the processed marrow, together with close inspection of both the morphology and colony-forming characteristics of the cells removed from the marrow.

Secondly a suitable preparation for efficient purging is one depleted as far as possible of mature cells such as erythrocytes and granulocytes that might interfere mechanically with optimal target cell/magnetic bead interaction. This entailed a great deal of preparative bench work to find cell separation procedures that would achieve a balance between desired depletion of unwanted cells and minimum non-specific loss of stem cells, at the same time as maintaining a strictly sterile preparation.

The use of automated cell separators instead of simple centrifugation allows for preferential concentration of progenitor cells with increased elimination of other haemic cells (Weiner et al, 1976; Linch et al, 1982; Gilmore and Prentice, 1986). Either intermittent flow (Linch, 1982; Duguid et al, 1988) or continuous flow (Gilmore et al, 1983; Faradji et al, 1988) cell separators have been described, with elimination of granulocytes and erythrocytes by the addition of density separation media such as Ficoll-Hypaque (Gilmore et al, 1986) or sedimenting agents such as hydroxyethyl starch (Baker et al, 1991).

Thirdly, the entire bone marrow processing time must be kept to a minimum, both to preserve cell viability and to reduce the risk of bacterial contamination.

The final method chosen to process and purge myeloma marrow that attempted to meet the above criteria was performed by Mr. Peter Baker and Dr. Jennifer Duguid (Baker et al, 1991). Initial concentration of mononuclear cells was achieved with the

lymphocyte collection programme on a Haemonetics V50 cell separator (Haemonetics, Braintree, Essex). Further depletion of erythrocytes and granulocytes was effected by a density gradient centrifugation step using the Ficoll programme of the Haemonetics V50. The processed marrow was then transferred in a standard sterile blood pack to a Class 1 sterile work station for magnetic bead separation.

6.3 METHODS

6.3.1 Patients.

Four patients with myeloma were studied. Patient characteristics are shown in Table XV: the initial disease had been treated with a variety of widely-used chemotherapy schedules and only patients whose bone marrow showed less than 20% plasma cells were considered for high-dose therapy and rescue with purged autologous bone marrow transplant.

Table XV

Characteristics of patients undergoing bone marrow harvest.

Patient number	1	2	3	4
Age (years)	51	39	44	50
Serum paraprotein g/l	53	66	BJP only	29
BM plasma cells at diagnosis (%)	30	7	11	45
BM plasma cells at harvest (%)	6	13	4	15
Prior therapy	VAMP	ABCM	VAD	VAD

Abbreviations:- VAD/MP - vincristine, adriamycin, dexamethasone/methylprednisolone; ABCM - adriamycin, BCNU, cyclophosphamide, melphalan.

6.3.2 Bone marrow harvest

Written informed consent was given by each patient to under go bone marrow harvest under general anaesthetic. Bone marrow was aspirated in multiple 3-5ml volumes from the posterior iliac crests and transferred to a 2000ml blood collection bag containing 150ml Acid/Citrate/Dextrose (Formula A) (ACD-A). A leucocyte-enriched product was prepared on a Haemonetics V50 cell separator; 40ml buffy coat were collected at each pass using the lymphocytapheresis two-arm procedure programme, and 240ml was collected in a total of 6 passes. 180ml of

plasma was collected at this stage for addition to the cells prior to cryopreservation. Bone marrow mononuclear cells were further concentrated by a density centrifugation step using Lymphoprep (Pharma,Oslo) to a total of 150ml. Bone marrow buffy coat cells were pumped into the centrifuge bowl and after addition of 50ml Lymphoprep the centrifuge speed was increased from 3000 to 5600 rpm in 2000rpm increments with at least 15 seconds in between. The pump was then restarted to add the remaining 100ml of Lymphoprep and product collection commenced.

At the next stage the MNC were washed on the Haemonetics V50 employing the washing protocol. The cells were transferred into the centrifuge bowl spinning at 4000rpm and were washed to remove the Lymphoprep, firstly by the addition of sterile physiological saline, then by 500ml of 4.5% human albumin solution (BPL, El stree) and finally in 500ml IMDM containing 15% v/v ACD-A and 20% v/v 4.5% human albumin solution. The final volume of washed MNC was 200ml.

6.3.3 Magnetic beads

Dynabeads (M-450 uncoated beads and anti-CD19-coated immuno magnetic beads) were obtained from Dynal, UK Ltd. In order to prepare them for plasma cell purging, the uncoated beads were coated with PNA by the method outlined in the previous chapter. 90mg uncoated Dynabeads were washed twice in phosphate buffered saline (PBS) and resuspended in 6.6ml PBS containing 5mg PNA for 16 hours at 4°C with end-over rotation. A final 16-hour incubation was carried out at 4°C in PBS containing 0.1% human albumin before the beads were resuspended at 28.5mg/ml in PBS/0.1% albumin. Plasma was omitted since previous studies had shown that its inclusion at this stage greatly reduced purging efficiency, presumably

because of the presence of PNA-binding glycoproteins in normal plasma (Ching and Rhodes, 1988).

Lectin coating efficiency was assessed by adding 10µl of washed PNA-coated beads to 40µl of a 2% suspension of neuraminidase-treated red cells. The mixture was incubated for 30 minutes at 22°C with gentle agitation and then observed microscopically to ensure that all the PNA-coated beads had bound cells.

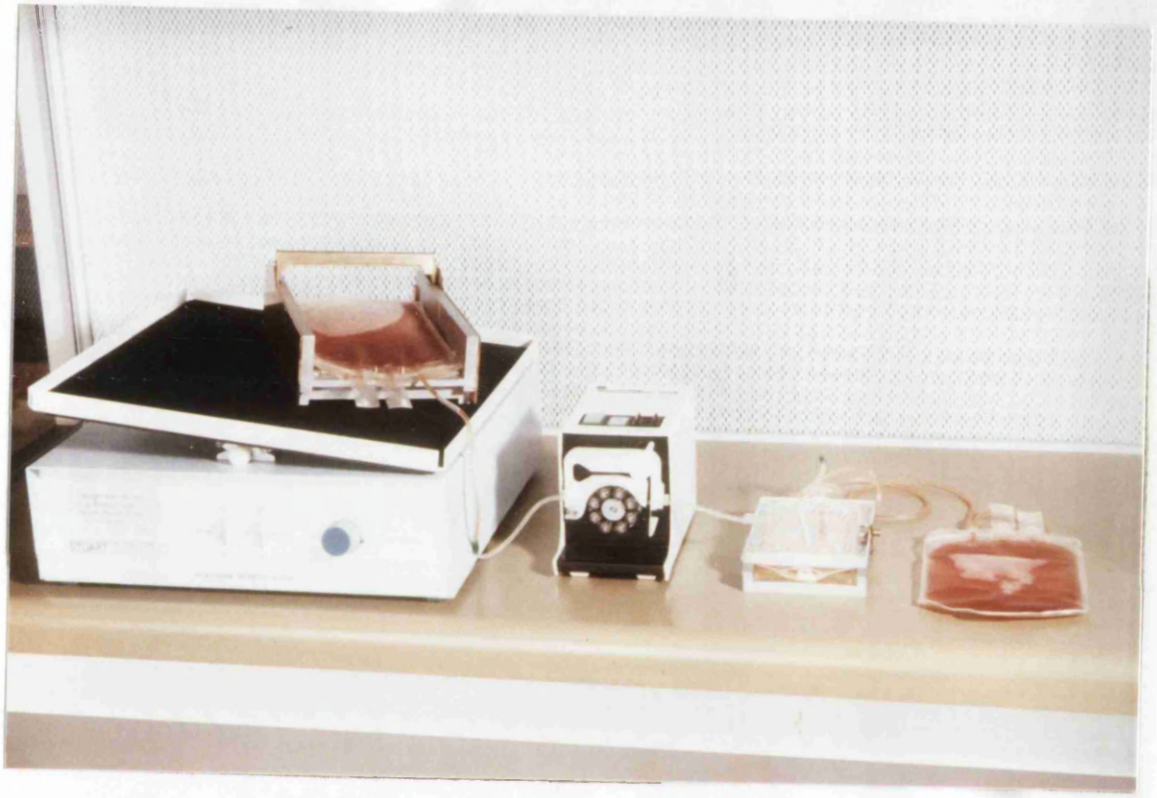
Supernatant from the lectin-coated magnetic beads was tested for the presence of endotoxin by means of the Limulus test (Marine Biologicals Inc., New Jersey, USA, via Lysate and Radio pharmaceutical Consultancy, Bradford upon Avon, UK).

6.3.4 Purging with PNA- and CD19- coated magnetic beads

The coated magnetic beads were washed three times in sterile saline and suspended in 100ml IMDM. PNA- and CD19-coated beads were each added to the bone marrow MNC in the blood collection bag at a bead to target cell ratio of 40:1 and the mixture was incubated at 22°C for one hour with constant gentle agitation. The bag containing bone marrow cells and magnetic beads was then loaded onto a Dynal Magnetic Separation Unit, separated from the magnet by a removable iron plate (Figure 17). After removing the plate the platform was lowered in 4 stages, 5 minutes for stages 1 and 2, 3 minutes each for stages 3 and 4. Immediately the bag had reached the lowest stage the free cells were pumped out of the bag by a peristaltic pump (Ismatec SA, Zurich, FRG) at 10ml/minute. After passing through a small magnetic trap (Dynal) the unbound cells were collected into a 400ml Fenwal transfer pack. Samples of purged bone marrow were taken for cytochemical analysis, bone marrow culture, and microbiological culture. The remaining bone marrow

sample was then centrifuged at 400g for 5 minutes at 4°C. The supernatant was discarded and 100ml autologous plasma was added to the bone marrow cells.

Figure 17



The set-up used for large-scale purging showing the magnet supported on a rocking deck with a peristaltic pump for removal of non-adherent cells.

6.3.5 Cryopreservation

Operating in a Class 1 sterile work station, the bone marrow/plasma mixture was supported on ice, and 100ml plasma containing 20% v/v dimethyl sulphoxide (DMSO) was gradually added at 10ml/min with thorough mixing. The final purged bone marrow/plasma/DMSO mixture was transferred into 2 Gambro hemofreeze bags (Gambro Dialysatoren, Hechingen, FRG) and the entry ports sealed. The bags were then frozen using the Planar Kryo 10 controlled rate freezer, and transferred to liquid nitrogen (Hagenbeek et al, 1989).

6.3.6 Monoclonal antibody staining

Monoclonal antibodies (Mab) were detected by the APAAP technique in cytocentrifuge slides (10^5 cells per slide), and the following Mab were employed:- anti-CD19 (Leu 12), anti-kappa, anti-lambda, (Becton Dickinson, Cowley, UK) and anti-plasma cell antibody (RFD6) (Dept of Immunology, Royal Free Hospital, London). In order to detect cytoplasmic immunoglobulin, cytospin preparations containing 10^5 cells were fixed in acetone for 10 minutes and washed in PBS. The cells were incubated with 1µl FITC-labelled goat anti-human kappa and 5µl TRITC-conjugated goat anti-human lambda (Southern Biotechnology Associates, Seralab) in 50µl PBS (previously titrated), for 30 minutes at 22°C. After washing in PBS, preparations were examined under UV light using a Leitz microscope.

6.3.7 Detection of PNA^+ cells

Staining of marrow preparations for PNA was performed by the immunoalkaline phosphatase technique on cytocentrifuge preparations, outlined

previously. Negative controls were prepared by incubating the cells with PNA in the presence of 0.2M D-galactose.

6.3.8 Normal progenitor assays

Granulocyte/macrophage colony forming units (CFU-GM)

CFU-GM were assayed in IMDM containing 0.3% (wt/vol) agar, 20% FCS and 10% medium conditioned by C 5637 bladder carcinoma cell line, as described in the previous chapter.

Granulocyte/erythrocyte/macrophage/megakaryocyte colony forming units (CFU-GEMM)

$1x10^5$ cells were suspended in 0.8% (wt/vol) methylcellulose in IMDM and supplemented with 30% FCS, 10% C 5637 CM, 2.5u/ml erythropoietin. In this series of experiments, 10% bovine serum albumin (previously batch tested) was substituted for the serum of a patient with severe aplastic anaemia, and PHA-LCM was omitted. The full method was described in the previous chapter.

Long term bone marrow culture (LTBMC)

Bone marrow MNC were cultured according to the method of Dexter in IMDM containing 10% FCS, 10% horse serum, 10^{-4}M 2-mercaptoethanol and 10^{-6} hydrocortisone. Cultures were fed weekly by replacing half the medium and non-adherent cells with fresh culture medium as described in the last chapter.

6.4 RESULTS

6.4.1 Cell recoveries during processing and purging

The mean volume of bone marrow aspirated was 1.47 +/- 0.311 (SD) (Table XVI) with a mean absolute white blood cell count (WBC) of 12.35 +/- 4.76 x 10^9/l. After the initial leucocyte concentration stage using the Haemonetics V50 there remained 9.09 (mean) +/- 3.63 x 10^9/l WBC, and after further processing with Lymphoprep the final absolute WBC prior to purging was 3.18 +/- 1.03 x 10^9. After incubation with combined PNA- and CD19-coated magnetic beads and separation on a magnet, the mean absolute WBC was 1.78 +/- 0.69 x 10^9 (Table XVI).

Table XVI

Characteristics of bone marrow harvest

Volume harvested (l)	WBC* in harvested BM	WBC in concd BM	WBC post-ficoll	WBC post-purge
1.47 +/- 0.31	12.35 +/- 4.76	9.09 +/- 3.63	3.18 +/- 1.03	1.78 +/- 0.69

* Absolute white blood cell count (x 10^9), as measured on a Coulter StkS automated machine.

Results shown are mean +/- standard deviation.

Percentage recovery of bone marrow nucleated cells at each stage of the processing is shown in Table XVII. The final purged product yielded 14.4% of the original nucleated cell preparation.

There was a 26.7% cell loss during the initial leucocyte concentration stage and a 64.9% cell loss of the leucocyte concentrate after the Lymphoprep processing.

Examination of cytocentrifuge preparations (Romanowski stain) showed mainly mononuclear cells with very few granulocytes. The purging procedure itself resulted in a 44% cell loss.

Table XVII

Numbers of cells recovered at each stage of processing

	After concentration	After ficoll separation	After purging
Nucleated cell recovery (mean+/- SD%)	72 +/- 9.1	28 +/- 4.2	20 +/- 3.7
(range)	60 - 80%	25 - 34%	15 - 23%

6.4.2 Phenotyping studies

Cytocentrifuge preparations were made of the bone marrow at each stage and stained for cells reactive with PNA, CD19 Mab and cytoplasmic kappa and lambda. The proportion of cells reacting with PNA prior to processing the bone marrow was 18.4% +/- 9.5 (mean +/- SD; range 12-35%). After processing and purging the bone marrows, PNA^+ cells were not detected by immunoalkaline phosphatase (Table XVIII). Also, cytoplasmic immunoglobulin was absent in the bone marrow samples after purging. $CD19^+$ cells were 5.0% +/- 3.0 (mean +/- SD; range 2-10%) in samples prior to processing, but were undetectable in the final product after purging (Table XVIII).

Table XVIII

Efficiency of removal of target cells from bone marrow harvests.

	PNA^+ (mean+/-SD%)	$CD19^+$ (mean +/- SD%)
Before purging	18.7 +/- 10.0	5.2 +/- 3.0
After purging	none detected	none detected

6.4.3 Recovery of normal haemopoietic progenitors

Recovery of normal haemopoietic progenitors after purging the bone marrow harvests is shown in Table XIX and Figure 19. In each case numbers of CFU-GM and CFU-GEMM were estimated from aliquots of bone marrow withdrawn after the density gradient separation stage (pre-purge) and after the purging procedure (post-purge). A mean of 42.5 +/- 20.3 CFU-GM per 2 x 10^5 cells plated was obtained before purging, and 87.5 +/- 56.4 per 2 x 10^5 after purging. When adjusted for cell loss there was (78.2%) recovery of CFU-GM following purging. A mean of 11.0+/-4.9 (+/- 1SD) CFU-GEMM per 1x10^5 plated cells was obtained before purging, and 15.5+/-6.0 per 1x10^5 cells plated after purging. When adjusted for nucleated cell loss there was a 79% CFU-GEMM recovery following purging. Calculations based on patient's body weight predicted a mean CFU-GM dose of 0.86 +/- 0.32 x 10^4/kg recipient (range 0.5-1.3 x 10^4/kg).

Table XIX

Recovery of normal haemopoietic progenitors after purging

a)

	CFU-GM per 2 x 10^5 cells plated (mean +/- SD)	CFU-GEMM per 10^5 cells plated (mean +/- SD)	Total nucleated cells (x 10^9) (mean +/- SD)
Before purging	42.5 +/- 20.3	11.0 +/- 4.9	3.3 +/- 1.1
After purging	87.5 +/- 56.4	15.5 +/- 6.0	2.0 +/- 0.6

b)

	CFU-GM	CFU-GEMM	Nucleated cells
Percent recovery	78.2	79.0	61.0

6.5 DISCUSSION

A method for removing all plasma cells and B lymphocytes in vitro from large volumes of myeloma bone marrow is described here. A mononuclear cell fraction enriched for haemopoietic progenitors and depleted of erythrocytes and mature granulocytes was obtained via a two-step procedure using the Haemonetics V50 cell separator. The initial concentration stage reduced the number of erythrocytes which facilitated the second stage of density gradient separation. The density gradient stage is desirable first because the efficiency of purging is reduced

in the presence of large numbers of erythrocytes and secondly PNA is known to bind some granulocytes. After density gradient separation fewer PNA-coated magnetic beads are required, thus reducing the cost and also improving efficiency.

Initial work involving small-scale experiments on normal peripheral blood and bone marrow spiked with tumour cell lines, and on myeloma marrow, had shown that efficient removal of plasma cells and $CD19^+$ lymphocytes could be achieved at a magnetic bead:target cell ratio of 40:1 without serious loss of haemopoietic progenitors. In the present series of experiments on large volumes of bone marrow, the numbers of magnetic beads required to achieve a 40:1 ratio were calculated on bone marrow aspirates taken from the patients just prior to the main purged bone marrow harvest. No detectable PNA^+ or $CD19^+$cells were found in the final product of the four cases. On this basis the magnetic bead:target cell ratio of 40:1 as well as the incubation times and temperature were not altered in subsequent procedures.

Haemopoietic progenitor cells (as assessed by CFU-GM and CFU-GEMM) were concentrated in the final MNC product, whereas colony assays of cells bound to magnetic beads yielded less than 3% of total CFU-GM (data not shown). The haemopoietic potential of the final product calculated as CFU-GM dose per kilogram body weight was greater than $0.75x10^4$/kg in four out of the six cases. The one patient who was harvested twice yielded $0.5x10^4$/kg CFU-GM on both occasions.

On the basis of the preliminary work presented so far two patients with myeloma have undergone autologous bone marrow transplantation using autologous marrow purged with the lectin/Mab combination to rescue them from high-dose chemoradiotherapy. The treatment and response of these patients is described in the next chapter.

Summary

This chapter described the techniques used to convert small-scale purging experiments using PNA- and CD19- coated Dynabeads to large scale methods suitable for clinical application without losing too many normal progenitor cells. The key features were the use of density gradient separation to improve the efficiency of purging, and a fairly simple automated cell separator programme to improve sterile conditions. By this means it was predicted that adequate numbers of normal haemopoietic progenitors could be preserved after purging to repopulate an irradiated autologous bone marrow transplant recipient marrow. The next stage of the work entailed devising a suitable protocol for applying the lectin/Mab purging procedure to patients with myeloma.

CHAPTER 7

AUTOLOGOUS BONE MARROW TRANSPLANTATION FOR MYELOMA PATIENTS USING PNA- AND CD19- PURGED MARROW RESCUE

7.1 Aims

To initiate a pilot study to assess the efficacy of treating patients with myeloma by purged ABMT.

7.2 Introduction

Following the preparative in vitro work outlined in the preceding chapters, a single-blind controlled trial was set up to compare purged with non-purged autologous bone marrow transplant (ABMT) in patients with myeloma who had previously received conventional cytotoxic agent treatment to the point where the plasma cell content of bone marrow was less than 20%. The latter proviso was dictated mainly by the financial cost of magnetic beads but also by the greater purging efficiency achievable by a relatively small target cell population.

Although initially it was the intention to randomise patients in a controlled single blind trial, not all patients with myeloma have PNA-reactive plasma cells (5-10% are PNA^-, and in a further small minority (approx. 1%), bone marrow plasma cells are a mixture of PNA^+ and PNA^-). Therefore patients who had PNA- unreactive plasma cells were allocated to the unpurged arm of the trial and all otherwise eligible patients with PNA-reactive plasma cells were considered for a purged ABMT.

A further difficulty encountered during these studies was obtaining an

adequate cell yield at bone marrow harvest. It has been our experience that myeloma bone marrow is relatively hypocellular (even when plasma cell numbers and serum paraprotein levels are low) at harvest compared with patients with non-Hodgkins lymphoma undergoing ABMT in this department. Although the 2 patients described below had satisfactory cell yields at bone marrow harvest, others have required 2 or even more harvests to obtain an adequate number of haemopoietic stem cells, and some patients have not been considered for ABMT on the basis of marrow hypocellularity.

Because of the highly experimental nature of the proposed treatment (PNA has not been used to treat patients in this way before), it was important not only to spend a lot of time with the patients to explain the procedure and experimental nature of the treatment, but also to obtain their consent for an initial unpurged "back-up" bone marrow harvest of adequate cellularity and haemopoietic quality. This was to be cryopreserved and stored for use in the event of failure of engraftment of the purged bone marrow.

The pre-transplant conditioning regime chosen (melphalan 140mg/m^2 plus 850cGy total body irradiation) was described by Barlogie et al, (1987) and designed to be marrow ablative, as opposed to high-dose melphalan alone as used in this context by Professor McElwain and colleagues at the Royal Marsden Hospital, (McElwain et al, 1983).

In view of recent evidence of the possible benefit of α-interferon in delaying the onset of disease relapse in patients with plateau-phase myeloma (Mandelli et al, 1990), and in view of the current MRC myeloma trial with an arm for α-interferon, it was not thought justifiable to deny our patients this therapy; all patients, whether

receiving a purged or nonpurged marrow would be given α-interferon treatment after ABMT when their peripheral blood count had recovered sufficiently.

7.2.1 ABMT trial protocol

The following is an abbreviated outline of the trial protocol:-

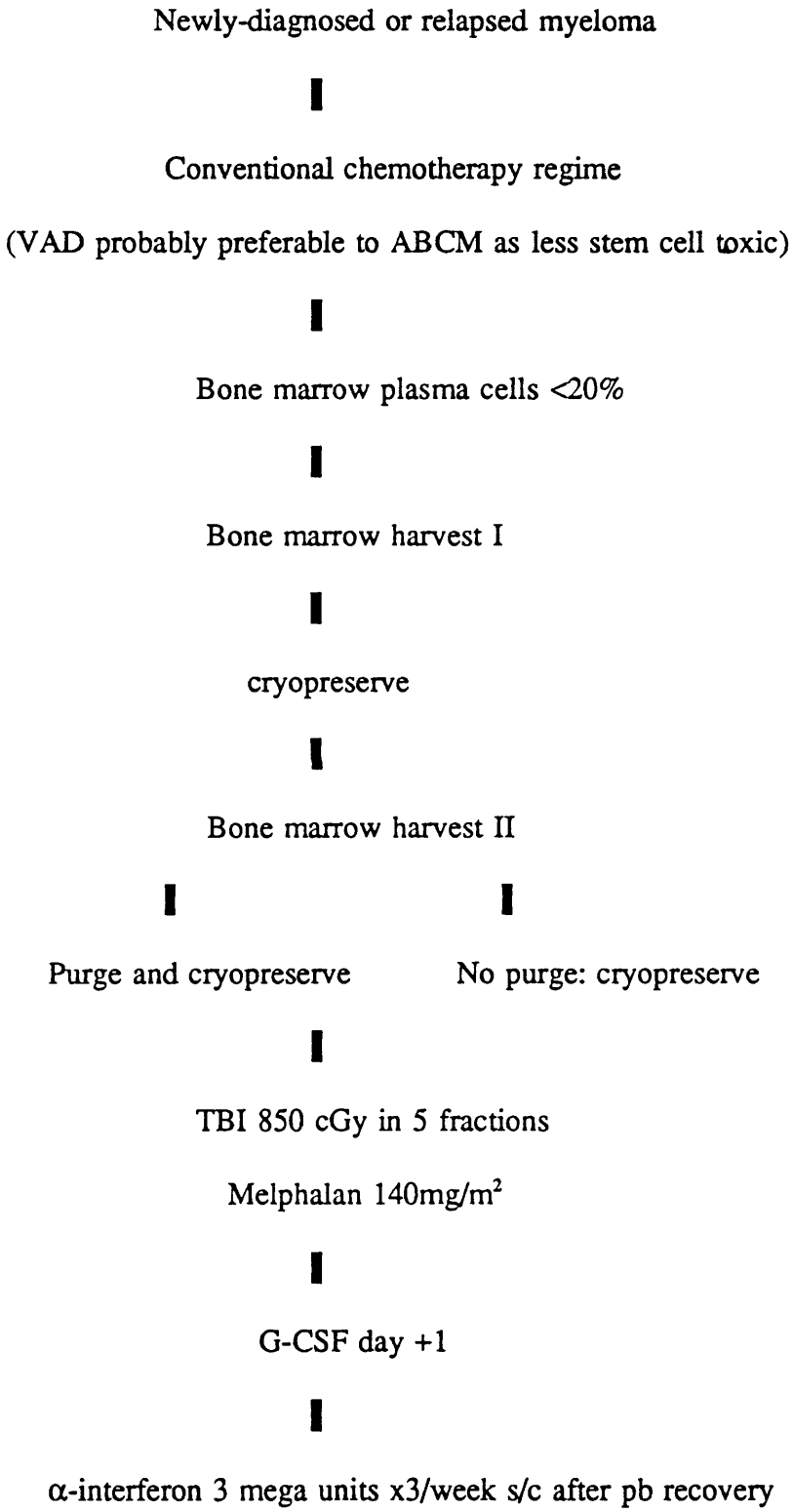
Inclusion criteria-

- patients under 55 years of age
- adequate marrow function as defined by:
  - peripheral blood neutrophil count $>2x10^9/l$
  - peripheral blood platelet count of $>100x10^9/l$
  - marrow cellularity >25% on trephine biopsy
  - marrow plasmacytosis <20%
- creatinine clearance >60ml/min
- reactivity of bone marrow plasma cells with PNA lectin (purged arm only)
- able and willing to conform to the requirements of the study
- witnessed written consent given

Exclusion criteria

- life expectancy severely limited by other disease
- symptomatic cardiac disease
- significant renal impairment (creatinine clearance <60ml/min)
- liver dysfunction: (bilirubin >50mmol/l; ALT >40iu/l

7.3 STUDY DESIGN



Patient 1 (RF)

A 38 year old male HGV driver developed rapidly progressing weakness of the legs in March 1989. He was found to have spastic paraparesis due to a spinal lesion at D 6/7. Investigations showed:- Hb 12.7 g/dl; WBC $7.7x10^9/l$, normal differential; platelets $400x10^9/l$. Serum immunoglobulins were:- IgG 25 g/l, IgA 1.2 g/l, IgM 0.3 g/l with a monoclonal band revealing an IgG kappa paraprotein in the serum. Renal function was normal and no Bence Jones proteinuria was detected. Bone marrow aspirate revealed 7% plasma cells with relatively well-preserved normal haemopoietic tissue. X-ray of the spine showed collapse and sclerosis of the seventh thoracic vertebra and a myelogram showed complete spinal block in the dorsal region.

He was treated with emergency surgical decompression in the D 7/8 region, followed by fractionated irradiation to the dorsal spine to a total of 400 cGy in April and May, 1989. This was followed by a total of 5 courses of the ABCM chemotherapy regime between May and December 1989, comprising adriamycin (doxorubicin) $30mg/m^2$ iv and BCNU (carmustine) $30mg/m^2$ iv, both on day 1, then melphalan $6mg/m^2$/day and cyclophosphamide $100mg/m^2$/day both for 4 days on days 22 - 25 for each cycle,repeated at 6-weekly intervals.

He recovered full use of his legs and in July 1989 was referred to the Royal Liverpool Hospital for consideration of ABMT. Bone marrow plasma cells (13% of nucleated cells) were reactive with PNA and in July 1989 a "back-up" bone marrow harvest was performed, the marrow being cryopreserved without treatment. In

September 1989 a second bone marrow harvest was per formed, and this marrow was purged in vitro with combined PNA- and anti-CD19- coated magnetic beads, then cryopreserved, as outlined in the previous chapter. In this, the first purged harvest for clinical use, bone marrow MNC were suspended in medium supplemented with autologous plasma during processing, and this was believed to be the cause of the incomplete removal of all detectable plasma cells. As can be seen in Table XX, plasma cells were reduced from 13% to 2% during purging, and all detectable $CD19^+$ cells were removed. Haemopoietic progenitor assays are shown in Table XXI, and CFU-GM colonies grown from cryopreserved pilot tubes of purged marrow predicted a final yield of $0.91x10^4$/kg.

In January 1990 the patient received total body irradiation (850 cGy in 8 fractions) (day -6 to -3): on day -1 he was given melphalan 140mg/m^2 by slow iv injection, with forced diuresis according to the MRC VIth Myelomatosis Trial protocol. Unfortunately on day 0 the patient developed signs of superior venacaval thrombosis secondary to thrombosis of the Hickman line. The latter was removed and heparin, 40,000iu per day by continuous iv infusion for 3 days, was administered. On day 0 the cryopreserved purged bone marrow was thawed rapidly in a 40°C water bath at the patient's bedside, and infused via a peripheral vein. The patient was nursed in a laminar airflow room with full anti-microbial precautions and irradiated blood product support as required.

On day +1 recombinant human G-CSF (Chugai Ltd), 15u/kg/day, was commenced and was given daily by iv injection until peripheral blood neutrophils had remained > $1.0x10^9$/l for 3 consecutive days. Apart from the thrombotic episode the patient remained free of serious complications.

Peripheral blood neutrophils reached $0.5x10^9/l$ by day +14 and the patient was well enough to leave hospital on day +17; platelets were $50x10^9/l$ by day +37, and on day +45 the patient began regular α-interferon 3 mega units s/c x3/week which has continued for the ensuing 22 months.

The patient himself remains extremely well and is back at work. Examination of the bone marrow at serial intervals following the ABMT has consistently shown less than 3% plasma cells, although overall cellularity has been moderately reduced in all cell lines - possibly an effect of α-interferon therapy. A monoclonal band is present in the serum; regular blood tests have shown a steady decline in the level of the paraprotein from 66 g/l immediately prior to ABMT, to 18 g/l 17 months later. This is the only indication that the myeloma has not been eradicated (Figure 20).

Patient 2 (CR)

This 50 year old woman presented to another hospital in acute renal failure in 1986. She was found to have thrombosis of the inferior vena cava and right renal vein. Further investigations showed:- Hb 12 g/dl, WBC $19x10^9/l$, and platelets $65x10^9/l$. Serum immunoglobulins were:- IgG 20 g/l, IgA 0.6 g/l, IgM 0.2 g/l with an IgG lambda paraprotein. No Bence Jones proteinuria was detected. Bone marrow examination revealed 25% plasma cells, but no bony lesions were found. She underwent total nephrectomy of the right kidney and commenced chemotherapy with the VAD regime outlined above (vincristine, adriamycin and dexamethasone). She received a total of 9 courses of this chemotherapy at which time she was deemed to have achieved plateau phase of the disease (serum paraprotein 7 g/l, bone marrow plasma cells 6%). No further chemotherapy was given after September 1988, and she

was referred to the Royal Liverpool Hospital in July 1989 for consideration of ABMT. Bone marrow plasma cells (6% of nucleated cells) were reactive with PNA and in July 1989 a "back-up" bone marrow harvest was taken and cryopreserved without in vitro treatment.

In June 1990 another bone marrow harvest was performed and purged in vitro with combined PNA- and anti-CD19- coated magnetic beads according to the method outlined in Chapter 6; the purged marrow was cryopreserved and characteristics of the procedure are shown in Table XX. No detectable PNA^+, $CD19^+$ or cytoplasmic immunoglobulin positive cells remained after purging, and sampling of a cryopreserved pilot tube containing purged marrow predicted a CFU-GM dose of $0.75x10^4$/kg.

In October 1990 the patient was admitted for ABMT. She was subjectively well, but renal function was compromised as evidenced by an EDTA clearance of 47 ml/minute. Serum paraprotein was 29 g/l and bone marrow plasma cells were 15% of nucleated cells. On day -6 total body irradiation was commenced (850 cGy in 8 fractions) followed by melphalan 140 mg/m^2 on day -1, with special care being taken to ensure a forced alkaline diuresis. The purged bone marrow was thawed rapidly on day 0 and infused via a Hickman catheter uneventfully. rhG-CSF was given daily from day +1 until neutrophils had been $>1.0x10^9$/l for 3 consecutive days. The patient suffered serious infections in the early days after ABMT requiring intensive treatment with anti-bacterial and anti-fungal agents. Peripheral blood neutrophils reached $0.5x10^9$/l by day 20, after which strict isolation measures could be relaxed, and peripheral blood platelets reached $50x10^9$/l by day + 30. Treatment with α-interferon was started on day 50 (3 mega units s/c x3 per week) but was

discontinued after 4 weeks because of intolerance by the patient. She has remained entirely well and symptom-free in the 9 months following ABMT: periodic estimations of serum and urine immunoglobulins and bone marrow showed no evidence of myeloma until 6 months after the ABMT when an IgG paraprotein was detected in the serum (Figure 18), and subsequently disappeared again. The patient remains well and has a normal bone marrow aspirate 13 months following purged ABMT.

Table XX

Characteristics of purged bone marrow harvests.

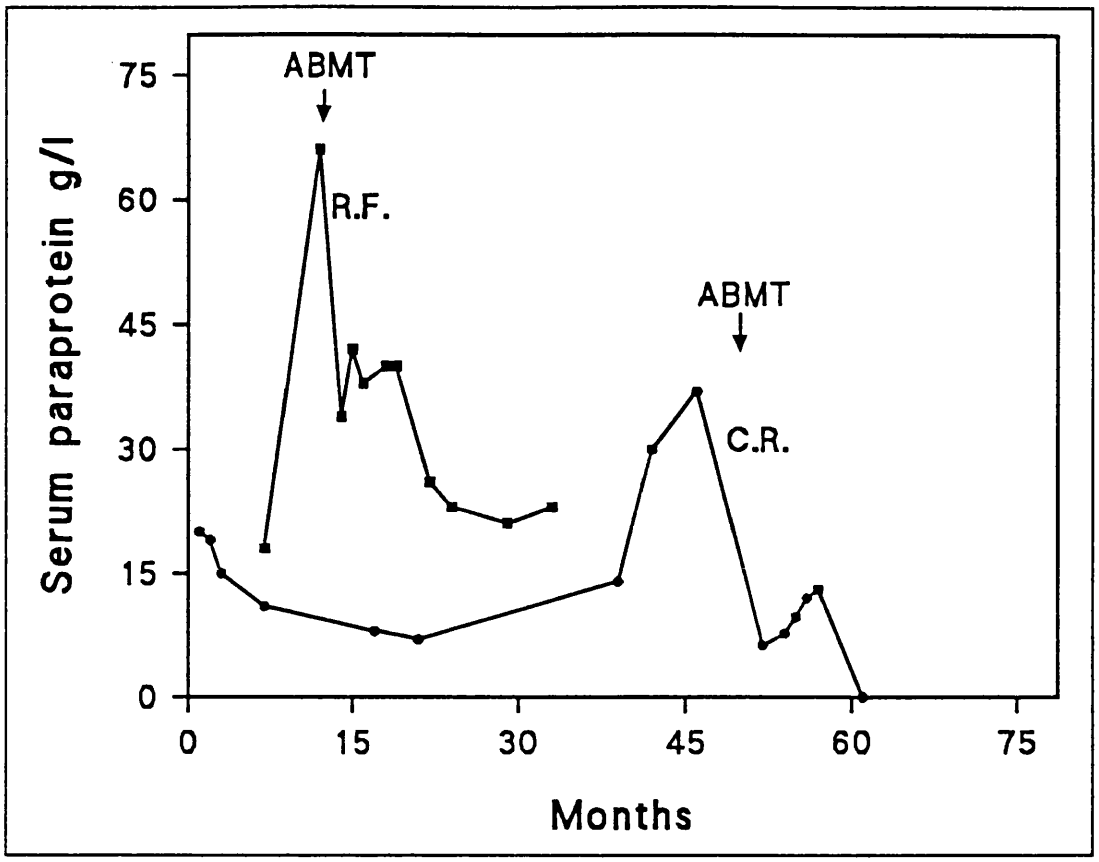
Patient	Volume bm harvested (l)	WBC $x10^9$ bm harvest	post-concn WBC $x10^9$	post ficoll WBC $x10^9$	post purge WBC $x10^9$
RF	1.4	8.2	7.1	2.5	1.02
CR	1.5	10.3	7.1	2.6	1.7

Table XX1

Efficiency of purging and normal progenitor cell recovery.

Patient	PNA^+ %	$CD19^+$ %	cytopl Ig %	CFU-GM per $2x10^5$ cells	CFU-GEMM per 10^5 cells	nucl. cell/kg	CFU-GM/kg
RF pre-purge	17	4	Not done	66	4	Not done	Not done
RF post-purge	3	<1	2%	166	7	0.11 $x10^8$	0.91 $x10^4$/kg
CR pre-purge	13	4	Not done	41	18	Not done	Not done
CR post-purge	<1	<1	<1	52	24	0.25 $x10^8$	0.75 $x10^4$/kg

Figure 18



Serial measurements of serum paraprotein from diagnosis to the present. It is apparent from the figure that both patients were showing signs of relapse at the time of ABMT.

7.5 DISCUSSION

The preceding section outlines the clinical course followed by two patients with myeloma who underwent autologous bone marrow transplantation and received marrow purged in vitro with PNA- and anti-CD19-coated magnetic beads.

The first patient was still receiving chemotherapy at the time of transplant and had evidence of a considerable tumour load as seen in the high serum paraprotein level of 66g/l, although bone marrow plasma cells were less than 20%. It is clear that purging of myeloma cells was incomplete as 2% plasma cells and cells bearing cytoplasmic immunoglobulin remained after purging. Because this was the only large-scale procedure using the bead:target ratios outlined previously in which incomplete removal of target cells was observed, and because this was the only procedure in which the bone marrow cells were suspended in medium containing autologous plasma prior to treatment with magnetic beads, we were led to conclude that plasma glycoproteins had interfered with the interaction of PNA with plasma cells, probably by direct competition (Ching and Rhodes, 1988).

There was a rapid and sustained engraftment of purged mar row, with no decline in peripheral blood counts on stopping rhG-CSF. This suggests that an adequate number of pluripotent haemopoietic stem cells had been infused, despite the very low number of nucleated cells ($1.1x10^7$ cells per kg). It also suggests that the CFU-GM content of the marrow ($0.91x10^4$/kg in this patient) may be the only useful predictor of successful engraftment in this situation.

This first patient has never achieved complete remission following high dose therapy, as evidenced by the persistence of a serum paraprotein. The level of the

paraprotein has steadily declined since the transplant, and it is difficult to say whether the decrease is due to the anti-tumour action of α-interferon or whether the ABMT has caused the malignant cells to lose their growth advantage in some way. Also, it is impossible to tell whether the presence of disease following high-dose therapy is due to inadequate conditioning therapy, or whether the incompletely purged marrow autograft is the source of malignant cells.

The second patient was in clinical plateau phase at the time of transplant, having received no treatment for two years. She posed a special problem with her compromised renal function, and I am grateful to Professor McElwain for advice concerning the high-dose melphalan in this context (he advised going ahead!) and also to Dr Brian Cottier, Consultant Radiotherapist at Clatterbridge Hospital, for his expertise in minimising the risks of radiation nephritis.

This patient also had sustained bone marrow engraftment after stopping rhG-CSF and after a period of 6 months during which no signs of myeloma could be detected, a serum paraprotein reappeared transiently.

The one definite conclusion that can be drawn from these results is that removal of a large number of cells reactive with PNA does not remove haemopoietic capability from bone marrow. This is powerful evidence that PNA does not bind haemopoietic cells in the human, despite previous reports that it does. Reisner et al (1978) effected haemopoietic stem cell transplantation in lethally irradiated allogeneic mice by means of cells positively selected with peanut agglutinin. Bone marrow cells were incubated with PNA, followed by the addition of rabbit erythrocytes, which formed aggregates with PNA-coated cells and could be separated by density centrifugation. Cells from the aggregated fraction were used successfully to

reconstitute the marrow of irradiated mice.

One can only infer from the totally different findings of this report from those presented in this project, that either a species difference accounts for different binding specificities of PNA, or that the method of cell separation does not only enrich for PNA^+ cells.

Summary

Two patients with myeloma have received autologous bone marrow transplantation in which their bone marrows have been purged *in vitro* with PNA and CD19 magnetic beads. Both patients showed prompt haemopoietic engraftment and are alive and well 29 and 19 months later. These results demonstrate the feasibility of purging bone marrow with this lectin/monoclonal antibody combination.

CHAPTER 8

CONCLUSIONS AND IMPLICATIONS FOR FUTURE WORK

A. Conclusions

This project has spanned nearly 5 years during which time considerable experience has been gained in treating myeloma patients with ABMT and some experience with purged ABMT (reviewed by Barlogie, 1990). No radically new therapeutic strategies have emerged and the majority of patients (>90%) relapse within 2 years of receiving high-dose therapy. It is clear that although major multi-centre trials of conventional treatment are essential, there is a definite role for small studies of new therapeutic options - which we are attempting to conduct in Liverpool.

The main conclusions of the project are listed below:-

1. After setting up established techniques for the culture of normal haemopoietic progenitor cells in vitro a new method for short-term in vitro growth of myeloma cells was developed using serum-free medium in liquid culture. Examination of the myeloma colonies revealed two main cell populations:- plasma cells and $CD19^+$ B lymphocytes. These two cell types were therefore targeted in the further development of a purging strategy for myeloma.

2. The novel observation was made that the lectin PNA shows specificity for bone marrow plasma cells, and does not appear to bind to haemopoietic progenitor cells.

Thus PNA is a candidate anti-plasma cell agent for purposes of purging myeloma marrow.

3. Two methods were developed for separating plasma cells from myeloma bone marrow by means of surface-bound PNA: an erythrocyte rosetting technique which relies on PNA binding to exposed T antigen on red cells, and a magnetised microsphere technique whereby PNA was bound to uncoated Dynabeads. This last method was further developed for purging purposes.

4. Model experiments were devised to formulate optimal conditions for removing all plasma cells using PNA-coated Dynabeads: these experiments were repeated using commercially available CD19-coated Dynabeads to remove B lymphocytes. Further experiments were aimed at combining PNA- and CD19-coated beads to remove all detectable plasma cells and $CD19^+$ lymphocytes from bone marrow without removing normal haemopoietic progenitors.

5. The model experiments were repeated on a large scale comparable to a bone marrow harvest: this involved experimenting with different bone marrow processing techniques, both manual and automated until the best balance was obtained between effective purging and preservation of normal haemopoietic progenitors.

6. Four bone marrow harvests from patients with myeloma were successfully purged with PNA and CD19 and were cryopreserved. A further bone marrow harvest was purged using autologous plasma as a protective agent during processing: incomplete

removal of plasma cells was achieved (2% remaining plasma cells). This incompletely purged, and one successfully purged, bone marrow was used as a source of stem cell rescue for two patients receiving high-dose chemoradiotherapy.

7. Both patients who received a purged ABMT showed prompt haemopoietic engraftment and are extremely well 19 and 29 months after the treatment. One patient, however has detectable serum paraprotein.

8. Large numbers of patients are needed to be able to show the benefit or otherwise of this novel treatment, but results so far are sufficiently encouraging for the study to continue.

B. Implications for future work

Further work on this area of research into myeloma can be divided into four categories: improving and extending *in vitro* culture of myeloma progenitors; examination of PNA-binding structures on plasma cells; exploring more sophisticated means of detecting small numbers of clonogenic myeloma cells in purged marrow; and examining the role of purged ABMT in myeloma in large numbers of patients.

Myeloma bone marrow culture

There is still a need for improved reliability of short-term *in vitro* culture of myeloma cells in order that a) the method can be used to monitor the effectiveness of purging, and b) larger numbers of cells can be examined phenotypically for the purposes of identifying progenitor cells.

Although the serum-free culture system described in this thesis has been useful in identifying important targets for purging it has the disadvantage of a low plating efficiency (only 17/32 bone marrow samples grew) and also of yielding too few cells for more detailed examination.

In order to improve clonogenic efficiency, further work is proposed using recombinant growth factors such as IL-6 (Fiedler et al,1990), GM-CSF (Zhang et al, 1990), TNF-α and IL-1 (Carter et al, 1990). Myeloma colonies should be isolated and fractionated according to cell surface phenotype. Immunoglobulin gene heavy-chain rearrangements should be examined in the different populations in order to assess the self-renewal properties of the different cell types. This would help to address the vexed question of whether plasma cells are tumorigenic - if not there may be no point in removing them from autografts.

Examination of PNA-binding structures on plasma cells

It will be useful to identify the membrane structures on plasma cells that bind PNA. The aim of this would be firstly to examine the role of surface membrane structures on plasma cells in the distribution of myeloma cells in the body (does the PNA-binding property of plasma cells contribute to their localisation within the bone marrow?): and secondly to use purified PNA-binding structures to raise further anti-plasma cell monoclonal antibodies.

Preliminary work on the PNA-binding K620 myeloma cell line (provided by Dr A Karpas, Cambridge) since the work described in this thesis has revealed that PNA binds to the CD44 membrane receptor (manuscript submitted). There is much interest currently in the role of CD44 as a homing receptor, and further work is

planned using native plasma cells to expand on these early findings.

Detection of minimal residual disease

In assessing whether autologous bone marrow transplantation can cure myeloma there remains the problem of determining whether all clonogenic myeloma cells have been removed from the autograft. The methods of detecting residual myeloma described in this thesis are based on models using H342-labelled cells in which 1 in 10^5 target cells can be detected in small scale experiments with even lower efficiency likely in large scale purging procedures.

All cases of myeloma are characterised by unique clonal rearrangements of the immunoglobin heavy chain gene. The CDR-III gene sequence acts effectively as a unique clone-specific marker for each patient's tumour, as has been found in acute lymphoblastic leukaemia (Brisco et al 1991). At the time of writing polymerase chain reaction techniques for amplifying clone-specific sequences of immunoglobin heavy chain rearrangements are at an early stage, and there are problems to be resolved, particularly when attempting quantitative analysis of minimal residual disease (Veerken et al 1991; Liang et al 1991). It is to be anticipated that molecular techniques such as these will be applied to purged autografts and to the follow-up of patients treated with autologous bone marrow tranplantation for myeloma.

Another important question to be answered is whether relapse following autologous bone marrow tranplantation originates from the autograft or from residual disease in the host that escaped conditioning therapy. One potentially effective way of addressing this is to add marker genes to the purged autograft before infusion.

The role of purged autologous bone marrow tranplantation in myeloma patients

There is at present insufficient data to assess the long-term benefits of *in vitro* purging of autografts in myeloma. The best results have been reported from the Dana-Farber Institute who report that 4 out of 11 patients have continued in complete remission from 12-29 months after purged autologous bone marrow transplantation. Their purging regimen comprises a combination of the monoclonal antibodies PCA1, CD20 and CD10 (Anderson et al 1991). It is too early to say whether this represents an improvement on unpurged autologous bone marrow tranplantation (the median duration of remission was less than 2 years in 28 patients autografted by the Royal Marsden group (Gore et al 1989)) and it is clear that more data are required. In the hands of the author PCA1 has not proved as effective an anti-plasma cell agent as PNA, but it may be that a combination of anti-B lymphocyte monoclonal antibodies would be more effective than CD19 alone. Again, more sensitive techniques for detecting residual malignant cells are required to assess this.

8.2 PUBLICATIONS ARISING FROM WORK DESCRIBED IN THIS THESIS

Rhodes EGH and Flynn MP. (1989) Peanut agglutinin shows specificity for bone marrow plasma cells. Br J Haematol 71: 183-187.

Rhodes EGH, Olive C, Flynn MP. (1990) A serum-free culture method for myeloma progenitors in vitro: proliferative and immunophenotypic characteristics. Exp Hematol 18: 79-83.

Rhodes EGH, Baker PK, Rhodes JM, Davies JM, Cawley JC. (1991) Peanut agglutinin in combination with CD19 monoclonal antibody has potential as a purging agent in myeloma. Exp Hematol 19: 833-837.

Rhodes EGH, Baker PK, Duguid JKM, Davies JM, Cawley JC. (1992) A method for clinical purging of myeloma bone marrow using peanut agglutinin as an anti-plasma cell agent, in combination with CD19 monoclonal antibody. Bone Marrow Transplantation (accepted for publication).

Rhodes EGH, Baker PK, Duguid JKM, Cawley JC. (1992) PNA purging and magnetic microspheres. Progresses in Clinical and Biological Research (ed S Gross, A Gee, D Worthington-White) (accepted for publication).

REFERENCES

Abdou NI, Abdou NL (1975) The monoclonal nature of lymphocytes in multiple myeloma. Effects of therapy. Ann Intern Med, 83, 42-45.

Alexanian R, Barlogie B, Tucker S. (1990) VAD-based regimens as primary treatment for multiple myeloma. Am J Hematol, 33, 86-89.

Alexanian R, Barlogie B, Dixon D. (1986) High-dose glucocorticoid treatment for resistant myeloma. Ann Int Med, 105, 8-11.

Anderson K, Barut B, Takvorian T et al. (1989) Monoclonal antibody (MoAb) purged autologous bone marrow transplantation for multiple myeloma (MM). Blood, 74, 202a.

Anderson KC, Park EK, Bates MP, Leonard RCF, Hardy R, Schlossman SF, Nadler LM. (1983) Antigens on human plasma cells identified by monoclonal antibodies. J Immunol, 130, 1132-1138.

Anderson KC, Jones RM, Morimoto C, Leavitt P, Barut BA (1989) Response pattern of purified myeloma cells to haemopoietic growth factors. Blood, 73, 1915-1924.

Anderson KC, Barut B, Ritz J et al. (1991) Monoclonal antibody purged autologous bone marrow transplantation therapy for multiple myeloma. Proceedings of the III International Workshop on Multiple Myeloma. 140-141.

Anonymous (1965) Malignant neoplasms of lymphatic and haemopoietic tissues: Multiple Myeloma (203). WHO Epidemiol Vital Stat Rep, 18, 414.

Aub JC, Tieslau C, Lankester A. (1963) Reactions of normal and tumor cell surfaces to enzymes 1. Wheat germ lipase and associated mucopolysaccharides. Proc Natl Acad Sci USA, 50, 613-619.

Baker PK, Rhodes EGH, Duguid JKM (1992) Effective concentration of bone marrow mononuclear cells using density gradient separation within an automated cell separator. Transfusion Sci, 13, 353-356.

Barlogie B and Gahrton G. (1991) Bone marrow transplantation in multiple myeloma. Bone Marrow Transplant, 7, 71-79.

Barlogie B, Epstein J, Selvanayagam P, Alexanian R. (1989) Plasma cell myeloma - new biological insights and advances in therapy. Blood, 73, 865-879.

Barlogie B, Smith L, Alexanian R. (1984) Effective treatment of advanced multiple myeloma refractory to alkylating agents. N Engl J Med, 310, 1353-1356.

Barlogie B, and Gahrton G. (1991) Bone marrow transplantation in multiple myeloma. Bone marrow transplantation, 7, 71-79.

Barlogie B, Alexanian R, Dicke KA, Zagars G, Spitzer G, Jagannath S, Horwitz L. (1987) High-dose chemoradiotherapy and autologous bone marrow transplantation for resistant multiple myeloma. Blood, 70, 869-872.

Barlogie B, Epstein J, Selvanayagam P, Alexanian R (1989) Plasma cell myeloma - new biological insights and advances in therapy. Blood, 73, 865-879.

Barlogie B, Hall R, Zander A, Dicke K, Allexanian R (1986) High Dose Melphalan with autologous bone marrow transplantation for multiple myeloma. Blood, 67, 1298-1301.

Barlogie B, Alexanian R, Smallwood L et al (1988) Prognostic factors with high dose melphalan for refractory multiple myeloma. Blood, 72, 2015-2019.

Barlogie B (1990) Management of multiple myeloma. Blut, 60, 1-7.

Barlogie B, Alexanian R, Dicke KA et al (1987) High-dose chemoradiotherapy and autologous bone marrow transplantation for resistant multiple myeloma. Blood, 70, 869-872.

Barlogie B, Alexanian R, Pershouse M, Smallwood L, Smith L (1985) Cytoplasmic immunoglobulin content in multiple myeloma. J Clin Invest, 76, 765-769.

Barlogie B, Jagannath S, Dixon DO, Cheson B, Smallwood L, Hendrickson A, Purvis JD, Bonnem E et al (1990) High-dose melphalan and granulocyte-macrophage colony-stimulating factor for refractory multiple myeloma. Blood, 76, 677-680.

Bast E, Van Camp B, Reynart P, Wiringa G, Ballieux R (1982) Idiotypic peripheral blood lymphocytes in human mmonoclonal gammopathy. Clinical Experimental Immunology, 47, 677-682.

Bast BJEG, BoomSE, Ballieux RE (1982) Characterization of the colony-forming cell in monoclonal gammopathies. Blood, 60, 608-612.

Bataille R, Jourdan M, Zhang X-G, Klein B (1989) Serum levels of interleukin 6, a potent myeloma cell growth factor, as a reflect of disease severity in plasma cell dyscrasias. J Clin Invest, 84, 2008-2011.

Bataille R, Grenier J, Sany J (1984) Beta-2-microglobulin in myeloma: optimal use for staging, prognosis, and treatment - a prospective study of 160 patients. Blood, 63, 468-476.

Belitsos PC, Hildreth JEK, August JT. (1990) Homotypic cell aggregation induced by anti-CD44 (Pgp-1) monoclonal antibodies and related to CD44 (Pgp-1) expression. J Immunol, 144, 1661-1670.

Bence Jones H. (1848) On a new substance occurring in the urine of a patient with Mollities Ossium. Phil Trans Roy Soc, 138, 55-62.

Berenson J, Wong R, Kim K, Brown N, Lichenstein A (1987) Evidence for peripheral blood B lymphocyte but not T lymphocyte involvement in multiple myeloma. Blood, 71, 1550-1553.

Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D,Knitter G, Buckner CD, Bernstein ID (1988) Antigen CD34 marrow cells engraft lethally irradiated baboons. Journal of Clinical Investigation, 81, 951-955.

Bergiu L, Schena M, Gardano G, Riva M, Caligaris-Cappio F (1989) Interleukin-3 and interleukin-6 synergistically promote the proliferation and differentiation of malignant plasma cell precursors in multiple myeloma. J Exp Med, 170, 613-618.

Bergsagel DE (1989) Is aggressive chemotherapy more effective in the treatment of plasma cell myeloma. Eur J Cancer Clin Oncol, 25, 159-161.

Bergsagel DE (1990) Plasma cell myeloma. Hematology, ed Williams and Beutler, 1114-1140.

Bergsagel DE, Bailey AJ, Langley GR et al (1979) The chemotherapy of plasma cell myeloma and the incidence of acute leukaemia. New Eng J Med, 301, 743-748.

Bergsagel DE, Sprague CC, Austin C, Griffith KM (1962) Evaluation of new chemotherapeutic agents in the treatment of multiple myeloma. IV: L-phenylalanine mustard. Cancer Chemother Rep, 21, 87-99.

Bernson J, Wong R, Kim K, Brown N, Lichtenstein A (1987) Evidence of peripheral blood B lymphocyte but not T lymphocyte involvement in multiple myeloma. Blood, 70, 1550-1553.

Bezkorovainy A, Springer GF, Desai PR. (1971) Physicochemical properties of the eel anti-human blood group H(O) antibody. Biochemistry, 10, 3761-3764.

Bird GWG (1964) Anti-T in peanuts. Vox Sang, 9, 748-749.

Boccadoro M, Marmont F, Tribalto M et al (1991) Multiple myeloma: VMCP/VBAP alternating combination chemotherapy is not superior to melphalan and prednisone even in high-risk patients. J Clin Oncol, 9, 444-448.

Boyd WC, Shapleigh E. (1954) Specific precipitating activity of plant agglutinins (lectins). Science, 119, 419-422.

Boyd WC, Reguera RM. (1949) Haemagglutinating substances for human cells in various plants. J Immunol, 62, 333-339.

Brenner MK, Vyarkarnam A, Reittie JE, Wimperis JZ, Grob JP, Hoffbrand AV, Prentice HG. (1987) Human large granular lymphocytes induce immunoglobulin synthesis after bone marrow transplantation. Eur J Immunol, 17, 43-47.

Brisco MJ, Condon J, Sykes PJ et al. (1991) Detection and quantitation of neoplastic cells in acute lymphoblastic leukaemia by use of the polymerase chain reaction. Br J Haematol, 79, 211-217.

Brooks ST and Melbye J. (1967) Skeletal lesions suggestive of Pre-Columbian multiple myeloma. Museum of Northern Arizona, Flagstaff, Arizona. Paleopathology 1, no. 7. Wade WD, editor, 23-29.

Brown G, Bunce CM, Howie AJ, Lord JM (1987) Stochastic or ordered lineage commitment during haemopoiesis. Leukaemia, 1, 150-155.

Buckner CD, Fefer A, Bensinger WI et al (1989) Marrow transplantation for malignant plasma cell disorders: summary of the Seattle experience. Eur J Haematol, 43, 186-190.

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

Caligaris-Cappio F, Bergui L, Gregoretti MG, Gaidano G, Gaboli M, Schena M, Zallone AZ, Marchisio PC (1991) Role of bone marrow stromal cells in the growth of human multiple myeloma. Blood, 77, 2688-2693.

Carter A, Merchow S, Sylvian-Draxler I, Tatarsky I. (1990) The role of interleukin-1 and tumour necrosis factor alpha in human multiple myeloma. Br J Haematol, 74, 424-431.

Ching CK, Rhodes JM (1988) Identification and partial characterisation of a new pancreatic cancer related glycoprotein by SDS-PAGE and lectin blotting. Gastroenterology, 95, 137-142.

Chiu EK, Ganeshaguru K, Hoffbrand AV, Mehta AB. (1989) Circulating monoclonal B lymphocytes in multiple myeloma. Br J Haematol, 72, 28-31.

Clofent G, Klein B, Commes J, Vincent C,Ghanem N, Lenoir G, Lefranc MP, Bataille R (1988) Limiting dilution cloning of B cells from patients with multiple myeloma. Int J Cancer, 43, 578-586.

Clofent G, Klein B, Commes T, Vincent C, Ghanem N, Lenoir G, Lefranc MP, Bataille R (1988) Limiting dilution cloning of B cells from patients with multiple myeloma: emergence of non-malignant B-cell lines. Int J Cancer, 43, 578-586.

Cooper EH, Forbes MA, Crockson RA et al (1984) Proximal renal tubular function in myelomatosis: observations in the fourth Medical Research Council trial. J Clin Pathol, 37, 852-858.

Cooper HS, Reuter VR (1983) Peanut lectin binding sites in polyps of the colon and rectum. Adenomas, hyperplastic polyps and adenomas with in situ carcinoma. Lab Investigation, 49, 655-659.

Cozzolini F, Torcia M, Aldinucci D, Rubartelli A, Miliani A Shaw R, Lansdorp AR, Di Guglielmo R. (1989) Production of interleukin-1 by bone marrow myeloma cells: Its role in the pathogenesis of lytic bone lesions. Blood, 74, 380-387.

Cuzick J, Cooper EH, MacLennan ICM. (1985) The prognostic value of serum beta2 microglobulin compared with other presentation features in myelomatosis (a report to the MRC working party on leukaemia in adults). Br J Cancer, 52, 1-6.

Cuzick J (1981) Radiation-induced myelomatosis. N Eng J Med, 304, 204-210.

Dalrymple J (1846) On the microscopical character of Mollities Ossium. Dublin J Med Sci, ii, 85-95.

Delamore IW (1982) Hypercalcaemia and myeloma. Br J Haematol, 51, 507-509.

Dewald GW, Kyle RA, Hicks GA,Greipp PR (1985) The clinical significance of cytogenetic studies in 100 patients with multiple myeloma, plasma cell leukaemia, or amyloidosis. Blood, 66, 380-390.

Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the proliferation of haemopoietic stem cellsin vitro. Journal of Cell Physiology, 91, 335-344.

Dinota A, Barbieri C, Gobbi M, Tazzari PL, Rizzi S, Bontadini A, Bolognesi A, Tura S, Stirpe F (1989) An immunotoxin containing momordin suitable for bone marrow purging in myeloma patients. Br J Cancer, 60, 315-319.

Dorshkind K (1986) In vitro differentiation of B lymphocytes from primitive haemopoietic precursors present in long term bone marrow cultures. J Immunol, 136, 422-429.

Durie BGM and Salmon SE (1982) The current status and future prospects of treatment for multiple myeloma. Clin Hematol, 11, 181-210.

Durie BGM, Grogan TM (1985) CALLA-positive myeloma: an aggressive subtype with poor survival. Blood, 66, 229-232.

Durie BGM, Salmon SE (1975) A clinical staging system for multiple myeloma. Cancer, 36, 842-854.

Durie BGM, Young LA,Salmon SE (1983) Human myeloma in vitro colony growth: Interrelationships between drug sensitivity, cell kinetics, and patient survival duration. Blood, 61, 929-934.

Durie BGM (1985) The malignant cell in multiple myeloma and Waldenstrom's macroglobulinaemia. Radl J, Higmans W, van Camp B (eds).

Durie BGM, Stock-Novak D, Salmon SE, Finley P, Beckord J, Crowley J, Coltman CA (1990) Prognostic value of pretreatment serum beta 2 microglobulin in myeloma: A Southwest Oncology Group study. Blood, 75, 823-830.

Durie BGM, Dalton WS (1988) Reversal of drug-resistance in multiple myeloma with verapamil. Br J Haematol, 68, 203-206.

Ehrlich P. (1889) Deutsch Med Wochenschr, 17, 976-979.

Elfstrand M (1898) Ueber Strychnos Ianceolaris Miq., die Stammpflanze des Blay-Hitam.. Arch. d. Pharm., Berl., 236, 100-104.

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

Erber WN, Pinching AJ, Mason DY (1984) Immunocytochemical detection of T and B cell populations in routine blood smears. Lancet, 128, 1042-1046.

Fauser AA, Messner HA (1978) Granuloerythropoietic colonies in human bone marrow, peripheral blood and cord blood. Blood, 52, 1243-1248.

Fauser AA, Messner HA (1979) Identification of megakaryocytes, macrophages and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood, 53, 1023-1027 .

Fefer A, Cheever MA, Greenberg PD (1986) Identical-twin (syngeneic) marrow transplantation for hematologic cancars. J Natl Cancer Inst, 76, 1269-1273.

Fermand JP, Levy Y, Gerota J, et al. (1989) Treatment of aggressive multiple myeloma by high-dose chemotherapy and total body irradiation followed by blood stem cell autologous graft. Blood, 73, 20-23.

Fermand J-P, Levy Y, Gerota J, Benbunan B, Cosset JM, Castaigne S, Seligmann M, Brouet J-C (1989) Treatment of aggressive multiple myeloma by high-dose chemotherapy and total body irradiation followed by blood stem cell autologous graft. Blood, 73, 20.

Fiedler W, Weh HJ, Suciu E, Wittlief C, Stocking C,Hossfeld DK. (1990) The IL-6 gene but not the IL-s receptor gene is occasionally rearranged in patients with multiple myeloma. Leukemia, 4, 462-465.

Fisher AG, Bunce CM, Toksoz D, Stone PCW, Brown G (1982) Studies of human myeloid antigens using monoclonal antibodies and variant lines from the pre-myeloid cell line HL-60. Clin Exp Immunol, 50, 374-381.

Gahrton G, Tura S, Ljungman P et al. (1991) Allogeneic bone marrow transplantation in multiple myeloma. N Eng J Med, 325, 1267-1273.

Gahrton G, Ringden O, Lonnqvist B, Lindquist R, Ljungman P (1986) Bone marrow transplantation in three patients with multiple myeloma. Acta Med Scand, 219, 523-527.

Gahrton G, Tura S, Belanger C et al (1989) Allogeneic bone marrow transplantation in patients with multiple myeloma. Eur J Haematol, 43, 182-185.

Gahrton G, Belanger C, Cavo M et al (1991) Allogeneic bone marrow transplantation in multiple myeloma-An EBMT registry study. Bone Marrow Transplant, 7, Suppl 2, 32.

Gallagher G, Christie JF, Stimson WH, Guy K, Dewar AE (1987) T-24 B-cell differentiation factor induces immunoglobulin secretion in human B cells without prior cell replication. Immunology, 60, 523-529.

Gallagher RP, Spinelli JJ, Elwood JM, Skippen DH (1983) Allergies and agricultural exposure as risk factors for multiple myeloma. Br J Cancer, 48, 853-857.

Garrett IR, Durie BGM, Nedwin GE et al (1987) Production of lymphotoxin, a bone-resorbing cytokine, by cultured human myeloma cells. N Eng J Med, 317, 526-532.

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.

Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H (1984) Cell-cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol, 133, 1710-1715.

Gianni AM, Siena S, Bregni M et al (1989) Granulocyte-macrophage colony-stimulating factor to harvest circulating haemopoietic stem cells for autotransplantation. Lancet, ii, 580-584.

Gobbi M, Cavo M, Tazzari PL et al. (1989) Autologous bone marrow transplantation with immunotoxin purged marrow for advanced multiple myeloma. (Suppl 51). Eur J Haematol, 43, 176-181.

Gobbi M, Caligaris-Cappio F, Campna D, Tazzari L, Bergui L, Cavo M, Tura S (1984) Functional behavior and immunological phenotype of circulating B lymphocytes in multiple myeloma. Studies with pokeweed mitogen. Clin Exp Immunol, 58, 625-630.

Goldstein IJ, Hughes RC, Monsigny M, Osawa J, Sharon N. (1980) What should be called a lectin? Nature, 285, 66-70.

Gonchoroff NJ, Katzman JA, Garton JP, Ruiz-Arguelles GJ, Eilers CR, Greipp PR, Kyle RA. (1986) A monoclonal antibody reactive with a subset of human plasma cells. Br J Haematol, 62, 619-630.

Gore ME, Selby PJ, Viner C et al (1989) Intensive treatment of multiple myeloma and criteria for complete remission. Lancet, 2, 879-882.

Gore ME, Selby PJ, Viner C, Clark PI, Meldrum M, Millar B, Bell J, Maitland JA, Milan S et al (1989) Intensive treatment of multiple myeloma and criteria for complete remission. Lancet, 2, 879-882.

Gorin NC, David R, Stachowick J et al (1981) High-dose chemotherapy and autologous bone marrow transplantation in adult leukaemias, malignant lymphomas and solid tumours. A study of 23 patients. Eur J Cancer, 17, 557-568.

Gould J, Alexanian R, Goodacre A, Pathak S, Hecht B, Barlogie B (1988) Plasma cell karyotype in multiple myeloma. Blood, 71, 453-456.

Grogan TM, Durie BGM, Spier CM, VAla E, Baum V, Rodriquez MA, Frutiger Y (1989) Myelomonocytic antigen positive multiple myeloma. Blood, 73, 770-776.

Grogan TM, Durie BGM, Lamen C, Spier C, Wist DP, Nagle R, Wilson GS, Richter L, Vela E et al (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.

Haimovitz A, Fuks Z, Galili N, Treves AJ (1982) Changes in peanut agglutinin binding to human monocytes during their maturation to macrophages. J Reticuloendothelial Soc, 31, 187-192.

Hall A, Proctor SJ, Harris AL (1986) Melphalan resistance in myeloma. Br J Haematol, 63, 1-6.

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

Hamilton MS, Barker HF, Ball J, Drew M, Abbot SD, Franklin IM (1991) Normal and neoplastic human plasma cells express bc(l)-2 antigen. Leukaemia, 5, 768-771.

Hansen OP, Galton DAG. (1985) Classification and prognostic variables in myelomatosis. Scand J Haematol, 35, 10-19.

Harousseau JL, Milpied N, Garand R, Bourhis JH (1987) High dose melphalan and autologous bone marrow transplantation in high risk myeloma. Br J Haematol, 67, 493.

Hewell GM, Alexanian R (1976) Myeloma in young persons. Ann Intern Med, 84, 441-443.

Horii Y, Muraguchi A, Suematsu S, Matsuda T, Yoshizaki K, Hirano T, Kishimoto T (1988) Regulation of BSF-2/IL-6 production by human mononuclear cells: Macrophage-dependent synthesis of BSF-2/IL-6 by T cells. J Immunol, 141, 1529-1535.

Horowitz MM,Gale RP, Sondel PM (1990) Graft-versus-leukaemia reactions after bone marrow transplantation. Blood, 75, 555-562.

Howard DR, Ferguson P, Batsakis JG (1981) Carcinoma associated cytostructural antigenic alterations: detection by lectin binding. Cancer, 47, 2872-2877.

Ichimaru M, Ishimaru T, Mikami M, Matsunaga M (1982) Multiple myeloma among atomic bomb survivors in Hiroshima and Nagasaki, 1950-76: Relationship to radiation dose absorbed by marrow. JNCI, 69, 323-328.

Ishimaru T, Finch SC (1979) More on radiation exposure and multiple myeloma. N Eng J Med, 301, 439-440.

Izaguirre CA, Minden MD, Howatson AF, McCulloch EA (1980) Colony formation by normal and malignant human B lymphocytes. Brit J Cancer, 42, 430-437.

Jacobson DR, Zolla-pazner S (1986) Immunosuppression and infection in multiple myeloma. Semin Oncol, 13, 282-290.

Jagannath S, Barlogie B, Dicke K et al. (1990) Autologous bone marrow transplantation in multiple myeloma: identification of prognostic factors. Blood, 76, 1860-1866.

Jalkanen ST, Bargatze RF, Herron LR, Butcher EC. (1986) A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. Eur J Immunol, 16, 1195-202.

Jeanpierre M (1987) A rapid method for the purification of DNA from blood. Nucleic Acids Res, 15, 9611.

Johnson WJ, Kyle RA, Dahlberg PJ. (1980) Dialysis in the treatment of multiple myeloma. Mayo Clin Proc, 55, 65-72.

Jonsson B. (1944) Blutengruppenstudien mit Japanischen und Schwedischen aalseren.. Acta Pathol Microbiol Scand Suppl, 54, 456-464.

Kaiser H, Stuart RK, Brookmeyer R, Beschorner WE, Braine HG et al. (1985) Autologous bone marrow transplantation in acute leukaemia: a phase I study of in vitro treatment of marrow with 4-hydroperoxycyclophosphamide to purge tumour cells. Blood, 65, 1504-1510.

Kaplan ME, Clark C (1974) Improved rosetting assay for detection of human T lymphocytes. J Immunol Methods, 5, 131-135.

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

Kawano M, Tanaka H, Ishikawa H, Nobuyoshi M, Iwato K, Asaoku H, Tanabe O, Kuramoto A (1989) Interleukin-1 accelerates autocrine growth of myeloma cells through interleukin-6 in human myeloma. Blood, 73, 2145-2148.

Kawano M, Yamamoto I, Iwato H, Asaoku H, Tanabe O, Ishikawa H, Nobuyushi M, Ohmoto Y et al (1989) Interleukin-1 beta rather than lymphotoxin as the major bone resorbing activity in human multiple myeloma. Blood, 73, 1646-1649.

King MA, Wells V (1981) Paraproteinaemia: use of idiotypic determinants as specific markers for tumour activity. Clin Exp Immunol, 45, 544-551.

King MA, Nelson DS (1989) Tumor cell heterogeneity in multiple myeloma: Antigenic, morphologic, and functional studies of cells from blood and bone marrow. Blood, 73, 1925-1935.

Kinkade P, Lee G, Pietrangeli CE, Hayashi SH, Gimble J (1989) Cells and molecules that regulate B lymphopoiesis in bone marrow. Ann Rev Immunol, 7, 111-115.

Klein B, Zhang X-G, Jourdan M, Content J, Houssian F, Aarden L, Piechaczyk M, Bataille R (1989) Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin 6. Blood, 73, 517-526.

Kubagawa H, Vogler CB, Capra JTJ, Conrad ME, Lawton AR, Cooper MD (1979) Studies on the clonal origin of multiple myeloma. J Exp Med, 150, 792-807.

Kurihara N, Bertolini D, Suda T, Akiyama Y, Roodman GD (190) IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. Immunol, 144, 4226-4230.

Kvalheim G, Sorenson O, Fodstad O, Funderad S, Kiesel S, Dorken B, Nustad K, Jakobsen E, Ugelstad JA (1988) Immunomagnetic removal of B-lymphoma cells from human bone marrow: a procedure for clinical use. Bone Marrow Transplantation, 3, 31-41.

Kyle RA, Nobrega FT, Kurland LT. (1969) Multiple myeloma in Olmstead County Minnesota, 1945-1964. Blood, 33, 739-745.

Kyle RA (1975) Multiple myeloma: Review of 869 cases. Mayo Clin Proc, 50, 29-40.

Kyle RA (1978) Monoclonal gammopathy of undetermined significance: Natural history in 241 cases. Am J Med, 64, 814-826.

Landsteiner K, Raubitschek H. (1909) Biochem Z, 15, 33-51.

Latov N, Sherman WH, Nemni R, Galassi G, Shyong JS, Penn AS, Chess L,Olarte MR, Rowland LP, Osserma. (1980) Plasma-cell dyscrasia and peripheral neuropathy with a monoclonal antibody to peripheral nerve myelin. N Eng J Med, 303, 618-621.

Lauwereys M, Foriers A, Sharon N, Strosberg AD. (1985) Sequence studies of peanut agglutinin. FEBS Lett, 181, 241-244.

Lehman TP, Cooper HS, Mulholland SG (1984) Peanut lectin binding sites in transitional cell carcinoma of the urinary bladder. Cancer, 53, 272-277.

Levin S, Russell EC, Blanchard D, McWilliams NB,Maurer HM, Mohanakumar T (1980) Receptors for peanut agglutinin (Arachis hypogaea) in childhood acute lymphocytic leukaemia: possible clinical significance. Blood, 55, 37-39.

Lewinsohn DM, Nagler A, Ginzton N, Greenberg P, Butcher EC (1990) Haematopoietic cells expression of the H-CAM (CD44) homing-associated adhesion molecule. Blood, 76, 589-595.

Liang R, Chan V, Chan TK et al. (1991) Detection of immunoglobulin gene rearrangement in acute and chronic lymphoid leukaemias of B-cell lineage by polymerase chain reaction. Am J Haematol, 38, 189-193.

Lis H, Sharon N (1986) Lectins as molecules and as tools. Annual Review of Biochemistry, 55, 35-67.

Lotan R, Skutelsky E, Darion D,Sharon N (1975) The purification, composition and specificity of the Anti-T lectin from peanut (Arachis hypogea). J Biol Chem, 250, 8518-8523.

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

MacIntyre W, (1850) Case of Mollities and Fragilitas Ossium, accompanied with urine strongly charged with animal matter. Med Chir Trans, 33, 211-233.

MacLennan ICM, Kelly K, Crockson RA, Cuzick J, Chapman C. (1988) Results of the /MRC myelomatous trials for patients entered since 1980. Hematol Oncol, 6, 145-158.

MacLennan ICM, Chapman C, Kanis J, Dunn J. (1991) Report on the MRC myelomatosis trials.

MacLennan ICM and Cusick J (1985) Objective evaluation of the role of VCR in induction and maintenance therapy for myelomatosis. Medical Research Council Working Party on leukaemia in adults. Br J Cancer, 52, 153-158.

Mandelli F, Avvisati G, Amadori S, Boccadoro M, Gernone A, Lauta VM, Marmont F, Petrucci MT et al. (1990) Maintenance treatment with recombinant interferon alpha 2b in patients with multiple myeloma responding to conventional induction chemotherapy. N Eng J Med, 322, 1430-1434.

Martin NH (1961) The incidence of myelomatosis. Lancet, 1, 237-239.

McNiece IK, Kriegler AB, Quesentissy PJ (1989) Studies on the myeloid synergistic factor from 5637: comparison with interleukin-1 alpha. Blood, 73, 919-923.

Medical Research Council working party on leukaemia in adults (1980) Prognostic features in the third myelomatosis trial. Br J Cancer, 42, 831-840.

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

Meltzer P, Shadle K, Durie BGM (1987) Somatic mutation alters a critical region of the c-myc gene in multiple myeloma. Blood, 70, 985A.

Millar B, Bell JBG, Lakhani A, Ayliffe MJ, Selby PJ, McElwain TJ (1988) A simple method for culturing myeloma cells from human bone marrow aspirates and peripheral blood in vitro. Brit J Haematol, 69, 197-203.

Miller RL (1983) Purification of peanut (Arachis hypogea) agglutinin isolectins by chromafocussing. Anal Biochem, 131, 438-446.

Morecki S, Paulotzky F, Margels P, Slavin S (1987) Purging breast cancer cells in preparation for autologous bone marrow transplantation. Bone Marrow Transplantation, 1, 357-260.

Morse D, Dailey RC, Bunn J (1974) Prehistoric multiple myeloma. Bull N Y Acad Med, 50, 447-458.

Morse D (1969) Ancient Disease in the Mid-West. Reports of investigations, no. 1. Springfield, Illinois State Museum, 144.

Najarian T, Castleman B (1979) Is low dose radiation associated with myeloma?. N Eng J Med, 300, 1278.

Nemunaitis J, Andrews FDF, Mochizuki DY, Lilly MB, Singer JW (1989) Human marrow stromal cells: response to interleukin-6 (IL-6) and control of IL-6 expression. Blood, 74, 1929-1935.

Neurohr KJ, Young NM, Mantsch HH. (1980) Determination of the carbohydrate binding properties of peanut agglutinin by ultraviolet difference spectroscopy. J Biol Chem, 255, 9205-9209.

Nowell PC. (1960) Phytohaemagglutinin: An initiator of mitosis in cultures of normal human leucocytes. Cancer Res, 20, 462-466.

Oken MM, Kyle RA, Greipp PR, Kay NE, Tsiatis A, O'Connell MJ (1990) Chemotherapy plus interferon (rIFN alpha2) in the treatment of multiple myeloma. Proc Am Soc Clin Oncol, 9, 288.

Pearce NE, Smith AH, Fischer DO (1985) Malignant lymphoma and multiple myeloma linked with agricultural occupations in a New Zealand Registry-based study. Am J Epidemiol, 121, 225-237.

Pereira MEA, Kabat EA (1976) Immunochemical studies on blood groups. Fractionation of hog and human A,H and AH blood group active substances on insoluble immunoabsorbents of Dolichos and Lotus lectins. J Exp Med, 143, 422-436.

Picker LJ, Medeiras LJ, Weiss LM, Waruke RA, Butcher EC (1988) Expression of lymphocyte homing receptor antigen in non-Hodgkin's lymphoma. Am J Pathol, 130, 496-504.

Pike BL, Robinson WA (1970) Human bone marrow colony growth in agar gel. J Cell Physiol, 76, 77-84.

Pilarski L, Mant MJ, Ruether BA (1985) Pre-B cells in peripheral blood of multiple myeloma patients. Blood, 66, 416-422.

Pilarski LM Andrews EJ, Mant MJ, Ruether BA (1986) Humoral immune deficiency in multiple myeloma patients due to compromised B-cell function. J Clin Immunol, 6, 491-501.

Pueppke SG (1981) Multiple molecular forms of peanut lectin: Classification of isolectins and isolectin distribution among genotypes of the genus Arachis.. Arch Biochem Biophys, 212, 254-261.

Reece DE, Barnett MJ, Connors JM et al (1989) Intensive therapy with busulfan, cyclophosphamide and melphalan (bucy + mel) and 4-hydroperoxycyclophosphamide (4HC) purged autologous bone marrow transplantation for multiple myeloma. Blood, 74, 754a.

Reiffers J, Marit G, Boiron JM. (1989) Autologous blood stem cell transplantation in high-risk multiple myeloma. Br J Haematol, 72, 296-298.

Reiffers J, Marit G, Boiron JM (1989) Autologous blood stem cell transplantation in high-risk multiple myeloma. Lancet, ii, 1336.

Reisner Y, Gachetin G, Dubois P, Nicholas JF, Sharon N, Jacob F. (1977) Develop Biol, 61, 20-27.

Reisner Y, Itzicovitch L, Meshorer A, Sharon N. (1978) Haemopoietic stem cell transplantation using mouse bone marrow and spleen cells fractionated by lectins. Proc Natl Acad Sci USA, 75, 2933-2936.

Reisner Y, Sharon N (1984) Fractionation of sub-population of mouse and human lymphocytes by peanut agglutinin or soybean agglutinin. Methods in Enzymology, 108, 168-179.

Reisner Y, Kapoor N, Hodes MZ, O'Reilly RJ, Good RA (1982) Enrichment for CFU-C from murine and human bone marrow using soybean agglutinin. Blood, 59, 360-363.

Renkonen KO (1948) Studies on haemagglutinins present in seeds of some representatives of the family of leguminoseae. Ann Med Exp Fenn, 26, 66-72.

Reynolds CP, Black AT, Woody JN (1986) Sensitive method for detecting viable cells seeded into bone marrow. Cancer Res, 46, 5878-5881.

Reynolds CP, Seeger RC, Vo DD, Black AT, Wells J, Ugelstad J (1986) Model system for removing neuroblastoma cells from bone marrow using monoclonal antibodies and magnetic immunobeads. Cancer Res, 46, 5882-5886.

Rhodes, EGH, Flynn, MP (1989) PNA shows specificity for bone marrow plasma cells. British Journal of Haematology, 71, 183 - 187.

Rhodes EGH, Olive C, Flynn MP (1990) A serum - free culture method for myeloma progenitors in vitro: proliferative and immunophenotypic characteristics. Experimental Hematology, 18, 79-83.

Richter LC, Grogan TM, Trent JM (1985) Establishment of two new myeloma cell lines from bilateral pleural effusions: Evidence for in vivo clonal change. Blood, 66, 548-552.

Rinsky R, Smith H, Hornung R, Fillon T, Young R, Okun A, Landrigan P (1987) Benzene and leukaemia. N Eng J Med, 316, 1044-1050.

Ritchie WA, and Warren SL (1932) The occurrence of multiple bony lesions suggesting myeloma in the skeleton of a pre-Columbian Indian. Amer J Roentgen, 28, 622-628.

Rosenberg M, Chitayat D, Tzehoval E, Waxdal MJ, and Sharon N. (1985) Detection and enumeration of monocytes in human blood with peanut agglutinin. J Immunol Methods, 81, 7-13.

Ruiz-Arquelles G, Katzman JA, Greipp PR, Gonchoroff NJ,Garton JP, Kyle RA (1984) Multiple myeloma: Circulating lymphocytes that express plasma cell antigens. Blood, 64, 352-356.

Salmon SE (1973) Immunoglobulin synthesis and tumour kinetics of multiple myeloma. Semin Hematol, 10, 135-142.

Salunke DM, Swamy MJ, Khan MI, Mande SC, Surolia A, Vijayan M. (1985) Arrangement of subunits in peanut lectin. J Biol Chem, 260, 13576-579.

Schedel I, Peest D, Stunkel K, Friche M, Eckert G, Deicher H (1980) Idiotype-bearing peripheral blood lymphocytes in human multiple myeloma and Waldenstrom's macroglobulinaemia. Scand J Immunol, 11, 437-444.

Selvanayagam P, Blick M, Narni F, van Tuinen P, Ledbetter D, Alexanian R, Saunders GF, Barlogie B (1988) Alteration and abnormal expression of the c-myc oncogene in human multiple myeloma. Blood, 71, 30-35.

Shimazaki C, Wisniewski D, Scheinberg DA, Atzpodien J, Strife A, Gulati S, Fried J, Wisniewolski R (1988) Elimination of myeloma cells from bone marrow by using monoclonal antibodies and magnetic immunobeads. Blood, 72, 1248-1254.

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol, 98, 503-517.

Spiegelberg HL, Fishkin BG (1976) Human myeloma IgA half-molecules. J Clin Invest, 58, 1259-1265.

Stillmark H (1889) In: Arbeiten des Pharmakologischen Institutes zu Dorpat. R Kobert (ed). (pp 57-62). Enke: Stuttgart.

Sullivan PW, Salmon SE (1972) Kinetics of tumour growth and regression in IgG multiple myeloma. J Clin Invest, 51, 1697-1708.

Sumegi J, Hedberg T, Bjorkholm M, Godal T, Mellstedt H, Nilsson MG, Perlman C, Klein G (1985) Amplification of the c-myc oncogene in human plasma cell leukaemia. Int J Cancer, 36, 367-371.

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

Tazzari PL, Gobbi M, Dinota A et al. (1987) Normal and neoplastic plasma cell membrane phenotype: studies with new monooclonal antibodies. Clin Exp Immunol, 70, 192-196.

Tedder TF, Clement LT, Cooper M (1984) Discontinuous expression of a membrane antigen (HB-7) during B lymphocyte differentiation. Tissue Antigens, 24, 140-149.

Terao T, Irimura T, Osawa T. (1975) Purification and characterisation of a haemagglutinin from Arachis hypogea. Hoppe-Seylers Z Physiol Chem, 356, 1685-1692.

Thomsen O (1927) Z Immunitaetsforsch, 52, 85-107.

To LB, Haylock DN, Kimber RJ, Juttner CA. (1984) High levels of circulating haemopoietic stem cells in very early remission from acute non-lymphoblastic leukaemia and their collection and cryopreservation. Brit J Haematol, 58, 399-410.

Tong AW, Lee JC, Stone MJ. (1987) Characterization of a monoclonal antibody having selective reactivity with normal and neoplastic plasma cells. Blood, 69, 238-245.

Tong AW, Lee JC, Fay JW, Stone MJ. (1987) Elimination of clonogenic stem cells from human multiple myeloma cell lines by a plasma cell-reactive monoclonal antibody and complement. Blood, 70, 1482-1489.

Tsuchiya H, Epstein J, Selvanayagam P, Dedman J, Gallick G, Alexanian R, Barlogie B (1988) Correlated flow cytometric analysis of H-ras, p21 and nuclear DNA in multiple myeloma. Blood, 72, 796-800.

Uhlenbruck G, Pardoe I, Bird GWG. (1969) On the specificity of lectins with a broad agglutination spectrum II. Studies on the nature of the T-antigen and the specific receptors for the lectin of Arachis hypogea (ground nut). Z Immunit Allerg Klin Immunol, 138, 423-433.

Van Beek W, Tulp A, Bolscher J, Blanken G, Roozendahl R, Egbers M (1984) Transient versus permanent expression of cancer-related glycopeptides on normal versus leukaemic myeloid cells coinciding with marrow egress. Blood, 63, 170-176.

van Camp B, Durie BGM, Spier C, De Waele M, Van Riet I, Vela E, Frutiger Y, Richter L, Grogan TM (1990) Plasma cells in multiple myeloma express a natural killer cell-associated antigen: CD56 (NKH-1; Leu-19). Blood, 76, 377-382.

van Riet I, Heirman R, Lacor P, de Waele M, Thielemans K, van Camp B (1989) Detection of monoclonal B lymphocytes in bone marrow and peripheral blood of multiple myeloma patients by immunoglobulin gene rearrangement studies. Br J Haematol, 73, 289-295.

Veelken H, Tycko B, Ritz J et al. (1991) Sensitive detection of clonal antigen receptor gene rearrangements for the diagnosis and monitoring of lymphoid neoplasms by a polymerase chain reaction-mediated ribonuclease protection assay. Blood, 78, 1318-1326.

Veerman AJP, Hogeman PHG, Huismans DR, Van Zantwijk CH, Bezeemer PD (1985) Peanut agglutinin, a marker for acute lymphoblastic leukaemia with a good prognosis. Cancer Res, 45, 1890-1893.

Walker L, Johnson GD, MacLennan ICM (1983) The IgG subclass responses of human lymphocytes to B-cell activators. Immunol, 50, 269-272.

Warburton P, Joshua DE, Gibson J, Brown RD (1989) CD10- (CALLA)- positive lymphocytes in myeloma: evidence that they are a malignant precursor population and are of germinal centre origin. Leuk and Lymphoma, 1, 11-20.

Warner TFCS, Krueger RG (1978) Circulating lymphocytes and the spread of myeloma. Review of the evidence. Lancet, 1, 1174-1176.

Watkins WM, Morgan WTJ. (1952) Neutralization of the anti-H agglutinin in eel serum by simple sugars. Nature, 169, 825-826.

Wearne A, Joshua DE, Kronenberg H (1986) CALLA-positive lymphocytes in the peripheral blood during progressive disease. Proc XXI Congress Int Soc Haematol, 313, 1457-1461.

Wysocki LJ, Sato VL (1978) "Panning" for lymphocytes. A method for cell selection. Proc Natl Acad Sci USA, 75, 2844-2848.

Zhang X-G, Bataille R, Jourdan M, Saeland S, Banchereau J, Mannoni P, Klein B (1990) Granulocyte-macrophage colony-stimulating factor synergizes with interleukin-6 in supporting the proliferation of human myeloma cells. Blood, 76, 2599-2605.

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Experimental Hematology

A Serum-free Culture Method for Myeloma Progenitors in vitro: Proliferative and Immunophenotypic Characteristics

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Abstract. Methods for forming multiple myeloma (MM) colonies are difficult because nonproliferative, but viable, plasma cells can survive for several weeks in culture and because MM cells tend to clump readily, forming pseudocolonies. The present study describes a method for growing pure myeloma colonies in serum-free conditions in which genuine myeloma growth is unequivocally demonstrated. Growth was observed in 17 of 32 MM bone marrow samples. After a delay of 3–5 weeks, during which most cells died, Ig light-chain-restricted colonies emerged, expanded for approximately 3 weeks, and then showed no evidence of further proliferation. Cell doubling time was 8–10 days, and a significant number of cells in all cultures expressed Ki-67, having earlier lacked this nuclear proliferation antigen. In addition, colony formation was abrogated by irradiation, and two of eight cultures were successfully replated in 0.8% methylcellulose. Phenotypic analysis revealed a mixed population of plasma cells (RFD6$^+$) and B-lymphocytes (CD19$^+$, CALLA$^-$), and cells were consistently Epstein-Barr virus negative. Culture of myeloma bone marrow by this serum-free method will allow appraisal of the role of various recombinant growth factors.

Key words: Human myeloma — Serum-free medium — Clonal proliferation — RFD6$^+$ — CD19$^+$

A tumor stem cell assay is an important tool in any malignancy for providing both a means of identifying growth-promoting factors and for yielding information about the tumor progenitor. With recent progress in the treatment of myeloma with ablative chemoradiotherapy accompanied by autologous bone marrow transplantation (ABMT) [1], the development of a reliable myeloma clonogenic assay has become even more relevant. Such an assay would aid in the selection of appropriate purging agents, and it would be of use in the detection of residual bone marrow disease following purged ABMT.

Techniques for growing multiple myeloma (MM) colonies in vitro are beset by particular difficulties. Myeloma cells can survive in vitro for a prolonged period in a nonproliferative state; they have a propensity to clump and form pseudocolonies. Early methods using specific batches of conditioned medium from mouse spleen cells have proved difficult to repeat [2, 3]. Other methods do not unequivocally demonstrate myeloma growth or exclude clumping [4, 5]. A later method of Takahishi et al. [6] demonstrated myeloma growth but has proved difficult to repeat and was not selective because the growth of mixed hemopoietic progenitors was also promoted. The very recent method of Millar et al. [7], which employs a feeder layer, yielded, by 3 weeks, lymphoplasmacytoid/plasma cell colonies in which cells expressed surface immunoglobulin and the common acute lymphoblastic leukemia antigen (CALLA; CD10) and PCA1 antigen.

In the present paper we describe a culture method enabling unequivocal growth of pure myeloma colonies with different growth and phenotypic characteristics from those of Millar et al. [7]. Our method, which employs serum-free conditions, has potential both for testing the effect of growth factors on myeloma cells and for monitoring the efficacy of purging techniques.

Materials and methods

Patients. Bone marrow was derived from diagnostic aspirates from patients with newly diagnosed untreated myeloma ($n = 25$) and from patients with established myeloma who had relapsed following a period of at least 3 months without treatment. One patient had a solitary plasmacytoma of bone, and a further patient had monoclonal gammopathy of undetermined significance (MGUS). Normal bone marrow samples were taken at the time of sternotomy from fully informed, consenting, hematologically normal patients undergoing coronary artery bypass surgery. Ethical committee approval from Broadgreen Hospital had been granted.

Cell preparations. Bone marrow samples were taken into a small volume of Iscove's modified Dulbecco's medium (IMDM) containing preservative-free heparin at a final concentration of 10 μm/ml. Density gradient centrifugation over Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) (density = 1.077 g/cm^3) yielded mononuclear cells, which were washed twice.

Monocytes and T-lymphocytes were removed by plastic adherence and double AET-treated sheep erythrocyte rosetting (B-lymphocytes >95% by CD19 staining; monocytes <1% by morphology and CD14 reactivity [8]).

Izaguirre's method. Bone marrow was cultured for myeloma colonies as described by Izaguirre et al. [4] with minor variations as follows: mononuclear cells were suspended in concentrations ranging from 5×10^3/ml to 5×10^5/ml in IMDM with 0.8% methylcellulose (1500 cp; Sigma Chemicals Limited, Poole, UK), 30% fetal calf serum, and 5%–20% conditioned medium from phytohemagglutinin (PHA)-stimulated T-lymphocytes (PHA-TCM) (see below). The suspensions were mixed thoroughly in a vortex mixer before being plated out in 0.1-ml aliquots into wells of 96-well microtiter plates (Northrumbrian Biologicals Limited, Cramington, UK). Five rep-

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licates were made for each experiment. The plates were covered and incubated at 37°C in a humidified atmosphere enriched with 5% CO_2 in air.

Growth promoters. PHA-TCM. PHA-TCM was prepared as described previously [4] by separating T-lymphocytes with a sheep erythrocyte rosetting technique and incubating them at a concentration of 10^6/ml with 1% PHA in IMDM at 37°C for 3 days. The culture supernatants were collected, filtered through 0.3-μm filters, and stored at −20°C.

Helper (H^+) T-cell-conditioned medium (H^+-TCM). H^+-TCM was prepared from PHA-stimulated $OKT4^+$ T-helper lymphocytes obtained by an indirect panning technique [9]. Briefly, bacteriological grade 35-mm plates (Sterilin) were coated with 5 μg/ml goat anti-mouse Ig (Serotec, Kidlington, UK) in 10 ml of 50 mM Tris buffer, pH 9.5, for 1 h at room temperature. The plates were washed three times with phosphate-buffered saline (PBS) and once with PBS supplemented with 1% heat-inactivated fetal calf serum (FCS). Erythrocyte-positive rosetted T-lymphocytes (3×10^6/ml) from normal human peripheral blood were incubated at room temperature for 20 min with 200 μg/ml Leu2 (anti-T-suppressor cell; Becton Dickinson). The cells were washed three times in ice-cold PBS and resuspended in PBS supplemented with 5% FCS. Cells from this suspension (10^7) were plated in 2 ml onto the antibody-coated polystyrene dishes and incubated for 1 h at 4°C, with occasional gentle mixing. The cells were decanted by tilting the plates and gently washing and swirling these twice with 3 ml PBS/5% FCS to remove all nonadherent cells; this yielded >90% $Leu3^+$ cells. $Leu3^+$ T-helper cells were incubated with 1% PHA as above to make H^+-TCM. This was added to various myeloma cultures at 10%–20% vol/vol instead of PHA-TCM.

Myeloma culture supernatants. Supernatants from myeloma bone marrow cells cultured for 48 h in IMDM with 5% FCS were collected, filtered, and added at 10%–20% vol/vol to various myeloma cultures.

Low molecular weight B-cell growth factor (LMW BCGF). Partially purified LMW BCGF (12 kd) was obtained from Cellular Products (Buffalo, New York) and added to myeloma bone marrow in liquid cultures. For a positive control it was established that 10% LMW BCGF induced optimal proliferation in normal tonsil purified B cells activated with suboptimal doses of insoluble anti-μ (10 μg/ml).

Conditioned medium from C5637 and T-24 cell lines. Conditioned media from the C5637 and T-24 bladder carcinoma cell lines (kindly given by Dr. Fogh, Sloan Kettering Memorial Center, New York, and Dr. R. Callard, Institute of Child Health, London, respectively) were added to various cultures of myeloma bone marrow cells. The C5637 supernatant acts on primitive hemopoietic cells and contains a synergistic factor (SF-1) which, together with macrophage colony-stimulating factor, is capable of stimulating high proliferative-potential colony-forming cells [10]. The T-24 supernatant contains a B-cell differentiation factor (BCDF) that can stimulate immunoglobulin secretion in normal human B cells without necessarily causing clonal expansion [11].

The conditioned media were added at 10%–20% vol/vol both singly and in combination to liquid cultures of Ficoll-separated myeloma bone marrow cells (see below) and to feeder layer cultures as described.

Liquid cultures. Ficoll-separated myeloma bone marrow cells were seeded at 5×10^5/ml to 10^6/ml in 25-mm^3 tissue culture flasks (Flow Laboratories, Rickmansworth, UK) in IMDM supplemented with various concentrations of serum and/or growth promoter. At intervals the entire culture was centrifuged, and the cells were resuspended using a fine-gauge needle before counting (using trypan blue dye exclusion to count viable cells) or replating as required.

Feeder layer techniques. Irradiated (20 Gy) autologous T-lymphocytes, with and without adherent cells, were immobilized in 0.5% agar (Difco) containing IMDM supplemented with 10%–30% FCS. Feeder layers prepared from normal bone marrow samples using methyl prednisolone [12] and by the method of Dexter et al. [13] were prepared for overlayer with myeloma cells. T-lymphocyte-depleted and adherent cell-depleted myeloma bone marrow cells were mixed with 0.8% methylcellulose, IMDM, and various growth promoters such as PHA-TCM, H^+-TCM, or C5637- or T-24-conditioned medium, and various sources of serum. Cultures were incubated at 37°C; colonies forming in the methylcellulose were picked off individually using a finely drawn Pasteur pipette and stained with a Romanowsky stain.

A further feeder layer technique was adapted from the murine long-term bone marrow cultures of Dorshkind [14], in which B-lymphocytes were induced to grow by transferring the cultures to Whitlock conditions after 2 weeks. Bone marrow aspirate cells from normal or myeloma bone marrows were cultured at 2×10^6 cells/ml in a 25-cm^3 tissue culture flask (Falcon) containing 5 ml IMDM supplemented with 10% FCS (Biological Industries, Glasgow, UK), 10% horse serum (Imperial Laboratories, Salisbury, UK), 10^{-6} M hydrocortisone sodium succinate, 5×10^{-5} M 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin. The flasks were cultured at 37°C in a humidified incubator enriched with 5% CO_2. After 2 weeks, or when an adherent layer was established, all culture medium was removed, and the nonadherent cells were resuspended in either RPMI-1640 medium supplemented with 5% FCS or DCCM.1 culture medium (Biological Industries). Thereafter cultures were fed at weekly intervals by removing 50% of the medium and replacing it with fresh medium.

Low serum conditions. Ficoll-separated mononuclear cells from myeloma bone marrow were suspended at 5×10^5 to 2×10^6 cells/ml in DCCM.1 medium supplemented with 2% bovine serum albumin (BSA, fraction V; Sigma) and transferrin (25 mg/liter) in 25-cm^3 flasks and cultured at 37°C. Weekly cell counts were performed, and cytospin preparations were made to examine morphological and phenotypic changes over time.

Epstein-Barr virus (EBV) studies. Cultures that yielded myeloma colonies were examined by fluorescence for the presence of the Epstein-Barr nuclear antigen (EBNA). These studies were kindly performed by Dr. C.G. Woodward at Leeds Public Health Laboratories.

Alkaline phosphatase–anti-alkaline phosphatase (APAAP) staining technique. Cytospin preparations (10^5 well-separated cells per slide) were fixed in acetone for 2 min and stained by APAAP [15] with the following first-layer monoclonal antibodies at concentrations previously determined to give optimal results: anti-K, anti-λ, LeuM3 (anti-monocyte), Leu12 (anti-CD19), Leu3 (anti-T cell) (all from Becton Dickinson); anti-CD38 was a kind gift from Dr. Tedder, Dana Farber Institute; and anti-RFD6 was used by kind permission of Professor Janossy, Royal Free Hospital, London. Irrelevant control first-layer antibodies of the appropriate isotype were always included. The second-layer and third-layer antibodies consisted of rabbit anti-mouse Ig and mouse anti-alkaline phosphatase–alkaline phosphatase conjugates, respectively. Alkaline phosphastase activity was then detected with naphthol AS-MX phosphatase (9.2 mg/ml) substrate and fast red TR (1mg/ml) as capture agent; levamisole (0.2 M) was added to inhibit endogenous alkaline phosphatase activity. Cells were formally counterstained with hematoxylin and eosin.

Immunoglobulin gene rearrangement studies. Genomic DNA was extracted from peripheral blood lymphocytes or Ficoll-separated bone marrow cells by the method of Jeanpierre [16]. The concentration and purity were determined by spectrophotometry.

Genomic DNA (10 μg) was digested with the restriction enzymes ECOR1 and BamH1 and subjected to electrophoresis in 0.8% agarose slab gels according to the method of Southern [17]. Hybridization was carried out at 65°C in a solution of 5× saline sodium phosphate ethylenediaminetetraacetate containing 0.1% sodium dodecyl sulfate (SDS), 0.1% bovine serum albumin (BSA), 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 100 μg/ml denatured herring sperm DNA, and 0.05–0.10 μg of the following nick-translated ^{32}P-labeled probes: J_H, C_K, and C_λ (kind gift of Professor T. Rabbits, MRC Unit, Addenbrookes Hospital, Cambridge, UK). Filters were washed at 65°C for 30 min each in the following solutions: 2× saline sodium citrate (SSC), 2× SSC plus 0.1% SDS, and finally 0.1× SSC and 0.1% SDS.

Results

Pseudocolony formation by MM marrow

When marrow was cultured at high density (>5×10^4 cells/ml in 200 μl) with PHA-TCM in round-bottomed microtiter

plates according to the method of Izaguirre et al. [4], rapid formation of apparent myeloma colonies occurred, sometimes within 48 h. Such colonies consisted of light-chain-restricted plasma cells, and persisted for up to 4 weeks. Furthermore, the colonies were apparently replatable in that colonies reformed from a single-cell suspension of pooled colonies.

However, when marrow was cultured at low density (1–5 $\times 10^3$ cells/ml in 200 μl), no colony formation was observed. Furthermore, in cytocentrifuge preparations pooled from the apparent colonies, no dividing cells were seen morphologically and none expressed the nuclear proliferation antigen detected by Ki-67. Irradiation (20 Gy) failed to abrogate colony formation in two of three experiments; in the third, cells failed to form colonies but appeared nonviable.

It was therefore concluded that the "colonies" observed at high cell density in this method resulted from clumping rather than from cell division, a conclusion recently also drawn by Boom et al. [18]. Further culture models were therefore explored.

Culture conditions unsuccessful in producing myeloma growth

Growth promoters. No evidence of selective myeloma growth was obtained with any of the growth promoters (see *Materials and methods*) tested. Some clumps/colonies of plasma cells were observed, but either T-cell or myeloid colonies were so numerous that accurate assessment of putative myeloma colonies was not possible.

Serum. None of the different sources and batches of serum produced evidence of myeloma growth, although markedly different numbers of myeloid colonies were observed.

Different feeder layers. Again, there was no selective growth of myeloma cells. Individual clumps/colonies containing plasma cells were observed among myeloid and T-cell colonies, but when such clumps were examined morphologically they never consisted exclusively of light-chain-restricted myeloma cells.

Under conditions similar to the Whitlock-Witte model for murine B-lymphopoiesis [14], selective growth of B-cell/plasma cell colonies was observed in one case of MGUS, one solitary plasmacytoma, and one MM. In these three cases, long-term bone marrow cultures were initiated, and after 2 weeks, by which time a confluent stromal layer had formed, nonadherent cells were discarded and the culture medium replaced with RPMI-1640 medium plus 5% FCS (MGUS) or DCCM.1 (plasmacytoma and MM bone marrow cultures). After a delay of 6–11 weeks, small clusters followed by colonies of 50–100 cells appeared in the nonadherent fraction. Analysis of pooled colonies, which were negative for EBNA fluorescence, revealed that >95% of cells were of B-cell origin, but were composed of both K-, and λ-expressing cells. It was thought that this might represent an outgrowth of normal B-cell clones; this prompted a study of low-serum conditions in MM.

Successful growth of myeloma colonies

Growth under low-serum conditions. When Ficoll-separated myeloma bone marrow cells were incubated in DCCM.1 nutrient medium containing 2% BSA and transferrin (25 mg/liter), 17 of 32 samples formed what proved to be pure myeloma colonies.

The pure clonal nature of growth. In APAAP preparations, the colonies clearly consisted of light-chain-restricted B-/plasma cells, with no myeloid and very few T cells (Table 1).

In the three cultures studied before and after culture, identical JH and light-chain rearrangements were observed after culture.

A small proportion of T-lymphocytes (1.5% ± 2%) was present in the majority (13 of 17) of the cultures during the proliferative phase. In no case was there evidence of T-cell colony formation, and there was an average 90% reduction in relative numbers of T-lymphocytes during the period of culture. However, in 5 of 32 myeloma bone marrow samples, the Ficoll-separated cells were rigorously T-cell depleted prior to culture, and none of these 5 samples subsequently showed any signs of clonal growth.

Characteristics of the myeloma cell growth

All 17 cultures in which growth was observed displayed similar growth patterns, with an initial lag phase of 3–4 weeks during which there was a rapid diminution in cell numbers (Fig. 1). At this stage, when viable cells comprised mainly lymphocytes and plasma cells, and no adherent layer had formed, clusters and clumps (>3 cells) appeared and slowly grew in size to colonies of approximately 30–70 cells. After a further 3 weeks, there was no additional increase in colony size, the numbers of viable cells rapidly diminished, and the culture died (Fig. 1). During the period of active cell growth, cells doubled in number in 8–10 days. During the first 3–4 weeks of culture the pH of the culture remained constant and the medium was not changed; thereafter, the cultures became acid, and the medium was changed weekly.

The observed myeloma cell growth was attributable to cell proliferation and not to clumping. During the period of active cell growth (i.e., after approximately 4 weeks in culture), an overall increase in cell numbers was recorded in each case.

Irradiation (20 Gy) of bone marrow at the outset of culture completely abrogated growth, whereas at the same time myeloma grew from the nonirradiated controls (three experiments) after the usual 4-week lag phase.

Mitoses/Ki-67. Mitotic figures (2%–5%) were consistently observed in 13 of 17 cultures. In APAAP preparations these mitoses were shown to be in cells of B lineage and no mitoses were observed in the occasional T cell present in the culture.

There was a marked increase in the numbers of cells in growth cycle, as reflected in increased numbers of Ki-67-positive nuclei. Some of these Ki-67 cells were undoubtedly plasma cells, but others could have been earlier in B-cell development.

Replating. In 3 of 17 cultures, cells from developing colonies were withdrawn and pooled so that colonies were broken up; the cells were then recultured at 5×10^5/ml in the same medium. The recultured cells were counted at intervals and

Table 1. Phenotype of myeloma bone marrow cells before and after culture in serum-free medium

	Phenotype of colonies in 17 samples (percent)						
	Morphological plasma cells	Dominant light chain	RFD6	CD19	CALLA	CD3	Ki-67
Before culture	47 ± 17	56 ± 14	46 ± 16	8 ± 7	2 ± 6	11 ± 6	4 ± 5
After culture (4–6 weeks)	66 ± 14	94 ± 6	68 ± 14	26 ± 14	1 ± 4	1 ± 1	15 ± 9

Note the increase in percent of $CD19^+$ and $Ki\text{-}67^+$ (proliferative) cells and the low expression of the CALLA. The $CALLA^+$ cells were all from one case of plasma cell leukemia that had 15% $CALLA^+$ cells in the bone marrow at diagnosis. After 5 weeks in culture there were still $CALLA^+$ cells in the colonies analyzed.

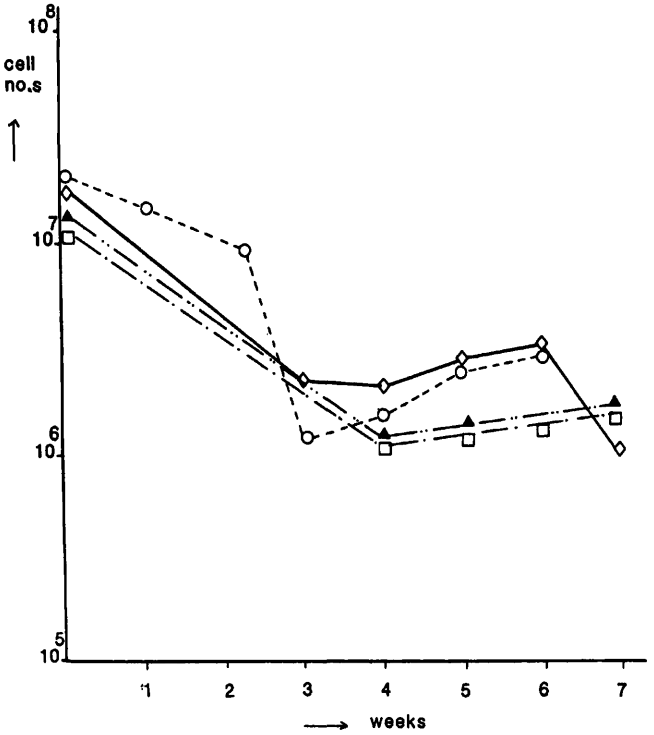

Fig. 1. Cell numbers during culture of MM bone marrow (four representative experiments). Total cell numbers fell markedly during the first 3 weeks of culture as the non-B cells died. After ~3 weeks, a modest increase in cell numbers was observed, and these cells were clonal B/plasma cells.

were shown to have a doubling time of 8–10 days. Eight of 17 cultures were replated into 0.8% methylcellulose, but only 2 of these formed colonies after a further 3 weeks. Two additional cultures were replated in limiting dilutions, but no colonies ensued. It appeared to be crucial to replate cultures early on in the growth phase because no culture was successfully replated after the first week of the growth phase.

EBV studies.

Colony cells from 5 out of the 17 growing myeloma cultures were pooled and examined for EBNA membrane fluorescence, and they were all negative. In the five cultures examined, all cells were negative for EBNA. These cultures were very similar in their growth and phenotypic characteristics to those not examined for EBNA.

In 3 of the 15 samples in which MM growth was not obtained, colonies with very different growth characteristics emerged. Their growth rate was considerably faster (doubling time 2–4 days) and the colony size was much greater, frequently attaining >500 cells. One hundred percent of the cells from these three cultures were strongly positive for EBNA, and the cultures have continued to grow vigorously ever since (>6 months). The differences between the EBV-positive and -negative cultures were strikingly obvious: a shorter lag period before colonies appeared, larger colony size, greater proliferative rate, and infinite self-renewal potential. Phenotyping studies showed expression of CD19 and PNA on the same cells, whereas in myeloma colonies cells were either PNA^+ $CD19^-$ or vice versa. Finally, cytoplasmic Ig light- and heavy-chain expression was different from that of the original myeloma samples, suggesting that EBV transformation of normal B-lymphocytes had occurred.

Phenotype of MM colonies

When clusters and colonies first appeared, the cultures showed a mixture of $CD19^+$ lymphocytes and plasma cells staining with RFD6 and CD38. After 3 weeks of proliferation >90% of the cells had the morphological appearance of plasma cells and were $CD19^-$. $CALLA^+$ cells were only detected in one culture (no. 9, Table 1) from a patient with plasma cell leukemia who had 15% $CALLA^+$ cells in the bone marrow at diagnosis.

Discussion

The present study clearly shows that nonfractionated bone marrow from approximately 50% of patients with untreated myeloma will grow in vitro under low serum conditions, without a feeder layer and without exogenous growth factors. Great care was taken to demonstrate that growth involved clonal plasma cells, that cell proliferation rather than clumping was actually taking place, and that cells were not transformed by EBV. Clonality was established by the demonstration of both light-chain restriction and clonal gene rearrangement. Actual proliferation occurred because increasing cell numbers were directly demonstrated, because cells were shown to contain mitoses and the Ki-67 nuclear proliferation antigen, and because irradiation abolished growth.

We are not certain why the present culture system succeeded in producing pure myeloma cell growth when other systems have not clearly done so. The absence of T-cell growth factors was clearly important in preventing the outgrowth of T cells, but the absence of inhibitory factors present in serum or in the T-cell-conditioned medium used by many other authors may also have contributed. It is also possible that the serum-free medium contains some factor essential for

myeloma cell growth. It is also perhaps worth noting that small numbers of T cells were consistently present in our cultures and it cannot be ruled out that they were able to produce factor(s) contributing to growth. Clearly, though, whatever the factor(s) contributing to successful growth, the culture conditions were not ideal because growth was demonstrated in only about half the cases tested. In this regard, it will be interesting to test the effect on our culture system of recombinant cytokines such as interleukin 6 (IL-6), IL-1, IL-4, IL-7, etc.

The present work indicates that plasma cells and B cells have proliferative potential in myeloma because both cell types containing the Ki-67 nuclear proliferation antigen were observed. Furthermore, because substantial numbers of non-plasmacytoid B cells were present at 4 weeks, whereas after 6 weeks most cells were plasma cells, we suggest that these B cells were myeloma precursors. Such cells, however, consistently lacked the CALLA and we therefore could find no evidence in support of previous suggestions that the myeloma progenitor is CALLA positive [19].

A notable feature of the present culture system was the long lag phase before colonies of B/myeloma cells emerged. The significance of this lag phase is not known, but it may indicate that production of growth factor by a minority cell type is necessary for successful growth. Also, of course, the consistent presence of this lag phase provided further evidence ruling out cell clumping as a possible explanation of our findings.

The culture method described here provides a means of examining the effect of a whole range of cytokines on myeloma cell growth and therefore of defining further the factors important in the control of B-cell growth and differentiation. Furthermore, particularly if the method can be improved (e.g., by addition of exogenous growth factors) to allow growth from the majority of cases of myeloma, it will be a valuable tool in assessing the efficacy of purging methods in autologous bone marrow transplantation programs for this disease.

References

1. Barlogie B, Alexanian R, Dicke KA, Zagars G, Spitzer G, Jagannath S, Horwitz L (1987) High dose chemoradiotherapy and autologous bone marrow transplantation for resistant multiple myeloma. Blood 70:869
2. Hamburger A, Salmon SE (1977) Primary bioassay of human myeloma stem cells. J Clin Invest 60:846
3. Durie BGM, Young LA, Salmon SE (1983) Human in vitro colony growth: interrelationships between drug sensitivity, cell kinetics and patient survival duration. Blood 61:929
4. Izaguirre CA, Minden MD, Howatson AF, McCulloch EA (1980) Colony formation by normal and malignant human B lymphocytes. Br J Cancer 42:430
5. 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
6. Takahishi T, Lim B, Jamal N, Tritchler D, Lockwood G, McKinney S, Bergsagel DE, Messner HA (1985) Colony growth and self renewal of plasma cell precursors in multiple myeloma. J Clin Oncol 3:1613
7. Millar BC, Bell JBG, Lakhani A, Ayliffe MJ, Selby PJ, McElwain TJ (1988) A simple method for culturing myeloma cells from human bone marrow aspirates and peripheral blood in vitro. Br J Haematol 69:197
8. Kaplan ME, Clark C (1974) Improved rosetting assay for detection of human T lymphocytes. J Immunol Methods 5:131
9. Wysocki LJ, Sato VL (1978) Panning for lymphocytes: a method for cell selection. Proc Natl Acad Sci USA 75:2844
10. McNiece IK, Kriegler AB, Quesentissy PJ (1989) Studies on the myeloid synergistic factor from 5637: comparison with interleukin-1 alpha. Blood 73:919
11. Gallagher G, Christie JF, Stimson WH, Guy K, Dewar AE (1987) T-24 B-cell differentiation factor induces immunoglobulin secretion in human B cells without prior cell replication. Immunology 60:523
12. Gordon MY, Hibbin JA, Kearney LU, Gordon-Smith EC, Goldman JM (1985) Colony formation by primitive haemopoietic progenitors in co-cultures of bone marrow cells and stromal cells. Br J Haematol 60:197
13. Dexter TM, Allen TD, Lajtha LG (1976) Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91:335
14. Dorshkind K (1986) In vitro differentiation of B lymphocytes from primitive haemopoietic precursors present in long term bone marrow cultures. J Immunol 136:422
15. Erber WN, Pinching AJ, Mason DY (1984) Immunocytochemical detection of T and B cell populations in routine blood smears. Lancet 128:1042
16. Jeanpierre M (1987) A rapid method for the purification of DNA from blood. Nucleic Acids Res 15:9611
17. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503
18. Boom SE, Lokhorst HM, Bast EJEG (1988) Myeloma colony formation in a semisolid fluid culture system [abstr]. Second Eurage symposium on monoclonal gammopathies, Brussels
19. Grogan TM, Durie BGM, Lamen C, Spier C, Wist DP, Nagle R, Wilson GS, 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

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A method for clinical purging of myeloma bone marrow using peanut agglutinin as an anti-plasma cell agent, in combination with CD19 monoclonal antibody

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Summary:

Previous studies have shown that the lectin peanut agglutinin (PNA) binds bone marrow plasma cells in the majority of patients with myeloma and does not bind to normal haemopoietic progenitors This lectin has been used in combination with anti-CD19 monoclonal antibody (moAb) in a system for purging myeloma bone marrow. This has now been scaled up for application to *ex vivo* treatment of large volumes of bone marrow suitable for autologous bone marrow transplantation. Four bone marrow harvests from patients with myeloma containing 9.5 ± 4.9% plasma cells were depleted of erythrocytes and mature granulocytes by Ficoll separation using the Haemonetics V50 cell separator. The mononuclear fraction was then purged with magnetic beads coated with PNA and anti-CD19 moAb. The system proved highly efficient with removal of all detectable plasma cells and CD19$^+$ cells. Average mononuclear cell recovery following purging was 71% of the concentrated marrow with 78% yield of CFU-GM. Normal progenitor recovery related to patients' weight is predicted to be adequate for haemopoietic reconstitution following ablative chemoradiotherapy. This system is therefore feasible for large-scale clinical purging.

Complete remission has been demonstrated in patients with myeloma treated with high doses of alkylating agents.[1] Autologous bone marrow rescue has since been used together with these more ablative regimes[2] to reduce the treatment-related morbidity. Unfortunately most patients relapse following autologous bone marrow transplant (ABMT) so attention has recently been focused on attempts to remove myeloma cells selectively from the autograft. Strategies for purging have included monoclonal antibodies (moAbs) against plasma cells such PCA-1[3] and MM4[4] and those such as 8A[5] whose specificity includes cells earlier in B cell development. The removal of plasma cells alone may not be adequate as there is evidence that pre-B cells[6,7,8] or even earlier stem cells[9] may be involved in the myeloma clone.

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We have previously shown that the lectin peanut agglutinin (PNA) binds to bone marrow plasma cells but not to haemopoietic progenitors.[10] Small-scale experiments using PNA bound to magnetic beads have shown that it is possible to remove all detectable plasma cells from myeloma bone marrow while sparing normal progenitors, and in combination with CD19-coated magnetic beads, all detectable B lymphocytes may also be removed.[11] We report here large-scale experiments on human myeloma bone marrow to test the feasibility of using PNA-coated and CD19-coated magnetic beads to purge myeloma marrow prior to re-infusion.

Methods

Patients

Four patients with myeloma were studied. Patient characteristics are shown in Table I. The initial disease had been treated with a variety of widely-used chemotherapy schedules and only patients whose bone marrow showed less than 20% plasma cells were considered for high-dose therapy and rescue with purged autologous bone marrow.

Bone marrow harvest

Written informed consent was given by each patient to undergo bone marrow harvest under general anaesthesia. Bone marrow was aspirated in multiple 3–5 ml volumes from the posterior iliac crests and transferred to a 2000 ml blood collection bag containing 150 ml acid/citrate/dextrose (Formula A) (ACD-A). A leucocyte-enriched product was prepared on a Haemonetics V50 cell separator; 40 ml buffy coat were collected at each pass using the lymphocytapheresis two-arm procedure programme, 240 ml being collected in a total of six passes.

Bone marrow mononuclear cells were further concentrated by a density centrifugation step using Lymphoprep (Pharma, Oslo) to a total of 150 ml. Bone marrow buffy coat cells were pumped into the centrifuge bowl and after addition of 50 ml Lymphoprep, the centrifuge speed was increased to 5600 rpm before restarting the pump and adding the remaining Lymphoprep.

Table I Characteristics of patients undergoing bone marrow harvest

Patient number	1	2	3	4
Age (years)	51	39	44	50
Serum paraprotein (g/l)	53	66	BJP	76
BM plasma cells at diagnosis (%)	30	7	11	45
BM plasma cells at harvest (%)	6	13	4	15
Prior therapy	VAMP	ABCM	VAD	ABCM

VAMP = vincristine, adriamycin, methylprednisolone; ABCM = adriamycin, BCNU, cyclophosphamide, melphalan.
VAP = vincristine, adriamycin, dexamethasone.

The mononuclear cells were then washed by means of the Haemonetics V50 Cell Washing Protocol, first with 500 ml normal saline, then with 500 ml human albumin solution (BPL, Elstree) and finally with 500 ml Iscove's Modified Dulbecco's Medium (IMDM) containing ACD-A and human albumin solution (20% vol/vol). The final volume of washed mononuclear cells was 200 ml.

Magnetic beads

Dynabeads M-450 Pan B magnetic microspheres were obtained from Dynal UK Ltd. These bind B lymphocytes bearing the CD19 antigen which is present on lymphocytes from early B cell maturation, up to but not including plasma cells. Small-scale purging experiments in our laboratory had shown that a 4-log removal of $CD19^+$ lymphocytes from normal and myeloma bone marrow could be successfully achieved at a magnetic bead to target cell ratio of 40:1.[11]

In order to remove plasma cells, Dynabeads M-450 (Dynal) were coated with peanut agglutinin (PNA) (Sigma) by a method modified from that of Morecki *et al.*[12]. A total of 90 mg uncoated Dynabeads were washed twice in phosphate buffered saline (PBS) and resuspended in 6.6 ml PBS containing 5 mg PNA for 16 h at 4 °C, with end-over rotation. A final 16-h incubation was carried out at 4 °C in PBS containing 0.1% human albumin, before the beads were resuspended at 28.5 mg/ml in PBS/0.1% albumin. Plasma was omitted since previous studies had shown that its inclusion at this stage greatly reduced purging efficiency, presumably because of the prescence of PNA-binding glycoproteins in normal plasma.

Lectin coating efficiency was assessed by incubating PNA-coated magnetic beads (PNA-IB) with neuraminidase-treated human red blood cells and observing rosetting which occurred with 100% of beads.

Purging with PNA- and CD19-IB coated magnetic beads

The coated magnetic beads were washed three times in sterile saline and suspended in 100 ml IMDM. PNA- and CD19-IB were added to the bone marrow mononuclear cells in the blood collection bag at a bead to target cell ratio of 40:1. The mixture was then incubated at 22 °C for 1 h with constant gentle agitation. The bag containing bone marrow cells and magnetic beads was then loaded onto a Dynal Magnetic Separation Unit, separated from the magnet by a removable iron plate. After removing the plate the platform was lowered in four stages, 5 min for stages 1 and 2, and 3 min each for stages 3 and 4. Immediately the bag had reached the lowest stage, the free cells were aspirated from the bag by a peristaltic pump (Ismatec SA, Zurich, Germany) at 10 ml/min and after passing through a small magnetic trap (Dynal) were collected in a 600 ml Fenwal pack. Samples of purged bone marrow were taken for cytochemical analysis, bone marrow and microbiological culture. The remaining bone marrow sample was then centrifuged at $2000 \times g$ for 5 min at 4 °C. The supernatant was discarded and 100 ml autologous plasma was added to the bone marrow cells.

Cryopreservation

The bone marrow/plasma mixture was supported on ice and 100 ml plasma containing 20% vol/vol DMSO was gradually added with thorough mixing in a Class 1 sterile work station. The final purged bone marrow/plasma/DMSO mixture was transferred to Gambro freezing bags. The bags were then frozen under controlled conditions using the Planer Kryo 10 freezer, and transferred to liquid nitrogen.

Monoclonal antibody staining

Monoclonal antibodies (moAbs) were detected by the APAAP technique[13] on cytocentrifuge slides (10^5 cells per slide). The following moAbs were employed: anti-CD19 (Leu 12), anti-kappa and anti-lambda (Becton Dickinson, Cowley, UK). To detect cytoplasmic immunoglobulin, cytospin preparations containing 10^5 cells were fixed in acetone for 10 min and washed in PBS. The cells were incubated with 1 μl FITC-labelled goat anti-human kappa and 5 μl TRITC-conjugated goat anti-human lambda (Southern Biotechnology Associates, Seralab) in 50 μl PBS (previously titrated), for 30 min at 22 °C. Preparations were examined under a Leitz microscope fitted with uv light after washing in PBS.

Detection of PNA^+ cells

Staining of marrow preparations for PNA was performed by an immunoalkaline phosphatase technique. Cytospin preparations were fixed in acetone for 5 min then incubated with PNA 10 mg/ml for 60 min at 22 °C.

After washing in 0.1 M Tris buffered saline (pH 7.6) rabbit anti-PNA antibody (Serotec, Kidlington, UK) was added for 30 min. After further washing, a sheep anti-rabbit IgG conjugated to alkaline phosphatase (Serotec) was added before incubation in alkaline phosphatase substrate, and counterstained with haematoxylin and eosin. Negative controls were performed by prior incubation of PNA with 0.2 M D-galactose (neutralizing sugar for PNA).

Normal progenitor assays

Granulocyte-macrophage colony forming cells (CFU-GM) were assayed in IMDM containing 0.3% agar (Difco), and supplemented with 20% fetal calf serum (FCS) (Biological Industries, Glasgow, Scotland) and 10% medium conditioned by bladder carcinoma cells (Dr Fogh, Sloan-Kettering, New York) grown in log phase for 7 days in the prescence of IMDM and 5% FCS. Cells were plated at 10^5/well in duplicate 35 mm wells (Sterilin) and scored for colonies (>50 cells) after 10–14 days of incubation at 37 °C in CO_2 in a fully humidified incubator.

Results

The mean volume of bone marrow aspirated was 1.5 litres (Table II) with a mean total nucleated cell count (WBC) of 14.8. After partial depletion of red blood cells (RBC) using the lymphocytapheresis programme, 9.3 (mean) $\times 10^9$/l WBC remained, and after further processing with Ficoll the final WBC prior to purging was 3.4×10^9/l. After incubation with combined PNA- and CD19-coated magnetic beads and separation on a magnet, the mean absolute WBC was 1.8×10^9/l (Table II). Percentage recovery of bone marrow nucleated cells after purging was 52%. The final purged product yielded 14.4% of the original nucleated cell preparation.

Cytocentrifuge preparations were made of the bone marrow at each stage and stained for cells reactive with PNA, CD19 moAbs and cytoplasmic kappa and lambda. The proportion of cells reacting with PNA prior to processing the bone marrow was 18.7% (mean); (range 12–35%). After processing and purging the four bone marrow harvests, no detectable PNA^+ cells were found in cytocentrifuge preparations (Table III). Cytoplasmic immunoglobulin was not detected in any of the four bone marrow samples after purging. $CD19^+$ cells were 5.0% (mean; range 2–10%) in samples prior to processing, but were undetectable in the final product after purging (Table III).

Recovery of normal haemopoietic progenitors after purging the bone marrow harvests is shown in Table IV. In each case, numbers of CFU-GM were estimated from aliquots of bone marrow withdrawn after the Ficoll separation stage (pre-purge) and after the purging procedure (post-purge). A mean of 41.5 CFU-GM per 2×10^5 cells plated was obtained before purging, and 73.0 per 2×10^5 after purging. When adjusted for cell loss there was 78% recovery of CFU-GM following purging. Calculations based on patient's body weight predicted a mean CFU-GM dose of $0.86 \pm 0.32 \times 10^4$/kg recipient (range $0.5–1.3 \times 10^4$/kg).

Discussion

This study reports a practicable method for *in vitro* removal of all detectable plasma cells and B lymphocytes from large volumes of myeloma bone marrow. A mononuclear cell fraction enriched for haemopoietic progenitors and depleted of erythrocytes and mature granulocytes was first obtained via a two-step procedure. The initial Hetastarch sedimentation reduced the number of erythrocytes which facilitated the next stage of Ficoll separation in an intermittent flow cell separator. The additional density gradient separation is desirable firstly because the efficiency of purging is reduced in the presence of large numbers of erythrocytes (small-scale purging experiments, not shown here, had confirmed this); secondly because PNA is known to bind to some granulocytes. When prior Ficoll separation is used fewer PNA-coated Dynabeads are required, thus reducing cost and also improving efficiency.

Table II Characteristics of bone marrow harvest

Volume harvested (l)	*Total nucleated cells ($\times 10^9$/l)*		*% recovery after purging*
	pre-purge	*post-purge*	
1.4	3.0	1.7	57
1.2	2.5	1.0	41
1.3	5.0	2.9	58
2.0	2.9	1.6	55

Table III Efficiency of removal of PNA^+ and $CD19^+$ cells

Before purging			*Plasma cells (Romanowski)*	*After purging*		
PNA+ (%)	*CD19+ (%)*	*cyIg*		*PNA+*	*CD19+*	*cyIg*
15	2	ND				
12	4	ND			None detected	
35	5	ND				
13	1	ND				

ND = not done

Table IV Recovery of normal haemopoietic progenitors after purging

Pre-purge CFU-GM[a]	*Post-purge CFU-GM*[a]	*% Recovery*[b]
38	45	68
66	166	102
21	30	60
4	52	83

[a]CFU-GM × 2 × 10^5 cells plated
[b]Adjusted for nucleated cell loss during purging

Extensive earlier small-scale experiments on normal peripheral blood, bone marrow spiked with tumour cell lines, and myeloma bone marrow had shown that efficient removal of plasma cells and B lymphocytes could be achieved at a magnetic bead to target cell ratio of 40:1 without serious loss of haemopoietic progenitors.[11] In the present study the numbers of magnetic beads required to achieve a bead to target ratio of 40:1 were calculated on bone marrow samples taken prior to the main harvest. Apart from the first purging procedure in which autologous plasma probably interfered with optimal purging efficiency (data not shown), no detectable PNA^+ or $CD19^+$ cells were detected in the final product in four of the five cases.

Haemopoietic progenitor cells as assessed by CFU-GM and CFU-GEMM were concentrated in the final mononuclear cell product, and colony assays of cells bound to magnetic beads yielded <3% of the total CFU-GM numbers (data not shown). The calculated CFU-GM dose/kg patient weight was >0.75 × 10^4/kg in three out of the four cases. One purged bone marrow harvest yielded only 0.5 × 10^4/kg and a further harvest will be necessary in this patient prior to ABMT.

The methods described above to detect residual myeloma in the purged bone marrow are relatively insensitive and more useful information about the potential advantages of purging myeloma bone marrow could be gained by application of a sensitive myeloma clonogenic assay and molecular techniques to detect immunogobulin gene rearrangements in the purged bone marrow. We attempted to grow myeloma colonies from the purged bone marrow (data not shown) using a serum-free culture method for clonogenic myeloma colonies,[8] but this method has an unacceptably low plating efficiency in this context, and in the three bone marrows tested, no myeloma colonies were obtained either before or after purging.

Much more promising is the technique described by Trainor *et al.*,[14] and Deane and Samson,[15] for detection of immunoglobulin gene rearrangements by polymerase chain reaction. This was not available to us at the time of performing the above experiments, but has obvious application for future work.

There is currently some debate as to whether it is even necessary to remove plasma cells when attempting to purge myeloma bone marrow. Until there is more evidence to show that myeloma plasma cells are not capable of initiating relapse, whether from residual disease that escapes conditioning therapy or from infused bone marrow, we feel it is desirable to eliminate plasma cells as well as other putative myeloma progenitors in the B lymphocyte compartment from ABMT. The reactivity of PNA with some cells of the monocyte and granulocyte series in addition to plasma cells may possibly prove an advantage if the early work Grogan *et al.*[9] showing myelomonocytic antigens on myeloma cell lines is substantiated.

In order to show that purged ABMT in myeloma leads to longer relapse-free survival than unpurged ABMT, large numbers of patients are required to participate in rendomized trials. A pilot study has commenced at this centre in which half the patients will receive purged and half unpurged bone marrow, and the feasibility of such treatment will be assessed.

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References

1. McELWAIN TJ, POWLES RL. High dose intravenous melphalan for plasma-cell leukaemia and myeloma. *Lancet* 1983, **8354**: 822–825
2. BARLOGIE B, ALEXANIAN R, DICKE KA *et al.* High-dose chemoradiotherapy and autologous bone marrow transplantation for resistant multiple myeloma. *Blood* 1987, **70**: 869–871.
3. SHIMAZAKI C, WISNIEWSKI D, SCHEINBERG DA *et al.* Elimination of myeloma cells from bone marrow using monoclonal antibodies and magnetic beads. *Blood* 1988, **72**: 1248–1254.
4. TONG AW, LEE JC, FAY JW, STONE MJ. Elimination of clonogenic stem cells from human myeloma cell lines by a plasma cell reactive monoclonal antibody and complement. *Blood* 1987, **70**: 1482–1487.
5. DINOTA A, BARBIERI C, GOBBI M *et al.* An immunotoxin containing momordin suitable for bone marrow purging in myeloma patients. *Br J Cancer* 1989, **60**: 315–319.
6. BAST E, VAN CAMP B, REYNART P, WIRINGA G, BALLIEUX R. Idiotypic peripheral blood lymphocytes in monoconal gammopathy. *Clin Experim Immunol* 1982, **47**: 677–682
7. WARBURTON P, JOSHUA DE, GIBSON J, BROWN RD. CD10-(CALLA)-positive lymphocytes in myeloma: evidence that they are a malignant precursor population and are of germinal centre origin. *Leuk Lymphoma* 1989, **1**: 11–13.
8. RHODES EGH, OLIVE C, FLYNN MP. A serum-free culture method for myeloma progenitors in vitro: proliferative and immunophenotypic characteristics. *Exp Hematol* 1990, **18**: 79–83.
9. GROGAN TM, DURIE BGM, SPIER CM, RICHTER L, VELA E. Myelomonocytic antigen positive multiple myeloma. *Blood* 1989, **73**: 763–765.
10. RHODES EGH, FLYNN MP. PNA shows specificity for bone marrow plasma cells. *Br J Haematol* 1989, **71**: 183–187.

11. RHODES EGH, BAKER PK, RHODES JM, DAVIES JM, CAWLEY JC. Peanut agglutinin in combination with CD19 monoclonal antibody has potential as a purging agent in myeloma. *Exp Hematol* 1991, **19**: 833–837.
12. MORECKI S, PAULOTZKY F, MARGELS P, SLAVIN S. Purging breast cancer cells in preparation for autologous bone marrow transplantation. *Bone Marrow Transplant* 1987, **1**: 357–360.
13. ERBER WN, PINCHING AJ, MASON DY. Immunocytochemical detection of T and B cell populations in routine blood smears. *Lancet* 1984, **i**: 1042–1046.
14. TRAINOR KJ, BRISCO MJ, WAN JH, NEOH S, GRIST S, MORLEY AA. Gene rearrangement in B- and T-lymphoproliferative disease detected by the polymerase chain reaction. *Blood* 1991, **78**: 192–194.
15. SAMSON D, GAMINARA E, NEWLAND AC *et al.* Infusion of vincristine and doxorubicin with oral dexamethasone as first-line therapy for multiple myeloma. *Lancet* 1989, **ii**: 882–885.

PNA PURGING AND MAGNETIC MICROSPHERES

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Introduction

Many centres are now treating myeloma patients with autologous bone marrow transplantation (ABMT), the majority using conditioning regimens comprising high dose melphalan (HDM) (or thiotepa) combined with total body irradiation (TBI). In contrast to allogeneic BMT, long-term survival is a rare occurrence following ABMT, with virtually all patients relapsing within 2 years.

Several groups have developed techniques for purging myeloma autografts: apart from the use of chemical purging in a minority of centres, most groups combine anti- B cell monoclonal antibodies (Mab) with either PCA-1 anti-plasma cell Mab (Anderson et al.,1991) or the broadly-reactive anti-B cell Mab 8A (Dinota et al., 1989): numbers are too small to judge the efficacy of this approach.

Effective purging strategies for myeloma have been hampered firstly because the target cells have not yet been clearly identified, and secondly because of the scarcity of suitable monoclonal antibodies that bind avidly to the surface of plasma cells. There is much evidence to implicate B lymphocytes in the malignant process (Barlogie B et al, 1989, Warburton P et al, 1989, Rhodes E et al, 1990), but no clear data to suggest that malignant plasma cells are or are not capable of initiating tumour growth. In an attempt to find better anti-plasma cell agents we examined a panel of lectins and observed that peanut agglutinin (PNA) binds powerfully to all detectable plasma cells

in a given bone marrow and, contrary to previous reports, does not bind to haemopoietic stem cells (Rhodes et al, 1989). Hence the decision to combine PNA with the pan-reactive anti-B cell Mab CD19 to purge myeloma autografts.

Small-scale experiments

Normal bone marrow and peripheral blood cells, myeloma bone marrow and normal cells mixed with tumour cell lines were used for model purging experiments (Rhodes et al., 1991).

Removal of PNA+ cells

The fluorescent supravital DNA label Hoechst 33342 (H342; Sigma Chemical Company) was used to tag tumour cells by the method of Reynolds et al (1986) prior to mixing them with normal peripheral blood or bone marrow cells. An EBV-transformed B-cell line (Kirk) grown originally from myeloma bone marrow was found to bind PNA. Magnetic microspheres (Dynabeads, Dynal UK, Ltd) were coated with PNA and washed extensively. Following incubation of normal peripheral blood cells spiked with tumour cells with PNA-coated Dynabeads at bead:target cell ratios of 1:10 to 1:100, bead-coated cells were separated for 2 minutes using a magnetic particle concentrator (Dynal). More efficient removal of PNA^+ cells was observed at 22^0C than 4^0C with 3.7-log removal at a ratio of 40:1 at 22^0C and 4-log removal at a ratio of 100:1 (Fig.1). Myeloma bone marrow samples were purged with PNA-coated beads at 22^0C and complete removal of plasma cells (but not B lymphocytes) was achieved at a bead to target cell ratio of 40:1 (one cycle) as assessed morphologically by Romanowski staining and APAAP staining for PNA.

B lymphocytes were separated by means of CD19-coated magnetic beads (M-450 Pan-B; Dynal UK Ltd). Model experiments comprised normal peripheral blood and bone marrow mixed with H342-labelled HCL cells that strongly expressed receptor for CD19. A 4.2-log removal of fluorescent tumour cells was achieved at a bead:tumour cell ratio of 40:1 at 4^0C.

PNA-coated and CD19-coated magnetized microspheres were added simultaneously to blood or bone marrow spiked with 10% each of the
PNA^+ and $CD19^+$ cell lines at a bead:target cell ratio of 40:1. After incubating the bead/cell mixture at room temperature for 1 hour and applying the magnet, a mean 4.3 log removal of fluorescent H342-labelled tumour cells was achieved (Table 1). When the incubation temperature was lowered to 4^0C some tumour cells were left behind, and when sequential purging or two cycles of purging were were attempted, non-specific cell loss was unacceptably high.

Small-scale experiments on myeloma bone marrow treated with one cycle of combined PNA- and CD19-coated Dynabeads resulted in a >3 log removal of target cells as assessed by a PNA-anti-PNA alkaline phosphatase technique and by staining for cytoplasmic immunoglobulin: satisfactory preservation of normal haemopoietic progenitors was observed.

Large-scale purging experiments

In order to increase the volume of bone marrow treated ex vivo for application to clinical purging, a mononuclear cell (MNC) preparation rich in normal stem cells and depleted of

mature red blood cells and PNA^+ granulocytes was required. Normal donor buffy coat preparations mixed with H342-labelled tumour cells were processed on the Haemonetics V50 automated cell separator with and without ficoll, or on the Cobe Spectra with and without hydroxyethyl starch (HES), prior to incubation with magnetic microspheres (Baker et al., 1991). These experiments were repeated using bone marrow harvests from patients with myeloma. It was found that although the Cobe Spectra (in the presence of HES) performed well in terms of MNC enrichment and granulocyte depletion, the haematocrit was not lowered sufficiently to ensure that red blood cells would not interfere with bead/tumour cell interaction. It was therefore decided to use the Haemonetics V50 cell separator with the lymphocyte enrichment programme followed by ficoll for cinical application (Rhodes et al., 1991).

Clinical purging

A clinical pilot study has been initiated at the Royal Liverpool Hospital for patients with myeloma who are less than 55 years and who have received conventional chemotherapy to the stage where bone marrow plasma cells are less than 20%. All patients have "back-up" bone marrow harvested and cryopreserved. Those patients whose bone marrow plasma cells are reactive with PNA undergo a second bone marrow harvest. This is processed as above and purged with combined PNA- and CD19-coated magnetic microspheres. Prior to cryopreservation, the purged marrow samples are assessed for normal haemopoietic progenitor content (CFU-GM, -GEMM, and BFU-E) and residual tumour cells ($CD19^+$ cells

by APAAP, PNA^+ cells by an alkaline phosphatase technique, and fluorescent cytoplasmic immunoglobulin using anti-kappa and -lambda monoclonal antibodies).

All patients then receive melphalan 140mg/m^2 followed by fractionated total body irradiation of 850cGy prior to infusing the autologous bone marrow.

Results

Five myeloma bone marrow harvests have been purged with PNA and CD19 and have been cryopreserved (Fig 2 and 3). Two patients have elected not to undergo ABMT and to remain off treatment for the time being. Too few stem cells were harvested from a third patient to ensure engraftment following purged ABMT, and he has been transferred to the unpurged arm of the study. Two patients, age 39 and 51 years, have received purged autografts following HDM and total body irradiation. The procedure was well tolerated and time to engraftment was 15 and 20 days for neutrophils >0.5x10^9/l, and 35 and 30 days for platelets >50x10^9/l respectively. Both patients remain in excellent health at 20 and 12 months post-ABMT, with a normal bone marrow.* Both patients still have detectable serum paraprotein, the first (this patient is on alpha interferon) showing a steady decline in level since ABMT, and the second (no interferon) has a low level of 12 g/dl.

Three patients received unpurged ABMT: two of these have died at 3 months (infection) and 21 months (relapse), while the third remained severely thrombocytopenic for 13 months but is currently in good partial remission at 20 months.

* As of Dec. '91 the 2nd. patient (no α-IFN) is in CR with no detectable paraprotein.

Conclusions

A novel purging regimen for myeloma bone marrow is described using PNA and CD19 bound to magnetic microspheres. Complete removal of all morphologically identifiable plasma cells and $CD19^+$ lymphocytes has been achieved in both small- scale and large-scale experiments with satisfactory preservation of normal haemopoietic progenitors. Two patients have received purged ABMT followed by prompt haemopoietic engraftment and are in good health 12 and 20 months later.

Although it is too early to predict whether this treatment will prove superior to current therapies we are encouraged by these preliminary results to continue the pilot study.

References

Anderson KC, Barut BA, Ritz J, Freedman AS, Takvorian T, Rabinowe SN, Soiffer R, Heflin L, Coral F, Dear K, Mauch P Nadler LM. (1991) Monoclonal antibody-purged autologous bone marrow transplantation therapy for multiple myeloma. Blood 77:712-720.

Baker PK, Rhodes EGH, Duguid JKM. (1991) Continuous flow cell separator use for bone marrow processing. Transfus. Sci. 12:183-187.

Barlogie B, Epstein J, Selvanayagam P, Alexanian R. (1989) Plasma cell myeloma: new biological insights and advances in therapy. Blood 73:865-871.

Dinota A, Barbieri C, Gobbi M,Tazzari PL, Rizzi S, Bontadini A, Bolognesi A, Tura S, Stirpe F. (1989) An immunotoxin containing momordin suitable for bone marrow purging in myeloma patients. Br J Cancer 60:315-319.

Reynolds CP, Black AT, Woody JN. (1986) Sensitive method for detectinng viable cells seeded into bone marrow. Cancer Res 46:5878-5881.

Rhodes EGH, Flynn MP. (1989) PNA shows specificity for bone marrow plasma cells. Br J Haematol 71:183-187.

Rhodes EGH, Olive C, Flynn MP. (1990) A serum-free cullture method for myeloma progenitors in vitro: proliferative and immu-

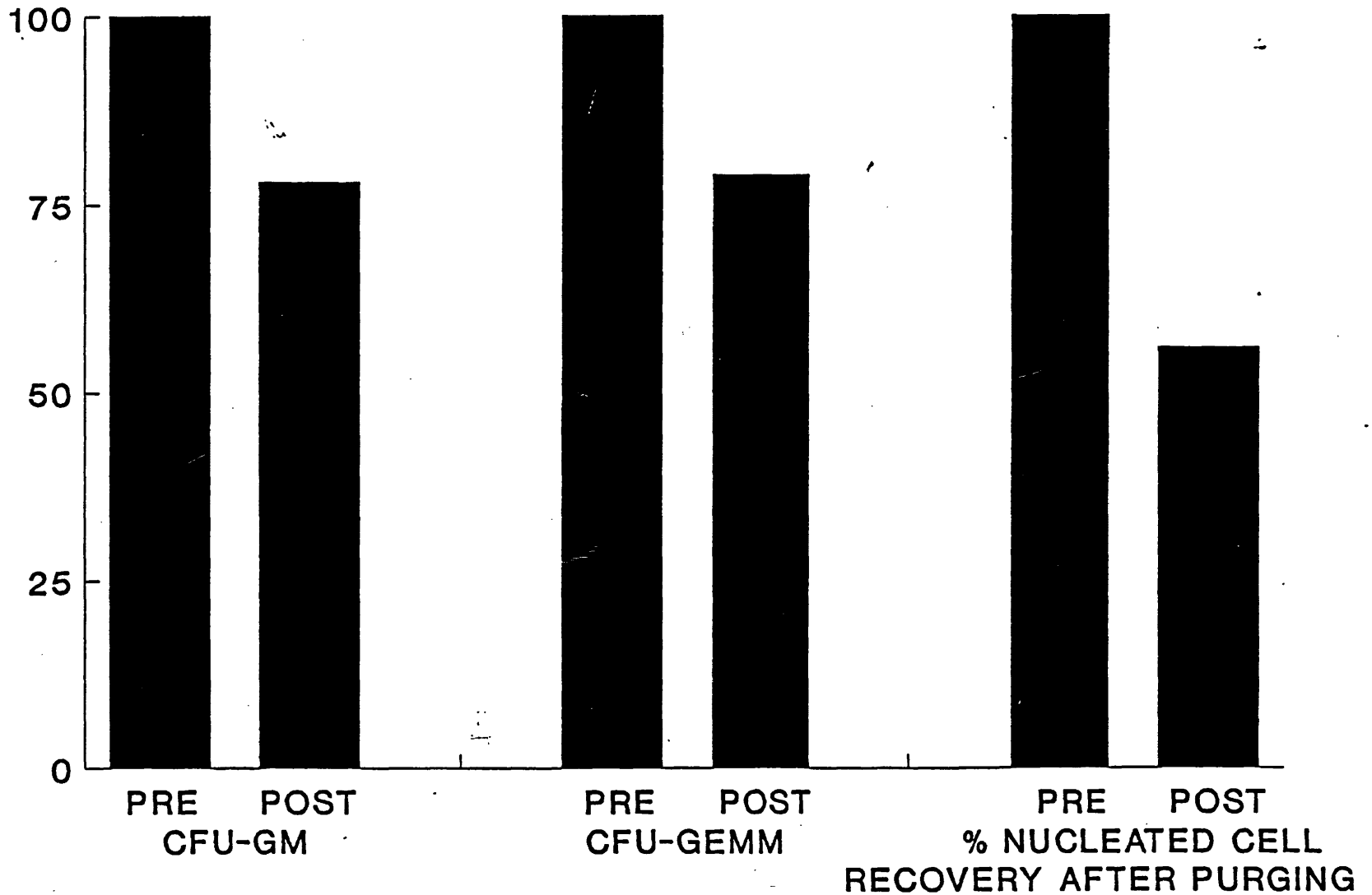
nophenotypic characteristics. Exp Hematol 18:79-84.

Rhodes EGH , Baker PK, Rhodes JM, Davies JM, Cawley JC. (1991) Peanut agglutinin in combination with CD19 monoclonal antibody has potential as a purging agent in myeloma. Exp Hematol 19:833-837

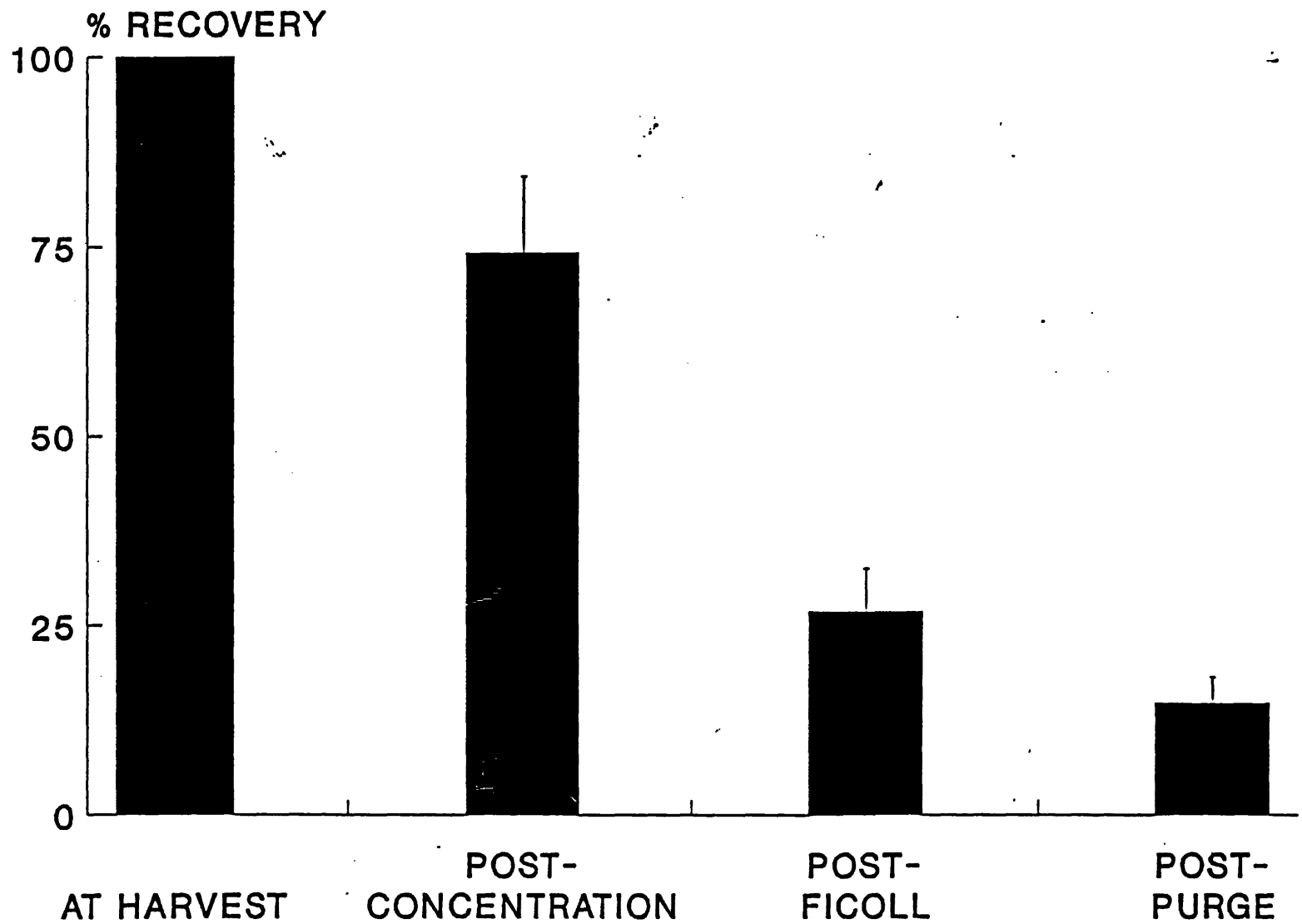
Rhodes EGH, Baker PK, Duguid JKM, Davies JM, Cawley JC. (1991) A method for clinical purging of myeloma bone marrow using peanut agglutinin as an anti-plasma cell agent, in combination with CD19 monoclonal antibody. Submitted for publication.

Warburton P, Joshua DE, Gibson J, Brown RD. (1989) CD10-(CALLA)-positive lymphocytes in myeloma: evidence that they are a malignant precursor population and are of germinal centre origin. Leuk Lymphoma 1:11-20.

% RECOVERY OF NORMAL HAEMOPOIETIC PROGENITORS AFTER PURGING



NUCLEATED CELL LOSS AT EACH STAGE OF PROCESSING



Combined PNA/CD19 purging: efficacy of removal of H33342-labelled PNA^+ (Kirk) and $CD19^+$ (HCL) cells

	Cell number	%PNA^+	%$CD19^+$	Bead: target	Log depl.
BM[a] spiked	$5x10^7$	25[b]	11[b]	40:1	4.3

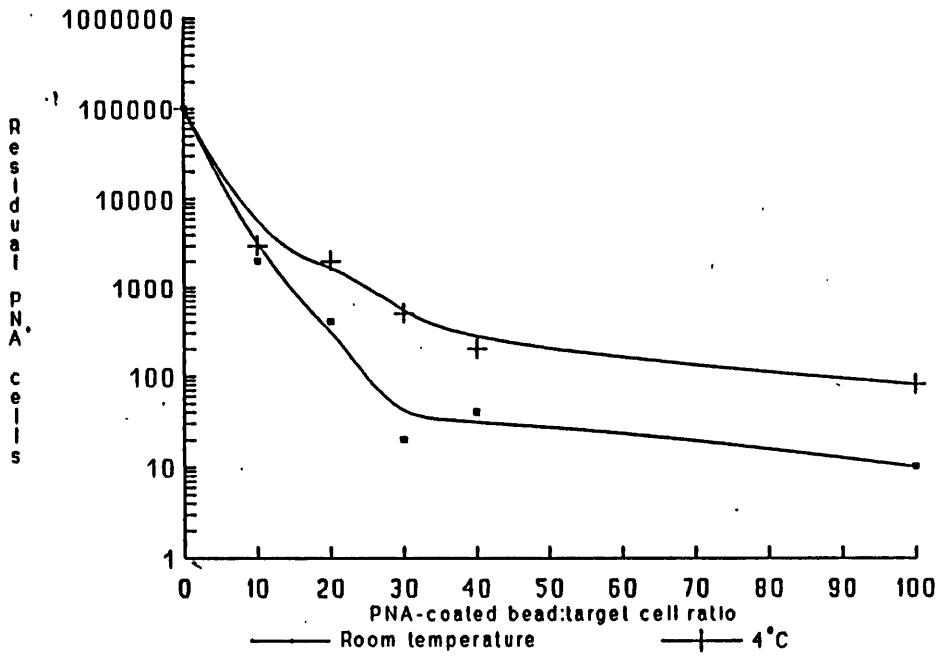
n=3

[a] normal bone marrow spiked with 5% H33342-Kirk and 5% H33342-HCL.

[b] number of unlabelled target cells assessed by APAAP prior to addition of tumour cells.

Normal bone marrow MNC were mixed 9:1 with H342-labelled PNA^+ and $CD19^+$ tumour cells: PNA-coated magnetic beads and anti-CD19-coated immunomagnetic beads were added at a bead:target cell ratio of 40:1 and the mixtures separated by magnet. Cytocentrifuge preparations were examined for fluorescence before and after magnetic bead treatment.

Removal of PNA^+ target cells with PNA-coated beads at varying bead:target cell ratios



Removal of PNA^+ tumour cells labelled with H342 from normal peripheral blood mononuclear cells: 1 $x10^6$ cells spiked with 10% H342-labelled Kirk cells were incubated with PNA-coated magnetic beads at varying bead:target cells ratios. Remaining PNA^+ cells were assessed by examining for fluorescent cells on cytocentrifuge preparations.

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Peanut agglutinin shows specificity for bone marrow plasma cells

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Summary. A panel of lectins was used to study surface carbohydrate expression on myeloma cells with the aim of finding a possible agent for *in vitro* bone marrow purging. Peanut agglutinin (PNA, galactose β1,3 N-acetylgalactosamine-binding) bound to all plasma cells in 33/34 bone marrow samples from myeloma patients and to all plasma cells in 11 bone marrows from patients with monoclonal gammopathy of undetermined significance and 10 normal bone marrow samples. Bone marrow and peripheral blood monocytes reacted weakly with PNA except in one case of acute monoblastic leukaemia and two of chronic myelomonocytic leukaemia in which monocytes were strongly positive. The only case of plasma cell leukaemia studied was PNA negative. All other bone marrow mononuclear cells were negative for PNA but became positive after sialidase treatment. Peanut agglutinin may have potential as an agent to be used in myeloma for *in vitro* marrow purging prior to autologous transplantation in combination with high dose chemotherapy.

The prognosis for patients with myeloma remains depressingly poor, but recently encouraging results have been obtained using high-dose melphalan either alone (McElwain & Powles, 1983) or in combination with marrow rescue by reinfusion of unpurged autologous bone marrow (Barlogie *et al*, 1987).

It seems probable that a better chance of cure would result if high-dose chemo/radiotherapy were followed by return of bone marrow which had been purged of malignant cells. Until recently, attempts to produce an agent with the ability to remove or destroy myeloma cells selectively have been unsuccessful, although a promising monoclonal antibody has been reported by Tong *et al* (1987). However, this is not generally available, and its efficacy is unknown.

Most of the surface antigens that distinguish haemopoietic cell types from each other are predominantly carbohydrate, and it seems possible that lectins, which have specificity for carbohydrates, might be useful purging agents (Reisner & Sharon, 1984). In the present study we examine a panel of lectins for their selective reactivity with plasma cells.

MATERIALS AND METHODS

Patients

Bone marrow (BM) or tonsil was obtained with informed consent (and local ethical committee approval) from haematologically normal patients undergoing either coronary artery bypass surgery or tonsillectomy.

The myeloma studies involved diagnostic BM aspirates from a total of 34 patients with typical multiple myeloma (MM), one patient with plasma-cell leukaemia, and 11 with monoclonal gammopathy of undetermined significance (MGUS). Peripheral blood (PB) or BM was obtained from normal volunteers (PB) or patients with a range of B-cell and monocytic haematological malignancies of differing maturities as specified in the text.

Cell preparation

Peripheral blood and bone marrow. Material was aspirated into heparin (30 u/ml blood/aspirate), diluted 1:1 with phosphate-buffered saline (PBS), and then centrifuged (400 *g* for 25 min at 22°C) over Ficoll-Paque (F/P) (Pharmacia, Uppsala, Sweden). Interface cells were washed twice in PBS before staining.

Tonsil. Lymphocytes were teased free from the solid tissue in PBS, washed twice in PBS and then isolated by F/P centrifugation as above. Monocytes and T lymphocytes were removed by plastic adherence and double AET-treated sheep erythrocyte (E) rosetting (B cells >95% by CD19 staining; monocytes <1% by morphology and CD14 reactivity) (Kaplan & Clark, 1974).

T cells. PB and BM T cells were purified by E rosetting followed by hypotonic lysis to remove the rosetting erythrocytes.

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Table I. Reactivity of F/P-separated bone marrow cells with fluoresceinated lectins

Lectin	Carbohydrate specificity	Optimal concn (μg/ml)	% positive cells (±1 SD) Normal (n=5; PCs 1·5±0·7%)	MGUS (n=3; PCs 11±4%)	MM (n=4; PCs 52±18%)
Ulex europeaus	αL-Fucose	20	41±15	43±12	15±8
Tetragonolobus purpurea	αL-Fucose	20	6±3	7±2	12±5
Bandeirea simplificolia I	αN-Acetylgalactosamine/α-glactose	12·5	2±2	2±1	
Bandeirea simplificolia II	αN-acetylglucosamine	20	52±4	48±18	26±8
Arachis hypogaea	Galactose-β1,3 N-acetylgalactosamine	10	5±2	16±3	56±20

PC=plasma cells; MGUS=monoclonal gammopathy of unknown significance; MM=multiple myeloma.

Desialylation. Cytospin preparations of normal BM were incubated (15 min at 37°C) with sialidase (*Clostridium welchii* Type V; Sigma) at a final concentration of 0·8 u/ml and then washed (PBS) three times before PNA staining.

Cell staining

FITC-labelled lectins. The five lectins employed are listed, together with their carbohydrate specificities, in Table I. All were directly fluoresceinated and obtained from Sigma. Cells suspended in PBS/azide (0·1%) were stained at 4°C in microtitre plates (5×10^5 cells/well in 200 μl) for 30 min. The optimal final concentration (Table I) of each lectin was determined in preliminary titration experiments.

Immunoalkaline phosphatase detection of PNA binding. Cytocentrifuge preparations (10^5 cells/slide) were fixed in acetone (5 min at room temperature (RT)), air dried, and washed before staining by an indirect immunoalkaline phosphatase method involving exposure first to PNA (10 μg/ml for 60 min at RT), then to rabbit anti-PNA immunoglobulin (30 min RT; 1/50 final dilution) and finally to sheep anti-rabbit Ig coupled to alkaline phosphatase (30 min RT; 1/20 final dilution) (all three reagents from Serotec, Kidlington, Oxford). Cells were washed three times between staining steps in Tris (0·1 M)-buffered saline (pH 7·6) and the alkaline phosphatase was detected with naphthol AS-MX phosphatase substrate (0·2 mg/ml) and fast red TR (1 mg/ml) as capture agent; levamisole (0·05 M) was added to inhibit endogenous alkaline phosphatase. Cells were counterstained with haematoxylin and eosin.

Negative controls were performed by omitting the PNA, by prior incubation of the lectin with 0·1 M galactose, or by substitution of the rabbit anti-PNA with a rabbit anti-lens culinaris antibody.

Double staining with PNA and monoclonal antibodies (Mab). FITC-PNA (final concentration 10 μg/ml) was added (30 min at 4°C) to cells (1×10^6/ml) suspended in PBS/azide. After washing (PBS/azide), the PNA-labelled cells were reacted in an indirect phycoerythrin technique with one of the following monoclonal antibodies (previously titrated for optimal concentrations): Leu12 (CD19), Leu4 (CD3), LeuM3 (CD14, anti-monocyte) and anti CALLA (CD10) (all from Becton Dickinson) and HB-7 (CD38; kind gift of Dr T. F. Tedder, Dana-Farber Cancer Institute). Cells were preincubated in goat serum (1:5 v/v dilution; 30 min at 4°C) to prevent non-specific binding of Mab to the PNA-coated cells and non-immune mouse IgG1 and 2a were included as class-specific negative first-layer controls.

After washing, Mab was detected with phycoerythrin-conjugated $F(ab')_2$ rabbit anti-mouse Ig (10 μg/ml; 30 min at 4°C). After further washing, the doubly labelled cells were examined in a FACS analyser setting the Mab and PNA thresholds such that <1% of cells were respectively reactive with the class-specific first layer control or with PNA previously exposed to galactose.

Table II. PNA immunoalkaline phosphatase staining of myeloma BM cells*

Source	% PNA-positive cells (±1 SD) Plasma cells Pos.	Plasma cells Neg.	Monocytes Pos.	Monocytes Neg.	Neutrophils
M† (n=32)	100	0	67±18	32±10	0
Normal (n=10)	100	0	67±10	30±19	2±1 in 3 samples
MGUS (n=11)	100	0	55±32	45±32	3±2 in 2 samples

* Ficoll-Paque separated.

† The single unreactive case of MM and the plasma-cell leukaemia patient excluded.

RESULTS

Screening of bone marrow cells with a panel of fluoresceinated lectins

Only *Arachis hypogaea* (peanut agglutinin, PNA) showed any specificity for myeloma cells (Table I). This lectin was therefore selected for further immunocytochemical study.

PNA-immunoalkaline phosphatase staining of fixed myeloma cells

All plasma cells in 33/34 bone marrow samples from myeloma patients were PNA positive (Table II). The plasma cells in normal and MGUS bone marrows also showed strongly positive PNA binding. The one negative myeloma

Table III. FACS analysis of bone marrow cells* doubly stained with PNA and various Mabs

		% positive cells					
Source	Reactivity	CD38	CD19	CD10	CD14	CD3	PNA
Normal ($n=3$)	Total†	33±14	7±5	0·7±0·4	6±2·4	9·5±3	5·6±3
	Double	13±4	0	0	4·5±3	0	
MM ($n=3$)	Total	19±4	2·8±2	0·6±0·3	4±2	2·5±2	31±12
	Double	6±4	0	0	1·9±1	0	

* Ficoll-Paque separated.

† Total = all cells stained with particular Mab or with PNA; Double = cells stained with PNA + particular Mab.

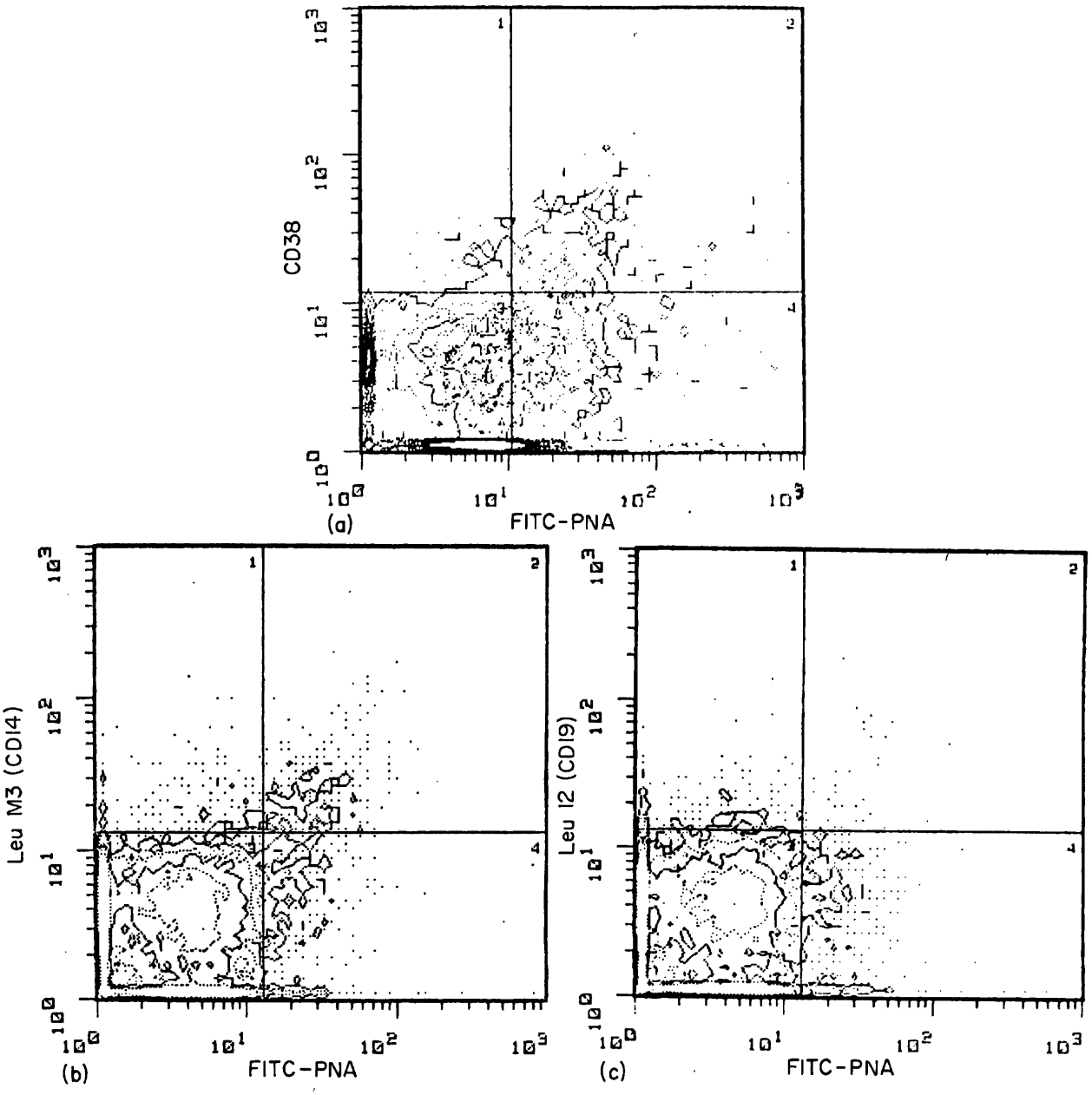

Fig 1. Two-colour FACS of FITC-PNA (horizontal axis) versus staining with CD38 (a), LeuM3 (b) and CD19 (c) on the vertical axis: the Mab staining was detected by an indirect method employing phycoerythrin-linked $F(ab')_2$ rabbit anti-mouse Ig. A significant population of both $CD38^+$ PNA^+ and $LeuM3^+$ PNA^+ cells is demonstrated, while $CD19^+$ cells are shown to be PNA negative.

bone marrow expressed IgG λ in plasma cell cytoplasm, and apart from being PNA negative, was indistinguishable from other myeloma samples. In addition, the plasma cells were unreactive with PNA in the single case of plasma cell leukaemia examined.

The only other cells to react consistently with PNA were monocytes and macrophages (Table II), although the reaction was less strong than with plasma cells. A small proportion of neutrophils reacted in some samples (Table II). Control slides were consistently negative.

PNA-immunoalkaline phosphatase staining of normal and malignant lymphocytes and monocytes, and sialidase-treated mononuclear cells

Purified tonsil B lymphocytes were PNA negative ($n=2$). In addition, malignant cells from bone marrows of 8/10 lymphoma patients, acute lymphoblastic leukaemia ($n=1$), and peripheral blood samples from hairy-cell leukaemia ($n=2$) and chronic lymphocytic leukaemia ($n=5$) were all negative. Bone marrow lymphoid cells in two cases of follicular lymphoma reacted weakly with PNA. Purified normal T cells from blood and marrow were consistently unreactive.

Normal PB monocytes were weakly PNA positive ($71\pm13\%$, $n=5$). In acute monocytic (M5) (94% cells positive; $n=1$) and chronic myelomonocytic leukaemias (62% positive; $n=2$), monocytes and their precursors were reactive with PNA but positivity was greater in earlier forms.

Sialidase treatment of normal bone marrow cells ($n=5$), prior to staining with PNA, resulted in all nucleated cells becoming PNA positive. This was expected since PNA binds to the Thomson-Friedenreich (or T) antigen (galactose β1,3 N-acetylgalactosamine) which on most cell types is masked by sialic acid.

FACS analysis of bone marrow cells doubly stained with PNA and a panel of Mab

PNA-positive cells were unreactive with CD3, 10 and 19, confirming that T cells and non-plasmacytoid B cells lack PNA receptors (Table III). In contrast, PNA-positive cells were reactive with anti-plasma cell (CD38) and anti-monocyte (CD14) Mab (Table III, Fig 1). However, as expected, some PNA- and CD38-positive cells were singly stained since not all plasma cells are CD38 positive and since not all CD38-positive cells are plasma cells. The FACS data confirmed that not all monocytoid cells react with PNA (Tables II and III).

DISCUSSION

This study clearly shows that all plasma cells from normal and myeloma bone marrows are consistently reactive with PNA. The only other bone marrow cells found to react with PNA were from the monocyte/macrophage series. However, staining was in general less strong than for plasma cells. Since immature monocytes were more strongly reactive with PNA than were mature forms, it is possible that heterogeneity of staining may reflect monocyte maturity as previously suggested by Rosenberg *et al* (1985). B and T lymphocytes were unreactive with PNA.

PNA has been shown in recent years to bind a wide variety of epithelial tumours, including those of gastrointestinal (Cooper & Reuter, 1983), breast (Howard *et al*, 1981) and urinary bladder (Lehman *et al*, 1984) origin. More relevant to the studies reported here have been numerous reports of PNA binding to normal and malignant lymphoid cells. For example, immature B (from sites other than bone marrow) and T cells, both normal and malignant, have been shown to react with PNA (Levin *et al*, 1980; Reisner *et al*, 1979; Veerman *et al*, 1985). However, few authors have commented on the PNA reactivity of plasma cells; Rose *et al* (1981) note in passing that a plasmacytoma was PNA positive but do not investigate the general reactivity of plasma cells. The present findings concerning the PNA positivity of plasma cells therefore do not conflict with previous studies.

The present work also confirms the observation of Haimovitz *et al* (1982) that peripheral blood monocytes react with PNA. Haimovitz also reported that, upon maturation *in vitro*, PNA positivity disappeared from most of the cells. Our work supports this in that monoblasts from a patient with acute myelobastic leukaemia (FAB classification M5A) reacted strongly with PNA, whereas mature monocytes in normal marrow and CMML reacted weakly or not at all.

The demonstration that PNA specifically stains a very high proportion of myelomatous plasma cells raises the possibility that the lectin may be a useful purging agent in autologous bone marrow transplantation for multiple myeloma. However, the fact that bone marrow B cells are unreactive with PNA and yet may contain at least a proportion of myeloma progenitor cells (Epstein *et al*, 1988) may mean that PNA purging will need to be combined with some additional method of removing bone marrow B cells. These possibilities are being actively pursued in this laboratory.

ACKNOWLEDGMENTS

We are indebted to Mrs Pamela Knowles for excellent secretarial assistance. We would also like to thank Mr W. Meade and Mr Fabry (Broadgreen Hospital) for helping us to obtain normal bone marrow samples, and Dr V. Clough (Countess of Chester Hospital) for myeloma samples.

REFERENCES

Barlogie, B., Alexanian, R., Dicke, K.A., Zagars, G., Spitzer, G., Jagannath, S. & Horwitz, L. (1987) High-dose chemoradiotherapy and autologous bone marrow transplantation for resistant multiple myeloma. *Blood*, **70**, 869–872.

Cooper, H.S. & Reuter, V.R. (1983) Peanut lectin binding sites in polyps of the colon and rectum. Adenomas, hyperplastic polyps and adenomas with in situ carcinoma. *Laboratory Investigation*, **49**, 655–659.

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

Haimovitz, A., Fuks, Z., Galili, N. & Treves, A.J. (1982) Changes in peanut agglutinin binding to human monocytes during their maturation to macrophages. *Journal of Reticuloendothelial Society*, **31**, 187–192.

Howard, D.R., Ferguson, P. & Batsakis, J.G. (1981) Carcinoma

associated cytostructural antigenic alterations: detection by lectin binding. *Cancer*, **47**, 2872–2877.

Kaplan, M.E. & Clark, C. (1974) Improved rosetting assay for detection of human T lymphocytes. *Journal of Immunological Methods*, **5**, 131–135.

Lehman, T.P., Cooper, H.S. & Mulholland, S.G. (1984) Peanut lectin binding sites in transitional cell carcinoma of the urinary bladder. *Cancer*, **53**, 272–277.

Levin, S., Russell, E.C., Blanchard, D., McWilliams, N.B., Maurer, H.M. & Mohanakumar, T. (1980) Receptors for peanut agglutinin (*Arachis hypogaea*) in childhood acute lymphocytic leukaemia: possible clinical significance. *Blood*, **55**, 37–39.

McElwain, T.J. & Powles, R.L. (1983) High-dose intravenous melphalan for plasma-cell leukaemia and myeloma. *Lancet*, **ii**, (8354), 822–824.

Reisner, Y., Biniaminov, M., Rosenthal, E., Sharon, N. & Ramov, B. (1979) Interaction of peanut agglutinin with normal human lymphocytes and with leukaemic cells. *Proceedings of the National Academy of Sciences of the United States of America*, **76**, 447–451.

Reisner, Y. & Sharon, N. (1984) Fractionation of sub-population of mouse and human lymphocytes by peanut agglutinin or soy bean agglutinin. *Methods in Enzymology*, **108**, 168–179.

Rose, M.L., Habeshaw, J.A., Kennedy, R., Sloane, J., Wiltshaw, E. & Davies, A.J.S. (1981) Binding of peanut lectin to germinal-centre cells: a marker for B-cell subsets of follicular lymphoma? *British Journal of Cancer*, **44**, 68–74.

Rosenberg, M., Chitayat, D., Tzehoval, E., Waxdal, M.J. & Sharon, N. (1985) Detection and enumeration of monocytes in human blood with peanut agglutinin. *Journal of Immunological Methods*, **81**, 7–13.

Tong, A.W., Lee, J.C., Fay, J.W. & Stone, M.J. (1987) Elimination of clonogenic stem cells from human multiple myeloma cell lines by a plasma cell-reactive monoclonal antibody and complement. *Blood*, **70**, 1482–1489.

Veerman, A.J.P., Hogeman, P.H.G., Huismans, D.R., Van Zantwijk, C.H. & Bezeemer, P.D. (1985) Peanut agglutinin, a marker for T-cell acute lymphoblastic leukaemia with a good prognosis. *Cancer Research*, **45**, 1890–1893.

Exp. Hematol. 19:833–837 (1991)

Experimental Hematology

Peanut Agglutinin in Combination with CD19 Monoclonal Antibody Has Potential as a Purging Agent in Myeloma

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Abstract. Administration of high-dose chemotherapy to patients with myeloma, followed by rescue with autologous bone marrow transplantation (ABMT), sometimes induces complete disease remission but relapse is usual. We have attempted to reduce the risk of relapse by selective in vitro removal of myeloma cells from the autologous graft. A combination of the (gal-galNac)-binding lectin peanut agglutinin (PNA), which binds all plasma cells, and the pan-B monoclonal antibody CD19 was assessed for purging marrow of myeloma cells and their putative precursors using a magnetic bead method. Preliminary experiments performed on peripheral blood mononuclear cells spiked with fluorescent-labeled PNA$^+$ Kirk tumor cells showed that a magnetic bead : target cell ratio of 40:1 resulted in a >3-log reduction in PNA$^+$ cells. This technique was then applied to 17 samples of myeloma bone marrow and to 18 samples of normal bone marrow spiked with PNA$^+$ Kirk cells and CD19$^+$ hairy cell leukemia cells. In each case all detectable plasma cells and CD19$^+$ lymphocytes were effectively removed, and normal hemopoietic progenitor cell recovery was >55%. This purging system deserves further study as a means of reducing relapse rates in myeloma patients treated by a combination of high-dose chemotherapy and ABMT.

Key words: Peanut agglutinin (PNA) — CD19 Mab — Myeloma

Attempts to purge myeloma bone marrow (BM) as part of autologous bone marrow transplant (ABMT) have been hampered by the scarcity of suitable monoclonal antibodies (Mabs) that react with the surface of plasma cells and by uncertainty as to the identity of the clonogenic fraction of the malignant cells. Several studies have recently been reported using Mabs directed towards plasma cell antigens [1, 2] or towards antigens on both plasma cells and earlier B-lineage cells [3], using a variety of purging techniques. Whether the myeloma stem cell is a pre-B cell [4] or an earlier hematopoietic stem cell [5] is still unresolved.

We have previously shown that the lectin peanut agglutinin (PNA) reacts relatively specifically with plasma cells [6], although some monocytes and granulocytes also bind to the lectin. Preliminary studies suggested that PNA does not react with normal hemopoietic progenitors [6] and warranted further examination as a potential purging agent. In addition, attempts to grow myeloma colonies with a view to identifying the clonogenic fraction led to the observation of colony formation after a 4- to 6-week incubation of myeloma bone marrow cells in a serum-free medium [7]. Preliminary analysis of these colonies revealed a mixture of plasma cells and CD19$^+$ lymphocytes.

On the basis of these initial data we have developed a purging regimen for myeloma BM comprising PNA- and CD19-coated magnetic beads. We report here the results of small-scale experiments aimed at developing a system for removing plasma cells and B-lymphocytes from normal and myeloma bone marrow.

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Materials and methods

BM cells

Normal sternal BM (5–10 ml) was harvested from hematologically normal patients (n = 18) undergoing coronary artery bypass surgery, with informed consent and with the approval of Broadgreen Hospital Ethics committee. Myeloma samples were diagnostic aspirates from patients (n = 17) with untreated or relapsed myeloma. BM (5–10 ml) was aspirated into 2 ml Iscove's modified Dulbecco's medium (IMDM) containing 15 U/ml preservative-free heparin. Mononuclear cells (MNC) were collected at the interface after centrifugation over Ficoll–Hypaque (FH; density 1.077 g/ml) and washed twice in IMDM.

Cell lines used for labeling and spiking experiments

An Epstein-Barr virus (EBV)-transformed B-cell line (Kirk) grown originally from myeloma BM was found to bind PNA; the cells were cultured in IMDM and 10% fetal calf serum (FCS) at 10^5 cells/ml with twice weekly replacement of half the culture medium. Tumor cells that strongly expressed receptor for CD19 were purified from the BM of a patient with hairy-cell leukemia. Purification of CD19$^+$ cells involved depletion of monocytes by incubation at 37°C for 1 h in a Falcon flask and depletion of T-lymphocytes by double Z-amminoethylisothiouronium bromide (AET)–sheep red cell rosetting. The resulting cell suspension was >95% CD19$^+$ by immunofluorescent testing.

Fluorescent supravital DNA labeling of tumor cells

The fluorescent supravital DNA label Hoechst 33342 (H342; Sigma Chemical Company) was used to tag tumor cells by the method of Reynolds et al. [8] prior to mixing them with normal peripheral blood or BM cells. The H342-stained target cells can be detected to a level of 1 viable target cell per 1 × 10^6 normal cells; this is a useful method for assessing the efficiency of removal of tumor cells from BM. H342 was dissolved in distilled water at 1 mg/ml and added at 2 μg/ml to 10^6 tumor cells/ml in RPMI-1640 medium/15% FCS for 1 h at 37°C. After washing twice in RPMI-1640/15% FCS the cells were further incubated in RPMI-1640/FCS for 2 h at 37°C before seeding the labeled tumor cells into normal peripheral blood or BM MNC in ratios of 1:20 or 1:10. After purging the cell mixtures

with PNA- or CD19-coated beads, or both together, cytocentrifuge preparations of 10^5 purged cells were made and examined using a Leitz fluorescent microscope equipped with a UV excitation filter (350 nm). For each target cell/normal cell mixture, triplicate Cytospins were examined for bright nuclear fluorescence, and the effect of varying the bead to target cell ratios from 10:1 to 100:1 was examined.

Removal of PNA$^+$ cells by means of PNA-coated magnetic beads

Dynabeads M-450 (Dynal UK Limited, Wirral, UK) were coated with PNA (Sigma Chemical Company) using a method modified from that of Morecki et al. [9]. Uncoated Dynabeads (90 mg) were washed twice in phosphate-buffered saline (PBS) and resuspended in 6.6 ml PBS containing 5 mg PNA for 16 h at 4°C with end-over-end rotation. A final 16-h incubation was carried out at 4°C in PBS containing 0.1% human albumin before the beads were resuspended at 30 mg/ml in PBS/0.1% albumin. Following incubation of cells with labeled magnetic beads at bead:target cell ratios of 1:10 to 1:100, bead-coated cells were separated for 2 min using a magnetic particle concentrator (Dynal UK Limited).

CD19 purging with immunomagnetic beads

Dynabeads commercially coated with IgM Mab against the CD19 antigen (M-450 Pan-B; Dynal UK Limited) were used. Ficoll-separated BM MNC (2×10^7 cells) were washed twice and resuspended in 5 ml PBS/10% plasma protein fraction (PPF) (BPL, UK) at 4°C. Various bead:target cell ratios were tested, and bead-coated cells were removed as described earlier. The CD19-depleted BM MNC were washed three times in PBS/10% PPF and examined for remaining CD19$^+$ cells and for their ability to grow in culture (granulocyte–macrophage colony-forming units [CFU-GM] and granulocyte erythrocyte macrophage megakaryocyte colony-forming units [CFU-GEMM]).

Mab staining

The following Mabs were employed: anti-CD19 (Leu 12), anti-CD10 common acute lymphoblastic leukaemia antigen (CALLA), anti-κ, and anti-λ (Becton Dickinson, Cowley, UK). Anti-plasma cell antibody (RFD6) was obtained from Professor Janossy (Royal Free Hospital, London). All of these Mabs are of the IgG1 subclass except CALLA (IgG2). Mabs were detected using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [10] on cytocentrifuge slides (10^5 cells/slide). In order to detect cytoplasmic immunoglobulin, cytospin preparations containing 10^5 cells were fixed in acetone for 10 min and washed in PBS. The cells were incubated with 1 μl fluorescein isothiocyanate (FITC)-labeled goat anti-human κ and 5 μl TRITC-conjugated goat anti-human λ (Southern Biotechnology Associates, Seralab) in 50 μl PBS (previously titrated), for 30 min at 22°C. After washing in PBS, preparations were examined using a Leitz microscope fitted with UV light.

Detection of PNA$^+$ cells

FITC-conjugated PNA, at a concentration of 10 μg/ml, was incubated for 30 min at 22°C with normal and myeloma BM MNC at 10^5 cells/ml in Tris-buffered saline (TBS; pH 7.4), containing Ca^{2+} and Mg^{2+} (both at 0.01 M). Washed cells were then examined in suspension by flow cytometry (Becton Dickinson fluorescence-activated cell sorter [FACS] analyzer) or by manual fluorescence microscopy in cytocentrifuge preparations.

After establishing the surface location of the PNA receptor using the above methods, routine staining of marrow preparations for PNA was performed by an immunoalkaline phosphatase technique so as to identify individual cells more easily. In brief, cytospin preparations were fixed in acetone for 5 min, then incubated with 10 μg/ml PNA for 60 min at 22°C. After washing in TBS, rabbit anti-PNA antibody (Serotec, Kidlington, UK) was added for 30 min. After further washing, a sheep anti-rabbit IgG conjugated to alkaline phosphatase was added before incubation in alkaline phosphatase substrate and counterstaining with hematoxylin and eosin. Negative controls were performed by omitting the lectin, or by substitution of the rabbit anti-PNA with a rabbit anti-lens culinaris (irrelevant lectin) antibody.

Normal progenitor assays

CFU-GM. CFU-GM were assayed in IMDM containing 0.3% agar (Difco) and supplemented with 20% FCS (Biological Industries, Glasgow, Scotland) and 10% medium conditioned by 5637 bladder carcinoma cells (Dr. Fogh, Sloan-Kettering, New York) grown in log phase for 7 days in the presence of IMDM and 5% FCS. Cells were plated at 10^5/well in duplicate 35-mm wells (Sterilin) and scored for colonies (>50 cells) after 10–14 days of incubation at 37°C in CO_2 in a fully humidified incubator.

Mixed-lineage colony-forming cells (CFU-GEMM). A modification of the method of Fauser and Messner [11] was used for assay of CFU-GEMM. Cells (1×10^5) were suspended in 0.8% methylcellulose (1500 centipoise made up in IMDM) and supplemented with 10% FCS, 20% plasma from a patient with severe aplastic anemia, 5% medium conditioned for 7 days by peripheral blood leukocytes in the presence of 1% phytohemagglutinin (PHA; PHA-LCM), 10^{-4} M 2-mercaptoethanol, and 2.5 U/ml erythropoietin (Terry Fox Laboratories, Vancouver, Canada). Duplicate cultures were incubated at 37°C with 5% CO_2. Mixed colonies containing at least erythroid and granulocytic cells, as judged by their in situ appearance, were scored at 14 days. Erythroid bursts (erythroid burst-forming units, BFU-E) containing >500 hemoglobinized cells were scored at 14 days.

Long-term BM cultures (LTBMC). BM MNC were cultured [12] at 4×10^6/ml in 5 ml of IMDM containing 10% FCS, 10% horse serum (Imperial Laboratories, Salisbury, UK), 10^{-4} M 2-mercaptoethanol, and 10^{-6} M hydrocortisone (Upjohn Limited, UK). Cultures were fed weekly by replacing half the medium and nonadherent cells with fresh culture medium. The nonadherent cells were assayed for CFU-GM at weekly intervals for up to 6 weeks.

Results

Removal of PNA$^+$ cells from normal peripheral blood and myeloma BM using magnetic beads linked to PNA

When 10^6 normal peripheral blood cells were mixed with 10^5 PNA$^+$ Kirk cells labeled with H342 and incubated with increasing concentrations of PNA-linked magnetic beads (PNA-MB), more efficient removal of PNA$^+$ cells was observed at 22°C than 4°C with 3.7-log removal at a ratio of 40:1 at 22°C and 4-log removal at a ratio of 100:1 (Fig. 1 and Table 1). When two cycles of depletion were tested in six subsequent experiments with different cell proportions, no fluorescent cells were detectable after purging on any occasion when bead to target cell ratios were >20:1 (at 22°C).

Myeloma BM samples ($n = 5$) were purged with PNA-MB at 22°C, and removal of plasma cells was assessed morphologically by Romanowski staining and by APAAP staining for RFD6, PNA, and CD19 positivity (Table 2). Fluorescent cytoplasmic immunoglobulin staining was performed on two samples and no positive cells were seen after purging. Complete removal of plasma cells was achieved at a bead to tumor cell ratio of 40:1 (one cycle), but the numbers of CD19$^+$ B-lymphocytes were unchanged after purging.

Removal of CD19$^+$ cells from normal and myeloma BM using immunomagnetic beads

In an experiment involving spiking of a normal BM MNC preparation with H342-labeled CD19$^+$ tumor cells (HCL)

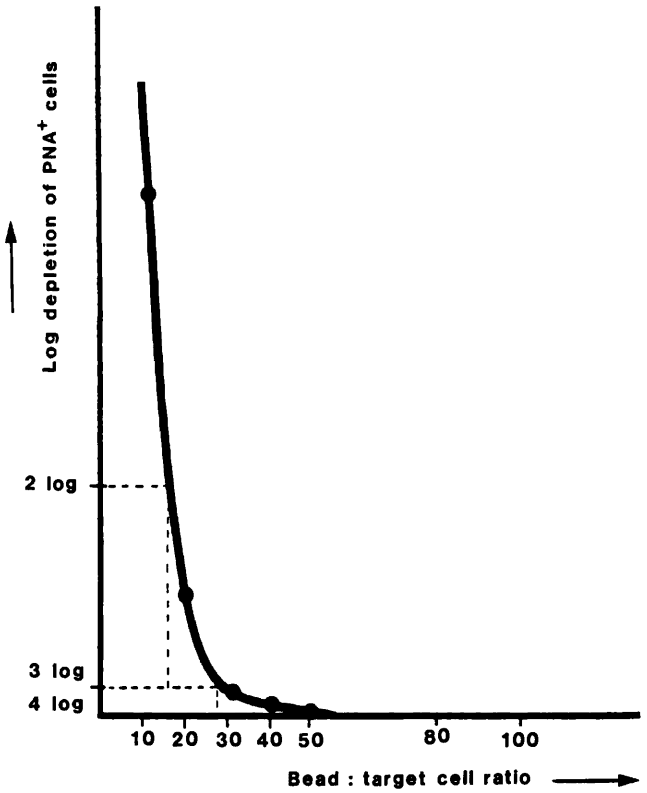

Fig. 1. Removal of PNA$^+$ tumor cells labeled with H342 from normal peripheral blood MNC: 2 × 10^6 cells spiked with 10% H342-labeled Kirk cells were incubated with PNA-MB at varying bead: target cell ratios. Remaining PNA$^+$ cells were assessed by examining for fluorescent cells on cytocentrifuge preparations.

and using a range of CD19-linked magnetic bead (CD19-MB): tumor cell ratios, a ratio of 40:1 at 4°C produced a 4.2-log removal of fluorescent tumor cells (Table 3). Similar results were obtained when purging with CD19$^+$ tumor cell-spiked peripheral blood MNC preparations (Table 3). Incubation was at 4°C.

When 2 × 10^6 myeloma BM cells were purged on four occasions with CD19-MB, CD19$^+$ cells were effectively removed by one cycle of purging at a bead: target cell ratio of 40:1 (CD19$^+$ cells mean 4%, range 2%–5%, of total cells before purging, 0%–0.1% after purging in all cases as assessed in APAAP-stained cytocentrifuge preparations [Table 3]).

Combined CD19- and PNA-MB purging on normal and myeloma BM

Preliminary experiments showed that combined purging with PNA-MB and CD19-MB at 22°C was as effective as sequential purging with CD19 at 4°C followed by PNA-MB at 22°C and resulted in lower nonspecific cell loss. These studies had also shown that a bead: target cell ratio of 40:1 was a suitable compromise between effective purging and cell loss. Further experiments were therefore performed by incubating a combination of CD19-MB and PNA-MB with BM at 22°C with bead: target cell ratios of 40:1.

When this system was applied to normal BM MNC preparations (n = 3) spiked with 5% H342-labeled CD19$^+$ HCL and 5% H342-labeled PNA$^+$ Kirk cells, a mean 4.3-log removal of fluorescent cells (range 3.5–4.6) was achieved (Table 4).

Table 1. Effect of bead: tumor cell ratio on the removal of PNA$^+$ tumor cells from normal peripheral blood MNC by PNA-MB

Temperature	PNA-MB: target cell ratio				
	10:1	20:1	30:1	40:1	100:1
	Log removal				
4°C	1.8	1.9	2.5	2.9	3.2
22°C	1.9	2.6	3.9	3.7	4.0

Normal peripheral blood MNC containing 10% H342-labeled PNA$^+$ cell line were mixed with PNA-MB and separated by magnet. Cytocentrifuge preparations of cells before and after treatment were examined for fluorescence, and the log removal of tumor cells was calculated.

Table 2. Efficacy of removal of PNA$^+$ cells from myeloma BM MNC (2 × 10^7 cells, mean of five samples) by PNA-MB

PNA purging	Percent positive cells (mean ± SD)			
	Morphological[a] plasma cells	PNA$^+$	RFD6$^+$	CD19$^+$
Before	20 ± 31	25 ± 26	20 ± 32	5 ± 3
After	<1	<1	<1	5 ± 4

Myeloma BM MNC (2 × 10^7) were incubated with PNA-MB at a bead: target ratio of 40:1 for 30 min at 22°C before separation by magnet. Cytocentrifuge preparations (10^5 cells/slide) were examined before and after separation.

[a] Morphology in Romanowsky- or APAAP-stained cytocentrifuge preparations.

Myeloma BM (n = 3) was purged similarly with one cycle of the combined CD19- and PNA-MB treatment, and a >3-log removal of target cells (range 3–4) was demonstrated by APAAP staining for PNA$^+$ and CD19$^+$ cells. Fluorescent cytoplasmic immunoglobulin staining was performed on one myeloma sample, and a 4-log removal of target cells was demonstrated again, with no identifiable immunoglobulin-containing cells remaining after purging.

Toxicity studies of PNA on normal hemopoietic cells

PNA had no direct inhibitory effect on CFU-GM formation when added to normal BM cultures (n = 5) over a range of final concentrations from 25 to 100 μg/ml. Further experiments were performed at 100 μg/ml of PNA under a range of culture conditions, and no inhibitory effect on CFU-GM, CFU-GEMM, and CFU-GM generated in LTBMC was observed.

Effect of purging on normal hemopoietic cells

Hemopoietic cell recovery after purging experiments is shown in Table 6. Removal of CD19$^+$ cells from myeloma BM resulted in 92% and 83% recovery of CFU-GM and CFU-GEMM, respectively. Progenitor cell recovery was at least 67% after purging with PNA-MB alone, and at least 51% with combined PNA-MB and CD19-MB purging.

Table 3. Removal of CD19+ cells from spiked normal and myeloma BM

Cell population	Bead:target cell ratio				Assessed by
	10:1	20:1	40:1	100:1	
Normal peripheral blood MNC plus 10% HCL	2.1	4.2	5	5	APAAP
Normal BM plus 10% HCL		4	5		APAAP
Normal BM plus 10% H342–HCL	2.3		4.2	5	Fluorescence
Myeloma BM			4.5		APAAP
Myeloma BM			4.7		Cytoplasmic Ig

Shown is the log removal of CD19+ cells with increasing bead:target cell ratios. The CD19+ cells HCL were mixed 1:9 with Ficoll-separated normal peripheral blood and BM and myeloma BM cells. In one set of experiments HCL cells were labeled with H342 prior to mixing with normal BM. The cell mixtures were incubated with CD19-MB at 4°C for 30 min before separation by magnet. Cytocentrifuge preparations (10^5 cells/slide) were examined for remaining CD19+ cells by APAAP, fluorescence, or the presence of cytoplasmic Ig.

Table 4. Combined PNA/CD19 purging: efficacy of removal of H-342-labeled PNA+ and CD19+ cells simultaneously from normal BM (mean of three samples)

Normal BM	Number of cells	Percent PNA+	Percent CD19+	Bead:target ratio	Log removal
plus 5% Kirk-H342 plus 5% HCL-H342	5×10^7	25[a]	11[a]	40:1	4.3

Normal BM MNC were mixed 9:1 with H342-labeled PNA+ and CD19+ tumor cells: PNA-MB and CD19-MB were added at a bead:target cell ratio of 40:1, and the mixtures were subjected to separation by magnet. Cytocentrifuge preparations were examined for fluorescence before and after magnetic bead treatment.

[a] Number of target cells assessed by APAAP prior to addition of tumor cells.

Table 5. Removal of plasma cells and CD19+ lymphocytes from myeloma BM by combined PNA-MB and CD19-MB

	Number of cells[a]	Percent plasma cells	Percent CD19+ cells	Bead:target ratio	Log removal of target cells
Myeloma BM	10^7	33	7	40:1	>3

Myeloma BM MNC were incubated with a combination of PNA-MB and CD19-MB at 40:1 and separated. Target cells were assessed by APAAP.

[a] Mean of three samples.

Discussion

This study has shown that combined purging of myeloma BM with PNA-MB and CD19-MB can be used to achieve efficient removal of plasma cells while retaining >50% of normal progenitor cells.

This efficiency of removal of plasma cells is considerably greater than that previously reported for magnetic bead separation using the monoclonal antibody PCA-1 [2]. In that study Shimazaki et al. recommend a magnetic bead:target cell ratio of 500:1 using PCA-1 as the anti-plasma cell agent [2], whereas in our study comparable efficiency of purging with PNA is achieved at a magnetic bead:target cell ratio of 40:1.

This study has also shown that PNA does not have a direct toxic effect on hemopoietic progenitors (CFU-GM, CFU-GEMM, and CFU-GM generated in LTBMC) when added in concentrations in excess of those needed for purging. We have previously demonstrated that the CD34+ phenotypic hematopoietic progenitors that are capable of repopulating irradiated BM [13] are not reactive with PNA [6].

Although the PNA reactivity of blood and lymphoid organs has been extensively investigated [14], little work has been done on PNA staining or fractionation of human BM. Reisner et al. [15] showed in the mouse that the BM fraction agglutinated by PNA contains hemopoietic progenitors, but they did not directly demonstrate staining of early cells, and they also found progenitors in the nonagglutinated fraction. The apparent discrepancy between the work of Reisner et al. [15] and the present study may be a species difference or may simply reflect nonspecific entrapment of mouse progenitors in the PNA-induced agglutinates; our studies were performed at lower nonagglutinating concentrations of PNA.

CD19+ B-lymphocytes still remain after removal of PNA+ cells, and several observations suggest that these cells are involved in the malignant clone. For example, they express immunoglobulin of the same idiotype as the myeloma cell [16] and have undergone identical clonal gene rearrangements [17]. In addition, phenotypic analysis of myeloma colonies revealed a mixture of mature plasma cells and CD19+ cells [7], further suggesting that some B-lymphocytes are involved in the clonogenic process.

We therefore removed CD19+ cells with an established immunomagnetic bead method [18] and showed that this, in combination with the PNA purging method, removed both plasma cells and B-lymphocytes without impairing normal progenitor growth. More recently, Grogan et al. [19] have identified coexpression of myelomonocytic antigens with plasma cell antigens in myeloma. It is tempting to speculate that the reactivity of PNA with some cells of myelomonocytic [6] origin in addition to plasma cells may increase its purging efficiency.

More sensitive techniques for detecting residual malignant cells after purging are being developed, using DNA technology to find clonal gene rearrangements, but until amplified gene products are available the detection of cells bearing cytoplasmic immunoglobulin, as in this study, is probably the best indicator of the thoroughness of purging.

In conclusion, we have described a method for removing cells of B lineage from myeloma BM. Because the precise stage of normal B-cell development at which malignant transformation occurs in myeloma is unknown, the purging agents described here are intended to eliminate both earlier B cells

Table 6. Normal hemopoietic progenitors remain after purging

	Cells removed	CFU-GM[a]		Percent recovery[b]	CFU-GEMM[c]		Percent recovery[b]
		Before	After		Before	After	
Normal BM[d]	PNA$^+$	105 ± 28	161 ± 19	67 ± 13	8 ± 3	20 ± 4	72 ± 12
Normal BM[d]	PNA$^+$ CD19$^+$	93 ± 15	148 ± 21	51 ± 8	9 ± 3	14 ± 6	56 ± 11
Myeloma BM[e]	PNA$^+$ CD19$^+$	67 ± 21	150 ± 42	57 ± 3	6 ± 3	16 ± 9	80 ± 21

[a] CFU-GM: colonies per 2×10^5 cells plated.
[b] Data collected for nucleated cell loss during purging and expressed as percentage of pre-purge colony numbers.
[c] CFU-GEMM: colonies per 10^5 cells plated.
[d] Ten million BM MNC spiked with 10^6 tumor cells (three experiments).
[e] Ten million myeloma BM MNC (four experiments).

and plasma cells. Whether the latter are capable of initiating tumor relapse is also unknown, and further myeloma BM culture studies together with gene rearrangement analysis are required.

The present findings relate to small-scale experiments. The application of this technique to patients receiving ablative chemoradiotherapy combined with rescue with purged BM transplant is currently being assessed.

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References

1. Tong AW, Lee JC, Fay JW, Stone MJ (1987) Elimination of clonogenic stem cells from human myeloma cell lines by a plasma cell reactive monoclonal antibody and complement. Blood 70:1482
2. Shimazaki C, Wisniewski D, Scheinberg DA, Atzpodien J, Strife A, Gulati S, Fried J, Wisniewolski R, Wang CY, Clarkson BD (1988) Elimination of myeloma cells from bone marrow using monoclonal antibodies and magnetic beads. Blood 72:1248
3. Dinota A, Barbieri C, Gobbi M, Tazzari PL, Rizzi S, Bontadini A, Bolognesi A, Tura S, Stirpe F (1989) An immunotoxin containing momordin suitable for bone marrow purging in myeloma patients. Br J Cancer 60:315
4. Warburton P, Joshua DE, Gibson J, Brown RD (1989) CD10-(CALLA)-positive lymphocytes in myeloma: evidence that they are a malignant precursor population and are of germinal centre origin. Leuk Lymphoma 1:11
5. Barlogie B, Epstein J, Selvanayagam P, Alexanian R (1989) Plasma cell myeloma: new biological insights and advances in therapy. Blood 73:865
6. Rhodes EGH, Flynn MP (1989) PNA shows specificity for bone marrow plasma cells. Br J Haematol 71:183
7. Rhodes EGH, Olive C, Flynn MP (1990) A serum-free culture method for myeloma progenitors in vitro: proliferative and immunophenotypic characteristics. Exp Hematol 18:79
8. Reynolds CP, Black AT, Woody JN (1986) Sensitive method for detecting viable cells seeded into bone marrow. Cancer Res 46:5878
9. Morecki S, Paulotzky F, Margels P, Slavin S (1987) Purging breast cancer cells in preparation for autologous bone marrow transplantation. Bone Marrow Transplant 1:357
10. Erber WN, Pinching AJ, Mason DY (1984) Immunocytochemical detection of T and B cell populations in routine blood smears. Lancet i:1042
11. Fauser AA, Messner HA (1978) Granuloerythropoietic colonies in human bone marrow, peripheral blood and cord blood. Blood 52:1243
12. Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91:335
13. Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, Bernstein ID (1988) Antigen CD34$^+$ marrow cells engraft lethally irradiated baboons. J Clin Invest 81:951
14. Lis H, Sharon N (1986) Lectins as molecules and as tools. Annu Rev Biochem 55:35
15. Reisner Y, Itzicovitch L, Meshorer A, Sharon N (1978) Hemopoietic stem cell transplantation using mouse bone marrow and spleen cells fractionated by lectins. Proc Natl Acad Sci 75: 2933
16. Bast E, Van Camp B, Reynart P, Wiringa G, Ballieux R (1982) Idiotypic peripheral blood lymphocytes in monoclonal gammopathy. Clin Exp Immunol 47:677
17. Berenson J, Wong R, Kim K, Brown N, Lichenstein A (1987) Evidence for peripheral blood B lymphocyte but not T lymphocyte involvement in multiple myeloma. Blood 70:1550
18. Kvalheim G, Sorenson O, Fodstad O, Funderud S, Kiesel S, Dorken B, Nustad K, Jakobsen E, Ugelstad J, Pihl A (1988) Immunomagnetic removal of B-lymphoma cells from human bone marrow: a procedure for clinical use. Bone Marrow Transplant 3:31
19. Grogan TM, Durie BGM, Spier CM, Richter L, Vela E (1989) Myelomonocytic antigen positive multiple myeloma. Blood 73: 763