THE EFFECT OF HIGH DOSE CHEMOTHERAPY ON HAEMOPOIETIC COLONY STIMULATING ACTIVITY IN THE PLASMA OF PATIENTS WITH MULTIPLE MYELOMA

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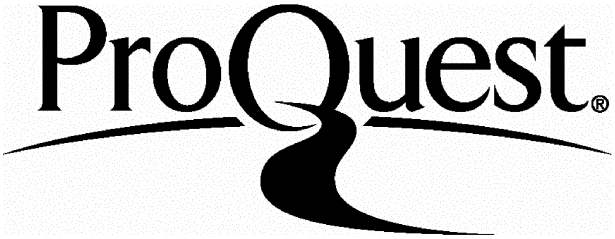

ABSTRACT

Following intensive chemotherapy with high dose meiphalan (HDM), piasma from patients with multipie myeloma (MM) has been tested for the presence of activity that can promote the growth of granulocyte-macrophage colonies (GM-CFUc) from normal human bone marrow mononuclear cells (BMMNC) *in-vitro*. Colony stimulating activity (CSA) in plasma has been compared with the recovery of peripheral blood leucocytes, platelets and haemopoietic progenitors (PB GM-CFUc). The presence of individual cytokines in plasma has been determined by the use of enzyme-linked immunoabsorbent assays (ELISA) and radioimmunoassy (RIA).

Peripheral blood GM-CFUc and CSA decreased after a priming dose of cyclophosphamide given before HDM, but numbers of GM-CFUc in the bone marrow increased in the same individuals. Numbers of PB GM-CFUc before priming predicted numbers of GM-CFUc that could be harvested from patients bone marow 5-9 days later.

After HDM, CSA increased and was independent of disease status, previous chemotherapy, the use of autologous bone marrow rescue (ABMR) or recombinant human granulocyte colony stimulating factor (rhG-CSF). CSA was not related to the acute phase response, as indicated by a lack of correlation with changes in plasma levels of interleukins 1 or 6 (IL-1 or IL-6) or C-reactive protein, but was correlated with the development and duration of leucopenia after treatment. Interleukin-4 (iL-4) could augment plasma CSA *in-vitro* and this was shown to be due to the presence of

endogenous G-CSF.

Interleukin-3 (IL-3) and GM-CSF (granulocyte-macrophage-CSF) were not detected in plasma but CSA could be inhibited *in-vitro* by the addition of antibody to GM-CSF.

The origins and physiology of CSA are discussed with reference to results that suggest that this activity does not arise from haemopoietic tissue.

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TO MAC

LIST OF CONTENTS

CHAPTER 2: MATERIALS AND METHODS

CHAPTERS 3-5: RESULTS

CHAPTER 3

CHAPTER 4

LIST OF TABLES

APPENDIX I

LIST OF FIGURES

CHAPTER 5

APPENDIX II

LIST OF PLATES

CHAPTER 1

INTRODUCTION

1,1 THE PROBLEM

Multiple myeloma is an incurable and invariably fatal malignant disease. Although most patients will respond to treatment with drugs, remissions, which result in a reduction in the bulk of their disease and an improvement in their symptoms and quality of life, are short lived. Eventually the disease becomes resistant to chemotherapy.

At the Royal Marsden Hospital (RMH) patients undergoing treatment for MM are given induction chemotherapy with VAMP (Vincristine, Adriamycin (Doxorubicin) and Methyl-Prednisolone - See Below) with or without cyclophosphamide followed by consolidation with high dose melphalan (HDM) (see below). Dose escalation of melphalan to levels that are associated with severe and life threatening haematological toxicity has been achieved by the use of autologous bone marrow rescue (ABMR), which has resulted in a reduction in the morbidity due to Infection and in the rate of mortality.

In recent years recombinant cytokines, which are involved in the control of haemopoiesis, have been used in further attempts to reduce haematological toxicity and infective mortality following high dose chemotherapy. Recombinant factors such as human granulocyte and human granulocyte-macrophage colony stimulating factors (rhG-CSF & rhGM-CSF) raise the level of circulating granulocytes *in-vivo* and can reduce the time taken to recover adequate levels of circulating granulocytes after chemotherapy.

They may also reduce infective morbidity and the length of hospitalization after intensive chemo/radiotherapy. However, the benefits of these agents has been less than anticipated and there has been no impact on the level or period of thrombocytopenia, which has its own morbidity and risk of mortality, or on the requirement for platelet transfusion.

Despite the administration of recombinant growth factors to cancer patients little has been published on the endogenous levels of these molecules either in steady state conditions or in response to intensive therapy.

Although serum from patients with malignancy or aplasia contains colony stimulating activity (CSA) which stimulates the proliferation of bone marrow progenitors *in-vitro*, the composition of this activity is unknown and it is not clear which tissues are responsible for its production. Furthermore, after chemotherapy, it is unclear whether CSA is related to disease status or to a response to treatment or to a combination of these factors. This activity is not present in the serum of normal donors.

This study was designed to ascertain the effect of intensive chemotherapy on CSA and to determine which cytokines are present in vivo after high dose therapy in patients with MM, and whether the levels of cytokines are influenced by high dose chemotherapy, by ABMR or by the administration of recombinant growth factors. The aim of the study was to suggest potential strategies that may enhance haemopoietic recovery after intensive therapy, for example, the administration of different cytokines before or after high dose chemotherapy and stem cell rescue.

1,2 MULTIPLE MYELOMA

1,2.1 DEFINITION

Multiple myeloma is a malignant disease of the B-cell lineage characterised by infiltration of the bone marrow by monoclonal plasma cells and the production of a monoclonal immunoglobulin or paraprotein.

1,2.2 HISTORY

The first report of MM described a patient with symptoms of bone pain, fatigue, urinary frequency and proteinuria [Macintyre, 1850]. The same patient was reported by Bence Jones in 1848 who described the precipitation of the urinary protein (immunoglobulin light chains) that now bears his name [Bence Jones, 1848]. The term "multiple myeloma" was coined in 1873 [Rustizky, 1873] and in 1899 the association of the disease with high serum protein and erythrocyte sedimentation rate was described [Ellinger, 1899]. In 1933 the plasma cell was identified as the malignant cell [Wright, 1933] and in 1953 protein electrophoresis was used to identify the paraprotein band characteristic of the disease [Grabar & Williams, 1953]. The earliest report of a response to systemic therapy occurred in 1947 [Alwall, 1947] when 15 per cent of patients responded to urethan.

1,2.3 AETIOLOGY AND EPIDEMIOLOGY

MM currently accounts for approximately 1.1 per cent of all malignant neoplasms and 1.5 per cent of cancer deaths in the United kingdom [CRC Factsheet 1.2/1.3 1990 and 3.2/3.3 1989]. The incidence is approximately 4.3 new cases per 100,000 per year in females and 4.6 per 100,000 in males in the United Kingdom and is similar in the United States of America. The NCI SEER program in the USA [Young et al, 1981], over the period 1973-77, identified a rate of 4.3 per 100,000 in whites but nearly twice that rate in blacks. There has been an increase in the incidence of MM over the last thirty to forty years and although some of this increase represents an increased awareness of the disease and better methods of detection there has probably been a true rise in the incidence.

The incidence of MM reaches a peak in the seventh decade (21/100,000) with a steady rise from the third decade of life (the incidence is 0.1/100,000 between age 25-34). Cases of MM before age twenty are extremely rare. There is a slight excess of males to females (<1.5:1) amongst MM patients. The age distribution of MM is similar to that of two related conditions, Waldenströms Macroglobulinaemia (see below), and monoclonal gamopathy of undetermined significance (MGUS). MGUS is usually detected by the presence of an elevated but stable paraprotlen in the serum. The incidence of MGUS in 6995 samples from an adult population was 2 per cent in the eighth decade and 6 per cent in the ninth [Axelsson et al, 1966].

The distribution of MM between racial groups suggests a genetic component in

the aetiology. Familial evidence for a genetic contribution to the aetiology of MM suggests that there may be an increase of certain HLA groups in patients with myeloma and an increased incidence of myeloma in relatives of patients with MM and MGUS. In one study [Blattner et al, 1981] a recessive mode of inheritance was implied, however, the data might equally suggest that common exposure to environmental factors is important.

Much of the pathogenesis of MM has been determined from murine plasmacytomas which develop spontaneously in inbred mice such as the C57BL/Ka strain and with increasing frequency with age [Radi et al,1978]. Although the incidence of plasmacytomas is low in Balb/c mice they are induced by peritoneal injection of mineral oils [Potter & Boyce,1962]. The rate of tumour production can be enhanced by virus infection [Anderson, 1970] and tumourgenicity is reduced in mice that have been reared in a germ free environment and subsequently treated with intraperitoneal promoters [McIntire and Princier, 1969]. These studies suggest that antigenic exposure of neonates may prime susceptible cells, making them vulnerable to a second mitogenic stimulus.

Various epidemiological studies have been undertaken in man to determine whether exposure to chemicals increases the risk of developing MM. One such study showed an increase risk of MM in people exposed to petroleum products [Linet et al, 1987] and recently a study of 34,000 seventh day adventists [Mills et al, 1990] showed a 3.01 fold risk of developing MM for ex-smokers compared to people who had never smoked. Furthermore, amongst ex-smokers there was a relationship between the

number of cigarettes smoked and length of time smoking, and the risk of developing MM. One of the constituents of cigarette smoke is benzene which has been implicated as a carcinogen in the petroleum industry. Farmers have also been found to be at increased risk of developing MM. A prospective study of 77.000 volunteers [Boffetta et al, 1989] produced 282 cases of MM. When compared to matched controls there was an increased risk of MM for farmers of 2.7 which rose to 4.3 times the expected rate if there was, additionally, a proven history of exposure to pesticides. However exposure to pesticides alone was not a significant risk factor.

Radiation has been implicated in man in various reports. The most important observations have been from studies on the effects of the atomic weapons used at the end of the second world war. These revealed a latent period of twenty years at which time there was an increased rate (4.7 fold) of MM detected in survivors who had received 100cGy [Ichimaru et al,1979, Cuzik, 1981]. Evidence of risk from exposure to low doses of radiation has come from studies showing a slightly increased risk of MM in radiologists and workers in nuclear plants [Lewis, 1963, Matanoski et al, 1975, Mancuso et al, 1977].

Evidence for a requirement of more than two events in the pathogenesis of MM may come from the related condition, MGUS, in which there is a paraproteinaemia and an expanded but non malignant clone which may persist for many years and then progress to MM. Among 241 MGUS patients attending the Mayo Clinic for a period in excess of 10 years, 38 per cent remained stable, 33 per cent died of unrelated causes, 9

per cent had a rise in paraprotein without malignant change and 17 per cent developed malignancy, of which 68 per cent developed myeloma and 32 per cent, non Hodgkins lymphoma or Waldenströms Macroglobulinaemia. One well reported anecdotal case [Seligmann et al, 1973], concerns a man who developed MGUS after Inoculation with horse antiserum to tetanus toxin. This persisted for thirty years following which he developed MM in which his paraprotein was found to be an IgG immunoglobulin directed against horse alpha-2-macroglobulin.

Many chromosome abnormalities have been described in MM, but none have been described in all or even the majority of cases. However, the *c-myc* oncogene which is over-expressed in murine plasmacytomas has been detected in up to twenty five per cent of human MM [Selvaney et al, 1988]. In murine plasmacytomas *c-myc* is frequently involved in translocations from chromosome 15 to chromosome 12 in proximity to the heavy chain locus suggesting that heavy chain gene activation may lead to over-expression of this oncogene .

1,2.4 PATHOLOGY

The plasma cells which characterise MM are large with abnormal and often multiple nuclei. These nuclei are usually eccentric and often display condensed chromatin and prominent nucleoli. In some instances the morphology of the malignant clone may be indistinguishable from the normal plasma cell population. The cell cytoplasm stains blue or blue/purple with Romanovsky type stains, revealing a prominent pale or non staining perinuclear hof. The blue staining cytoplasm reflects the high

concentration of rough endoplasmic reticulum and the perinuclear hof indicates the prominent golgi apparatus involved in glycosylating and packaging immunoglobulin for export from the cell. The mitotic rate in bone marrow aspirates or biopsies of MM is low, usually less than 3% reflecting the comparatively low proliferation index of between one and three per cent and the long cell cycle time of between one and three days. There is frequently an associated increase in isotypic cells that are phenotypically earlier in the B-cell lineage. Immunoglobulin of the specific type for the tumour can be detected within the cytoplasm but not the cell membrane in mature plasmacytoid myeloma cells. In common with earlier B-cells and lymphocytes, more primitive cells of the myeloma clone may retain membrane immunoglobulin. Various leucocyte markers can be identified on the surface of myeloma cells but none is a universal marker of MM, nor is there any other surface marker which is unique to MM. Thus there is no means of isolating tumour cells from normal bone marrow elements by positive selection. The most common markers found in MM are CD10 (CALLA) and CD38 (anti leu-17) with a lower incidence of B cell differentiation markers, B1,B2 & B4 (CD 20,21,19 (anti leu-16,anti CR2, anti leu-12)). Myeloid and T-cell antigens can also sometimes be identified on MM cells.

Both Plasma cells and isotypic lymphocytes have been detected and cultured from the peripheral circulation of MM patients [Millar et al, 1988, Caligaris-Cappio et al, 1991]. Evidence that isotypic lymphocytes and plasma cells in bone marrow and blood belong to the malignant clone has been provided by the use of anti-idiotype antibodies [Stevenson and Thomson, 1988]. Circulating clonal cells can also be detected by specific heavy chain gene rearrangements [Bell et al, 1990].

Although it has not been shown that isotypic lymphocytes or lymphoplasmacytoid cells

mature to plasmacytoid myeloma cells [Millar et al, 1988], it seems likely that both cell types are progenitors.

Bone marrow infiltration with localised collections of plasma cells in particular regions corresponding to the lytic lesions in bone are characteristic of MM. As the disease progresses plasmacytomas expand into surrounding tissues including the spinal canal, and may cause spinal cord compression. In some cases of myeloma isolated soft tissue deposits occur, for example in the skin or breast tissue.

The commonest class of immunoglobulin secreted in MM is IgG followed by IgA and IgM in that order of frequency. IgD myeloma is rare and IgE exceptionally so. There is an excess of kappa light chains compared to lambda. In a significant proportion of cases light chains only are secreted and in a small proportion no secreted protein can be detected in the serum or urine. These cases are known as non-secretory although the tumour cells usually contain cytoplasmic immunoglobulin.

The amount of protein secreted in relation to tumour bulk is highly variable between individuals, but within an individual the paraprotein level can be used as a marker of tumour bulk [Salmon, 1973]. It is not uncommon for the paraprotein to be lost or for the secretion to change to light chains only. This may occur at relapse or in patients whose disease has become resistant to chemotherapy. Similarly cells may acquire a more primitive morphology that is indicative of poor prognosis [Bartl, 1988].

The secretion of paraprotein in MM may be associated with renal failure which is

also an important indicator of poor prognosis. The principle lesion is selective tubular damage related to a toxic effect of immunoglobulin light chains (Lambda light chains are more nephrotoxic than kappa) which reduces the ability of the kidney to reabsorb this protein resulting in the appearance of light chains in the urine (Bence Jones protein (BJP)) and deposition of casts containing BJP, albumin and intact immunoglobuiin. These further impair renal function. However, the renal failure of MM is multifactoriai and other important contributing factors are hypercalcaemia, dehydration and infection. Amyloid deposition in the renal vasculature and tubular basement membrane is involved in a minority of cases, as is renai infiltration by plasma celis.

Hypercalcaemia arises from the activation of osteoclasts around myelomatous deposits. Osteoclast activating factor (OAF) which is secreted by myeloma cells has been implicated [Mundy et al, 1974, Valentin-Opran et al, 1982], although other lymphokines such as tumour necrosis factor, iymphotoxin and interleukin 1, which are active in bone resorption assays and have been detected in the culture supernatants of MM cells, may be involved [Garrett et ai, 1987, Lichtenstein et al, 1989].

Bone marrow infiltration by plasma cells may result in bone marrow failure characterised by a normochromic anaemia (which is of prognostic significance see below), thrombocytopenia, and neutropaenia which increases the susceptibility of these patients to infection. Additionally humoural immunity is reduced by a generalised immunoparesis which occurs also in some cases of MGUS. In mouse models of MM factors have been identified that directly and indirectly inhibit normal B-cell activation and antibody secretion [Ullrich et al, 1982]. It is likely that a similar mechanism is operable in

man.

1,2.5 KINETICS AND CONTROL OF TUMOUR GROWTH

Multiple myeloma has a low growth fraction and was thought initially to undergo a twenty or thirty year lag period, for IgA and IgG MM respectively, between malignant transformation and clinical disease [Hobbs, 1969]. However, improved mathematical modelling suggested a preclinical phase spanning only one to three years [Sullivan and Salmon, 1972]. A recent anecdotal case, however, supports the former hypothesis. Two identical twins have developed myeloma with the same IgGk paraprotein within one year of each other and ten years after allogeneic transplantation of bone marrow from one brother to the other for treatment of chronic myelogenous leukaemia (CML) [Stewart et al, 1993].

In some instances periods of non expansion of the malignant clone occur, spontaneously, or often, after successful chemotherapy suggesting that there are mechanisms which control cell proliferation. In this "plateau phase" bone marrow infiltration and serum paraprotein remain stable for periods extended, sometimes, to years. During these periods myeloma colonies can be grown from bone marrow aspirates *in-vitro* suggesting that the mechanism(s) which inhibit growth *in-vivo* is absent when tumour cells are removed from the body [Millar et al, 1988]. Similarly colonies have been grown *in-vitro* from bone marrow aspirates taken from patients receiving alpha-interferon to maintain remission after intensive therapy (unpublished observation, B.C.Millar).

1,2.6 RELATED CONDITIONS

Several conditions exist in which the presence of isotypic immunogiobulin or paraprotein indicates monoclonal B-cell proliferation. These include MGUS and Waldenströms Macroglobulinaemia, solitary plasmacytoma of bone, extramedullary plasmacytoma and plasma cell leukaemia (PCL).

In plasmacytoma there are one or more isolated lesions consisting of cells identicai to the plasma cells of MM, a paraprotein may be present but there is no generalised dissemination of plasma cells throughout the bone marrow and systemic effects such as hypercalcaemia and renal failure are rare. Although these diseases may respond to local treatment, in most cases of plasmacytoma of bone patients develop MM within five years. Extramedullary plasmacytoma is very rare and more likely to appear in younger age groups. It usually presents in the mucous membranes, gastrointestinal tract, breast or lymph nodes and may metastasise vla the lymphatics or blood.

PCL is rare and is usually an aggressive dlsease characterised by bone marrow failure, circulating plasma blasts and splenomegaly. Serum paraprotein and urinary light chains help to distinguish this condition from other poorly differentiated leukaemias. PCL may occasionally arise as a transformation in a patient with existing MM.

Waldenströms Macroglobulinaemia is characterised by earlier cells of the B-cell lineage than MM. The morphology of the cells usually resemble those of lymphoplasm-acytoid non-Hodgkins lymphoma (NHL) and the clinical features of lymphadenopathy, splenomegaly and bone marrow infiltration without destructive bone lesions resemble the

behaviour of NHL rather than MM. The Immunoglobulin produced in this tumour is usually an IgM molecule which can result in hyperviscosity.

1,2.7 CLINICAL FEATURES

The most frequent symptom at presentation is bone pain, often associated with pathological fractures. Spinal lesions result, characteristically, in loss of height due to vertebral collapse. Expansion of these deposits into the spinal canal can result in symptomatic and/or occult cord lesions, which are most sensitively detected by magnetic resonance imaging [Joffe et al, 1988].

Hypercalcaemia is the second most common feature. Symptoms include thirst, polyuria, constipation, nausea and, later, drowsiness and impaired consciousness.

Recurrent or severe bacterial and viral infections due to neutropaenia and reduced humoural immunity are often prominent in the history. Symptoms of anaemia may indicate bone marrow failure. Abnormal bleeding and easy bruising may occur due to thrombocytopenia or the impairment of clotting factor activity by a high level of serum paraprotein. Hyperviscosity syndrome affects four percent [Selby, 1987] of all myeloma patients particularly those with IgM myeloma. The syndrome consists of cardiac failure, malaise, neurological phenomena and retinal vein dilatation.

Characteristic laboratory findings include a raised erythrocyte sedimentation rate (ESR) and rouleaux on examination of the blood film. Beta-2-microglobulin, derived from the light chain of the HLA antigen, is raised in patients with MM and its level reflects cell turnover and impaired renal function.

1,2.8 STAGING AND PROGNOSIS

Important prognostic factors within most staging systems for MM are haemoglobin, renal function, serum calcium and performance status. The most widely used staging system has been that described by Salmon and Durie, which relates these variables to total tumour cell mass. The main features are described in Table 1,2. below.

TABLE 1,2.

Staging System of Salmon & Durie

Stage I All of the following Haemoglobin > 10g/dl Serum Calcium < 3mmol/l Solitary or no plasmacytoma on skeletal survey (X-rays) Low paraprotein IgG < 50g/l IgA < 30g/l BJP < 4g/24hrs Equivalent to low cell mass < 0.6×10^{12} cells/m^2	**Stage III** One or more of the following Haemoglobin < 8.5g/dl Calcium > 3mmol/l 2 or more lytic lesions on skeletal survey High paraprotein IgG > 70g/l IgA > 50g/l BJP > 12g/24hrs Equivalent to high cell mass > 1.2×10^{12} cells/m^2
Stage II Intermediate between stage I and stage III Equivalent to Intermediate cell mass	Renal Function determines allocation to stage A or B in each of stages I-III Serum Creatinine A = < 177µmols/l B = > 177µmols/l

[Modified from Durie & Salmon, 1975]

More recently beta-2 microglobulin has been found to be the single most important predictor of prognosis in myeloma. Patients with levels of β2-microglobulin, at

presentation, below 6 μg/ml had a significant advantage of survival at five years compared to patients with levels above 6 μg/ml [Bataille et al,1986]. In this study low serum albumin was also found to be a predictor of poor prognosis particularly when combined with high levels of β2-microglobulin. More recently it has been suggested that interleukin-6 may be an autocrine or paracrine growth factor for MM and that its concentration in the serum of patients may be of prognostic significance [Bataille et al, 1989]. This is discussed further in chapter 1,4.2.g (Interleukin-6).

1,2.9 CRITERIA FOR RESPONSE TO TREATMENT

The criteria used for assessment of response to treatment that are used in this study and those of most investigators are as defined by Gore et al, [1989].

For a patient to have achieved complete remission (CR) four criteria were required: No paraprotein should be measurable by scanning densitometry of serum proteins separated on cellulose acetate membrane by electrophoresis and stained with ponceau S; no detectable BJP on electrophoresis of neat urine stained with colloidal gold; 5% or fewer of plasma cells of normal morphology on bone marrow aspiration; and these criteria had to be fulfilled for at least three months. Patients were regarded as having achieved partial remission (PR) if there was a 50% decrease in measurable paraprotein (IgG or IgA MM) or bone marrow infiltration (non-secretory or Bence-Jones Myeloma) which was sustained for a month or more.

1,2.10 DEVELOPMENT OF CHEMOTHERAPY FOR MM

a) Single agents and steroids alone and in combination

Alkylating agents were first found to have activity in MM. Melphalan, the L-isomer of phenylalanine mustard, was first reported to produce a thirty per cent remission rate by the South Western Oncology Group (SWOG) in 1962 [Bergsagel et al, 1962]. Cyclophosphamide was shown to be effective in 1964 [Korst et al, 1964] and prednisolone, a glucocorticoid, in 1967 [Salmon et al, 1967]. Melphalan and prednisolone were studied in combination in the early 1970s [Alexanian et al, 1972, Costa et al, 1973]. The single agent efficacy of doxorubicin (Adriamycin) and BCNU (Carmustine) were described in the following years [Alberts and Salmon, 1975, Salmon, 1976]. Limited single agent activity, usually 10% or less, has been described for other agents such as epirubicin, etoposide and platinum compounds.

Response rates to cyclophosphamide or melphalan either alone or in combination with steroids are between thirty and seventy per cent in previously untreated patients and the median survival of patients treated with these drugs is increased from a median of one year to 24 months (18 - 36 months)[Salmon and Cassady, 1989]. In the United Kingdom the Medical Research Council (MRC) has conducted several large trials in the treatment of MM. The first found no difference in survival (18 months) between patients treated with daily oral melphalan or cyclophosphamide. The second showed a 20 month median survival for both melphalan with prednisolone or cyclophosphamide alone, suggesting that prednisolone did not add to the efficacy of alkylating agents. In the third

MRC trial intermittent oral melphalan was compared to intravenous cyclophosphamide for which there was a small survival advantage [MRC1, 1971, MRC2, 1980, MRC3, 1980, MacLennan et al, 1988].

b) Combination therapy

Multi-drug combination therapy was developed with the aim of combining drugs with different modes of action and different spectra of toxicity in order to increase response rates and survival without exceeding acceptable levels of toxicity. The first major protocol published was the M2 regimen of the Memorial hospital in 1977 [Case et al, 1977]. Vincristine, melphalan, cyclophosphamide, BCNU and prednisolone were used in a four week cycle. This regimen has been compared to melphalan and prednisolone (M/P) in several trials and although there is an increase in response rate there is no survival advantage. In the study by the East Coast Oncology Group (ECOG) in the USA [Oken et al, 1984] the response rates were 72% for M2 (VBCMP) and 51% for M/P. There was no increase in median survival of patients receiving M2, although reports suggest that patients with stage III disease had a small survival benefit. The SWOG included adriamycin in a drug regimen that alternated VMCP with VBAP or VMCP with VCAP (vincristine, melphalan, cyclophosphamide, prednisolone/vincristine, BCNU, adriamycin, prednisolone (VCMP/VBAP) or vincristine, cyclophosphamide, adriamycin, prednisolone VCMP/VCAP). In this regimen (which is still widely used) each four-drug combination is given in a three week cycle but the combination of drugs is alternated each three weeks. Switching each nine weeks, after three cycles of the same four drugs, has also been used. Both increased response rates, and an increased median survival

especially in stage III patients were reported [Salmon et al, 1983, Durie et al, 1986], however, in other studies using this protocol the duration of remissions for alternating therapy and M/P were not different [Alexanian & Driecar, 1984].

The fifth MRC trial in the UK also used an alternating system in its ABCM regimen (adriamycin, BCNU, cyclophosphamide, melphalan). The essential difference from the SWOG alternating regimen is the omission of prednisolone and vincristine. In this trial ABCM was compared with 7 day oral melphalan (M7). A higher proportion of patients treated with ABCM responded or developed a stable plateau (minimal symptoms, transfusion independent and paraprotein stable for 3 months - 61% compared to 49] and there was a significant survival benefit for the combination treated patients ($p=0.0003$, $n=630$), for example, median survival of the two groups were (ABCM) 42 and (M) 32 months [MacLennan et al, 1992].

The survival curves for the SWOG study and MRC V are almost superimposable [Salmon and Cassady, 1989] suggesting that vincristine and prednisolone contribute little to these regimens. This is consistent with the findings of the second MRC trial with regard to steroids and the fourth trial in which no difference was found in survival between patients treated with M/P or MVP (melphalan, vincristine, prednisolone). A further MRC trial compares ABCM with ABCM plus prednisolone (ABCMP). At an interim analysis ABCMP resulted in a more rapid response but there was no evidence of a better response rate or survival [Chapman et al, 1989].

VAD (Vincristine and adriamycin infused via central venous cannulation over 5 days with oral high dose dexamethasone, repeated every 21 days) was first reported as

an effective treatment for refractory myeloma in 1984 [Barlogie et al, 1984], when a 70% response rate was reported in 20 patients who had not responded to conventional combination therapy. VAD was more effective in refractory relapsing patients than in primarily resistant patients in which high dose dexamethasone (HDDex) was shown to be the important agent when VAD was compared to HDDex [Alexanian et al, 1989]. Alexanian et al reported that primarily resistant patients had a response rate of thirty percent to VAD or HDDex. The response rates of resistant myeloma to VAD have been confirmed in other studies [Monconduit et al, 1986]

In untreated patients The Riverside Haematology group in London had an 84% response rate in 32 patients treated with VAD of whom 28% achieved a CR. The median duration of response was 18 months and projected survival 44 months [Samson et al, 1989]. At the Royal Marsden Hospital (RMH) concern about immunosuppression and a high incidence of proximal myopathy prompted the replacement of dexamethasone in VAD with high dose methyl prednisolone which had been shown to produce short responses in 25% of patients as a singie agent [Forgeson et al, 1988]. This regimen, known as VAMP, induces similar response and survival rates to VAD and is now being used (with the addition of cyclophosphamide (C-VAMP)) as induction therapy before consolidation with high dose therapy and ABMR (see below).

c) Treatment intensification

In 1983, using doses of melphalan between 100 and 140mg/m^2, McElwain and Powles reported responses in 7 patients with MM and one with PCL. These included

complete remissions in 3/5 previously untreated patients [McElwain and Powles, 1983]. In the follow up study of 58 patients [Selby et al, 1987] the overall response rate in 41 untreated patients was 78% (CR-27% and PR-51%) and 66% in 15 resistant patients with 2 patients achieving complete remissions. The median duration of remission of 19 months was associated with bone healing and a good quality of life. The median survival was 5 years. Addition of high dose methyl prednisolone (HDMP) did not alter the response rates. The ongoing sixth MRC study compares ABCM with HDM as initial therapy.

At the RMH the dose of melphalan has been increased to 200mg/m^2 in appropriate patients (see later) which requires ABMR to obviate the myelotoxicity seen with this dose of drug. To minimise tumour mass and clear the bone marrow sufficiently of tumour cells for harvesting, HDM was preceded by induction therapy with VAMP. In the first study of VAMP/HDM with and without ABMR [Gore et al, 1989] 50% of patients have achieved complete remission and 24% partial remission. Although the period of neutropaenia (less than 10^9/l WHO grade 4) was reduced from a mean of 30 days to 20 days by ABMR there was no reduction in the period of thrombocytopenia. The ongoing programme at the RMH incorporates cyclophosphamide into the VAMP regimen. This was based on *in-vitro* observations [Maitland et al, 1990] that cyclophosphamide increases the clonogenicity of MM and may thus activate tumour cells into cycle before each course of VAMP thereby increasing the cell kill. Verapamil has also been added to the regimen (VC-VAMP) in a randomised study to investigate this agent's role in overcoming drug resistance.

d) Interferon and maintenance of remission

After the first SWOG trial of combination therapy a randomised trial of maintenance M/P Vs "no treatment" until relapse was instituted in patients who had responded to treatment, however, there was no benefit in survival among patients who received maintenance M/P who suffered more infective episodes (SWOG, 1975). Further studies confirmed the lack of benefit [Belch et al, 1988, Alexanian, 1986] and maintenance chemotherapy was abandoned by most units.

In 1979 Mellstedt reported two complete and two partial responses in four patients using α-interferon as a single agent [Mellstedt et al, 1979]. Subsequently, in a randomised study in untreated patients, the Myeloma Group of Central Sweden reported a response rate of 14% in IFN treated patients compared to 44% for M/P treated patients. The duration of response was shorter for the IFN treated patients and igG myelomas appeared to be more resistant to interferon than IgA Myelomas.

In resistant patients IFN was tested in a joint study between Manchester and Texas [Costanzi et al, 1985]. In this small group there was a response rate of approximately 25% for relapsed patients but half this for primarily resistant patients.

Studies *In-vitro* showed that interferon synergised with melphalan and with melphalan in combination with prednisolone in the RPM-I 8226 cell line [Cooper & Weliander, 1987]. This has been tested recently, in patients, by Monturoro et al, who suggest a benefit for the addition of IFN to M/P in terms of survival and an increase in response rate in patients with stage III disease [Montuoro et al,1990]. However, it appears that patients receiving IFN/M/P may have received more prednisolone than those who received M/P only.

Mandelli et al first reported the use of α-interferon as maintenance therapy in 1987. Patients who responded to therapy with M/P or VMCP/VBAP were randomised to receive IFN or no treatment. In 1990 101 patients had entered the study. The duration of remission was longer in the IFN group (26 Vs 14/12, P=0.0002), Survival was longer in the treatment group (52 Vs 39/12) and deaths during the study period less (23/50 Vs 37/51) [Mandelli et al, 1990].

At the RMH patients have been randomised to treatment with Interferon-α or to observation alone after C-VAMP/HDM with ABMR. There is currently a significant progression-free survival benefit for the interferon treated patients (39 vs 27 months after HDM) in whom there have also been fewer deaths. The advantage relates, predominantly, to those patients in CR after HDM [Cunningham et al, 1993].

The seventh MRC trial will address the question of the benefit of intensification in patients who are to receive maintenance therapy with interferon. Patients will be randiomised to C-VAMP plus HDM or to ABCM and then treated with interferon-α in remission (plateau as defined for MRC V, page 18).

1,3 HIGH DOSE CHEMOTHERAPY AND BONE MARROW/STEM CELL TRANSPLANTATION

1,3.1 RATIONAL

Allogeneic bone marrow transplantation (ABMT) was developed to repopulate the bone marrow of patients with aplastic anaemia and immune deficiency. This procedure followed conditioning treatment to ablate the recipient's immune defences and prevent rejection of the graft by the host (HVG). The same concepts were applied to haematological malignancies such as leukaemia in which tumour cells are sufficiently sensitive to radiation and chemotherapy to allow eradication of the malignant cells. Successful ABMT is associated with some rejection of the host by the graft (Graft verses host disease (GVHD)) as well as graft verses tumour activity which produces long term remissions. The disadvantages of ABMT are the rejection processes, graft rejection and GVHD, which are major causes of mortality and morbidity, and also the consequent need for immunosuppressive agents. These factors can delay engraftment when compared to ABMR and prolong the period of susceptibility to infection due to cellular and humoural insufficiency. Graft rejection is reduced by choosing donors whose tissue type is matched by HLA (Human Leucocyte Antigens) type with that of the recipient. HLA represents the major histocompatibility complex (MHC) in man; a group of three classes of antigens, encoded on chromosome 6, involved in the processes of immune recognition of self and non-self and antigen presentation.

In contrast, autologous bone marrow rescue (ABMR) was developed as a means of reducing toxicity following intensive chemotherapy or radiotherapy at doses that would be unacceptable otherwise. The advantages of ABMR are the lack of rejection (GVH and GVHD), and the lack of the need for a donor. The major disadvantages are the presence of residual tumour cells in the graft which may permit the tumour to repopulate, and the collection of sufficient viable stem cells from patients who have been heavily pre-treated with bone marrow toxic agents.

1,3.2 HAEMOPOIETIC RECONSTITUTION

Neutrophils and reticulocytes usually reappear in the circulation between 10 and 20 days after ABMT or ABMR followed by platelets 5 to 10 days later. Recovery times are slightly longer in allogeneic transplantation. Despite the re-emergence of reticulocytes, red cell transfusions may be required for a few weeks after this period.

Several studies have examined bone marrow aspirates taken sequentially after transplantation and have attempted to correlate the recovery of circulating elements, bone marrow cellularity and progenitor cell levels with the numbers of infused mononuclear cells or colony forming units (CFU). After ABMT in patients with aplastic anaemia and haematological malignancy neither bone marrow cellularity nor progenitor cell numbers measured in bone marrow aspirates after transplant correlated with recovery of haematological indices [Arnold et al, 1986]. However, total transfused MNC (mononuclear cells) and CFUc (granulocytes), CFUe (Erythrocytes) and BFUe (burst forming units-erythrocytes) did correlate with time to recovery of reticulocytes. CFUc transfused correlated with the with recovery of granulocytes. Other studies after ABMT

have found similar relationships between neutrophil recovery times and the numbers of infused CFUc but not between recovery and total infused MNC [Faille et al, 1981, Jansen et al, 1983], although some have not [Torres et al, 1985]. A similar correlation between total infused CFUc and neutrophil recovery was found in patients with AML, treated with AMBR [Spitzer et al, 1980].

These studies suggest that there may be a relationship between the progenitor cell content of the graft and subsequent rate of engraftment. However, the CFUc content of the graft is not the only factor that determines engraftment since depletion of CFUc forming cells by marrow purging with cytotoxic drugs does not inhibit engraftment after ABMR [Yeager et al, 1986, Gorin et al, 1986]. Long term culture of bone marrow, sampled sequentially after ABMT, suggests that stromal cells may be of importance in the establishment of the graft [Keating et al, 1982].

In both animals and man circulating progenitors can be cultured *in-vitro* and can rescue animals from lethal doses of radiation [Debelak-Fehir et al, 1975]. In primates peripheral blood stem cells (PBSC) collected after induction by chemotherapy retained the ability to rescue animals from total body irradiation (TBI) [Abrams et al, 1981a, Abrams et al, 1981b]. Abrams et al showed an inverse correlation between Peripheral blood CFUc infused and time to recovery of neutrophils.

Since cancer patients have more circulating progenitors cells after chemotherapy than normal controls [Richman et al,1976], PBSC can be harvested after priming chemotherapy. Recently, recombinant growth factors have been used to increase the numbers of PBSC [Socinski et al, 1988, Gianni et al, 1989]. Successful transplantation with PBSC is associated with higher numbers of CFUc ($30x10^4$/kg) than are needed in

bone marrow transplantation ($1\text{-}2x10^4$/kg), as assessed by *in-vitro* culture of the harvests [Juttner et al, 1985, To et al, 1984, Bell et al, 1987, To et al, 1983], suggesting a qualitative difference between the sources of cells. The percentage of multi-lineage progenitors (CFU-GEMM) is higher in bone marrow than in blood of normal individuals [Ash et al, 1981].

Reconstitution of circulating neutrophils is generally faster with PB stem cells than with autologous bone marrow cells, suggesting that PBSC contain a larger number of committed progenitors which provide mature elements temporarily whilst earlier stem cells establish long term engraftment. Following ABMT or ABMR there is an initial phase of rapid expansion of commited precursors and end cells but a delay before the establishment of adequate numbers of primitive precursors in the bone marrow [Arnold et al, 1986]. Late graft failure in ABMT and ABMR is likely to be related to inadequate numbers of stem cells despite adequate numbers of committed progenitors.

The pleuripotent stem cell has not been identified, however, cells capable of maturation into each lineage carry the same membrane antigen, a 110-120 kd protein recognised by the My10 and HPCA-1 (CD34) antibodies [Brandt et al, 1988]. CD34 +ve cells comprise 1-4% of bone marrow cells in man and some primates and can provide complete haemopoietic reconstitution in lethally irradiated baboons [Berenson et al, 1988].

CD34+ve cells are increased in the circulation of patients treated with cyclophosphamide and contribute to the efficient engraftment seen after combined bone marrow and PBSC transplantation following intensive chemotherapy [Siena et al, 1989]. The number of circulating CD34+ve cells can be further increased by rhGM-CSF to levels

that provide greater numbers of CD34 +ve cells than can be harvested from bone marrow. The *in-vitro* growth characteristics of CD34+ve cells from PB and BM are similar although there is no evidence that these cells are the earliest pleuripotent stem cells that may be required for long term engraftment.

1.3,3 BONE MARROW TRANSPLANTATION IN MULTIPLE MYELOMA

The two largest studies reporting ABMR in MM are from the RMH [Gore et al, 1989] using the VAMP/HDM regimen (chapter 1,2.10.c) and Barlogie's group in the USA [Jagannath et al, 1990] in which melphaian 140/m^2 or thiotepa 750mg/m^2 was combined with TBI 850 cGy. In the latter study 5/14 patients with resistant relapse died early and there were no complete remissions. In the remaining 41 patients including those with primarily resistant disease, 30% achieved CR and the relapse free survival was 18 months with projected 80% survival at 4 years. In this study patients who received greater than >5x10^4/kg GM-CFUc/kg demonstrated faster haematological recovery.

Other major predictors of delayed engraftment, particularly in relation to platelets have been age and previous treatment [Barlogie et al, 1990, Jagannath et al, 1990, Selby et al, 1988].

Bone marrow purging using monoclonal antibodies (to CALLA, B1 and PCA-1 [Anderson et al, 1989]), or immunotoxin linked to monoclonal antibody ('8A' antibody which recognises cells of the B-lymphocyte lineage, and momordin, a ribosome inactivating protein [Gobbi et al, 1989]) or with 4-hydroperoxycyclophosphamide [Reece et al, 1989]) has been done to reduce the number of tumour cells reinfused during

ABMR. Engraftment has occurred in most patients and remissions obtained, however, relapses have occurred and as yet predictions cannot be made for long term survival or duration of remission.

The rational for bone marrow purging in MM is not accepted by some centres since it is unlikely that treatment intensification with ABMR will be able to eradicate the malignant clone in MM. At the RMH, HDMP is administered following reinfusion of the autologous bone marrow as a means of reducing the numbers of viable MM cells reinfused. The efficacy of this stratagem is unproven.

Recently autologous rescue with PBSC has been used in MM [Fermand et al, 1989, Reiffers et al, 1989, Ventura et al, 1990] resulting in successful engraftment and remission rates comparable with studies using ABMR. Long term follow up is not yet available but this technique may offer advantages in terms of ease of collection in MM patients, whose bone marrows may be difficult to harvest because of tumour cell infiltration, bone disease or previous treatment.

Allogeneic BMT is also being used in MM. The largest single study is from Seattle [Buckner et al, 1989] and the largest combined report from the EBMT (European Bone Marrow Transplant Registry) [Gahrton et al, 1990, Gahrton et al, 1991]. The European Data represents patients treated by different groups with different conditioning regimens and entry criteria. Unmatched transplants have done very badly, with early deaths in 5/6 patients (1990 data), however, most transplants have involved syngeneic or HLA identical sibling transplants. Engraftment occurred in 67/85 HLA matched patients, 39/67 of whom achieved CR. The actuarial survival of this group at 76 months was 40%.

The median relapse-free survival for patients who achieved CR was 48 months. Patients who had responded to induction therapy, those who had received induction therapy only and patients who presented with stage I disease were more likely to achieve CR following ABMT which was the best predictor of survival after ABMT. The choice of conditioning regimen did not influence outcome or survival. The Seattle data confirms the better prognosis in syngeneic transplants but overall the early death rate in this study which included matched and partially matched allografts is high. The best conditioning regimen for allografts in MM is not yet established, nor are satisfactory entry requirements. Barlogie and Gahrton [Barlogie & Gahrton, 1991] in their recent review of the data on all stem cell transplants in MM have been unable to make firm recommendations other than to suggest allogeneic transplantation be reserved for patients under 50 years of age (because of increasing GVHD with age) where transplant related mortality should not be expected to exceed 30% with HLA matched donors. Until the optimum conditioning regimen or type of stem cell graft is defined HDM with ABMR is likely to continue.

1,4 LYMPHOKINES AND HAEMOPOIETIC GROWTH FACTORS

1,4.1 INTRODUCTION

Peptide regulatory factors (PRF) are a diverse group of low molecular weight peptides which have a short or intermediate range of action, act on cells that have specific cell surface receptors and affect cellular differentiation and/or proliferation. They are distinct from endocrine hormones because their primary action is locally within micro-environments. Their activity is autocrine and/or paracrine.

Cytokines are PRF which have additional immunoregulatory activity as well as growth/differentiation promoting activity. Lymphokine refers to lymphocyte-produced-cytokines and monokine to monocyte-produced-factors. The term interleukin was adopted by the Sixth International Congress of Immunology in 1986 in an attempt to standardise the nomenclature of lymphokines. Initially lymphokines were named according to their first described biological function. Once the human amino acid sequence was known an interleukin (IL) number was assigned to the molecule and other names abandoned. The interleukin nomenclature only applies to molecules thought to have primarily immunoregulatory function, although it is becoming increasingly apparent that most have multifunctional activity, for example, interleukin 6 (IL-6) (chapter 1,4.2 (g))

In man, the genes encoding several cytokines (GM-CSF, M-CSF, IL-3,IL-4,IL-5, Platelet derived growth factor Rβ and the *c-fms* proto-oncogene, now known to be the

M-CSF receptor) are located in close proximity on the long arm of chromosome 5. It is, therefore, possible that some degree of coordination of transcription occurs.

There is considerable diversity of effects mediated by PRF on different cells and in different environments. This may be explicable by interactions at cell surface receptors which may have different affinities for a given cytokine on the same and/or different cell types. IL-1β, for example binds to both high and low affinity receptors on T-cells and structurally distinct receptors on B-lymphocytes. Some PRF inhibit ligand binding of other factor(s) either by virtue of a close association of receptors within the cell surface or by the sharing of a single surface receptor. This is thought to occur with a subpopulation of receptors that bind both IL-3 and GM-CSF [Gesner et al, 1988, Park et al, 1989]

1,4.2 CHARACTERISATION OF INDIVIDUAL FACTORS

a: Erythropoietin (Epo)

Erythropoietin was discovered over 80 years ago and its role in erythropolesis identified. The genes for human Epo were cloned in 1985 [Jacobs et al, 1985, Lin et al, 1985]. It is produced by the juxtatubular cells of the kidney predominantly but also by macrophages in the adult. In the foetus the liver is an important site of production. Secretion by the kidney is in response to low oxygen tension via sensitivity to the concentration of the deoxy-heme. Unlike the other factors, Epo is produced distantly from its site of action and is detectable in the circulation. Epo can be measured in the plasma and urine and rises, in hypoxia or anaemia due to blood loss or destruction, and

falls below expected levels, in anaemia due to renal disease.

Receptors are present on proerythroblasts and receptor density declines with increasing differentiation of erythrocyte precursors. Epo is required for the production and maturation of erythrocytes in vivo and stimulates committed progenitors (CFU-E and BFU-E) in vitro. Some studies suggest that Epo has activity in megakaryocytopoiesis but overall it is the most lineage specific of all the growth factors.

Control of erythropoiesis requires the action of multilineage factors such as IL-3, GM-CSF and IL-1.

b: Granulocyte Colony Stimulating Factor (G-CSF)

Murine G-CSF was identified in 1981 [Nicola & Metcalf, 1981], in conditioned medium, as a molecule which selectively stimulates neutrophil colony growth and differentiation of myeloid leukaemia cell lines. Early names for this factor included CSF-β [Nicola et al, 1985] and pluripoietin [Welte et al, 1985]. The factor was purified as a protein of molecular weight 19,600 Da from medium conditioned by the 5637 human bladder carcinoma cell line [Welte et al, 1985] and shown to be homologous to the murine equivalent. The cDNA for G-CSF was first expressed in Escherichia Coli [Souza et al, 1986]. The gene for G-CSF is located on the long arm of chromosome 17 and is proximal to the region involved in the translocation of t(15;17) (q23;21) seen in human promyelocytic leukaemia in which the encoding region for G-CSF remains intact [Simmers et al, 1987].

G-CSF is probably produced by many cells, both within the haemopoietic lineages and in 'stroma'.

Murine G-CSF DNA has 69% homology with the human form and the protein amino acid sequence 73% homology explaining the inter-species cross reactivity that has been observed.

Granulocytes have one high affinity receptor for G-CSF with approximately 560 binding sites/cell on human granulocytes and similar numbers in mice. Receptor numbers increase with increasing maturity of the neutrophil line [Nicola & Metcalf, 1986]. Promonocytes, monocytes and macrophages have only a few receptors and they are absent on eosinophils, lymphocytes and erythroid cells. Human placenta and human small cell lung cancer have also been shown to bind G-CSF, but with different affinities, suggesting the presence of a different receptor [Avalos et al, 1989, Uzumaki et al, 1988].

Several phosphorylation events have been described following G-CSF binding to its receptor as well as activation of the genes for *c-fos* and *c-myc* which may be essential for the promotion of cell division by G-CSF [Harel-Bellan & Farrar, 1988]

In murine studies G-CSF produced neutrophilia (up to 50x base levels) which could be maintained by continued treatment with G-CSF over several weeks [Moore, 1988, Moore, 1990]. This was associated with a small increase in circulating monocytes and lymphocytes. There was no change in marrow cellularity but clonogenic assays revealed a 3-6 fold increase in numbers of granulocyte-macrophage colony forming units (GM-CFUc) as well as increases in erythrocyte burst forming units (BFU-E), megakaryocyte-CFU (CFU-MK) and multilineage-CFU (CFU-GEMM) and CFU-S (Splenic CFU). Following treatment with myelotoxic drugs G-CSF accelerated the recovery of neutrophils in mice and to a lesser extent platelet recovery and haematocrit [Shimamura et al, 1987, Moore & Warren, 1987]. This was accompanied by an increase in splenic

granulopoiesis [Shimamura et al, 1987]. G-CSF also hastened haemopoietic recovery in mice supported by syngeneic bone marrow transplantation after sublethal doses of irradiation [Moore, 1988b]. Similar findings were reported in primates given G-CSF after cyclophosphamide [Welte et al, 1987]. This was associated with increased bone marrow cellularity, increased splenic granulopoiesis and some foci of extramedullary haemopoiesis.

In-vitro rhG-CSF promotes the growth of granulocyte colony forming units (G-CFUc) and GM-CFUc from human bone marrow mononuclear cells (BMMNC). Maximal stimulation from G-CSF can exceed that from IL-3 in day 7 CFUc but not in day 14 CFUc [Platzer et al, 1985, Ottmann et al, 1989]. There is some evidence that G-CSF can synergise with IL-3 in promoting early blast expansion [Ikebuchi et al, 1987] and with GM-CSF and M-CSF [Whetton & Dexter, 1989].

In-vitro G-CSF is synergistic with IL-4 in promoting the growth of G-CFUc [Broxmeyer et al, 1988]. Although it enables cells to respond more effectively to G-CSF, IL-4 alone is not a growth factor for myelopoiesis.

In man endogenous levels of G-CSF have been measured in serum [Watari et al, 1989]. In normal donors levels could only be detected (above the lower limit for the assay of 30pg/ml) in 7/56 people (range 33-163pg/ml). In patients with malignancies, myelodysplasia, aplasia and infection G-CSF was detected at levels between 46pg/ml and greater than 2ng/ml, the limit of detection for the assay. In patients with aplastic anaemia there was an inverse relationship between G-CSF levels and neutrophil levels. In the patients with malignant disease G-CSF levels could not be related to disease activity. However, following chemotherapy there was a direct relationship between the levels of G-CSF and neutrophils. In another study [Janowska-Wieczorek,et al, 1990]

levels of G-CSF have been found to increase to a maximum 6 days after bone marrow transplantation whilst those of colony stimulating factor-1 (CSF-1 (macrophage colony stimulating factor (M-CSF)) rose later and reached a peak around 11 days.

c: Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

Activity that was subsequently identified as GM-CSF was described in 1977 in medium conditioned by mouse lung [Burgess et al, 1986, Burgess et al, 1987]. Human GM-CSF was purified and cloned in 1984 [Wong et al, 1985, Gasson et al, 1984]. The gene is located in proximity to those of IL-3, IL-4, IL-5, M-CSF and the M-CSF receptor (*c-fms*) on the long arm of chromosome 5. It encodes a 144 amino acid sequence which is processed to form the 127 amino acid active protein. Human GM-CSF has 54% homology with the murine counterpart and a complex tertiary structure with internal disulphide bonds. The molecular weight is dependant on the degree of glycosylation (14 - 30kDa) and the high levels of glycosylation seen in native GM-CSF reduce the activity of natural human GM-CSF (or rGM-CSF expressed in mammalian cell lines), compared to recombinant factors expressed in bacteria, by reducing receptor binding [Kelleher et al, 1988, Clarke & Kamen, 1987].

Receptors for GM-CSF are found on human neutrophils, monocytes and eosinophils. Like receptors for G-CSF the number of receptors increases with differentiation [Williams et al, 1988, Park et al, 1986]. In leukaemic cells which express GM-CSF receptors, receptor numbers do not predict response to GM-CSF *in-vitro* [Park et al, 1989). There has been some controversy over the number of receptors with

different affinity for GM-CSF, probably because of differences in the affinity of GM-CSF produced in E.Coli or yeast. There is competition between IL-3 and GM-CSF for binding to at least one type of GM-CSF receptor [Gesner et al, 1988, Park et al, 1989].

Functionally GM-CSF is species specific although its activities are similar in murine and human systems *in-vitro*. In clonogenic assays GM-CSF promotes growth of mixed colonies of granulocytes, macrophages/monocytes and eosinophils from human bone marrow. GM-CSF stimulates BFU-E and mixed colonies (CFU-GEMM) in combination with erythropoietin. Megakaryocyte colonies are also produced, but in lower numbers than with IL-3 [Sieff et al, 1985, Ottmann et al, 1989, Lu et al, 1988]

In fully differentiated cells GM-CSF has important non-proliferative functions, for example, phagocytosis, chemotaxis and antibody-dependant cytotoxicity are all augmented by GM-CSF [DiPersio, 1990]. *In-vitro* GM-CSF production and/or transcription can be induced by the addition of interleukin-1 to cultures of endothelial cells [Broudy et al, 1987], fibroblasts [Zucali et al, 1986, Kaushansky et al, 1988] mononuclear cells [Fibbe et al, 1986] and lymphocytes [Herrmann et al, 1988]. The mechanisms of production and tissue of origin are not defined *in-vivo*.

GM-CSF increased neutrophils in a dose dependant manner and promoted the accumulation of inflammatory cells when injected into the peritoneum in mice [Metcalf et al, 1987, Donahue et al, 1986]. Following cytotoxic drugs GM-CSF promoted the early recovery of neutrophils, monocytes, lymphocytes and reticulocytes in primates [Donahue et al 1986, Donahue et al, 1989] and in lethally irradiated immune suppressed mice GM-CSF promoted the growth of human bone marrow xenografts

[Clutterbuck et al 1989].

d: Macrophage Colony Stimulating Factor (M-CSF (CSF-1))

Human M-CSF has been purified from urine as a heavily glycosylated protein dimer of two identical sub-units. Variations in glycosylation give molecular weights between 47 and 76 kDa. The gene, in man, was cloned from a pancreatic carcinoma cell line [Kawasaki et al, 1985] and is located on chromosome 5 in proximity to other growth factor genes. A common transmembrane sequence is shared by three proteins that result from mRNA products derived by alternative splicing during transcription. [Cerretti et al, 1988, Cosman et al, 1988].

The M-CSF receptor which is encoded by the *c-fms* proto-oncogene, also located on the long arm of chromosome 5 has been implicated in malignant transformation [Sherr, 1990]. The receptor is associated with a tyrosine specific kinase (Sherr, 1990) and shares considerable homology, including the tyrosine kinase, with the PDGF-Rβ receptor. *c-fms* mRNA expression increases with increasing maturity along the macrophage/monocyte lineage [Sariban et al, 1985]. In murine macrophages M-CSF may act by the induction of a cyclin (a protein involved in cell cycle control) to promote proliferation [North, 1991].

In-vitro human M-CSF promotes macrophage/monocyte colonies from human bone marrow which increase in number, size and maturity with the addition of IL-3 and IL-1 [Zhou et al, 1988]. In mice M-CSF induces activation of macrophages, antibody directed cytotoxicity and phagocytosis in which TNF, interferons and IL-2 are important.

M-CSF is detectable in normal human serum [Bartocci et al, 1987, Das et al, 1981], at higher levels, in serum of patients with myeloproliferative diseases [Gullbert et al,1987] and following bone marrow transplantation [Janowska-Wieczorek et al, 1990].

e: Interleukin-3 (IL-3) (Multi-CSF)

There is little homology between human and murine IL-3 but human and gibbon IL-3 differ in only 11 amino acids [Yang et al, 1986]. IL-3 is expressed in a primate cell line from which the gene was cloned and used to provide a probe for the human gene which was found on chromosome 5 (see above). Although it has been detected in the supernatant of human haemopoietic cells in long term bone marrow culture [Caligaris-Capio et al, 1991], IL-3 is not present in the serum/plasma normal individuals.

Unlike G and GM-CSF receptors, the receptors for IL-3 are at highest frequency on primitive haemopoietic cells, decreasing in number with increasing differentiation. Receptors have been identified on myeloid and some B-cell precursors, but not on the majority of lymphocytes, nor on cells of the erythroid series [Nicola & Metcalf, 1986, Elliot et al, 1989]. Some myeloid leukaemias and cell lines have receptors for IL-3 [Gesner et al, 1988, Park et al, 1989]. At least one type of IL-3 receptor has equivalent affinity for GM-CSF with which there is competition for binding [Budel et al, 1990].

IL-3 supports the growth of colonies of multiple, mixed and restricted lineage (CFU-GM, -GEMM, -M, -MK, BFU-E). Maximal stimulation of colony forming cells requires the addition of other factors [Messner et al, 1987, Lopez et al, 1987, Ottmann et al, 1989, Sieff et al, 1987]. IL-3 is less effective in promoting GM-CFUc than GM-CSF but

pre-treatment with IL-3 enhances colony formation when cells are subsequently exposed to GM-CSF. IL-3 also stimulates BFU-E but Epo is required for erythrocyte maturation. The effects of IL-3 on the proliferation of cells are augmented by IL-1 or IL-6 and, maximally, by IL-1 in combination with IL-6 [Moore, 1991].

In animal models IL-3 produced only a modest elevation of neutrophil count and platelets compared to the effects of G or GM-CSF. There was, however, a considerable elevation in circulating progenitor cells in mice and primates and increased splenic haemopoiesis in mice [Lord et al, 1986, Donahue et al, 1988, Geissler et al, 1989]. Administration of iL-3 to primates followed by GM-CSF resulted in a subsequent rise in circulating progenitors and neutrophils and similar increments in circulating stem cells were seen in primates after IL-1 and IL-3 were used in combination. In both mice and primates IL-3 has been shown to promote haemopoietic recovery from irradiation or cytotoxic drugs, but with less efficiency than G or GM-CSF.

f: Interleukin-1 alpha and beta (IL-1α & IL-1β)

Two forms of IL-1 are recognised in man [March et al, 1985]. iL-1α and IL-1β have 26% homology of amino acids and 45% homology of nucleotides. [March et al, 1985]. The genes for both proteins are located on the long arm of chromosome 2 (2q14) [Clark et al, 1986]. mRNA for IL-1β is expressed In 10-50 fold excess of IL-1α in human monocytes and this proportion is maintained in serum or conditioned media [March et al, 1985, Kovacs et al, 1989]. Both proteins are present in the cytoplasm as large molecules (31kDa) and are cleaved by serine proteases to form biologically active

proteins [Auron et al, 1987]. Both forms of secreted IL-1 have a similar tertiary structure and have equal affinity for the same receptor(s) [Dinarello & Savage, 1989, Dower & Urdal, 1988, MacDonald & Lowenthal, 1987]. There is also evidence for receptors on T and B lymphocytes with different affinities for IL-1α and β [Scapigliati et al, 1989].

IL-1 has a broad spectrum of activity within the immune and haemopoietic systems (see below). it is involved in the acute phase response [Andus et al, 1988] and in the control of endocrine function and in connective tissue metabolism and proliferation [Review - See Moore, 1990, Dinarello & Savage, 1989].

IL-1 interacts with IL-2 in the stimulation of thymocytes, T and B-cells and NK (natural killer) cells and enhances IL-2 production and IL-2 receptor expression [Aribia et al,1987, Moore, 1990].

An important activity of IL-1 is to stimulate the release of other haemopoietic and inflammatory cytokines (G-CSF, GM-CSF, M-CSF, IL-6 & PDGF) from several cell types [Haworth, 1989] including: monocytes [Fibbe et al, 1986], fibroblasts [Fibbe et al, 1988a, Zucali et al, 1986, Zucali et al, 1987], endothelial cells [Bagby et al, 1986, Fibbe et al, 1989], T-cells [Herrmann et al, 1988] and bone marrow stromal cells [Fibbe et al, 1988b].

In-vitro IL-1 synergises with GM-CSF, G-CSF and IL-3 to promote proliferation of human bone marrow progenitors but does not stimulate haemopoiesis directly [Schaafsma et al, 1989, Zhou et al, 1988, Moore & Warren, 1987, Moore et al, 1987a].

The broad range of activity of IL-1 *in-vitro* is reflected by its effects after administration to animals. In mice, intra-peritoneal injection of IL-1 (α or β) lead to a neutrophilia associated with a fall in BM neutrophil numbers and an increase in circulating G-CSF, M-CSF and acute phase proteins [Neta et al, 1988a]. The period of

neutropaenia in mice after treatment with 5-flurouracil (5-FU) was shortened by the administration of human IL-1 α or β [Moore & Warren, 1987, Moore et al, 1990]. In the same study, G-CSF or GM-CSF shortened the period of neutropaenia after a single dose of 5-FU, but after repeated weekly treatment with 5-FU, G or GM-CSF treated mice developed neutropaenia that was similar to control groups (receiving no cytokine therapy), however, mice that received IL-1β as well as G or GM-CSF recovered neutrophil counts faster and after three weeks of treatment had a significantly lower weight loss and mortality [Moore et al, 1990]. A dose dependant effect of IL-1α on neutrophil count was also seen in mice after administration of cyclophosphamide [Benjamin et al, 1989] and IL-1 could protect against the effect of cyclophosphamide when administered 20 hours before the drug [Castelli et al, 1988]. A similar protective effect was also seen when IL-1 was administered before irradiation [Neta et al, 1988a, Neta et al, 1988b].

In primates treated with 5-FU, IL-1 reduced the period of neutropaenia, however, prolonged administration of IL-1 resulted in delayed recovery of neutrophils and a reduction in the numbers of BM GM-CFUc. This was found to be due to the induction of a serum suppressor of GM-CFUc that was neutralised by the addition of antibodies to TNFα [Gasparetto et al, 1989].

g: Interleukin-6 (IL-6)

IL-6 is a multifunctional cytokine and was, consequently, identified by virtue of several diverse activities which explains the many names given to this molecule before it became known as IL-6; interferon-β2 [Weissenbach et al, 1980], B-cell differentiating factor/BSF2 [Hirano et al, 1986] and hybridoma/plasmacytoma growth factor [Van Snick

et al, 1988]. Human IL-6 consists of 184 amino acids with 42% homology with murine IL-6 [Hirano et al, 1986, Van Snick et al,1988]. Human IL-6 has some homology with G-CSF as a protein and in the arrangement of the gene with 5 similar sized exons and 4 introns [Yasukawa et al, 1987].

Receptors for IL-6 are found on most cells of the immune and haemopoietic systems and on hepatocytes. This reflects the diverse action of this cytokine which is involved in B-cell differentiation and immunoglobulin production [Muraguchi et al, 1988, Kishimoto, 1989], T-cell activation and proliferation (in association with IL-1) [Helle et al, 1988, Holsti & Raulet, 1989], cytotoxic T-cell differentiation [Takai et al, 1988], and the acute phase response [Baumann et al, 1988, Castell et al, 1989]. IL-6 is also a haemopoietic growth factor, acting directly to stimulate myelopoiesis in mice [Suda et al, 1988] and as a co-factor of haemopoiesis in man [Caracciolo et al, 1989, Montes-Borinaga et al, 1990].

When administered to mice [Hill et al, 1989, Ishibashi et al, 1989] or to primates [Zeidler et al, 1989, Asano et al, 1990, Stahl et al, 1991] the major effect of IL-6 *in-vivo* is an increase in thrombopoiesis. In rats [Ulich et al, 1989] there is an immediate (30mins) lymphopaenia followed by a later lymphocytosis (1-4 hours), a biphasic neutrophilia at 1.5 hours and between 4 and 12 hours and a retculocytosis at 12-24 hours. These changes are associated with myeloid and erythroid hyperplasia after 12 hours only, suggesting that the early changes in leucocyte numbers are the results of marginating or de-marginating effects. Interleukin-6 has been found to protect primates from thrombocytopenia after irradiation [Herodin et al, 1992] and a similar effect on platelet numbers, after chemotherapy, has now been observed *in-vivo* in man [Aronson et al, 1993, Chang et al, 1993].

It has been suggested that IL-6 may be an autocrine or paracrine growth factor for myeloma [Kawano et al, 1988, Klein et al, 1989, Klein & Bataille, 1991]. This question is, as yet, unresolved, for example, although IL-6 increased the incorporation of tritiated thymidine into myelomatous bone marrow cells [Kawano et al, 1988] it did not influence the numbers of MM colonies grown *in-vitro* [Montes-Borinaga et al, 1990]. Furthermore, the levels of IL-6 in MM patients bone marrow did not correlate with disease activity or influence clonogenicity of myeloma cells cultured *in-vitro* [Bell et al, 1991].

Data from studies of cell lines derived from patients with multiple myeloma that suggest an autocrine or paracrine role for IL-6 in proliferation of these cell lines are also conflicting (see below). Some studies suggest that commonly used cell lines are dependant on IL-6 for growth, whereas, others, using the same cell lines, show growth independence of IL-6. The major problem of using cell lines as models for myeloma in man is that phenotypic changes may occur during culture *in-vitro*, and/or sub-populations of cells may come to dominate the original parental cells. Cell lines may then have different requirements for cytokines and express different receptors than those associated with the parental tumour from which they were derived.

This is illustrated by three studies of IL-6 dependency in the RPMI 8226 and U226 cell lines. In one study neither of these cell lines was found to be dependant on IL-6 for growth, was inhibited by antibody to IL-6 or expressed mRNA for IL-6 [Klein et al, 1989]. In another study [Barut et al, 1992] both cell lines were sensitive to IL-6, demonstrated increased DNA synthesis, IL-6 receptor (IL-6R) expression and mRNA for IL-6R but did not secrete IL-6. In the third study [Jernberg-Wiklund et al, 1992], an early passage of U-266 (1970) was found to be dependant on feeder-cell production of IL-6 but a later

(1984) passage grew independently of exogenous IL-6. In contrast to the study of Klein et al [1989], both the early and late passage expressed mRNA for IL-6 and IL-6R but the mRNA for IL-6 was increased in the 1984 line which secreted IL-6, and proliferation of which was inhibited by antibody to IL-6, suggesting the development of an autocrine loop of IL-6 stimulation. A similar sequence of events has recently been demonstrated in the evolution of a new cell line, derived from myelomatous bone marrow [Scibienski et al, 1992], in which a stroma dependant (found to be due to IL-6) line became gradually independent of exogenous IL-6 and after a year in culture demonstrated mRNA for IL-6 and secreted measurable quantities of the cytokine.

Although some authors report that IL-6 dependency of MM *in-vivo* increases with time [Zhang et al, 1992], it cannot be assumed that the changes in IL-6 dependency of cell lines *in-vivo* reflects the development of MM *in-vivo*.

There is now good evidence that IL-6 is not an autocrine growth factor for myeloma since, although IL-6 levels have been found to be higher in the bone marrow of some patients with MM, it has been found, by culture of fractions of BMMNC and by detection of mRNA, that this property is related to the adherent cell fraction of the bone marrow, and not the plasma cell fraction [Klein et al, 1990]. More specifically, mRNA for IL-6 was found, in another study, to be derived from macrophages, and not tumour cells, from myelomatous bone marrow [Durie et al, 1990].

Several authors suggest that levels of IL-6 may be increased in the serum [Bataille et al, 1989, Ludwig et al, 1991, Nachbaur et al, 1991] and bone marrow [Klein et al, 1989] of patients with myeloma. These relationships have not been found by other

workers [Bell et al, 1991, Brown et al, 1992]. It remains controversial whether levels of this cytokine correlate with tumour burden and indicate prognosis.

The evidence that levels of IL-6 in plasma or serum is a specific consequence of myeloma tumour burden is questionable, since raised levels of serum/plasma IL-6 are found in other malignant conditions, such as in ovarian carcinoma where mRNA was absent from peritoneal tumour cells despite production of IL-6 in ascites [Scambia et al, 1993] and where IL-6 levels could predict prognosis [Berek et al, 1991]. Scambia et al [1993] also found raised levels of the cytokine in sera from patients with breast cancer. In patients with acute myeloid leukemia [Archimbaud et al, 1993] 78% of patients also had serum levels of IL-6 significantly greater than the range in normal individuals and concentrations of IL-6 were correlated with the presence of non-infective pyrexia and abnormalities of liver function. Similarly, in patients with Hodgkins and non-Hodgkins lymphoma [Kurzrock et al, 1993] raised levels of IL-6 were related to the presence of 'B'-symptoms but not other prognostic indicators although there was an independent prognostic correlation of serum IL-6 with survival in patients with Hodgkins disease. In a further study [Solary et al, 1992] levels of IL-6 were greater in patients with myeloma than in controls, but less than in patients with Hodgkins disease or acute leukemia. Similarly, patients with monoclonal gamopathy of unknown significance (MGUS) also had higher concentrations of serum IL-6 than patients with chronic lymphocytic leukemia (CLL) or MM, who had greater levels of IL-6 than controls [Pettersson et al, 1992], although raised levels were not found in MGUS in the original report on MM by Bataille et al [1989].

These data suggest that raised serum levels of IL-6 may be related to tumour mass but probably represent a non-specific response to malignancy reflecting a

combination of immune activity and inflammation. In diseases involving the bone marrow, the degree of bone marrow infiltration may also determine serum IL-6 concentrations.

A number of studies have demonstrated that IL-6 stimulates the incorporation of ^{3}H-thymidine into myelomatous bone marrow [Kawano et al, 1988, Anderson et al, 1989b, Tanabe et al, 1989] and may increase the number of cells in S-phase [Klein et al, 1989] in similar cell populations. Even when these cells are highly purified, such that plasma cells represent 95% of the cells present, it has been argued that contamination by myeloid cells may account for the observations [Montes-Borinaga et al, 1990, Nilsson et al, 1990]. However, some studies demonstrate that these observations are unlikely to be due to contamination by myeloid cells, since there was no increase in incorporation of ^{3}H-thymidine in response to known myeloid growth factors, such as G-CSF, M-CSF, GM-CSF, IL-1β or IL-3 [Anderson et al, 1989b]. These studies do not exclude the possibility that DNA synthesis in myeloma cells may not represent proliferation since many malignant plasma cells are multinucleate and are, thus, able to undergo mitosis without proliferating [Montes-Borinaga et al, 1990, Bell et al,1991].

One clinical study has been reported that suggests that IL-6 may function, *in-vivo* as a growth factor for plasma cell malignancies in man. A patient with drug resistant plasma cell leukemia has been treated with a combination of two murine anti-human IL-6 monoclonal antibodies over a 68 day period [Klein et al, 1991]. This resulted in a temporary reduction of tumour related hypercalcaemia and paraprotein secretion and a reduction in the percentage of tumour cells from the patients bone marrow that were in

S-phase. There was an associated decline in levels of C-reactive protein and complement factor-C3, indicating inhibition of IL-6 as an inflammatory cytokine, and a reduction of serum IL-6 bio-activity, measured as the ability of serum to stimulate the growth of an IL-6 dependant cell line *in-vitro*.

The Is some evidence, therefore, that IL-6 may promote the growth of myeloma and related neoplasms, and that, if so, this may be in a paracrine manner by production of this cytokine from bone marrow stroma. However, the role of IL-6 as a promoter and growth factor in myeloma is not yet fully defined.

h: Interleukin-4 (IL-4)

IL-4 was identified in the mouse as a T-cell derived protein with the ability to activate B-cells and stimulate immunoglobulin gene expression and was named B-cell Stimulating Factor-1 (BSF-1) [Howard et al, 1982, Rabin et ai, 1985]. The gene was cloned in 1986 [Lee et al, 1986, Noma et al, 1986]. The human IL-4 gene is located on chromosome 5 (5q31) in proximity to other cytokine genes [Arai et al, 1989, Van Leeuwen et al, 1989] and there is 70% homology with the DNA sequence in the mouse. At the amino acid level the first 90 amino acids have 50% homology with murine IL-4 but the remaining sequence (total 129 amino acids) has little homology with that of the mouse.

IL-4 receptors are present on most cell types (in the mouse) [Lowenthal et al, 1988] such as lymphocytes, macrophages and haemopoietic cells, in which IL-4 is known to be active, and on other cells such as muscle, brain and hepatocytes that are

not yet known to respond to this cytokine.

As well as its differentiating and proliferative effects on lymphocytes [Brown et al, 1988, Spitz et al, 1987] IL-4 inhibits IL-2 dependant NK and LAK cell induction [Gallagher et al, 1988, Jin et al, 1989].

In the mouse, the activity of erythropoietin and IL-6 in myeloid, erythroid and megakaryocyte colony formation is augmented by IL-4 [Rennick et al, 1989]. In man, IL-4 does not stimulate proliferation of stem cells directly [Sonoda et al, 1990, Jansen et al, 1990] but augments the activity of G-CSF [Broxmeyer et al, 1988a, Broxmeyer et al, 1988b] and inhibits the activity of IL-3 or GM-CSF [London & McKearn, 1990] in the formation of granulocyte-macrophage colonies.

1,4.3 CLINICAL USE OF GROWTH FACTORS

G-CSF

G-CSF has been used in clinical trials to promote neutrophil recovery after chemotherapy, and to increase the progenitor numbers from PBSC for harvest. Phase i/II studies have been conducted by several groups [Bronchud et al, 1987, Bronchud et al, 1988, Duhrsen et al,1988, Morstyn et al, 1988, Morstyn et al, 1989, Gabrilove et al, 1988]. A 24 hour IV infusion [Bronchud et al, 1987] of G-CSF promoted an initial fall in circulating neutrophils during the first minutes of infusion, followed by a rise within 1-4 hours which was dose dependant. Steady state was reached after 2-4 days with rises in neutrophil progenitors (myelocytes and promyelocytes) after 4-5 days. Small rises in lymphocytes and monocytes are also reported. Neutrophil counts fell rapidly after cessation of G-CSF infusion.

Similar results were reported when G-CSF was given as a continuous intravenous or continuous subcutaneous infusion [Duhrsen et al, 1988]. Bolus subcutaneous injections were less effective than IV infusions but more effective than intravenous bolus or short infusion. Duhrsen et al [1988] demonstrated large increases in the numbers of circulating stem cells and all studies showed an increase in bone marrow cellularity but could not confirm increased clonogenicity in the bone marrow.

Neutrophil mobility and bactericidal activity was normal in patients receiving G-CSF [Bronchud et al, 1988]

Phase II studies showed that in patients receiving G-CSF neutropenia could be

prevented or abrogated following combination chemotherapy [Bronchud et al, 1987, 1988, Gabrilove et al, 1988] or following single agent melphalan [Duhrsen et al, 1988, Morstyn et al, 1988, 1989]. This was associated with a reduction in the amount of antibiotics prescribed and in the number of infective episodes. Gabrilove [1988] found that patients receiving G-CSF for bladder cancer were able to receive subsequent courses of chemotherapy without scheduling delays due to neutropaenia. Chemotherapy related mucositis was also reduced in G-CSF treated patients.

The Australian group [Morstyn et al] extended their trial to ABMR (following busuiphan & cyclophosphamide for various malignancies - G-CSF 20μg/kg/day continuous s/c infusion) and demonstrated a reduction in the period of neutropaenia ($<0.5x10^9$/l 11 Vs 18 days) and a reduction in the period of antibiotic use (11 Vs 18 days) when compared to historical controls [Sheridan et al, 1989]. Several other studies have shown similar results after ABMR and ABMT [Peters, 1989, Kodo and Asano, 1989, Masaoka et al 1990, Taylor et al, 1989], and in conventional and high dose therapy without transplantation, for example, in bladder carcinoma [Kotake et al, 1991], in lung cancer [Eguchi et al,1990, Ota et al, 1990, Crawford et al, 1991, Trillet-Lenoir et al, 1993] and in non-Hodgkins lymphoma [Pettengell et al, 1992]. In a number of the randomised studies there have also been reductions in the number of documented infections, periods of pyrexia, use of antibiotics and days in hospital after conventional chemotherapy. G-CSF has also been used to intensify drug dosage where myelotoxicity is the major limiting toxicity. In most cases modest increases in drug dosage have been possible but thrombocytopenia and other toxicities become dose limiting [Bronchud et al, 1989].

Expansion of the PBSC compartment with G-CSF allowed up to eighty times more stem cell to be collected than can be collected in steady state conditions or following chemotherapy [Molineux et al, 1990, Sheridan et al, 1990]. The combined use of chemotherapy with G-CSF is most effective.

G-CSF has also been used in the treatment of non-malignant conditions such as cyclical neutropaenia [Hammond et al, 1989], myelodysplasia [Kobayashi et al, 1989, Negrin et al, 1989] and congenital agranulocytosis [Bonilla et al, 1989]. In these patients G-CSF has increased functional neutrophil levels. Although bone marrow cytogenetics remained abnormal in the myelodysplastic patients excess blasts were not induced in the circulation and in some patients the numbers of blasts declined on G-CSF.

The pharmacodynamics of G-CSF were studied during phase I and II studies. In intravenous bolus administration peak levels and initial elimination rates were dose dependant and the half life between 1.3 to 4.2 hours. Layton [Layton et al, 1989] suggested that one mechanism of elimination becomes saturated following doses above 10μg/kg. Subcutaneous bolus injection results in sustained high levels of G-CSF in plasma. 10μg/kg resulted in levels of 10ng/ml for more than 12 hours. Continuous subcutaneous infusion of G-CSF resulted in steady state levels being obtained but Layton [1989] describes a fall in this level in the last 2 days of a 5 day infusion. This pattern was not seen during prolonged s/c infusions following ABMT, where high levels were maintained until neutrophil levels increased. Layton et al [1989] suggested that sustained high levels of neutrophils ($>30x10^9$/l) induce a further clearance mechanism which may be important in homeostatic control of neutrophil numbers.

Some studies have attempted to investigate the mechanism(s) of increase in neutrophilia following G-CSF administration. Lord et al, [1989] have shown that G-CSF induces more rapid maturation of cells, hastens their passage into the circulation and expands the myeloblast compartment. Mature cells appeared in the circulation within 1 day compared to 4-5 days in studies of normal granulopoiesis. The half life of the circulating neutrophils did not differ from those of normal volunteers and there was no unusual sequestration of neutrophils.

GM-CSF

GM-CSF increases the numbers of circulating granulocytes, monocytes and neutrophils in patients with various cancers [Steward et al, 1989, Lieschke et al, 1989, Herrmann et al, 1989]. Intravenous and subcutaneous routes were effective. In some studies a threshold level for activity of 3μg/kg/day was described. At doses above 10μg/kg/day the rise in neutrophil count was dependant on the dose of GM-CSF [Steward et al, 1989]. The rise in neutrophils, like that seen in studies with G-CSF, was preceded by a transient drop in neutrophil numbers. This probably represents a marginating effect on circulating neutrophils. The earliest neutrophils to appear are usually immature suggesting the premature release of pre-formed cells. Later, more mature cells appear in the circulation implying that expansion of the neutrophil pool is responsible. A further rise in neutrophil numbers has been demonstrated after continued administration of GM-CSF beyond 5 days without an increase In the dose of GM-CSF [Steward et al, 1989, Lieschke et al, 1989]. This may represent the induction of other

cytokines, the production of endogenous GM-CSF or the up-regulation of GM-CSF receptors. Unlike G-CSF there is no evidence that the clearance of GM-CSF is increased after prolonged administration. All the studies have demonstrated a fall in neutrophil count when GM-CSF administration is stopped.

The effects of GM-CSF on cell kinetics in the bone marrow have shown that bone marrow cellularity is increased with an increase in the myeloid:erythroid ratio and an increase in more immature cells in the myeloid lineages [Antman et al, 1988, Herrmann et al, 1989, Lieschke et al, 1989, Broxmeyer et al, 1988]. Broxmeyer et al reported no increase in BM GM-CFUc but an increase in the number of CFUc in cycle in patients receiving GM-CSF. Another study [Aglietta et al, 1989] reported increases both in GM-CFUc and BFU-E. The differences may well be dose related. Aglietta et al used a dose of 8μg/kg/day whereas Broxmeyer et al used lower doses (15-60 $\mu g/m^2$).

Socinski described increases of circulating GM-CFUc by 18 fold and BFU-E by 8 fold after combined use of chemotherapy and infusion of GM-CSF [Socinski et al, 1988], with no concomitant increase in bone marrow clonogenicity. Similar findings have been made by other groups [Herrmann et al, 1989, Villeval et al, 1990]. GM-CSF infusion has been used to increase circulating PBSC for harvesting for autologous transplantation [Gianni et al, 1989, Peters et al, 1989]. Two patients with MM were grafted with PBSC in whom bone marrow harvests were inappropriate [Gianni et al, 1990]. GM-CSF promoted PB CFUc have been shown to have an enriched population of cells bearing the early progenitor marker CD34 [Sienna et al, 1989].

There have been mixed reports on the influence of GM-CSF on red cell dynamics

and platelets. In myelodysplasia one report showed an increase in red cell transfusion requirement after treatment with GM-CSF [Vadhan-Raj et al, 1987]. This was also found in a study of GM-CSF following bone marrow transplantation [Powles et al, 1990]. GM-CSF receptors have been demonstrated on megakaryocytes and GM-CSF increased the number of CFU-Mk in S phase *in-vivo* [Aglietta et al, 1991]. However, there was no increase in platelet production, suggesting that maturation required additional factors. During chemotherapy the effect of GM-CSF on megakaryocytopolesis is dependant on the schedule and dosage of the lymphokine [Edmonson et al, 1989].

Like G-CSF, GM-CSF has been shown to be effective in reducing the level and period of neutropaenia after conventional and high doses of chemotherapy without stem cell transplantation in various malignancies. [Antman et al, 1988, Morstyn et al, 1989, Steward et al, 1990]. In patients with colon cancer GM-CSF induced earlier recovery of platelets after HDM compared with historical controls [Steward et al, 1990]. The study by Morstyn et al [1989] showed a prolongation and worsening of the perlod of neutropaenia between successive cycles of chemotherapy despite the use of GM-CSF suggesting that it does not protect against cumulative marrow/stromal injury.

ABMR in combination with GM-CSF has been used in several studies. In patients with lymphoma GM-CSF shortened the period of neutropaenia ($n<0.5$ or $<1.0x10^9/l$) compared to historical or randomised controls [Brandt et al, 1988, Devereaux et al, 1989, Gorin et al, 1990, Link et al, 1992, Nemunaitis et al, 1988].

In the first phase I/II trial patients also had shorter times to platelet independence, less febrile days and bacteraemic episodes, and shorter stays in hospital [Nemunaitis et

al, 1988]. These effects were seen only in doses of GM-CSF above 60$\mu g/m^2$/day using yeast derived product and at these doses toxicity was minimal. At doses of greater than 19.2μg/kg/day (in a study using Chinese Hamster Ovary (CHO) derived GM-CSF) considerable toxicity was reported [Brandt et al, 1988], characterised by fluid retention, capillary leak and oedema. Equivalent doses of yeast or E.Coli derived GM-CSF were used by Devereaux et al [1989] without toxic side effects. However, neither of these studies found any enhancement of platelet recovery, reduction in febrile episodes or decreased hospitalisation. Use of different products and differences in dose and scheduling between these studies makes direct comparison difficult.

In a pooled phase III study reviewed by Rabinowe et al [1991] 128 patients were randomised to receive daily 2 hour infusions of 250$\mu g/m^2$/day of yeast derived GM-CSF or placebo in three centres in the USA. Neutrophil recovery was significantly accelerated and hospital stay and antibiotic use reduced in the treatment group. There was no benefit in terms of platelet recovery and no significant difference in the number of documented infections.

Overall, in ABMR, the data show that neutrophil levels are increased and the period of neutropaenia reduced with the use of GM-CSF. However, there is no effect on platelet recovery. Furthermore there is no advantage for the use of continuous infusion verses IV bolus injection. Long term follow-up (Median 774 days (455-982) of patients who received GM-CSF after ABMR showed no difference compared with controls in engraftment, late marrow failure, stem cell compromise or relapse rates [Nemunaitis et al, 1991a]. GM-CSF probably does not, therefore, either contribute to or interfere with early progenitor engraftment.

In ABMT for leukaemia [Powles et al, 1990] GM-CSF (8μg/kg/day, CHO derived, with a stated equivalence in potency to 5.5μg/kg of non-glycosylated GM-CSF, equivalent to approximately 300-500μg/day glycosylated GM-CSF) reduced the period of neutropaenia ($n<0.5$) for all patients from 16 to 13 days but was only significant for patients given total body irradiation (TBI) (13 Vs 18 days). As well as increased platelet and red cell requirements, the GM-CSF treated group had higher levels of serum urea, creatinine and bilirubin. This study included many poor risk patients but no differences could be identified between the randomly assigned treatment and placebo groups. Patients given GM-CSF developed higher lymphocyte levels during engraftment but this was not associated with a difference in the incidence or severity of GVHD for which all patients received cyclosporin as prophylaxis. There was no difference in infective morbidity although GM-CSF induced pyrexia may have prompted greater use of antibiotics in these patients. Hospital stay was not shortened.

Another study (in ABMT), comparing GM-CSF treated patients with historical controls, has shown more rapid recovery of neutrophils ($n>1$) and platelets (not statistically significant for platelets) using Yeast derived GM-CSF in increasing doses as a 2 hour daily infusion [Nemunaitis et al, 1991b]. The benefit was seen both in patients receiving and not receiving GVHD prophylaxis with methotrexate which can delay engraftment.

In randomised studies of rhGM-CSF after ABMR or ABMT reduced hospitalisation, reduction in the use of antibiotics and in the number of documented infections in patients treated with rhGM-CSF have each been reported in some but not all publications [Gorin et al, 1992, Link et al, 1992, Gulati and Bennett, 1992 and Advani et al, 1992]. All have demonstrated a faster neutrophil recovery in patients treated with rhGM-CSF rather than

placebo.

GM-CSF has also been used with benefit in patients with myelodysplasia, aplastic anaemia, congenital neutropaenia. Anti-tumour effects, usually tumour stabilisation, have also been noted in a minority of patients included in phase I and II studies. In the report of Steward et al [1989], a single patient with a heavily pre-treated liposarcoma achieved a partial remission. There have been no reports of tumour growth in response to GM-CSF and no reports of increases in relapse after ABMT or ABMR with GM-CSF.

IL-3

Results of early phase I and II studies with IL-3 are now available [Ganser et al, 1990a, Ganser et al,1990b, Ganser et al,1990c, Hoelzer et al, 1991, Oster et al, 1991]. In patients with normal haematopoietic function (but malignant disease) s/c bolus injection of IL-3 produced a multilineage leucocytosis (neutrophil, eosinophil, lymphocyte-with preservation of T4:T8 ratio) which was delayed and of less magnitude (2.8 fold at 500μg) than that reported after GM-CSF. The effect was dose dependant over the range 30-500$\mu g/m^2$/day. There was a concomitant increase in reticulocytes which was independent of dose and an increase in platelets which depended on the administered dose (1.3 fold at 60μg and 1.9 fold at 250μg). Although bone marrow cellularity increased there was no increase in CFUc but the proportions of CFUc in S-phase was increased. Circulating CFU-GM and CFU-GEMM were elevated after 7 days but fell to base line levels after continued treatment for 15 days.

In patients with impaired haematological function following therapy or with bone marrow infiltration by tumour, IL-3 increased the number of circulating leucocytes and platelets. Although the magnitude of these effects was greater when compared to patients with normal marrow function there was a delay before the peripheral count increased; neutrophil levels peaked at 3.7 fold at 19 days (median). The effect on thrombopoiesis was also greater than in normal controls. In 5/8 patients in whom a platelet effect was seen, increases of up to 6 fold occurred allowing discontinuation of platelet transfusions in 2/3 transfusion dependant patients [Hoelzer et al, 1991]. The effect of IL-3 on thrombopoiesis was prolonged, maintaining high and increasing levels in one, patient, beyond 50 days. Red cell transfusion requirements were not reduced.

Patients with myelodysplasia, (including patients with deletions of the long arm of chromosome 5) gained comparable benefits from IL-3 but results in patients with aplastic anaemia were less impressive.

These studies have also assessed the effect of GM-CSF administered for 10 days after IL-3 administration for 5 days (both at 250μg/m^2/day), in patients with normal haemopoiesis. The results of combined treatment cycles were compared with IL-3 alone for 15 days in the same patients. Increases in neutrophils were of the order predicted for the effect of GM-CSF (15 days) from previous studies and that for piatelets, comparable to the first arm of the treatment programme with IL-3 alone.

1,5 COLONY STIMULATING ACTIVITY IN PLASMA AND SERUM

Granulocyte-macrophage (GM) colonies were first grown in semi-solid agar from cells derived from the bone marrow of mice [Bradley & Metcalf, 1966, Metcalf et al, 1967]. It was apparent that growth of colony forming units (CFUc) required the presence of a source of growth factors (such as that derived from cell-line conditioned medium) other than the nutrients available from growth media. Such activity was present in the sera of mice [Robinson et al, 1967] but human serum contained an inhibitor of mouse GM-CFUc [Chan & Metcalf, 1970] that could be removed by treatment with heat or ether [Chan et al, 1971]. Furthermore, when serum from several species (including man) were added to cultures of murine BMMNC containing another source of growth factor activity (cell line conditioned medium) this activity was augmented, rather than inhibited, and this activity, that was due to a non-dialysable molecule, could also be inhibited by treatment with heat [Metcalf et al, 1975].

Human GM-CFUc could also be grown in semi-solid agar [Pike & Robinson, 1970, Senn et al, 1967] in the presence of colony stimulating factor(s) (CSF). It was found that human plasma (and urine) had a variable ability to stimulate the growth of human GM-CFUc *in-vitro* [Metcalf , 1974] and that the effect of a source of CSF was dependent on the species of the target cell population [Lind et al, 1974].

The measurement of CSA in human serum/plasma has been complicated by the use of different assays. The response of human BMMNC to colony stimulating activity (CSA) in human serum was found to be dose dependant [Francis et al, 1977], however,

the presence of adherent cells in BMMNC was found to differentiate between two types of serum CSA [Francis et al, 1977, Francis, 1980, Furusawa et al, 1978, Ishizaka et al, 1985], one that directly stimulated myelopoiesis and was elevated in patients with aplastic anaemia [Ishizaka et al, 1985] and another that was present in normal human serum but required the presence of adherent cells (Ishizaka et al, 1985, Francis, 1980] or peripheral blood leucocytes [Francis, 1980]. Entringer [Entringer et al, 1980] found significant CSA in the serum of 230 normal individuals using adherent cell depleted BMMNC. In another study [Tilly et al, 1986] normal serum promoted the growth of colonies only in the presence of adherent cells, but in this study the BMMNC were exposed to pure foetal calf serum (FCS) (and cultured in a high concentration of FCS), which has been shown to permit the growth of nonadherent BMMNC when a further CSF is added [Alley et al, 1983]. FCS was present in the assay of Francis et al [1980], but not in the assay of Entringer et al. In some studies heat inactivated serum has been used [Francis et al, 1976, Francis, 1980] and the method of collection and storage of BMMNC and serum samples have varied, as has the type of culture medium (with variable concentrations of FCS).

The effect of storage of serum was investigated by Entringer et al [1980] who found that CSA was stable at -15 and -66^{o}C for up to 80 days but decreased by 50% after 240 days. Repeat freeze/thaw cycles did not reduce the activity.

The assay of CSA clearly depends on a number of factors including the use of adherent cells, foetal calf serum and the treatment of the serum samples. In studies of levels of CSA it is clearly necessary to identify and/or standardise methods of analysis and to identify the relationship of levels in an abnormal population to that of normal individuals or to the same population prior to the event (such as chemotherapy) under

study.

Serum or plasma CSA has been detected in several pathological conditions: In patients with chronic myeloid leukaemia [Rao et al, 1987], patients with aplastic anaemia [Ishizaka et al, 1985, Adams & Barrett, 1982] and patients with multiple myeloma [Millar et al, 1990]. CSA has also been detected in plasma [Yamasaki et al, 1988b] and serum [Millar et al,1992] after bone marrow transplantation.

There are no publications in which investigators have attempted to measure the contribution of specific growth factors to CSA by the direct measurement of levels of cytokines in the same plasma/serum samples, but two studies have used antibodies to GM-CSF as a method of inhibition of CSA [Yamasaki et al, 1988b, Millar et al, 1990] and suggest that this molecule does contribute to CSA. The biological relevance, pathophysiology and control of CSA is not known.

CSA for megakaryocytopoiesis (Meg-CSA) has also been identified in several studies. Such activity is present in the serum of patients with aplastic anaemia [Adams & Barrett, 1982, Mazur et al, 1990] and in the serum/plasma of bone marrow recipients [Adams et al, 1990, Fauser et al, 1988, Yamasaki et al, 1988b]. Inhibition of this activity using antisera suggest that it is not due to the presence of GM-CSF or IL-3 [Mazur et al, 1990].

1,6 THE ACUTE PHASE RESPONSE

Following major trauma, for example sepsis, burns or necrosis, such as follows a myocardial infarction, there is a complex series of metabolic and physiological processes that give rise to fever, leucocytosis and a generalised catabolic state. This sequence of events is mediated by the rapid synthesis and secrection of a number of plasma proteins (and decreases in other protein fractions) and is termed "the acute phase response" [Pepys, 1987]. Most of the synthesis of acute phase proteins occurs in the liver and increases are seen in proteinase inhibitors such as α_1-Antitrypsin; coagulation proteins such as fibrinogen, prothrombin and factor VIII; complement proteins; transport proteins and a miscellaneous group of proteins that include serum amyloid-A protein and C-reactive protein (CRP).

C-reactive protein is synthesised by the liver and composed of 5 identical non-glycosylated polypeptide subunits of molecular weight 115000. CRP binds to a pneumococcal C-polysacharide (from which C-RP derives its name), as well as other materials containing similar phosphoryl choline residues, and also binds to chromatin and damaged (altered) cell membranes derived from autologous cells. The CRP-ligand complex activates the complement pathway. This is probably the physiological function of CRP, as part of a scavanging mechanism for dead or damaged cells and a defence against foreign organisms.

Synthesis of acute phase proteins, including CRP, by hepatocytes can be induced, *in-vitro*, by the addition of IL-1β [Pepys, 1987] or IL-6 [Sheldon et al, 1993, Whicher et al, 1991]. Increases of IL-6 can be detected in plasma within 2-3 hours of

trauma *in-vivo* and are followed after about 6 hours by increases in CRP [Whicher et al, 1991].

Normal levels of CRP in man are low (mean 0.8mg/l, range 0-20mg/l). In response to trauma, de-novo synthesisis by the liver results in secretion of large amounts of CRP within about 6 hours. Levels as high as 500mg/l have been observed after 50 hours but levels usually fall rapidly after removal of the stimulus. CRP is a sensitive indicator of infection, inflamation and the acute phase response [Hind & Pepys, 1987, Matson et al, 1991, Sheldon et al, 1991] and is used routinely as a marker of inflammation.

CHAPTER 2

MATERIALS AND METHODS

2,1 MATERIALS

2,1.1 5637 HUMAN BLADDER CARCINOMA CELL LINE

The 5637 cell line, derived from a human bladder carcinoma was supplied by Dr. J. Fogh, Sloane Kettering Institute for Cancer Research, Walker Laboratory, Rye, New York, USA. Medium conditioned by this cell line (medium in which the cell line has grown) supports the growth of normal human bone marrow cells in a clonogenic assay [Myers et al, 1984] and the gene for G-CSF was cloned from this cell line [Souza et al, 1986]. Culture methods are described below (2,1.4b)

2,1.2 PATIENT SAMPLES

a: Bone Marrow

Samples were collected, with the consent of the patient, in the out-patient department, at the same time as samples required for routine management, or at the time of bone marrow harvest.

In the out-patient department samples were aspirated from one or other posterior iliac crest or occasionally from the sternum when samples were unobtainable from the

iliac crest or this area had recieved previous therapeutic irradiation. Samples were collected under local anaesthesia with 5-10 ml of lignocaine (Lignocaine 2%, Antigen Ltd, U.K.) which was infiltrated into the skin, subcutaeous tissue and periosteum. A Salah needle was introduced into the bone marrow cavity and 2-5ml of bone marrow aspirated into a sterile plastic syringe (Gillette). Aliquots of bone marrow were dispensed for routine smears and for immunotyping and the remainder transfered immediately into a sterile universal container (Sterilin Ltd, Hounslow, Middlessex, U.K.) containing 2 or 3 drops of preservative free heparin sodium (PFH) (1000 Units/ml Monoparin, CP Pharmaceuticals Ltd, Wrexham, U.K.). Samples were transfered immediately to the laboratory.

Bone marrow harvest was performed under general anaesthesia and marrow aspirated through Islam needles into syringes previously heparinised by flushing with heparinised saline. Bone marrow was aspirated from both posterior iliac crests and occasionally from the anterior iliac crests or sternum. For this study 10-20 ml of bone marrow was dispensed into universal containers containing PFH at the begining of the harvest.

b: Peripheral Blood Samples

Samples were collected with the consent of the patient at the time of routine venepuncture in the out-patient department or from the patients central venous catheter, at the time of diagnostic aspirations, during their hospitalisation after high dose chemotherapy.

Samples were collected in sterile glass tubes under vacuum (Vacutainer Systems - Becton Dickinson U.K.) without preservative, containing no additives or lithium heparin (143 units/10ml). For some experiments sampies were collected into vacutainer tubes containing EDTA-K_3.

2,1.3 SAMPLES FROM NORMAL HUMAN DONORS

a: Bone Marrow

Bone marrow was obtained with informed consent from donors for allogeneic transplantation under general anaesthesia at the time of bone marrow harvest. The method of collection was as above.

b: Peripheral Blood

Peripheral blood was obtained, with informed consent, either from laboratory staff using the methods above or from donors for allogeneic bone marrow transplantation at the time of bone marrow harvest. These sampies were aspirated from the donors peripheral venous canula during general anaesthesia and handled as above.

2,1.4 CULTURE MEDIA

a: Alpha Modification of Eagles Medium (α-MEDIUM)

The alpha modification of Eagles medium (Tabie 2,1) (Flow Laboratories, Herts) was used in all clonogenic assays. It was supplied as a powder and reconstituited in

distilled water (1500ml) with the addition of extra vitamins (100ml Eagles MEM vitamins (Flow)) to produce a concentrated stock solution (α-stock) from which further modified culture medium was prepared.

In order to dilute α-medium with agar solutions but retain the correct final concentrations of its constituents and osmolality α-medium was prepared at double concentration (2xα).

100ml aliquots of 2xα were prepared in glass measuring cylinders by mixing the following stock solutions with 32ml of α-stock and completing the volume with distilled water:

Foetal calf serum (FCS) (Flow Laboratories) 40 or 10ml*

Sodium bicarbonate (7.5% - Gibco) 6ml

20mls 0f 10% bovine serum albumin (BSA) (Sigma)

Transferrin 1ml of 10mg/ml (Sigma)

vitamin C 1ml of 5mg/1ml (L-ascorbic acid, BDH, Poole, U.K.)

gentamicin 0.1ml of 80mg/2ml (Cidomycin, Roussel Laboratories Ltd, Uxbridge, U.K.).

*- early experiments used 20% FCS (final concentration) and later 5% was used - see results

2xα-medium was poured into sterile screw top glass bottles and stored at 4_oC.

Table 2.1 Alpha Modification of Eagles Medium (Flow)

AMINO ACIDS	Conc. mg/l	VITAMINS SALTS METABOLITES	Conc. mg/l
L-Alanine	25.0 *	Ascorbic Acid	50.0 *
L-Argenine HCL	126.4	Biotin	0.1 *
L-Asparagine H_2O	50.0 *	D-Ca Pantothenate	1.0
L-Aspartic Acid	30.0 *	Choline Chloride	1.0
L-Cysteine HCL	89.74 *	Folic Acid	1.0
L-Cystine Na_2 H_2O	30.22	i-Inisotol	2.0
L-Glutamic Acid	75.0 *	Nicotinamide	1.0
L-Glutamine	292.0	Pyridoxal HCL	1.0
Glycine	50.0 *	Riboflavin	0.1
L-Histidine $HCLH_2O$	41.9	Thiamin HCL	1.0
L-Isoleucine	52.5	Vitamin B12	1.36 *
L-leucine	52.5	$CaCl_2.2H_2O$	264.9
L-lycine HCL	73.06	KCl	400.0
L-Methionine	14.9	$MgSO_4.7H_2O$	200.0
L-Phenylalanine	33.02	NaCl	6800.0
L-Proline	40.0 *	$NaHCO_3$	2000.0
L-Serine	25.0 *	$NaH_2PO_4.2H_2O$	158.3
L-Threonine	47.64	D-Glucose	1000.0
L-Tryptophan	10.2	Lipoic Acid	0.2 *
L-Tyrosine Na_2	45.0	Phenol Red (Na)	10.0
L-Valine	46.9	Sodium Pyruvate	110.0

* - Not present in Minimal Essential Medium, Eagle.

b: 5637 Conditioned medium (5637CM)

5637CM was prepared by seeding 10^6 5637 cells (chapter 2,1.1) in 20ml of RPMI-1640 growth medium (Sigma, Poole, Dorset, UK) without Hepes buffer in 80 cm^2 plastic culture flasks (Nunc, Denmark). The cells form a monolayer in culture and were grown at 37° C in a humidified atmosphere with 5% CO_2. At 7 days the cells were passaged and resuspended at 3 x 10^6 cells in 60 ml RPMI 1640 in 175 cm^2 plastic culture flasks (Nunc, Denmark). The medium (5637 CM) was collected after 8 days and filtered through a 0,2 μm filter (Acrocap, Gelman Sciences, Ann Arbor, Michigan, USA), dispensed in 4 ml aliquots in sterile Bijou bottles and stored at -20°C. The cells were harvested and cryopreserved at a density of 10^6/ml in sterile plastic tubes (1.8ml Cryotubes - Nunc) in liquid nitrogen at -70°C for further use.

Each batch of 5637CM was tested for bone marrow colony stimulating activity against one or more previous batches of 5637CM in a clonogenic assay using normal and myelomatous BMMC (2,2.3a).

2,1.5 RECOMBINANT HUMAN GROWTH FACTORS AND LYMPHOKINES

a: Granulocyte Colony Stimulating Factor (rhG-CSF)

Recombinant human G-CSF manufactured by Chugai was a gift from G.H.Besselar Associates Ltd, Maidenhead, U.K. and Chugai Pharmaceutical Co. Ltd, Japan.

It is expressed in Chinese hamster ovary cells from cDNA derived from a human squamous cell carcinoma cell line (CHU-2) that produces G-CSF in culture. The recombinant product was indistinguishable from natural G-CSF. Data on specific activity *in-vitro* was not available but activity *in-vivo* in animals was comparable to published data on other recombinant human G-CSF products.

rhG-CSF was supplied as a sterile lyophilised powder, free of pyrogens, in vials 0f 100μg. It was reconstituted in 2ml of sterile water to produce a clear solution of pH 6.5-8.0. Aliquots were diluted in Dulbecco's phosphate buffered saline A (PBSA) containing 0.1% BSA in sterile plastic Bijou bottles and stored at -20^{o}C until required.

b: Granulocyte-Macrophage Colony Stimulating Factor (rhGM-CSF)

rhGM-CSF was supplied by the Genzyme Corporation, Boston, USA. It was derived from cDNA from activated human T-lymphocytes and expressed in yeast. The product is composed of compounds with three molecular weights (15.5,16.8,19.5 KDa), reflecting differences in glycosylation.

The product is 95% pure and is species specific with an activity of $5x10^7$ CFUc/mg measured as day 14 GM-CFUc in cionogenic assay of 10^5 normal human bone marrow cells.

It was supplied as a frozen solution of 1ml containing 100µg (5000 CFU). For use, aliquots of the stock solution were diluted in PBSA containing 0.1% BSA and stored, frozen, as above.

c: Interleukin-3 (rhIL3)

rhIL3 was supplied by Genzyme. It was expressed in yeast from cDNA cloned from activated human T-cells. It differed from natural human IL3 by an intentional substitution of aspartic acid for asparagine at positions 15 and 70 to reduce N-iinked glycosylation. The molecular weight was 15KDa and the stated activity was 10^8 CFUc/mg. It was supplied as a frozen solution of 1mi containing 50µg (5000 CFU). The stock solution was diluted and stored as above.

d: Interleukin-4 (rhIL4)

rhIL4 was supplied by British Bio-technology Ltd, Abingdon, Oxon., U.K.. it was expressed in E. Coli from a designer gene (BBG 15). The sequence differed from that of natural human IL4 by the presence of a N-terminal methionine.

Activity, measured by ^{3}H-thymidine incorporation into PB lymphocytes was 1-$3x10^6$Units/mg. It was supplied in vials containing 5µg, as a lyophilised powder, reconstituted in distilled water and diluted in PBSA containing 0.1%BSA as above.

e: Interleukin-6 (rhIL-6)

rhIL-6 was supplied by Genzyme and had a specific activity of 10^7 units/mg prolL-6. For radioimmunoassay (RIA) rhIL-6 was obtained from The National Institute for Bioiogical Standards and Controls (NIBSC - Code No 87/736).

f: Interleukin-1-alpha (IL-1α)

NiBSC supplied IL-1α (Code 86/632) for RIA.

g: Interleukin-1-beta (IL-1β)

IL-1β (Code 86/680) was supplied by NIBSC for use in RIA.

2,1.6 ANTIBODIES AND ANTISERA TO GROWTH FACTORS AND LYMPHOKINES

a: Monoclonal antibody to rhGM-CSF

Monoclonal murine IgG_1 anti human GM-CSF raised against rhGM-CSF expressed in CHO cells was supplied by Genzyme as a frozen solution in vials containing 1mg in 1ml. The neutralising activity of this antibody in colony forming assays was stated to be 1μg to neutralise, completely, 50 units of GM-CSF and 0.04μg to reduce the activity of

50 units by half. (1μg antibody ≡ 1ng GM-CSF)

Aliquots of stock solution were diluted and stored as above.

b: Polyclonal Antibody to rhGM-CSF

Rabbit polyclonal antibody to rhGM-CSF was supplied by Genzyme, frozen in glass vials containing 1mg/1ml in PBS. The antibodies were of mixed immunoglobulin classes (80% IgG and 20% IgM) and were raised against purified recombinant human GM-CSF of unstated source. The neutralising activity in a colony forming assay was said to be specific to GM-CSF and 1mg ≡ 1000 Units (500ng) GM-CSF. Aliquots were diluted and stored as above.

c: Antibody and Antiserum to Interleukin-6

Rabbit anti-human IL-6 was supplied by Genzyme. It had a neutralising activity per mg of 10^4 units (100ng) of IL-6. Goat antiserum to rhIL-6 was supplied by Dr S.Poole, NIBSC. 1μl neutralised 156 pg rhIL-6.

d: Antibody to IL-1α

Polyclonal sheep anti-serum to human IL-1α was supplied by Dr S.Poole, NIBSC. 1μl neutralised 1ng ofIL-1α.

e: Antibody to IL-1β

Polyclonal sheep anti-seum to human IL-1β also came from Dr S.Poole, NIBSC. 1μl neutralised 0.1ng IL-1β.

2,2 LABORATORY METHODS

All the work described was carried out In a class II microbiological safety cabinet (M.D.H. or Envair, U.K. Ltd.)

2,2.1 SEPARATION OF MONONUCLEAR CELLS FROM BLOOD AND BONE MARROW AND STORAGE OF PLASMA AND SERUM

a: Bone Marrow

Bone marrow sample volume was measured using a graduated pipette and then diluted with the same volume of PBSA. In some cases bone marrow plasma was separated from the cellular fraction before dilution with PBSA by the method descrlbed below for peripheral blood. 4-5ml aliquots of diluted bone marrow were layered onto 10 ml of Fycoll-Hypaque (Lymphoprep, Nicomed, Oslo, Norway) with a density of 1,077 g/cm^3 in sterile plastic universal containers and the cells separated according to density by centrifugation [Boyum, 1968] at 128*g* (IEC-CENTRA 7 - International Equipment Company, USA) for 20 mins.

The erythrocytes and granulocytes were deposited at the bottom of the container and the mononuclear cells MNC appeared as a white band at the interface between the lymphoprep and the aqueous fraction of the bone marrow sample. Cells were aspirated from the interface, transfered to a second universal container and diluted in approximately 15ml of PBSA before centrifugation at 450*g* for 10 mins. The supernatant

was discarded, the MNC resuspended using an electric/mechanical mixer (Whirlimixer, Fisons, Leicestershire) and diluted in 1-5 ml of single strength α-Medium.

The MNC count was determined by diluting 40μl of the cell suspension in 20ml of Isoton followed by the addition of 6 drops of Zaponin or Zap-Oglobin (both-Coulter Electronics) to lyse red cells. 500μl aliquots were counted as above and the cell concentration calculated by multiplication of the mean of two readings by a factor of 1000.

The total yield of MNC from the sample was calculated by multiplication of the total volume of the final MNC suspension by the cell count/ml.

b: Peripheral Blood Samples

Blood samples were collected and taken to the laboratory within one hour of removal from the patients or donors. Serum samples were allowed to clot at room temperature before separation of the serum from the cellular fraction. Plasma samples were separated as soon as possible (i.e. within one hour) except when comparison of activity(ies) was made with serum in which case both samples were separated at the same time, after clotting of the serum sample.

Separation of the cellular and aqueous components was done by centrifugation for 10 minutes at 200*g* (Centra-7). The serum or plasma was aspirated from the cellular fraction and transfered to a sterile 10ml plastic screw top test tube (Sterelin) in which it was stored at -20^{o}C until used.

Cells from plasma samples were diluted in twice the cell volume of PBSA and

separated on Lymphoprep as described (2,2.1a)

2,2.2 ENRICHMENT OF MONONUCLEAR CELLS FOR CD34 POSITIVITY

CD34 enrichment was achieved by use an immuno-separation technique employing a specific murine antibody to CD34 (Anti-PCA-1) (Becton Dickinson) and magnetic beads coated with sheep anti-mouse IgG (Dynabeads, Dynal A.S., Oslo, Norway).

Bone marrow MNC were obtained and counted using the methods described above. An aliquot of the ceil suspension containing $2x10^7$ MNC were transfered to a 10ml sterile plastic test tube (Sterilin) and diluted to a volume of 0.5mi after washing twice in PBSA. 40-100µl (initial experiments used 100 and later experiments 40µl) of anti CD34 Mab was added asceptically; the ceil suspension was mixed before incubating at 4^oC for 20mins. Cold PBSA was added (10ml) to remove excess antibody. The cells were centrifuged (3mins at 200*g*), the supernatant discarded and the MNC resuspended. This process was repeated before the cells were resuspended in 0.5 ml in PBSA. 45µl of Dynabeads were added and after mixing (Whirlimixer) the cells were incubated for a further 20mins at 4^oC.

The magnetic beads were captured on a magnetic particle concentrator (Dynal MPC-1) after diluting the sample to a volume of 5-10ml with PBSA. CD34+ve cells were retained against the magnet attached to the magnetic beads. The supernatant and unbound cells were aspirated and discarded. This process was repeated after removing the tube from the magnetic separator and diluting again with PBSA.

To dissociate the bound cells from the magnetic beads they were resuspended in 5ml of an equal mixture of single strength α-medium and plasma, from the same patient or donor where posible, and incubated in a humidified atmosphere containing 5%CO_2 at 37°C overnight.

The magnetic beads were then removed as before and the cell suspension was aspirated into a fresh sterile container. The beads were washed in PBSA and the process repeated. The cell suspension was concentrated by centrifugation for 5mins at 200*g* and resuspended in single strength α-medium (0.5-1ml). Total cell count was estimated as above (2,2.1a). An aliquot was examined for the presence of surface CD34 by immuno-staining, as described below (2,2.4b).

2,2.3 COLONY FORMING ASSAYS

a: Assay of GM-CFUc from Bone Marrow and Peripheral Blood Mononuclear Cells.

A double layer agar colony forming assay was used to assess GM-CFUc in MNC [Bradley et al, 1978]. Each assay was carried out in triplicate in 35mm sterile Petri dishes (Nunc, Denmark) to which 100μl aliquots of 5637CM (as above) were added. The underlay was prepared using equal volumes of 1% agar in distilled water (Agar noble, Difco Laboratories, Detroit, Michigan, USA) and 2xα medium. The agar was melted using a microwave oven (Finess 500) and was maintained at 56-60°C on a hot plate (Hot

plate/Stirrer PC351 - Corning). This temperature range was sufficient to maintain the fluidity of the agar without damaging the components of the medium. 1ml aliquots were dispensed into the Petri dishes and allowed to cool and solidify at room temperature.

The second agar layer containing the cells was prepared by dispensing mononuclear cells into sealable test tubes (Falcon 2058, Becton Dickinson) or universal containers. If the volume of cell suspension exceeded 100μl it was concentrated by centrifugation for 5 mins at 200*g*, the supernatant discarded and the cells resuspended, before the addition of growth medium (α) containing 0.3% agar (final concentration). 0.5ml aliquots of this cell suspension, containing 10^5 cells per plate for normal bone marrow and $2x10^5$ for myelomatous bone marrow [Millar et al, 1988], were dispensed onto each underlayer. PBMC were plated at $5x10^5$/plate.

Petri dishes were placed in plastic sandwich boxes (Stewart plastics - U.K.) containing a water reservoir. Boxes were sealed with PVC tape and gassed with a mixture of 5% CO_2, 10% O_2 and 85% N_2 through two holes which served as entry and exit ports (also then then sealed with PVC tape). Cultures were incubated (LEEC) at 37°C (in an atmosphere of 5% CO_2 in case of leakage from the box) for 12-14 days at the end of which colonies were counted using an inverted microscope (Nikon, Japan or Wilovert, Will, W.Germany). A colony consisted of 50 cells or more and 20-50 cells were considered to be a cluster.

b: Assay of Plasma and Serum for Colony-Stimulating Activity

Serum and plasma was first assayed for colony stimulating activity by methods described previously [Millar et al, 1990] using 0.5ml/test. In later experiments the volume of plasma was reduced to 0.3ml/test and incorporated into a double layer method for reasons described in Chapter 3,3.

b.i: Single layer method

Plasma or serum samples were thawed at room temperature. O.5ml aliquots were transfered in triplicate to 35mm plastic Petri dishes. Normal bone marrow MNC were processed as above and mixed with growth medium containing 0.5% agar (final concentration) in universal containers. 0.5ml aliquots containing $2x10^5$ MNC/ml were added to each Petri dish and mixed well with the test plasma/serum. The final agar concentration was 0.25%. After cooling the dishes were incubated under the conditions described for GM-CFUc from bone marrow (2,2.3a).

b.ii: Double layer method

300µl of each test plasma was transfered to triplicate 35mm Petri dishes as above. The underlayer was completed by the addition of 0.7ml of growth medium containing 0.75% agar. Thus, the final concentration of agar in the underlay was 0.52%. The overlay was prepared as described in chapter 2,2.3a incorporating 10^5 normal

BMMC as above.

Cultures were incubated at 37°C as above for 12-14 days.

c: Assay of cytokines and antibodies to cytokines within colony forming assays.

Solutions of the test factor were added to the Petri dishes in a total volume of ≤100µl before the addition of the underlayer or plasma. The assays were then prepared as described above.

2,2.4 IDENTIFICATION OF THE CELLS AND COLONIES GROWN IN CLONOGENIC ASSAYS

a: Light microscopy

Slides of individual colonies which had been removed from agar in a few drops of PBSA were prepared using a Shandon Cytospin (Shandon, USA) and allowed to dry in air at room temperature. The slides were fixed in 99.8% methanol (BDH) for 10 mins, dried at room temperature and stained with May-Grünwaid-Giemsa (Gurr, BDH Ltd, Pooole, U.K.) for ten minutes. After drying, slides were mounted in D.P.X (BDH), covered with a glass coverslip (Chance Propper Ltd, Smethwick) and examined using light microscopy (Axioscope, Zeiss, W.Germany).

b: Immuno-phenotyping

BMMNC were examined, without methanol fixation, for surface CD34 before and after enrichment for this phenotype (Chapter 2,2.2). Alliquots containing 10^6 cells were dispensed into small tubes (LP3 - Luckam, U.K.) and were washed with PBSA, centrifuged at 200*g* for 5mins and resuspended in 100µl of PBSA. 20µl of murine anti-CD34 (Anti-PCA-1 - Becton Dickinson) monoclonal antibodies was added to each tube. This was the same antibody used for enrichment of BMMNC for CD34+ve cells (Chapter 2,2.2).

After incubation, excess antibody was removed from the cells by washing in 2mls of PBSA followed by centrifugation at 200*g*/5mins. The supernatant was discarded and the process repeated. The cells were resuspended in 100µl of PBSA and 5µl of rabbit anti mouse igG congugated with flourescein or rhodamine (Dakopatts) was added to each sample. Samples were incubated for 20mins at 4^{o}C. The cells were washed as above and resuspended in 100µl of PBSA. Aliquots of cell suspension were applied to glass microscope slides and examined using flourescence microscopy (Axioskop).

2,2,5 ELISA ASSAYS FOR GROWTH FACTORS AND CYTOKINES

a: ELISA for Granulocyte colony-stimulating factor (G-CSF)

A quantitative enzyme linked immunoassay system was supplied by Oncogene Science, Inc (Manhasset, N.Y., USA.). The lower limit of detection of the assay was 50pg/ml, 100pg/ml giving approximately twice background levels of absorbance. The kit

is specific for G-CSF without cross-reactivity with a large range of cytokines including GM-CSF and interleukins 1,3,4 and 6.

A microtiter assay plate was supplied pre-coated with monoclonal mouse anti-human G-CSF. Standard curves of known concentrations of G-CSF mixed with buffer or normal donor serum, to correct for the effects of plasma, were prepared in duplicate each time the assay was done. Aliquots of 100µl of each standard or test sample was added to each microtitre well and the plate was incubated plate overnight at 4°C. Samples were removed by inversion and the plate was washed 4 times with buffer. 100µl of biotinylated polyclonal rabbit anti human G-CSF was added to the assay plate and incubation continued for 2 hours at 37°. Excess antibody was removed as before and the plate washed 4 times with buffer. Streptavadin-Horseradish peroxidase was added for 1 hour at room temperature. The plate was washed to remove unbound streptavidin-HRP. A mixture of two peroxidase substrates was added for 30 mins at which time absorbance of each well was read at 405nm using an automated plate reader (Titertek Multiscan MCC/340, Flow Laboratories ltd, Rickmansworth, Herts.).

Samples containing known concentrations of G-CSF were used to construct a standard curve of G-CSF against absorbance from which the G-CSF content of test samples was determined.

b: ELISA for Interleukin-4 (IL-4)

A quantitative ELISA (Quantikine) was supplied by Research and Diagnostics Systems, Minneapolis, USA. The assay was specific for IL4 (human) and the lower limit

of detection 3pg/ml. The method was similar to that for G-CSF.

Each microtitre plate supplied was coated with monoclonal murine antibody to IL4. The second antibody was polyclonal antibody (species not stated) against human IL4 conjugated to horseradish peroxidase. 200µl aliquots of standard concentrations of IL-4 and test samples were incubated for 2 hours at room temperature, as was the IL-4 conjugate. The third stage employed hydrogen peroxide and tetremethylbenzidine, mixed 15 minutes before use and a final stop solution of 2N sulfuric acid was used to terminate the reaction after 20 minutes. The optical density in each well was measured at 450nm as above. Standard solutions containing known concentrations of IL4 diluted in serum or synthetic protein based diluent were tested on each microtitre plate. Optical density was plotted against the standard IL4 concentrations on a log/log scale to derive IL4 levels for the samples tested.

c: ELISA for Interleukin-3 (IL-3)

A "Quantikine" kit supplied by R&D Systems (2,2.5b) was used for the quantification of IL3. The microtiter plate was supplied coated with a murine monoclonal antibody to IL3. The second antibody, conjugated to horseradish peroxidase was a polyclonal antibody (species not stated) to IL3. The remaining reagents were as described for the ELISA for IL4 and the method described by the manufacturers was employed. The lower limit of detection of IL3 in this assay was stated to be 7.4pg/ml.

d: ELISA for Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

This assay was developed by Dr S Denham, Institute of Cancer Research. Antibodies to human GM-CSF were obtained from Genzyme (2,1.6). Microtitre plates (Nunc ELISA plate) were coated with mouse monoclonal antibody to rhGM-CSF by incubating overnight with a solution of the antibody at 2.5-4μg/ml in PBSA pH 8.6 (by the addition of NaOH). The plate was washed as above with a solution of PBSA containing 0.5% Tween 20 (Sigma) and 2% BSA (2,1.4a)). 100μl of PBSA with 2% BSA was added to each well for 30mins at room temperature before use.

Standard concentrations of rhGM-CSF (Genzyme 2,1.5b) were prepared by adding a concentrated solution of rhGM-CSF (in PBSA with 1% BSA) to 2 wells and carrying out doubling or tripling dilutions of the factor in succesive wells containing 100μl PBSA with 1% BSA. The content of each well was mixed well. Standard concentrations for comparison with serum/plasma test samples were prepared in diluted heat inactivated FCS (2,1.4a) and/or pooled normal human serum collected from normal donors or laboratory staff or AB serum supplied by the Blood Transfusion Service (Tooting, London). At least one standard curve was prepared with each assay. One set of duplicate wells was left without standards or test samples in each assay plate.

Test samples were added to duplicate wells in volumes of 100μl. The samples and standards were incubated for 2 hours at room temperature. The wells were then washed 4 times with detergent solution.

The second antibody (Polyclonal rabbit anti rhGM-CSF - Genzyme, 2,1.6b) was added to each well as 100μl of a 3μg/ml solution in PBSA, and the plate incubated for two hours at room temperature. After removal of the second antibody followed by

washing the plate four times, as above, a biotinylated swine anti-rabbit Ig antibody (Dakopatts) was added, at a dilution of 1/1000 in PBSA. After 1 hour at room temperature excess was removed by inversion and four washes with detergent solution.

Avidin/ biotinylated Horseradish Peroxidase kit (ABComplex/HRP - Dakopatts) was prepared by adding 1 drop (45µl) of Avidin and 1 drop of biotinylated HRP to 5ml of 0.05M Tris/HCl buffer at pH 7.6 (TRIZMA Base - Sigma), mixing well and leaving for 30mins before use. 100µl aliquots were added to each well and incubated for 30mins at room temperature. The substrate for HRP was o-phenylenediamine (Sigma). For two assay plates 30mg was dissolved, in a brown glass bottle, because of photosensitivity, in 20ml of a citric acid phosphate buffer solution at pH 5.0, prepared by dissolving 0.511g of citric acid monohydrate (Sigma) and 1.841g of $Na_2HPO_4.12H_2O$(Sigma) in 100 ml of purified water. 7µl of hydrogen peroxide solution (100 volumes - FSA laboratories supplies, Loughborough, U.K.) was added just before use. 100µl of this substrate solution was added to each microtitre well and after 10-15mins 100µl of 4.5M $H_2SO_4$was added to stop the reaction.

Absorption was measured at 492nm in a Titertek Multiscan. A standard curve was prepared by plotting the absorbance of the standard solutions against their concentrations of GM-CSF on a log/log plot.

2,2.6 RADIOIMMUNOASSAYS (RIA)

The following assays (IL-1α/β, IL-6 & CRP) were performed by Dr P Riches in The Department of Immmunology, Charing Cross and Westminster Medical Schools, Westminster Hospital, London.

a: Interleukin-6 (IL6)

RhIL-6 was radloiodinated (Na ^{125}I,Carrier free, Amersham International, U.K.) using a mild chloramine-T method [Greenwood et al, 1963]. RIA was carried out at 4^{o}C in PBSA (0.02M $NaP0_4$, 0.01M EDTA, 0.145M NaCl, 0.1% (w/v) sodium azide) containing 0.5% BSA and 0.01% Tween 20 (pH7.4). rhIL-6 (NIBSC code No: 88/514) was used as a standard. Samples or standards (100μl) were added to assay diluent (200μl) and incubated for 24 hours with 100μl goat anti-IL-6 serum (Final dilution 1/133,333). ^{125}I-labelled IL-6 was added to each tube for a further 48-72 hours. Separation of bound cytokine from free was by addition of 100μl of antibody-coated cellulose (SACCEL) (Donkey anti sheep/goat ASac2, IDS, Tyne and Wear, U.K.). Reaction tubes were incubated at room temperature for 30min and then 0.5ml of water was added before centrifugation at 1000*g* for 15min. The supernatant was aspirated and discarded and bound radioactivity in the pellet quantified in a gamma counter (LKB-Wallac 1261 multigamma) [Montes-Borinaga et al, 1990]

b: Interleukin-1-alpha (IL-1α)

This assay employed the method described above (2,2.8 a.). The RIA standard was NIBSC 86/632. A polyclonal sheep IL-1α anti-serum at final dilution of 1/200,000 was used and had a neutralising ability of 1μl ≡ 1.0ng IL-1α

c: Interleukin-1-beta (IL-1β)

This cytokine was iodinated using a mild iodination protocol (using N-bromo-suucinimide (NBS)) to avoid loss of immunochemical activity that is seen with this molecule under oxidising conditions. Iodinated cytokine was recovered using a second isoelectric focussing gel [Poole et al, 1989]. RIA employed the same method as that for IL-6 and IL-1α, using NIBSC standard 86/680 and sheep anti-IL-1 with a neutralising activity of 1μl≡ 0.1ng IL-1β att a final dilution of 1/133,333.

2,2.7 BIOASSAY FOR INTERLEUKIN-6 (IL6)

Interleukin-6 was assayed by incorporation of tritiated (^{3}H) thymidine by the IL-6 dependant B9 mouse hybridoma cell line [Aarden et al, 1987]. The cell line was maintained in continuous growth in RPMI-1640 medium (Flow) containing 7.5% FCS, 20mM l-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin and 2% peripheral blood mononuclear cell conditioned medium (PBMC-CM) as a source of IL-6.

Samples of plasma and medium containing known concentrations of rhIL-6 were

added in 100µl aliquots to 96 well flat bottomed tissue culture plates in which B9 cells had been plated at $5x10^4$/100µl/well. Plates were incubated for 72 hours then pulsed with 1.86 MBq/well/20µl of ^{3}H-thymidine for the final 24 hours before removal of the free radioactive nucleotide and measurement of the incorporation of ^{3}H by the B9 cells in a scintillation counter.

2,2.8 ASSAY OF C-REACTIVE PROTEIN (CRP)

This protein was measured in plasma samples by rate nephelometry with an Array analyser (Beckman, High Wycombe, UK). There was a between-batch coefficient of variation at both normal and above normal concentrations of <7% and a measuring range of 1-600 mg/l.

2,3 CLINICAL METHODS

2,3.1 PATIENT DETAILS

a: Patient Population Studied

The patients whose details are reported in this study were all treated by the Myeloma Unit of the Royal Marsden Hospital between 1989 and 1992. Most received all of their treatment at the RMH but some were previously treated elsewhere and some continued their treatment at other centres but following the protocols of, and under the supervision of, the RMH. In all cases the high dose chemotherapy (HDM or HDBu with or without ABMR) was carried out at the RMH.

Patients treated at the RMH were seen every three weeks during induction therapy with one of the VAMP regimens. On each of these occasions they had a paraprotein measurement, blood count and biochemical profile. Diagnostic bone marrow aspirates were carried out at the begining of treatment and, usually, on alternate visits to monitor bone marrow tumour clearance. Bone marow aspirates were also carried out when serum PP appears to enter a plateau during treatment or fails to respond to chemotherapy. A bone marrow aspirate was always carried out before priming (see below) one week before bone marrow harvest. Patients were routinely monitored with *in-vitro* growth of GM and My-CFUc each time a bone marrow sample was obtained as they progressed through induction therapy, high dose chemotherapy and remission.

During high dose chemotherapy the patients were closely monitored in a specially

designated unit in four bed bays. In this unit, isolation in single rooms had not been found to reduce the incidence or severity of infective episodes during neutropenia after ABMR. All the patients had central venous canulae with two or more channels, either Hickman lines, inserted before induction therapy, or subclavian lines inserted at the time of bone marrow harvest. These facilitate the administration of chemotherapy, antibiotics, intravenous fluids and parenteral nutrition, when nessesary, and the sampling of venous blood for chemical and haematological monitoring.

b: Selection of Patients for Individual Studies

Patients were entered into ongoing studies as they presented to the unit or as they became eligable for a given randomisation.

Selection of patients for the post high dose chemotherapy studies described below was carried out by sequential recruitment of patients entering the high dose phase of their treatment, subsequent to their consent, irrespective of their previous history. A heterogeneous group of patients was, thus, recruited, some having received previous high dose therapy. Because of the routine monitoring of patients data on the clonogenicity of myeloma and normal myeloid precursors was available for the induction phase of their chemotherapy.

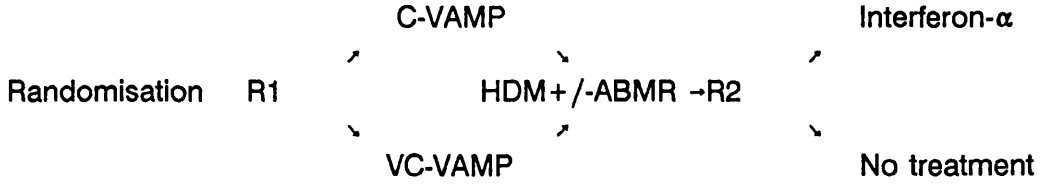
On a few occasions blood samples were not obtained before high dose chemotherapy commenced and these patients were excluded from the studies described.

For the study comparing plasma and serum CSA, patients attending one MM out-patient clinic were recruited in the phlebotomy department. All patients with a diagnosis

of MM who were due for routine blood samples were asked to allow extra samples to be taken for the study. Patients who consented were included in the study. As all stages of treatment were represented in the clinic a heterogeneous group of patients with different stages of disease was recruited.

2,3.2 TREATMENT PROTOCOLS

During the study period the protocol for new, previously untreated patients or patients resistant to other regimens with MM presenting to the RMH, who had a good performance status and adequate renal function (EDTA clearance>40ml/min), was as follows:



Patients with poor renal function but good performance status entered the first randomisation but recieved HDBu+ABMR if their EDTA clearance was < 30ml/min and if they obtained adequate clearance of tumour from their bone marrow. Patients with EDTA clearance ,<40, but >30ml/min received reduced dose (140mg/m^2) HDM+ABMR.

Patients who had relapsed from previous HDM +/- ABMR recieved C-VAMP and then HDM+ABMR or HDBu+ABMR depending on their previous response to HDM.

Patients with good performance status and renal function who did not obtain adequate bone marrow tumour clearance (<20%) received HDM (140mg/m^2) without

ABMR.

Elderly patients (70yrs) or patients with poor performance status received weekly cyclophosphamide alone or cyclical oral melphalan and prednisolone.

Details of these regimens and others that have been received by the patients described below, either previously at the RMH or elsewhere, are given below.

a: VAMP

The development of this regimen is described in chapter 1 (1,2.10b).

The VAMP regimen consists of the following drugs:

Vincristine	0.4 mg over 24hrs x 4 days
Adriamycin	9mg/m^2 over 24hrs x 4 days
Methlyprednisolone	1.5g daily for 5 days.

The Adriamycin and vincristine are administered, mixed together, intravenously, by a portable electric pump (e.g. Infumed, Neurotechnics, Thame, Oxfordshire, UK) via a central venous canula. The methylprednisolone is taken by mouth or given as an intravenous bolus. Each drug is started on day one of a 21 day cycle.

The following drugs were also administered at the RMH as prophylaxis against [1]oral candidasis, [2]respiratory infection, [3]peptic ulceration and [4]hyperuricaemia.

[1]Nystatin supension	1ml four times daily
[1]Amphoteracin Lozenges	1 four times daily
[2]Cotrimoxazole	960mg three times weekly
[3]Ranitidine	150mg twice daily
[4]Allopurinol	300mg daily (During maximal response)

The portable infusion pumps are worn on a belt and allow complete mobility for the patient. Apart from myelosuppression, the regimen is not particularly toxic and the pump reservoir size allows the patient to recieve treatment entirely at home.

b: C-VAMP

This regimen [Bell et al, 1990] employs VAMP with the addition of a weekly injection of 500 mg of cyclophosphamide given on days 1,8 and 15 of the VAMP treatment cycle. The ancillary drugs and methods of administration are as given for VAMP.

c: Verapamil-C-VAMP (VC-VAMP)

A randomised trial was in progress to determine whether verapamil (a calcium channel blocking drug) increases the response rate to VAMP by inhibition of drug resistance as has been shown in *in-vitro* cultures of MM cells [Millar et al, 1989].

Patients were randomised, at presentation, to recieve high (100mg/day) or low (10mg/day) dose verapamil in addition to C-VAMP (VC-VAMP) or C-VAMP alone. The verapamil was given as a contiuous intravenous infusion for 5 days, starting and finishing 4 hours before or after the adriamycin/vincristrine infusion. For the purpose of this study no distinction will be made between high and low dose verapamil.

d: HDM with or without ABMR

With ABMR the dose of melphalan was 200mg/m^2 and without ABMR it was 140mg. The number of bone marrow mononuclear cells required for ABMR was >2.0x10^8/kg with less than 20% infiltration with MM.

Patients received a "priming" dose of cyclophosphamide [Millar et al, 1975, Millar and McElwain, 1985] (400mg/m^2) 5-9 days (a further study was progress in which patients were randomised to recieve priming with different priming/HDM intervals) before bone marrow harvest and HDM.

Melphalan was administered to patients, on the day of bone marrow harvest (except when cryopreserved cells are used), after a period of hydration assesed by measurement of central venous pressure (CVP) and establishment of a diuresis of 20ml/min. The melphalan was prepared immediately before use and given over 5mins

into a fast running drip of normal saline. Adequate hydration, according to an established regimen, was given post melphalan to maintain an adequate CVP and a urine output of 20ml/min for the first hour and 500ml/hr for two further hours. Urine output was maintained with frusemide. 11 hours after melphalan patients received 1.5g of methylprednisolone intravenously followed at 12 hours by the return of their bone marrow harvest. If cryopreserved bone marrow was used this was covered by a forced alkaline diuresis.

Antiemetic prophylaxis was given with ondansetron (a $5HT_3$ antagonist) or a combinantion of a centrally acting antiemetic with dexamethasone and a benzodiazepine.

Methlyprednisolone (1.5g IV) was administered daily for five days as an anti-tumour agent for residual tumour cells in the harvest bone marrow.

Patients received prophylaxis against [1]oral candidiasis from day 1 after melphalan and against [2]systemic infection from day 5. Second[3] and third[4] line antibiotic regimens were employed for episodes of pyrexia without established microorganisms. Systemic treatment for suspected or possible systemic/pulmonary infection with [5]fungi, [6]herpes viruses and [7]Pneumocystis carinii was given with the drugs shown below (Table 2,3. page 94). Other antimicrobial agents were given when indicated for specific infections. Patients also received ranitidine for prophylaxis of peptic ulceration.

Table 2.3 DRUGS ADMINISTERED AFTER HDM

Nystatin suspension[1]	1ml p.o. qds
Amphoteracin Lozenges[1]	1 p.o. qds
Ranitidine	150mg i.v. bd
Gentamicin[2,3]	1.67mg/kg i.v.tds
Piperacillin[2]	4g i.v. qds
Flucloxacillin[2]	500mg i.v. qds
Ceftazidime[3]	2g 1.v. tds
Ciprofloxacin[4]	2g i.v. bd
Amikacin[4]	7.5mg/kg i.v. bd
Amphoteracin[5]	<1mg/kg/day i.v.
Acyclovir[6]	5-10mg/kg i.v. tds
High dose Co-trimoxazole[7]	60mg/kg i.v. bd

bd-twice daily, tds-three times daily, qds-four times daily,
i.v.-intravenous, p.o.-by mouth

==========

Antibiotic cover was maintained until patients were apyrexial with a neutrophil count of over $0.5x10^9$/l for three days.

Patients received irradiated blood products, as required to maintain their haemoglobin over 10g/l and their platelet count over $10\text{-}20x10^9$/l, or higher, if bleeding, or at risk of bleeding, due to severe infection or septicaemia.

e: HDBu and ABMR

Patients received 1mg/kg 6 hourly for four days (total 16mg/kg). Priming and eligability are discussed above.

Phenytoin, 100mg/day, to prevent convulsions that may be associated with high doses of busulphan, was given throughout treatment. Busulphan was administered with an alkaline diuresis maintained for 4 days (urine pH >8). ABMR was carried out 48 hours after the last dose of busulphan, on day 6. Management of the patients was as described for HDM, antibiotic prophylaxis comencing on day 5 after ABMR or when neutrophils fall below $1x10^9$/l, whichever occured soonest.

f: Recombinant Human Granulocyte Colony-Stimulating Factor (rhG-CSF)

Two trials of rhG-CSF, supplied by Chugai, were carried out during the period of study. Patients with MM and other malignancies recieving ABMR in several centres were included in the studies. Patients with MM who presented for HDM or HDBu during the period of these studies were included in the laboratory work described below.

The first study was a phase II study in which patients who consented to take part were randomised to recieve one of 4 doses of rhG-CSF (2,5,10 or 20μg/kg/day) or placebo as a 30min i.v. Infusion each morning from day 2 after ABMR until neutrophils were maintained at $>0.5x10^9$/l for three consecutive days. The trial was "single blind" in that the patients did not know which dose they reived but the nursing and medical staff did.

The second trial was a double blind phase III trial in which patients who

consented to be included were randomised to recieve 5µg/kg/day of rhG-CSF or placebo. The period and details of administration were the same as those in the phase II trial.

g: Weekly Cyclophosphamide (C-Wkly)

Weekly cyclophosphamide is given as a bolus i.v. injection of 500mg every 7 days until plateau phase or remission is obtained.

h: Oral Melphalan and Prednisolone (M/P)

Patients refered from other hospitals had recieved these drugs in combination but in various dose schedules. When this drug combination was used by the MM unit at the RMH it was given in a 21 day cycle with Melphalan 6mg/m^2 daily and prednisolone 60mg daily during days 1-5.

i: ABCM

Many patients refered to the Myeloma Unit had recieved ABCM as primary treatment without response. This protocol consists of the following drugs:

Adriamycin	i.v.bolus	30mg/m^2 day 1
BCNU	i.v. bolus	30mg/m^2 day 1
Cyclophosphamide	p.o.	100mg/m^2 days 21-24
Melphalan	p.o.	6mg/m^2 days 21-24

The cycle is repeated every 42 days.

j: Interferon-alpha (IFN)

Patients with MM entering remission after HDM or HDBu were asked to consent to a further treatment randomisation. When their peripheral blood count had recovered (WCC>2.5 and Plts>100x10^9/l) patients recieved either maintenance treatment with α-interferon or no maintenance. Interferon was given as a sub-cutaneous injection of 5mega-units of α-interferon three times weekly (Mon, Wed, Fri). This has been continued indefinitely or until disease progression. Patients who had recieved previous HDM are not eligable for this trial, however, those recieving high dose therapy for the second time, who had not previously recieved interferon, were offered maintenance interferon, as above. It is hoped that their previous duration of remission will serve as an internal control for the effect of IFN.

2,3.3 CLINICAL MEASUREMENTS AND INVESTIGATIONS

a: Paraprotein Measurement

Serum samples were collected from patients at each out-patient visit and at each admission to the ward. During the recovery period from HDM/Bu samples were collected each Monday for the duration of hospitalisation. Analyses are described in chapter 2,2.6.

b: Blood Samples After HDM/Bu

During the period after high dose chemotherapy patients had daily blood counts and assay of plasma biochemistry in the routine haematology and chemical pathology laboratories of the RMH. Samples were collected from the central venous canula at the same time each morning. In patients who were entered into the G-CSF trials samples were collected before the administration of G-CSF or placebo. Samples for the experiments described below were taken at the same time.

2,4 STATISTICAL METHODS

Where possible difference between the means of data groups have been compared using students "t" test. In many instances the groups were small and the data highly skewed, rather than following a normal distribution. In these circumstances non-parametric tests have been required and the median values of data in rank order have been compared by means of the Mann-Whitney "U" test or Wilcoxon's rank sum test. The probability "p" (that the data confirms to the null hypothesis that means or medians of the data are equal) has been given for 2-tailed tests (unless stated in the text). Significance has been accepted at the 5% level i.e. that $p \leq 0.05$.

In tests of correlation between between 2 variables, measured for individual patients and groups of patients, Pearson's correlation coefficient has been calculated. Spearman's rank correlation has been used for smaller groups of highly skewed data where relationships appeared likely but could not be demonstrated by relating the variables on an interval scale. In general, correlation coefficients (r) of ≥ 0.5 have been tested for significant deviation from the null hypothesis that the variables are unrelated ($r=0$) and $p \leq 0.05$ considered significant.

2,5 COMPUTER SOFTWARE

Preparation of the figures and word-processing were carried out on an IBM compatible personal computer (Elonex PC 386SXM/16) using the following software:

®WordPerfect Version 5.1	:Text and tables
®Fig P Version 5.0b & 6.0c	:Graphs and charts
®True Epistat	:Statistics
®Notebook II Version 3.02	:Reference sorting.

CHAPTER 3

PRELIMINARY EXPERIMENTS TO OPTIMISE AND EVALUATE THE METHODS FOR ASSESSING CSA IN SERUM AND PLASMA AND THE CLONOGENICITY OF PB GM-CFUc

3,1 THE EFFECT OF CELL NUMBERS PLATED ON THE CLONOGENICITY OF PBMNC GM-CFUc

PBMNC from patients with MM were incorporated into agar overlayers at concentrations between $5x10^4$ and $5x10^5$ per Petri dish in a double layer agar colony forming assay (Chapter 2,2.3a). 100µl of 5637CM was added to the underlay as a source of growth factors.

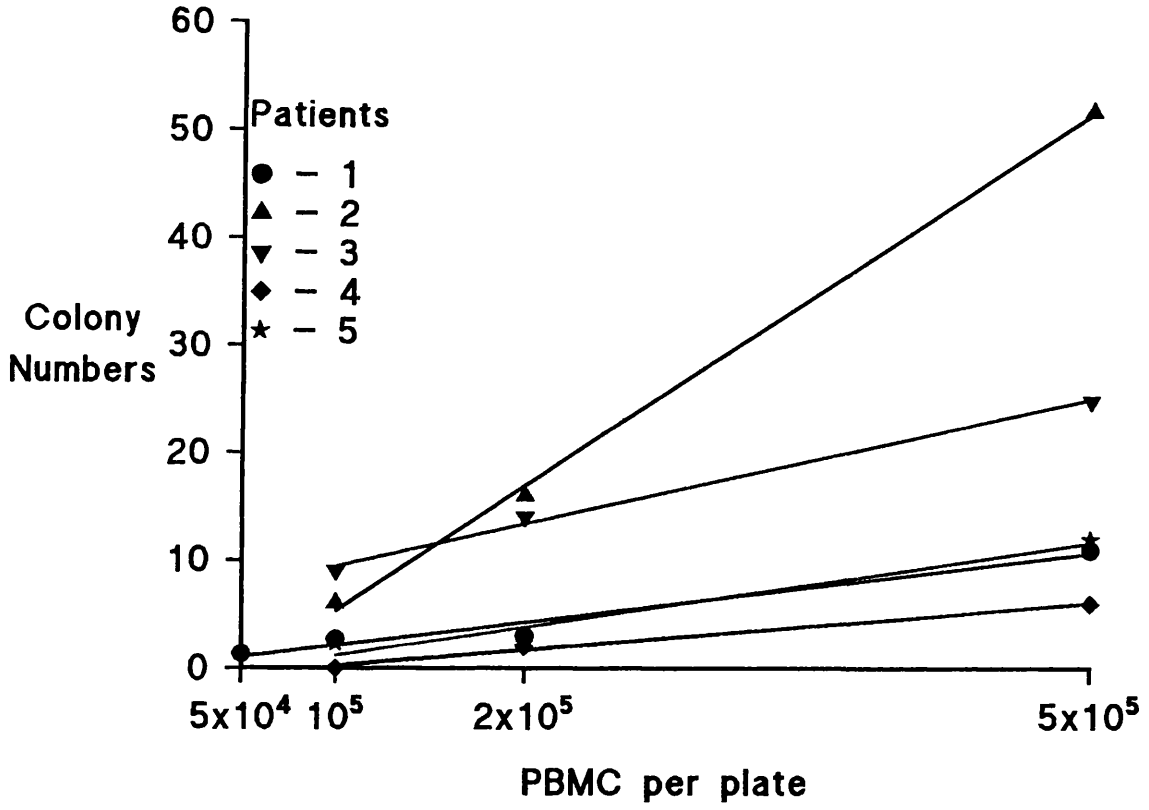
Only clusters of cells (10-50 cells) were grown at cell densities of less than 10^5 per plate. Plating was not done at cell densities greater than $5x10^5$ per plate since during the post HDM nadir adequate numbers of PBMNC are not available from 10-20ml of blood.

FIG 3,1.1 Page 105

There was a linear relationship between the number of cells plated and the number of GM-CFUc, although there was considerable variation in the numbers of colonies that were grown from the peripheral blood of different individuals.

Because the numbers of GM-CFUc in PBMNC were low and there was no evidence for auto-stimulation at a cell density of $5x10^5$ per plate, this concentration of cells was used in further experiments.

FIG 3,1.1



Plating efficiency of PBMC
from 5 patients using 5637CM

3,2 COMPARISON OF CSA IN PLASMA AND SERUM

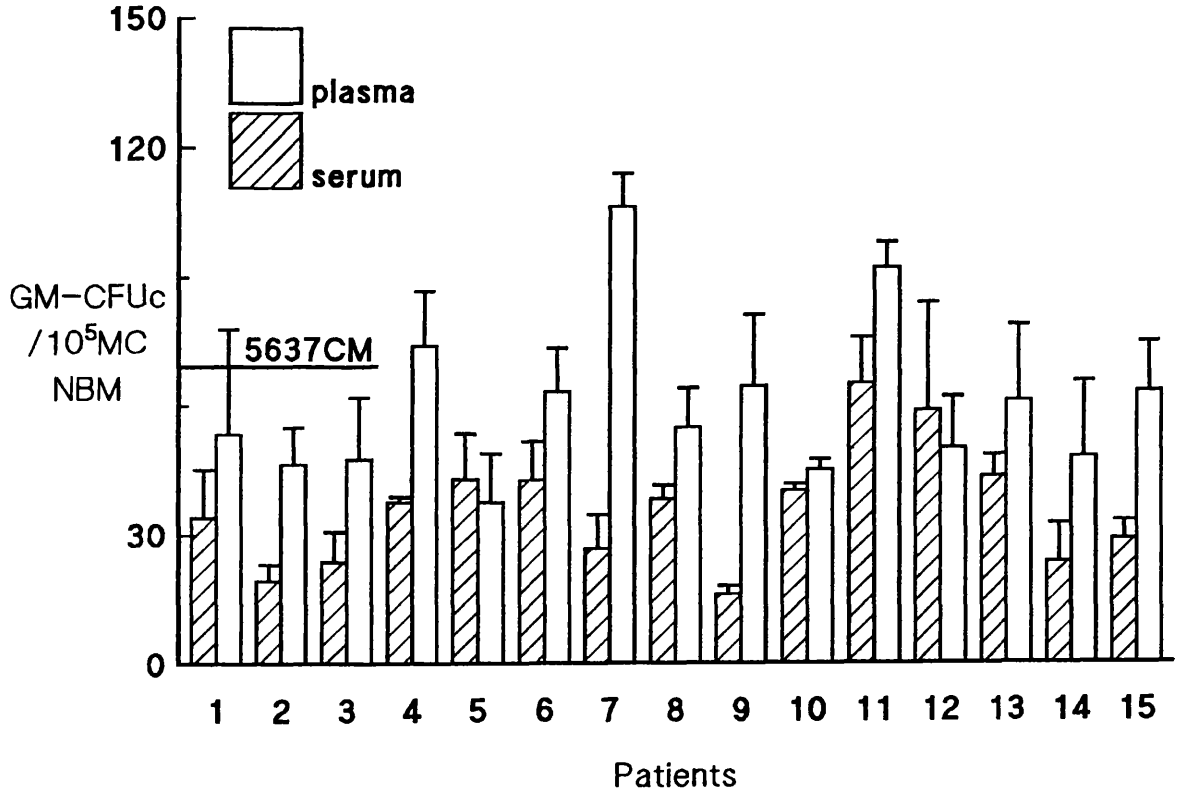
Most published data on CSA have been obtained from experiments using serum. To obtain MNC and samples for assessment of CSA from the same sample, blood was collected into heparin. Preliminary experiments were done to compare CSA in plasma and serum, and to determine whether CSA in plasma is maintained after freezing and thawing, as has been reported for serum [Entringer et al, 1980]. In each of the following experiments CSA in heparin-derived plasma was compared with serum immediately after the separation of samples (performed as soon as the time taken for the serum samples to clot) and after 28 days storage at -20^{o}C. The first two experiments employed a single layer of agar in the assay with 500µl of serum/plasma/plate (Chapter 2,2.3b.i) and the third was performed with the double layer assay, using only 300µl of serum or plasma/plate to make greater use of the avallable volumes of serum/plasma. (Chapter 2,2.3b.ii). Samples for each experiment were collected from patients with MM attending a single out-patient clinic (Chapter 2,3.1b). The patients' details are given in tables 3,2 A-C (Appendix I). Different patients were included in each experiment.

Plasma and serum from 5 normal donors was also compared, 4 against their own BMMNC and 1 against allogeneic cells.

FIG 3.2.1 page 107

In the first experiment CSA was detected in plasma and serum from 15/16 patients when tested immediately after separation from cellular components. In 13/15 patients CSA was greater in plasma than in serum. The difference between plasma and serum CSA was statistically significant. ($p=0.0004$ - Students' paired *t*-test).

Fig 3,2.1



COMPARISON OF CSA IN PLASMA AND SERUM WITHOUT FREEZING

Patient 16 – No CSA in P or S
5637CM – Colony numbers from 100µl of 5637 conditioned medium

The colonies grown with plasma and serum were the same, and consisted of granulocyte-macrophage colonies (GM-CFUc). There was no correlation between disease status and the level of CSA in either plasma or serum. However, the patient with no detectable CSA (pt 16) was in CR, 21 months following HDM and ABMR.

Fig 3.2.2 page 109

After freezing and storage for 28 days the same samples were thawed and tested against MNC from a different normal donor. Samples from 13 patients were evaluable, of which, 8/13 retained greater CSA in plasma than in serum. ($p=0.046$ - Students' paired *t*-test). As the fresh and frozen samples were tested against different BMMNC the colony numbers do not indicate absolute levels of CSA before and after freezing and storage. The difference between plasma and serum was less after freezing and thawing but it was not possible to tell if this was due to a loss of activity in plasma or an increase in the activity of serum.

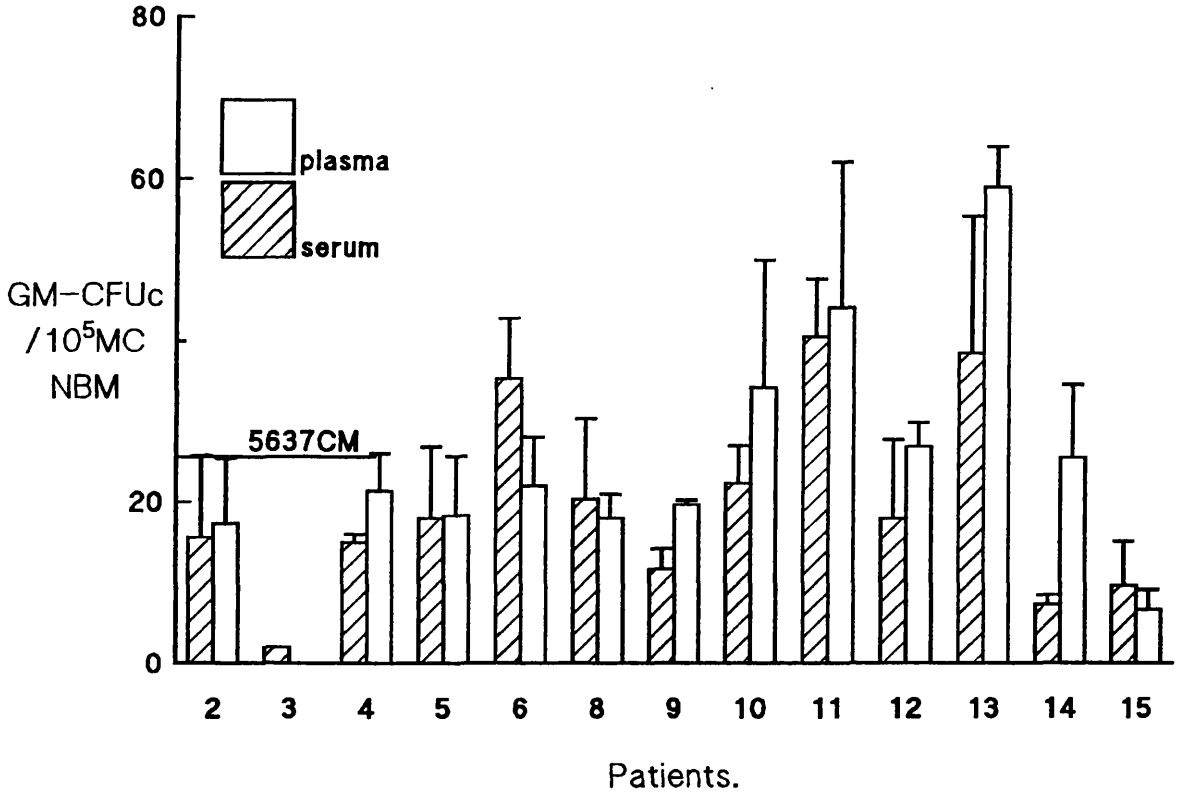
Fig 3.2.3 page 110

In the second experiment CSA in fresh serum was compared with plasma collected in heparin (H) or EDTA. These samples were tested on the day of collection without freezing or storage.

No colonies were grown in any of the plasma samples collected into EDTA. In this small group of patients 6/7 had greater CSA in H-Plasma than in serum.

To determine whether heparin might alter the pH of plasma/serum and thereby change the CSA, a further set of samples were collected for comparison. Heparin (Monoparin 1000U/ml) was added to aliquots of serum samples immediately after

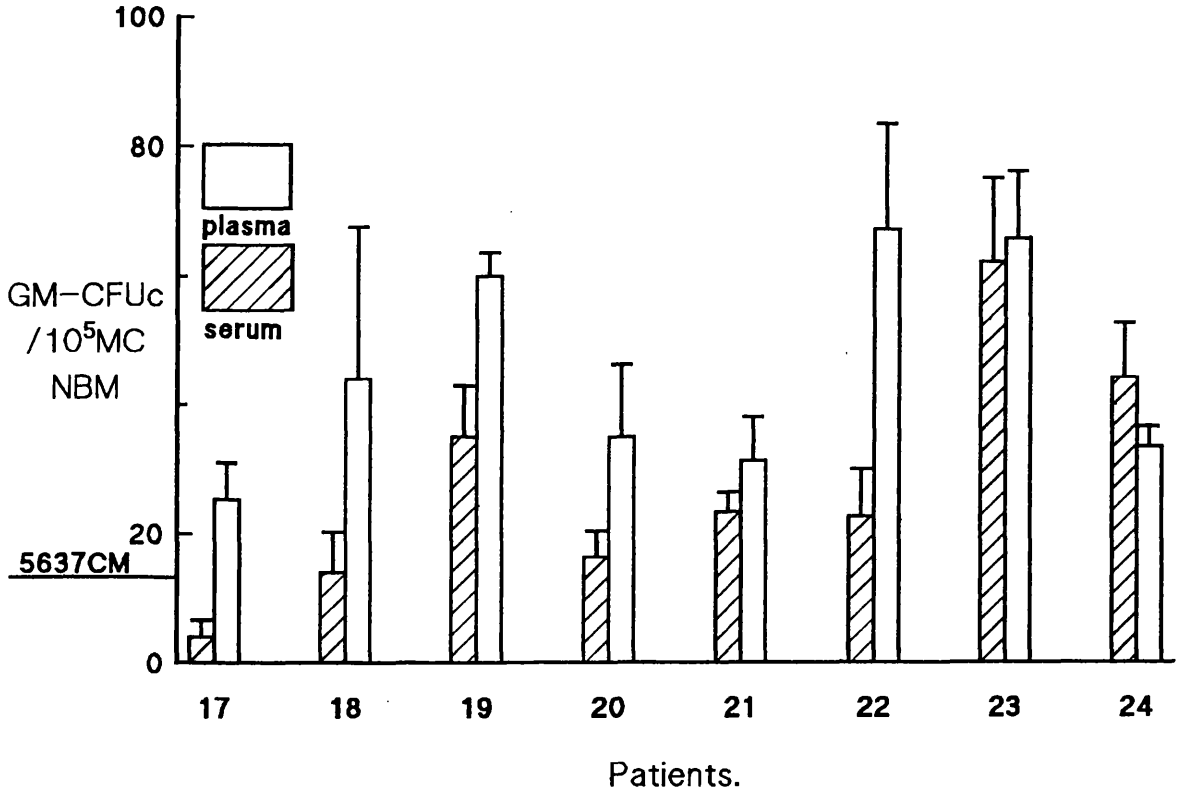
FIG 3,2.2



COMPARISON OF CSA IN PLASMA AND SERUM AFTER FREEZING

Pts 1&7 – samples contaminated
Pt 16 – No CSA
5637CM – 26 GM-CFUc

FIG 3,2.3



COMPARISON CSA IN FRESH PLASMA AND SERUM - SECOND EXPERIMENT

NO COLONIES GROWN IN EDTA PLASMA FROM ANY PATIENT

5637cm - 16 GM-CFUc

separation from the clot at a concentration of 14.3 U/ml (as in Vacutainer bottles). The pH of these samples was compared with that of heparin-free serum and with heparin-plasma, which had been collected at the same time, using narrow range reagent strips (BDH). There was no significant difference between the pH of the serum, heparinised plasma or serum to which heparin had been added. In each patient the pH of all three samples were the same. The mean pH of all samples was 7.5.

Aliquots of these samples were stored at either +4 or -20°C for 24 hours before assaying for CSA and for G-CSF and GM-CSF using ELISA (Chapters 2,2.7 & 5,1/2).

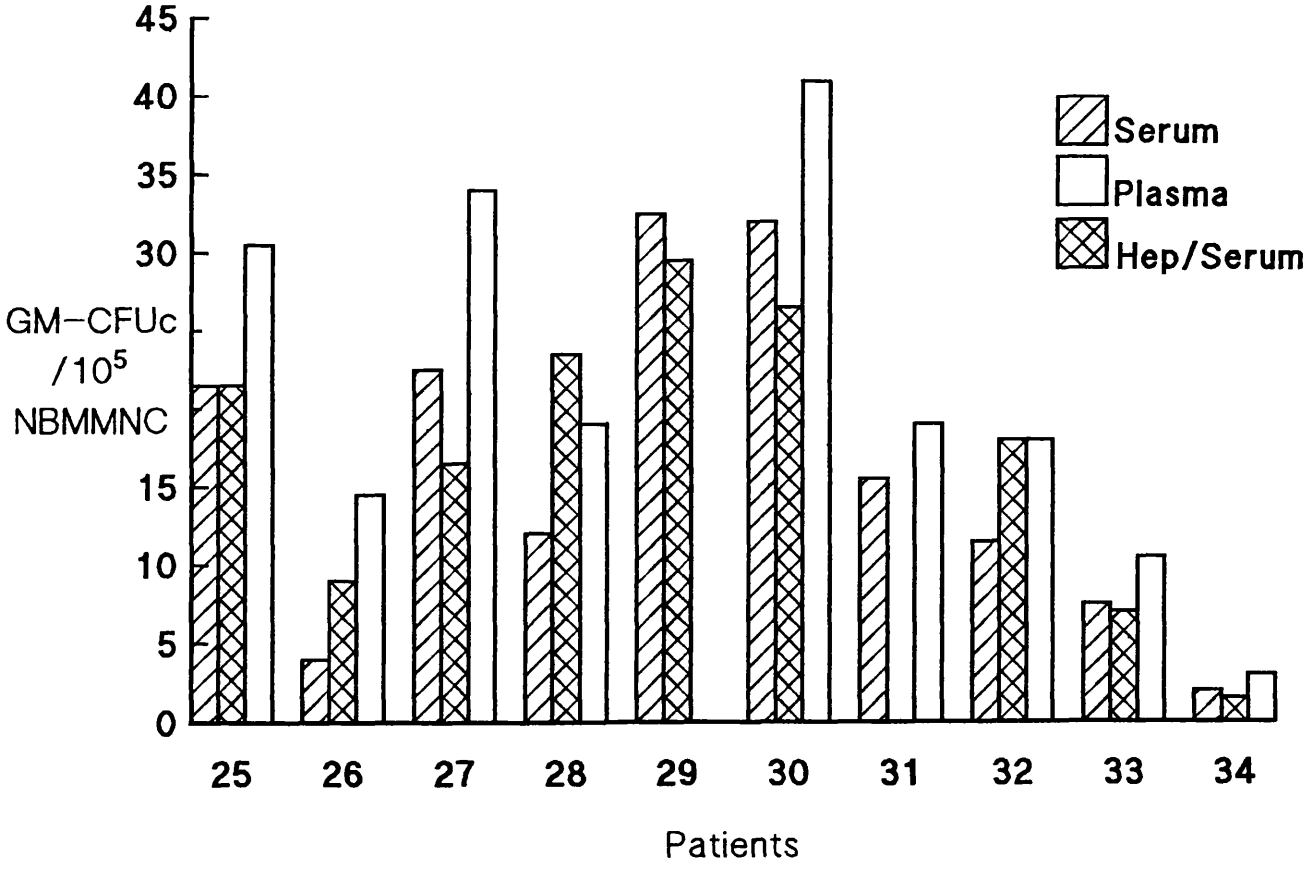
Fig 3,2.4/5 page 112/3

Heparin failed to produce any consistent effect on CSA when added to serum. Paired samples of serum and heparin-serum from 9 patients were evaluable in which there was no change in CSA in 4, an increase in 3 and a decrease in 2. CSA was greater in plasma (H) than in serum from 9/9 patients and this difference was maintained in 6/9 samples that had been frozen and thawed (Fig 3,2.5).

The amounts of G-CSF and GM-CSF in plasma and serum were measured using ELISA assays (Chapter 5,2). There was no detectable GM-CSF in any sample, whereas, low levels of G-CSF were detected in all the samples. However, there was no significant difference between the G-CSF content of plasma (mean(range) 368 (180-650) pg/ml) and serum (352 (160-755) pg/ml) (Students' paired *t*-test - 2 tailed $p=0.7$).

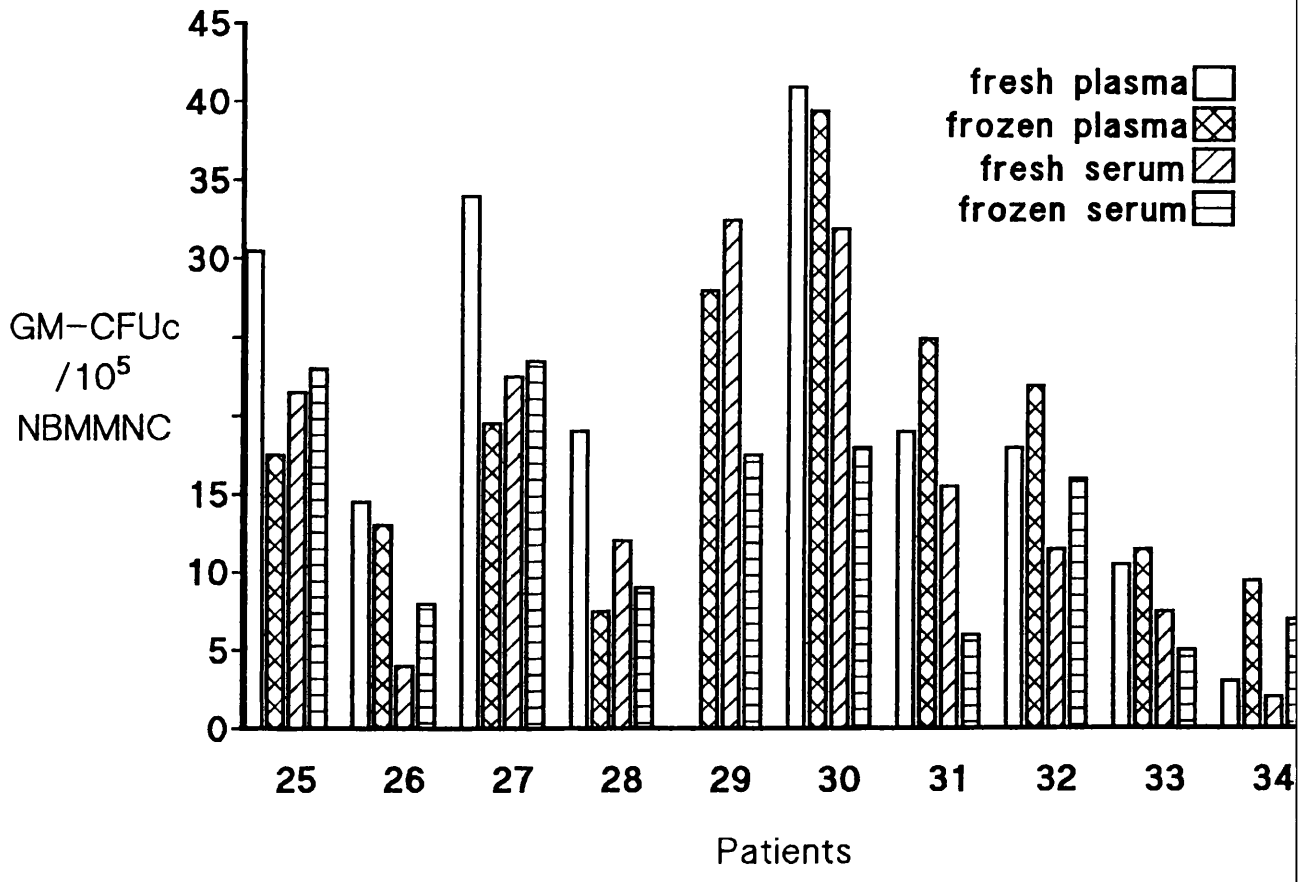
In the samples collected from normal donors there was more CSA in plasma than in serum in all the patients.

FIG 3,2.4



Comparison of CSA in fresh plasma, serum and heparinised serum

FIG 3,2.5



CSA of fresh and frozen plasma and serum tested against the same normal BMMNC

5637cm – 80 cols

DISCUSSION

CSA in plasma was greater than in serum when tested as soon as possible after collection and the difference was less when samples were compared after a cycle of freezing, storage and thawing. This observation was made in patients with MM and in normal donors. The CSA of serum has been shown previously to be stable over an extended period of storage at -20^{0}C [Entringer et al, 1980] but in these experiments the data did not indicate whether freezing and thawing resulted in a loss of activity in plasma or an increase in the CSA of serum.

The difference between plasma and serum tested without freezing and thawing could not be attributed to differences in concentrations of G-CSF or GM-CSF or pH and the difference between plasma and serum was seen using progenitor cells of each donor marrow that was used, suggesting that the difference observed was not due to an effect mediated by a particular BMMNC population.

The addition of heparin to serum did not increase colony numbers, but addition of heparin to whole blood may have a different effect. Plasma collected with EDTA rather than heparin did not support the growth of GM-CFUc, possibly because of a reduction in calcium ions. Thus, this possibility is unresolved.

Alternatively, the difference in CSA between plasma and serum may be related to the different levels of activity of proteins involved in haemostasis. In serum, enzymes of fibrinolysis may inhibit factors which promote haemopoiesis or activate inhibitors of haemopoiesis which remain inactive in plasma. One such molecule is transforming growth factor-β_1 (TGF-β_1) which inhibits the proliferation of early myeloid progenitors by inhibiting GM-CSF and IL3 [Keller et al, 1989], whilst potentiating the effect of G-CSF in chronic myeloid leukaemic cells [Mahendra et al, 1993] and normal myeloid progenitors

(Dr B.C.Millar - personal communication).

In-vivo TGF-β is bound in an inactive form to α2-macroglobulin (α2-M) [O'Connor-McCourt and Wakefield, 1987, Huang et al, 1988] and is activated *in-vitro* by acidification or heat [Roberts and Sporn, 1988] and by the action of enzymes such as plasmin [Lyons et al, 1990] Another source of inactive TGF-β is within platelets and this is released and activated by exposure to thrombin *in-vitro* [Assoian & Sporn, 1986].

Other mediators of haemopoiesis that may have different activities in serum and plasma include interleukins 1 and 6 (IL-1, IL-6) which are important co-factors in myelopoiesis and which may influence the response of progenitor cells to IL3, GM-CSF and G-CSF [Schaafsma et al, 1989, Montes-Borinaga et al, 1990, Moore, 1991]. Heparin inhibits the generation of IL1 from lipopolysacharide stimulated macrophages [Jones & Geczy, 1990], whereas, thrombin and factor Xa enhance IL-1 activity and release in the same system.

IL6 is released rapidly from PBMNC after collection of blood samples [Bell et al, 1991]. The production of IL-6 in blood samples, and also IL-1 and tumour necrosis factor (TNF), have been shown to be associated with the presence of endotoxin in the tubes used for collection [Riches et al, 1992, Riches & Gooding, 1990]. However, differences in IL6 concentration, by RIA, between serum and plasma samples collected simultaneously and separated from PBMNC within 1-2 hours were not found (Chapter 5,5) in this study.

Because of the complex interactions between mediators of haemopoiesis that are likely to occur, because of clotting ,fibrinolysis, or the presence of endotoxin, and which may vary in samples with time after collection, blood sample collection and processing was standardised for later experiments.

3,3 TITRATION OF PLASMA CSA

The effect of plasma concentration on the number of colonies produced was investigated to determine whether there was an optimal concentration of plasma to use in assays of CSA.

Plasma at concentrations between 50μl and 500μl was added to Petri dishes and the volume maintained at 500μl by the addition of single strength α-medium. BMMNC were added in a further volume of 500μl containing 0.5% agar (final concentration) as described in chapter 2,2.3.b. Plasma was pooled from samples collected from 4 patients after HDM & ABMR at the times indicated (Table 3,3.). Dose response curves for each pooled sample were constructed to determine the effect of plasma concentration on colony numbers at different times after ABMR.

Table 3,3

POOLED SAMPLES OF PLASMA

POOLED SAMPLE	from	DAYS (after ABMR)
0		0
1		1-3
2		4-6
3		7-9
4		10-12
5		13-15
6		16-18
7		19-21
8		22-24
9		35-27
10		28-31

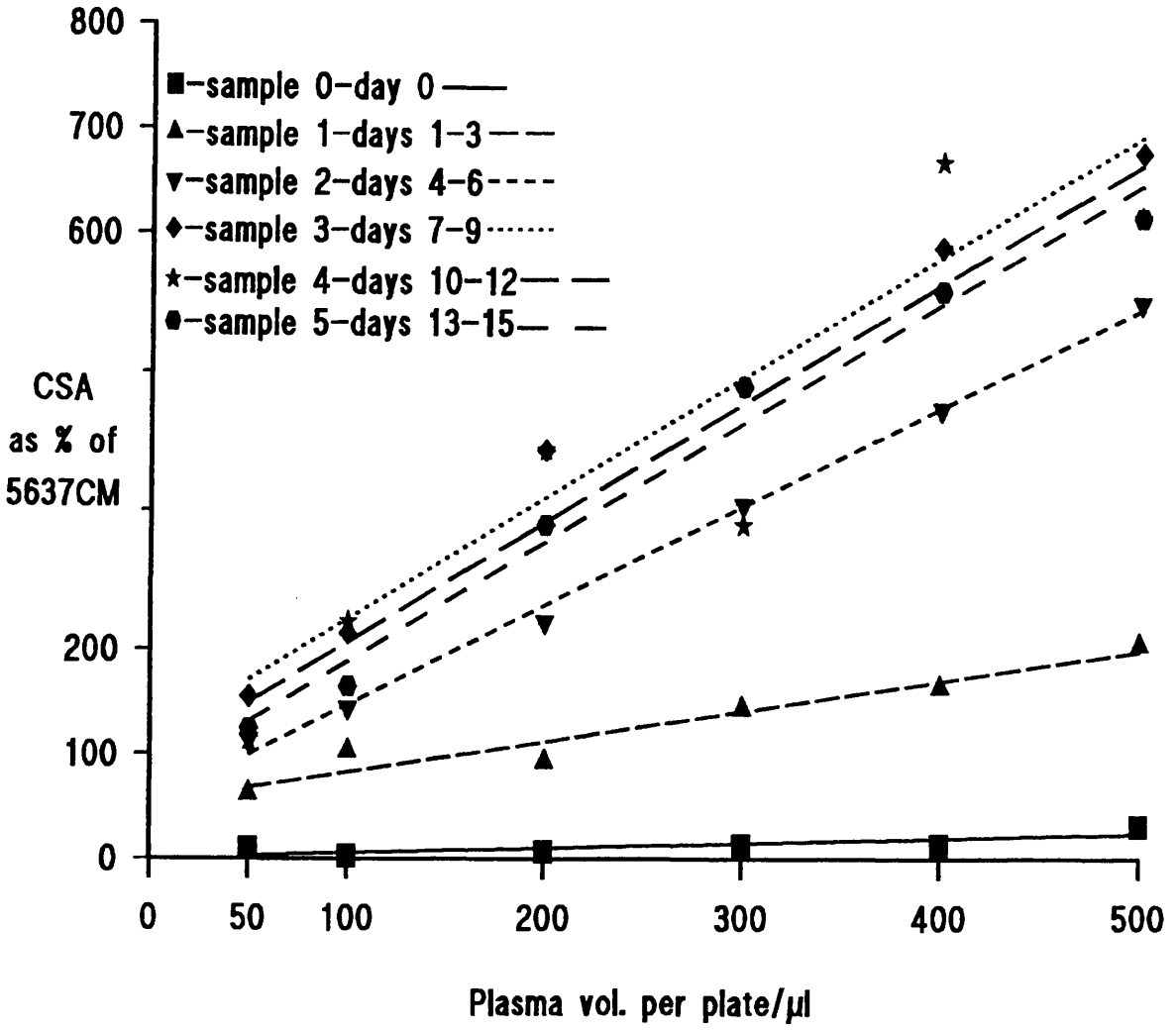
FIGS 3.3.1/2 page 118/9

For each pooled sample of plasma the number of colonies was proportional to the volume of plasma used. The relationship between the number of colonies and the plasma concentration was linear.

DISCUSSION

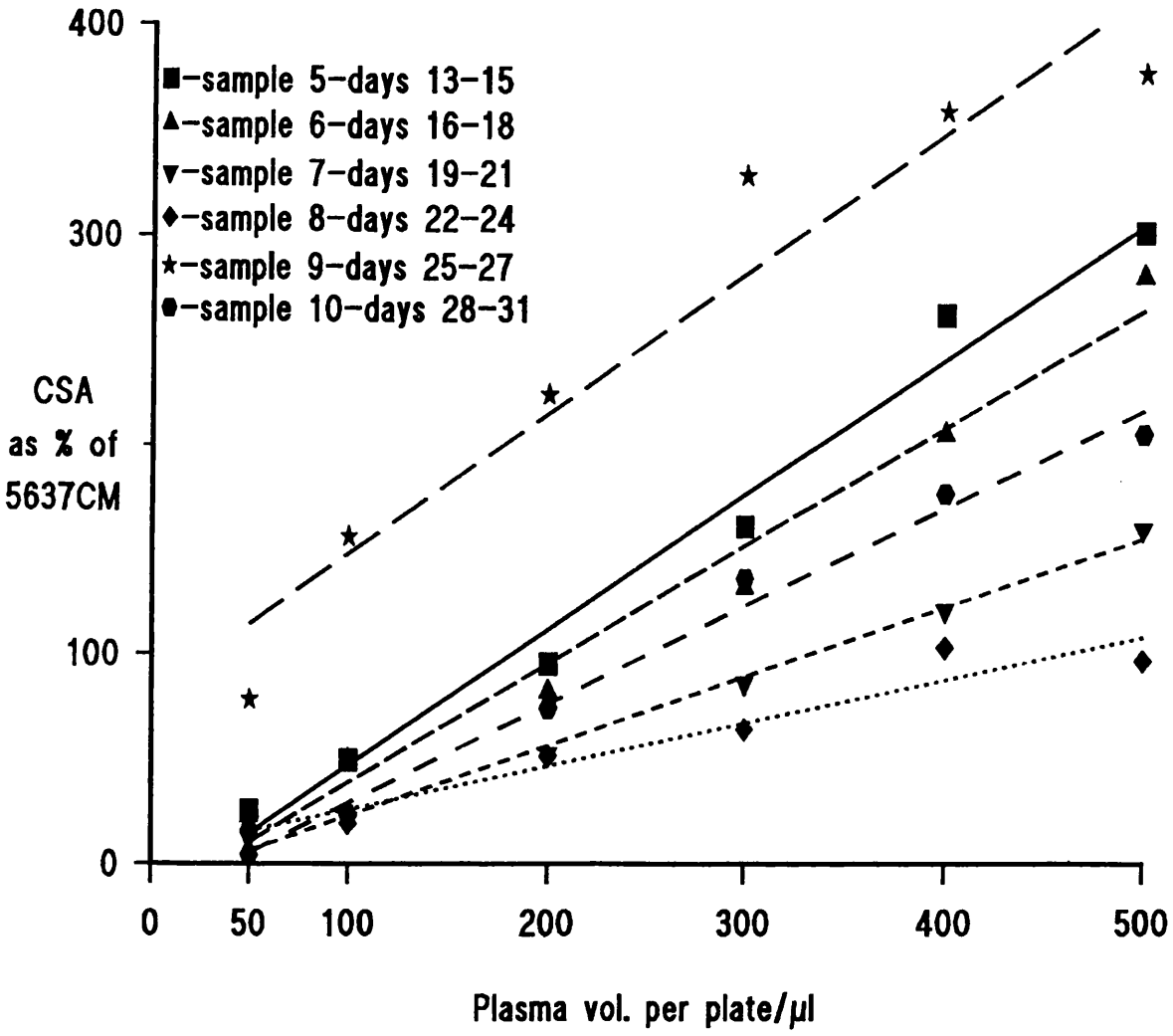
Because the number of colonies which grew as a function of plasma concentration was linear the amount of plasma was reduced to 300µl in subsequent experiments. This increased the number of tests which could be done using individual samples from each patient. Plasma was incorporated into an underlay and MNC added in soft agar to an overlay as described in chapter 2,2.3.b.ii.

FIG 3.3,1



Titration curves of plasma vol per plate against CSA for pooled plasma samples 1-5 tested against NBM (T)

FIG 3,3.2



Titration curves of plasma vol per plate against CSA for pooled plasma samples 5-10 tested against NBM (J)

CHAPTER 4

CSA BEFORE AND AFTER HIGH DOSE CHEMOTHERAPY

In a previous study [Millar et al, 1990] CSA was detected in the serum of patients with MM and persisted for up to 2 years after chemotherapy. In patients who had CSA in their serum no attempts were made to correlate this activity with: i) previous chemotherapy; ii) disease activity; iii) persistent deficits in mature circulating haemopoietic cells; or iv) proliferative potential of endogenous haemopoietic progenitor cells.

Experiments were designed to determine whether CSA is produced in response to high dose chemotherapy, whether its production is altered by the use of ABMR or recombinant G-CSF and whether the duration of elevated CSA is related to haemopoietic recovery.

4,1 PRIMING

Patients with MM receive a dose of cyclophosphamide (400mg/m^2) 5-9 days before HDM. Previous work has shown that this sequence of treatment, referred to as "priming" is associated with a more rapid haematological recovery [Millar et al, 1975] and reduced toxicity to the gastrointestinal tract (Millar et al, 1978] in animals. These observations have been confirmed in man, in patients with malignant melanoma who had accelerated recovery of peripheral leucocytes [Hedley et al, 1978], and in patients with various malignancies who had a reduction in gastrointestinal toxicity [Selby et al, 1987],

when HDM was preceded by a smaller dose of cyclophosphamide. The mechanism(s) of these effects is not known but may be mediated by changes in the number of stem cells in cycle [Millar & McElwain, 1985] which result in an accelerated recovery phase without entirely protecting the stem cells from the effect of subsequent exposure to intensive chemotherapy or irradiation. In patients with MM who received a "priming" dose of cyclophosphamide before HDM & ABMR the numbers of GM-CFUc were increased in their bone marrow 7 days later [Maitland et al, 1990].

In order to examine the effect of this "priming" dose of cyclophosphamide on the numbers of PB GM-CFUc and on CSA, peripheral blood and bone marrow samples were collected from patients before "priming" and then at the time of bone marrow harvest, on the day of treatment with HDM (Chapter 2,3.2.d/4,2).

Table 4,1.A, (page 123) shows that in 19 patients data were available for comparison of BM GM-CFUc before and after "priming". In 10/19 patients there was an increase in BM GM-CFUc after this treatment.

Among 13 patients for whom samples were evaluable before and after cyclophosphamide, colony forming progenitors were detected in peripheral blood before priming (6-8 weeks after treatment with induction chemotherapy regimens). PB GM-CFUc persisted after cyclophosphamide although the number of progenitors decreased in 9/13 patients (6,8,9,13,14,18,20,21,22) ($p<0.009$ - Wilcoxon's signed rank test). Amongst patients whose PB GM-CFUc decreased after priming with cyclophosphamide, the level of CSA also decreased in 5/9 patients.

Overall, CSA was detected in 13/14 patients before cyclophosphamide, and in 11/14 afterwards. CSA correlated with numbers of BM GM-CFUc in individual patients

bone marrow before priming (Pearson r= 0.650, p=0.012) but not after this treatment. CSA decreased in 10/14 patients after cyclophosphamide (1 tail p<0.004 - Wilcoxon's signed rank test).

Although one patient (22) failed to produce CSA before or after cyclophosphamide he still demonstrated an increase in bone marrow progenitors after priming and had circulating GM-CFUc before and after this treatment.

Although there was no correlation between numbers of PB GM-CFUc and BM GM-CFUc in individual patients measured either before or after treatment with a priming dose of cyclophosphamide, the numbers of circulating progenitors before priming correlated with the number of GM-CFUc grown from bone marrow on day 0, 5-9 days after priming (Pearson's r=0.758, p<0.005) and with the total number of GM-CFUc (GM/kg body weight (Table 4,3.B)) harvested (r=0.802, p= <0.0004). This relationship is shown in Figure 4,1.1.

TABLE 4,1.A

Changes in CSA and GM-CFUc of Bone Marrow and Peripheral Blood Pre and Post Priming with Cyclophosphamide

Pt	BM-CFUc Pre	BM-CFUc Post	PB-CFUc Pre	PB-CFUc Post	CSA Pre	CSA Post
1	-	165	-	1.3	-	7
2	21	55	-	21	-	-
3	154	0	3	7	-	4
4	12	97	-	4	-	60
5	152	33	-	6	-	69
6	48	42	6	0.3	65	25
7	90	30	2	3	103	6
8	6	65	11	3	15	18
9	71	36	2.3	1	9	0
10	23	29	0.3	0.6	37	0
11	105	99	-	19	75	25
12	46	61	-	1	50	40
13	128	217	92	22	200	118
14	50	-	36	6	75	101
15	-	76	-	12	-	7
16	23	67	-	1	-	113
17	-	23	-	12	-	40
18	71	66	7.7	3.3	90	35
19	59	40	3	3.7	85	55
20	83	106	109	51	10	10
21	75	112	8.5	6.7	19	6
22	20	60	7	3	0	0
23	107	166	-	2.7	-	2

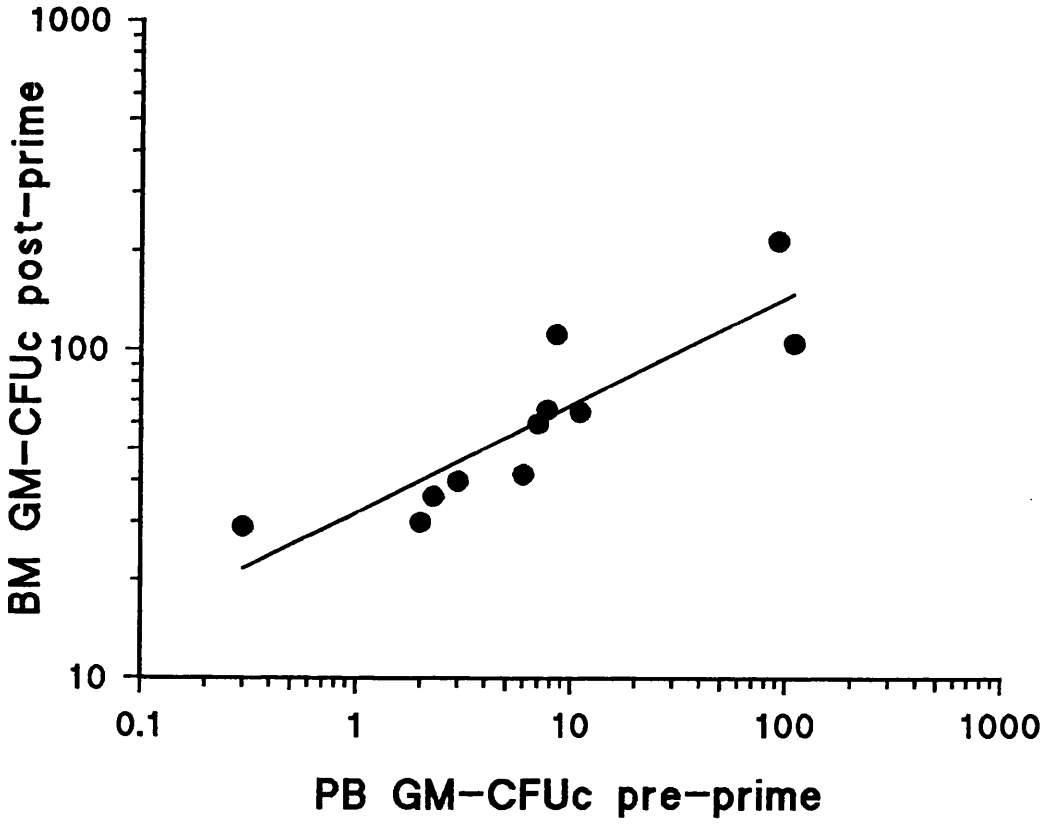
Pre/Post -Before/After priming with cyclophosphamide

BM/PB CFUc -Colony numbers per $2/5x10^6$ Bone Marrow/Peripheral Blood Mononuclear cells

CSA -Colony Stimulating Activity as % of 100µl 5637cm (see text)

Pt -Patient

Fig 4,1.1



Correlation between numbers of peripheral blood GM-CFUc before priming with cyclophosphamide and numbers of bone marrow GM-CFUc after priming

PB GM-CFUc - per $5x10^5$ PB mononuclear cells

BM GM-CFUc - per 10^5 BM mononuclear cells

DISCUSSION

The number of clonogenic PB cells was reduced 5-9 days after patients had received a "priming" dose of cyclophosphamide even though there was an increase in GM-CFUc in 10/19 BM aspirates taken at the same times. In 6/10 of these patients data were available for PB progenitors before and after priming (patients 8,10,13,20,21,22) and in 5/6 the was a concomitant decrease in the numbers of PB GM-CFUc. The exception is patient 10 in whom there were few circulating progenitors before or after treatment. The decrease in PB-GM-CFUc probably reflects the direct cytotoxic effect of cyclophosphamide, whereas the increase in BM GM-CFUc suggests recruitment of GM-CFUc from the stem cell pool following the cytotoxic insult. This suggests that recovery of progenitor cells in the bone marrow precedes that in the peripheral circulation by at least 5-9 days. When cyclophosphamide is used to stimulate the production of peripheral blood stem cells for autografting [Tarella et al, 1991] PB CFUc rise substantially 11-16 days after treatment. However, in the present study, patients received high dose melphalan 5-9 days after cyclophosphamide, thus, the data do not indicate whether the priming dose of 500mg is adequate to produce an increase in circulating progenitors since the ablative dose of melphalan was given before such a rise would have been predicted. Following HDM & ABMR (Chapters 4,3) the mean time to the re-emergence of PB GM-CFUc for these patients (Chapter 4,2) was 16 days (range 12-21 days). This interval is similar to that seen by Tarella et al (1991) and to that found in a study of circulating progenitors after ABMR and ABMT in 7 leukaemic patients [Kwong et al, 1989] and, following induction therapy for acute leukaemia [To et al, 1984] .

The observation that levels of PB GM-CFUc before priming can predict the

number of GM-CFUc that can be harvested after priming suggests that levels of PB GM-CFUc may reflect either proliferative activity in bone marrow or the proliferative competence of bone marrow stem cells. This measurement may be useful to predict which patients will obtain useful bone marrow harvests and might also be applicable to collection of peripheral blood stem cells.

Since normal donors have few if any circulating GM-CFUc, the presence of circulating progenitors prior to treatment with cyclophosphamide suggests that there was considerable activity within the bone marrow despite the fact that patients had not received any chemotherapy for 6-8 weeks. This suggests that after repeated doses of cytotoxic chemotherapy the proliferative activity that results from trauma to the haemopoietic system takes months to overcome and persists beyond the time taken to recover normal haematological indices. A similar observation was made by Millar et al (1990).

Intensive therapy induces severe haemopoietic toxicity associated with a rise in CSA (Chapters 4,2-4). In contrast, the dose of cyclophosphamide used for "priming" resulted in a decrease in CSA (in some patients) associated with an increase in proliferative activity in the BM, suggesting an inverse relationship between proliferative activity and CSA. Before "priming" there was a correlation between CSA and BM GM-CFUc measured in individual patients but this relationship was not apparent after treatment with cyclophosphamide when BM CFUc increased and CSA fell. These data suggest that although levels of CSA and proliferative activity in the BM may be interrelated, chemotherapy may disrupt this relationship or reset the mechanism(s) that control(s) it, depending on the severity of the toxic insult.

4,2 CSA AFTER HDM WITHOUT ABMR

The time course of CSA in 7 patients who received HDM (140mg/m^2) without ABMR was examined. These patients are a poorer prognostic group, compared to those that received HDM & ABMR (Chapter 4,3), because induction therapy has not reduced their bone marrow infiltration by MM sufficiently for bone marrow harvesting. They have relatively resistant MM and greater impairment of bone marrow function, because of tumour Infiltration and previous chemotherapy. The previous histories of this group of patients are summarised in table 4,2.A., below.

3/7 of these patients (Pts 25,27 & 29) died without recovering their peripheral counts. One patient (28) had delayed haematological reconstitution associated with a fungal osteomyelitis of the mandible and received prolonged treatment with intravenous amphotericin. Another patient (26) had a prolonged hospitalisation because of a mediastinitis related to perforation of the oesophagus, but nevertheless, engrafted and mounted a good granulocyte response to his infection. The remaining two patients (24 & 30) had relatively less toxicity and recovered without life-threatening events.

Samples of peripheral blood were collected 3 times weekly after HDM for measurements of CSA and PB GM-CFUc. Peripheral white cell counts (WCC) were performed daily. The data for each patient are shown in Appendix II, Figs 4,2.1-7, Appendix II and Table 4,2.B, below. An example, Figure 4,2.1, is shown on page 129

Data from all 7 patients were evaluable before and after HDM. All patients developed significant increases in CSA after HDM (150-600% of 5637CM) which reached a maximum 6-11 days after HDM in 5/7 patients. CSA peaked on day 19 in patient 30 (Fig 4,2.7), but this was not associated with a relative delay in engraftment, and in patient

TABLE 4,2.A DETAILS OF PATIENTS WHO RECIEVED HDM ALONE

Pt	Age/sex	Isotype	Disease Status	Previous Treatment
24	47/M	IgGl	PR	VC-VAMP x 5
25	50/F	IgGk	<PR	VC-VAMP x 4
26	38/M	IgGk	<PR	C-VAMP x 5
27	60/M	IgGk	<PR	VC-VAMP x 4 (<PR) EPIC X 2 (NR)
28	51/M	IgGk	RD	C-VAMP x 2 (NR) EPIC x 2 (NR)
29	52/F	IgGk	<PR	C-VAMP x 4 (<PR) EPIC x 2 (NR)
30	33/F	IgAk	RD	C-VAMP x 3

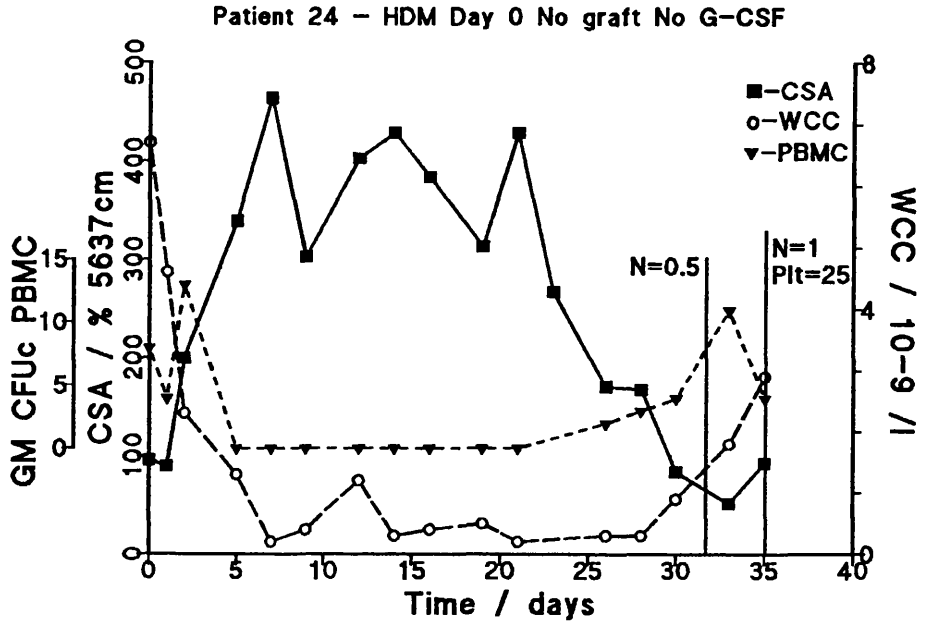
< -Less than
NR -No response
RD -Resistant disease
EPIC -Etoposide, Prednisolone, Ifosfamide, Cis-Platin
(Salvage chemotherapy regimen for resistant patients)
Remainder of key - See Table 3,2.A

TABLE 4,2.B RECOVERY TIMES FOR PATIENTS TREATED WITH HDM WITHOUT ABMR

Pt	Max CSA	N=0.5	N=1	Plt=25	PB-CFUc
24	7	32	35	35	26
25	11	D	D	D	D
26	6	27	29	27	20
27	10	39	D	D	39
28	8	55	62	65	55
29	D	D	D	D	D
30	19	27	29	27	23

Max CSA -Time taken to reach maximum levels of Colony Stimulating Activity
N=0.5 -Time to achieve neutriphils of $0.5x10^9/l$
N=1 -Time to achieve neutriphils of $1.0x10^9/l$
Plt=25 -Time to achieve platelets of $25x10^9/l$
PB-CFUc -Time to first reappearance of circulating GM-CFUc
D -Patient died before recovery of this variable
Times are in days after treatment with high dose melphalan (Day 0)

FIG 4.2.1



For this and subsequent figures:

GM CFUc PBMC = Colony numbers per $5x10^5$ PBMNC
– collected from the same blood sample as the plasma tested for CSA

CSA / % 5637cm = activity of 300 or 500µl of test plasma
– colony numbers are compared with those obtained from the same
– normal BMMNC stimulated by 100µl of medium conditioned by
– the 5637 bladder carcinoma cell line

WCC = Total peripheral white cell count x 10^9/litre

N=0.5 = day on which total neutrophil count reached and remained at $0.5x10^9$/l

N=1 = day on which total neutrophil count reached and remained at $1x10^9$/l

Plt=25 = day from which platelet numbers were maintained at $25x10^9$/l
– without support by platelet transfusions

29 CSA was still increasing at day 31 when she died without engrafting.

In the patients who survived, CSA was maintained until a rise in WCC occurred at which point CSA decreased. In 4/6 patients small unsustained rises in WCC were detected at earlier times after HDM (e.g. pt 24, day 9-13, Fig 4,2.1) but these were not associated with a subsequent rise in neutrophils (indicating engraftment) or a fall in CSA. The eventual increase in neutrophils was generally preceded by a sustained rise in lymphocytes and monocytes.

Re-emergence of circulating GM-CFUc occurred before or at the same time as the establishment of a neutrophil count of $0.5x10^9/l$.

DISCUSSION

CSA increased after HDM in conjunction with the fall in total WCC and reached a maximum approximately 8 days after chemotherapy. The duration of elevated levels of CSA showed an inverse relationship with total WCC. Prolonged periods of leucopenia were associated with prolongation of the period of elevated CSA.

Re-appearance of circulating GM-CFUc occurred shortly after the first elevations of total WCC but before establishment of circulating neutrophils of greater than $0.5x10^9/l$. It was not possible to correlate the timing of either the rise or fall of CSA with the recovery of PB GM-CFUc.

PB GM-CFUc were not grown after HDM in patients who failed to engraft and/or who did not show any evidence of recovery of neutrophils. In patient 29 (Fig 4,2.6) CSA increased until death from pulmonary Aspergillosis and Pseudomonas septicaemia. It is possible that factors associated with the acute phase response such as IL-1 and IL-6 may have contributed to this activity (Chapter 5,5). In contrast, in patient 25 (Fig 4,2.2), CSA decreased before death, which was also thought to be due to sepsis although no

organisms were isolated.

The possibility that drugs administered to the patients might have affected the pattern of CSA was also considered. Changes in CSA could not be attributed to any single drugs' administration or to administration of any of the routinely used combinations of drugs (which most patients received) including antibiotic/antifungal combinations with known potential haemopoietic toxicity.

Despite the fact that these patients had relatively resistant disease and greater bone marrow infiltration by myeloma, the pattern of CSA after HDM was similar to that seen after HDM & ABMR (Chapter 4,3), in patients who had had a better response to induction treatment with VAMP. However, as a group the 7 patients treated with HDM alone experienced considerably greater toxicity and more infective complications. This can be attributed to the longer period of neutropenia that is recognised in patients who do not receive an autograft and also to the persistence of impairment of humoural immunity due to the activity of the patients myeloma.

4,3 THE EFFECT OF ABMR AND rhG-CSF

Most patients with MM treated with C-VAMP or VC-VAMP obtain adequate bone marrow remissions to allow consolidation treatment with 200mg/m^2 of melphalan followed by ABMR (Chapter 2,3.2.d). Only patients who received ABMR were eligible for entry into the clinical studies of rhG-CSF.

In order to provide as large a group as possible for analysis, the results of all patients who received HDM & ABMR with or without rhG-CSF are described together to provide comparison with the data from patients treated with HDM alone. These results are discussed (chapter 4,3.1) and then sub-groups compared in chapter 4,3.2 to provide data on the effect of rhG-CSF.

4,3.1 THE EFFECT OF ABMR

23 patients were recruited to this study. 22 received 200mg/m^2 of melphalan and one received 140mg (pt 2 because of a poor harvest). All received an ABMR and 12/23 patients received rhG-CSF (Chapter 2,3.2.f) after ABMR. The previous histories of the patients are shown in Table 4,3.A. below.

22/23 patients survived the post transplant period. The numbers of BM GM-CFUc infused per Kg (from the bone marrow harvest) varied from 0.02 - 4.5x10^5/kg, median = 1.24x10^5/kg (Table 4,3.B below).

TABLE 4,3.A **DETAILS OF PATIEBTS WHO RECIEVED HDM & ABMR**

Pt	A/S	I	D.S.	Previous Treatment	G-CSF
1	58/M	IgAl	PR	VAMP x 5	0
2	48/F	IgGk	<PR	M/P x 4, VC-VAMP x 4	0
3	43/M	IgGl	PR	VC-VAMP x 4	0
4	50/F	BJk	PR	C-VAMP x 4	0
5	61M	IgGk	PR	VC-VAMP x 4	0
6	43/M	NS	CR	HDM/ABMR → (CR) C-VAMP x 4	0
7	38/M	IgGk	PR	VC-VAMP x 6	0
8	34/M	IgGk	CR	ABCM x 4/12, C-VAMP x4	0
9	45/F	IgGl	PR	VAMP x 6, C-VAMP x 3	0
10	63/M	IgGk	PR	VAMP x3(PR), VAMP x8 → HDM/ABMR → (CR) C-VAMP x 5	0
11	52/F	IgGk	PR	C-VAMP x 4	0
12	59/M	IgGl	PR	VC-VAMP x 6	2
13	45/M	IgGk	PR	C-VAMP x 5	2
14	47/M	IgGk	PR	VAMP x4/HDM/ABMR →(PR) PD → C-VAMP x 5	20
15	43/M	IgAk	CR	VAD x 6	10
16	45/F	IgAk	CR	HDC x2 ,HDM/ABMR →(CR) C-VAMP x 2	5
17	49/F	IgGl	CR	C-VAMP x 4	2
18	40/M	IgGk	PR	C-VAMP x 4	5
19	45/M	NS	CR	C-VAMP x 4	5
20	45/M	IgGk	PR	C-VAMP x 5	5
21	46/M	IgGl	PR	C-VAMP x 8	5
22	36/M	IgGk	PR	ABCM x6/12, C-Wkly x20 VAMP x 5	5
23	48/F	IgAl	CR	VAMP x 6	5

G-CSF - Dose of G-CSF given after ABMR (µg/kg/day)
NS - Non-secretor Remainder of Key- See Table 3,2.A

TABLE 4,3.B RECOVERY TIMES IN DAYS AFTER HDM & ABMR AND NUMBERS OF INFUSED GM-CFUc IN ABMR

Pt	PB CFUc	N 0.5	N 0.1	Plt 25	L 0.5	L 1.0	GM-CFUc/Kg $x10^5$	Max CSA
1	13	17	21	23	14	16	4.59	8
2	15	19	28	25	15	26	0.82	N/a
3	15	14	24	24	13	14	0.02	13
4	14	18	23	23	15	16	2.0	9
5	16	17	20	37	16	17	0.66	9
6	12	30	33	40	16	27	N/a	10
7	15	20	Dis	18	15	17	0.54	13
8	23	22	30	27	15	22	1.21	14
9	14	17	20	49	16	18	0.82	14
10	36	41	56	63	19	19	0.67	10
11	16	22	Dis	22	13	20	2.07	9
12	18	17	25	36	15	16	1.16	9
13	12	14	15	19	12	14	4.23	7
14	17	21	24	45	11	20	N/a	8
15	12	14	16	26	13	14	1.82	7
16	19	D	D	D	D	D	N/a	9
17	19	17	18	24	16	17	0.46	9
18	14	12	14	24	12	13	1.27	7
19	21	12	16	28	10	14	0.94	7
20	13	15	17	17	14	15	2.54	9
21	19	13	15	22	11	14	2.35	7
22	14	24	Dis	20	14	23	1.27	9
23	12	10	13	20	9	13	3.02	7

GM-CFUc/Kg - GM-CFUc per Kg body weight
N/a - Not available
L - Total leucocyte count ($0.5/1x10^9/l$)
Dis - Discharged before achieving the count
Max CSA - Time in days to maximum CSA
Remainder - See Table 4,2.B

To determine the effect of ABMR and rhG-CSF on CSA, samples were collected as described previously (Chapter 4,2). Examples of data from individual patients showing changes in CSA, PB GM-CFUc and total WCC are shown on page 136. Data for all patients are included in Appendix II (Figs 4,3,1-23) and in Table 4,3.B.

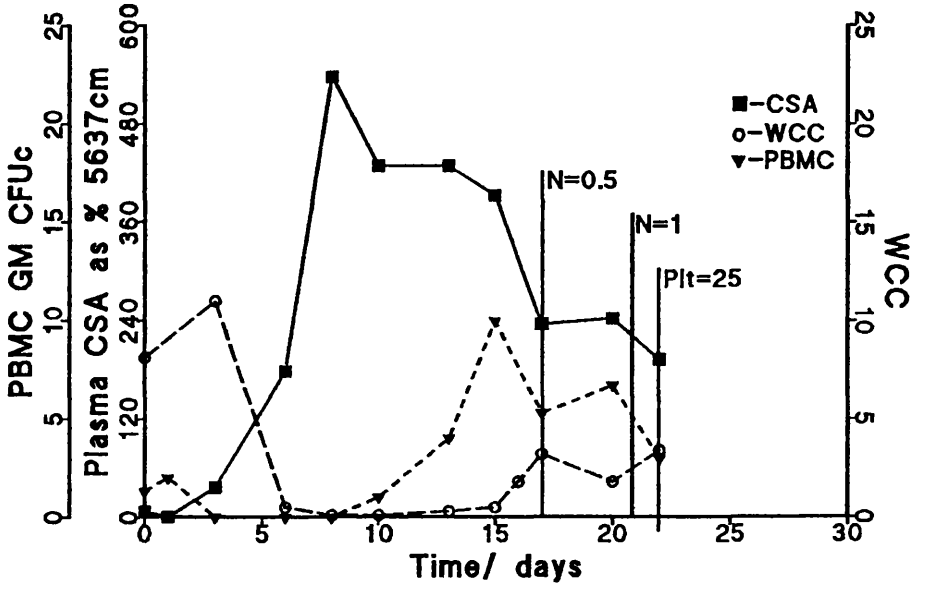
Pattern of CSA after HDM & ABMR

In most patients the time course of CSA with time was similar to that seen in patients who received HDM alone. During the first two days after HDM, CSA was low ($\leq$100% of 5637cm) and increased from day 3-4 with maximum levels of CSA (200-600% of 5637CM) being attained 7-9 days after HDM in most patients. The increase in CSA occurred in association with the fall in total WCC, the nadir of which was reached between days 5-8 in all patients. High levels of CSA were maintained, generally, for one to two weeks until a decrease occurred at the same time as a rise in circulating leucocytes (e.g. Fig 4,3.1). In 2 patients there was a steady increase in CSA until a rise in WCC occurred at which point CSA fell (Figs 4,3.7/23) and in 2 further patients there was a second peak of activity 1-2 weeks after the first peak (Figs 4,3.8/12).

Neither the time course nor the amounts of CSA could be predicted from any of the pre-HDM variables such as the clonogenicity of either the BM or PB before or after "priming", CSA levels at these times, disease status, age or previous treatment.

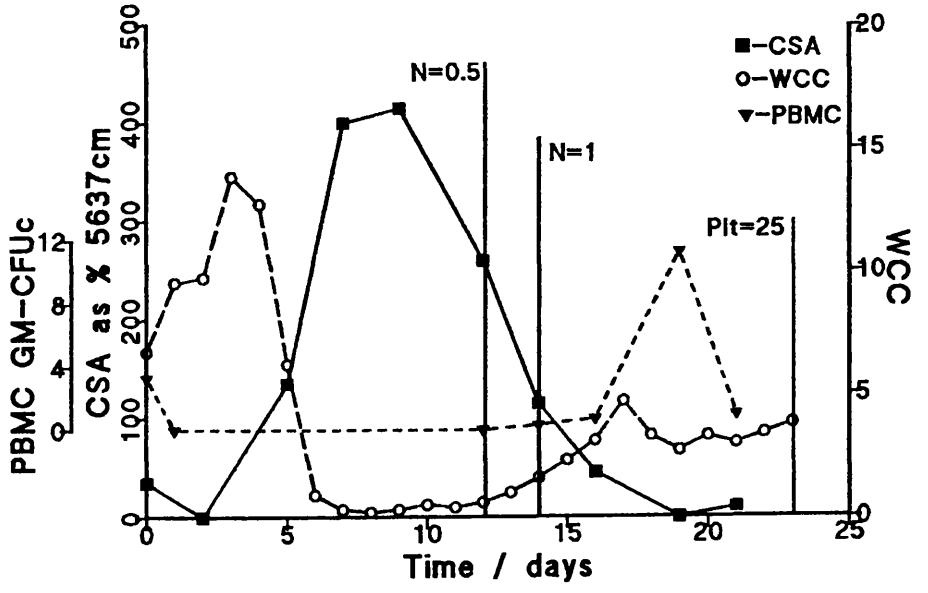
There was an inverse relationship for each patient between the curves of CSA/time and total WCC/time although the shapes of the curves of CSA Vs time varied between patients. Patients whose CSA took longer to reach maximal levels tended to take longer to recover their WCC to $0.5x10^9$/l. The marker events in neutrophil recovery (N=$0.5x10^9$/l and $1x10^9$/l) occurred after comparable rises in total WCC and after falls in CSA in most patients. It is possible that measurement of neutrophils at these levels may

FIG 4,3.1



Patient 1 - HDM & ABMR Day 0 - No G-CSF

FIG 4,3.18



Pt 18 - HDM & ABMR Day 0 + G-CSF 5μg

have been too late in the course of neutrophil recovery in terms of possible feed-back control mechanisms but accurate measurements of neutrophil numbers of less than this were not available.

Although there was an inverse relationship between CSA/time and WCC/time, the data do not prove that a low WCC is directly responsible for changes in CSA.

Recovery of circulating GM-CFUc

Peripheral blood GM-CFUc were grown from all patients at the time of bone marrow harvest, before HDM. PB GM-CFUc were grown again from 22 patients after HDM but not until some degree of WCC recovery had occurred. In 14/22 patients (64%) the first day that GM-CFUc could be recovered from the circulation after HDM & ABMR preceded the recovery in neutrophil count to $0.5x10^9/l$. In 8 patients PB GM-CFUc recovered after this stage of neutrophil recovery. 6/8 of these patients had received rhG-CSF.

The time at which PB GM-CFUc were first measurable after HDM & ABMR was not related to the time at which CSA reached a maximum level, but in common with neutrophil recovery, delays in recovery of circulating PB GM-CFUc appeared to be associated with prolongation of elevated levels of CSA.

Delays in engraftment

Recovery times to neutrophils of $0.5x10^9/l$ and $1x10^9/l$ and platelets of $25x10^9/l$ varied across the following ranges: N=0.5 - 12-41 days; N=1.0 - 14-56 days; Plt=25 - 17-63. Within this group of patients the numbers of GM-CFUc infused did not determine the time taken for haematological recovery (Table 4,3.B), however, a history of previous HDM and the age of patients both affected the recovery times of neutrophils and/or

platelets. In patients of $\geq$60 years of age the median time to attainment of platelets of $25x10^9/l$ was 36.5 days compared to 24.3 days in younger patients (p=0.04, Mann Whitney U test). This observation has been made before [Barlogie et al, 1990]. Patients who had received previous HDM also had significant delays in recovery times of neutrophils and platelets but not PB GM-CFUc, as shown below in Table 4,3,C.

Table 4,3.C.

Neutrophil (N) and platelet (P) recovery times after HDM and ABMR in patients with and without previous treatment with HDM in days

	Prev HDM	No prev HDM	Significance
$N=1x10^9/l$	33	19	p=0.007
$N=0.5x10^9/l$	30	17	p=0.0045
$P=25x10^9/l$	45	23.5	p=0.00075
PB GM-CFUc	18	15	p=0.16

Times are in days
p values are single tail values for M-W U test

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Although there were delays in recovery of mature elements there was no significant difference in the time taken for re-emergence of circulating GM-CFUc. Also, there was no difference in the pattern of CSA/time. The median times taken to reach maximum levels of CSA were 9.5 and 9 days in the previously treated and untreated groups respectively.

Previous HDM was a significant determinant for predicting delays in engraftment. All patients had received at least 3 cycles of combination therapy (table 4,3.A).

Colony Morphology

There was a change in the morphology of colonies that were grown in patient plasma with time after HDM & ABMR. At day 0 and until elevations of CSA after day 3 colonies consisted of low numbers of cells, 50-100, and there were frequently many clusters of cells (<50). Colonies could be divided according to shape and density and also depending on the size of cells within them. Initially colonies were of either large cells or small cells and generally quite diffuse in appearance. However, when CSA increased and was maintained at high levels the colonies became larger (100-200+ cells) and more condensed, suggesting a more rapid rate of division and a greater proliferative potential, and consisted predominantly of small cells (Plates 4,3.a-f).

The colonies of small cells consisted, predominantly of granulocytes and myeloid precursors (G-CFUc - granulocyte colony forming units), and the colonies of larger cells of macrophages (M-CFUc) or mixed macrophages and granulocytes (GM-CFUc). In many cases, macrophages were found after aspirating small cell colonies as well as granulocytes and their precursors suggesting that these were GM-CFUc. It proved difficult to select individual colonies for cytological examination (Chapter 2,2.4) and during processing most cells were lost when only one colony was aspirated from the plate. For this reason several colonies of similar morphology were selected from individual plates and this may have given rise to contamination from non-selected colonies.

Following the fall in CSA, remaining activity in plasma promoted the growth of a mixture of smaller colonies, of either small or large cells, from normal bone marrow MNC. Although granulocytes could be identified cytologically after selection of these

colonies there was a predominance of macrophages during this later stage of recovery from HDM.

No Megakaryocyte colonies were identified but on several occasions single ceils that resembled mature megakaryocytes were identified after selection of single colonies for histological staining. Thls occurred with plasma collected in the later stages of recovery (i.e.days 15-30) (Plate 4,3.g).

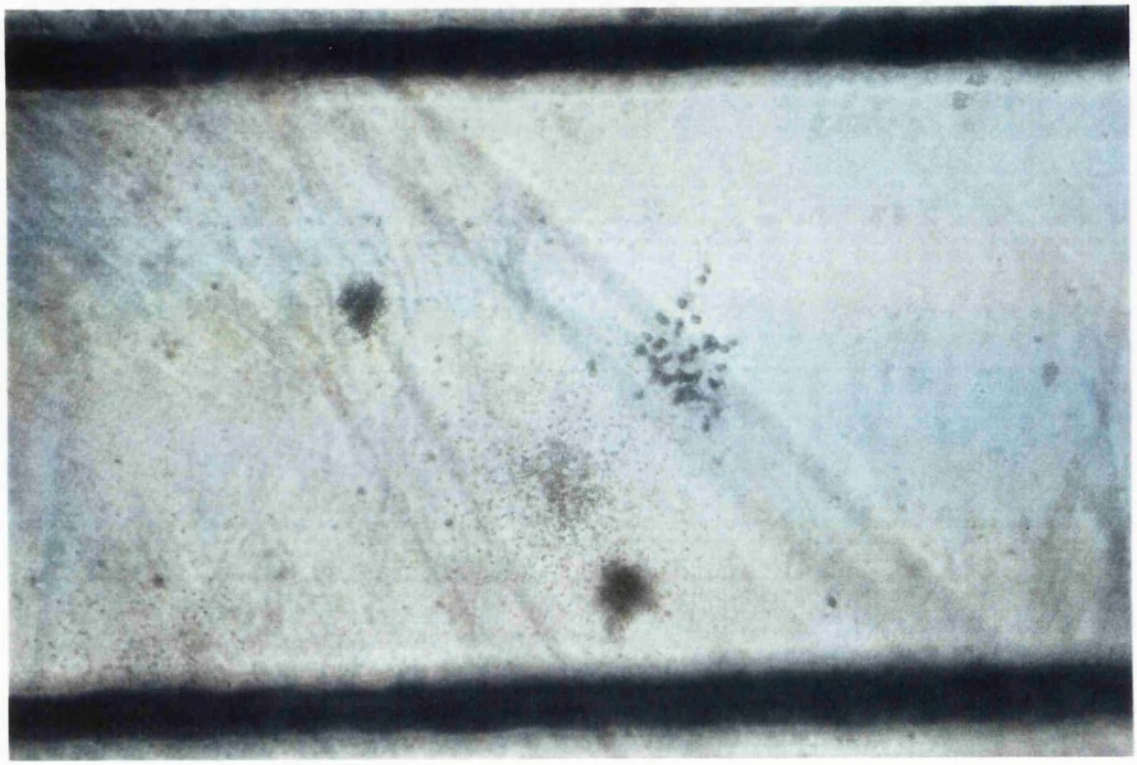

Plate 4,3.a Colonies of different morphology magnified x 40 as seen for counting (grown in plasma from patient 11, day 5)

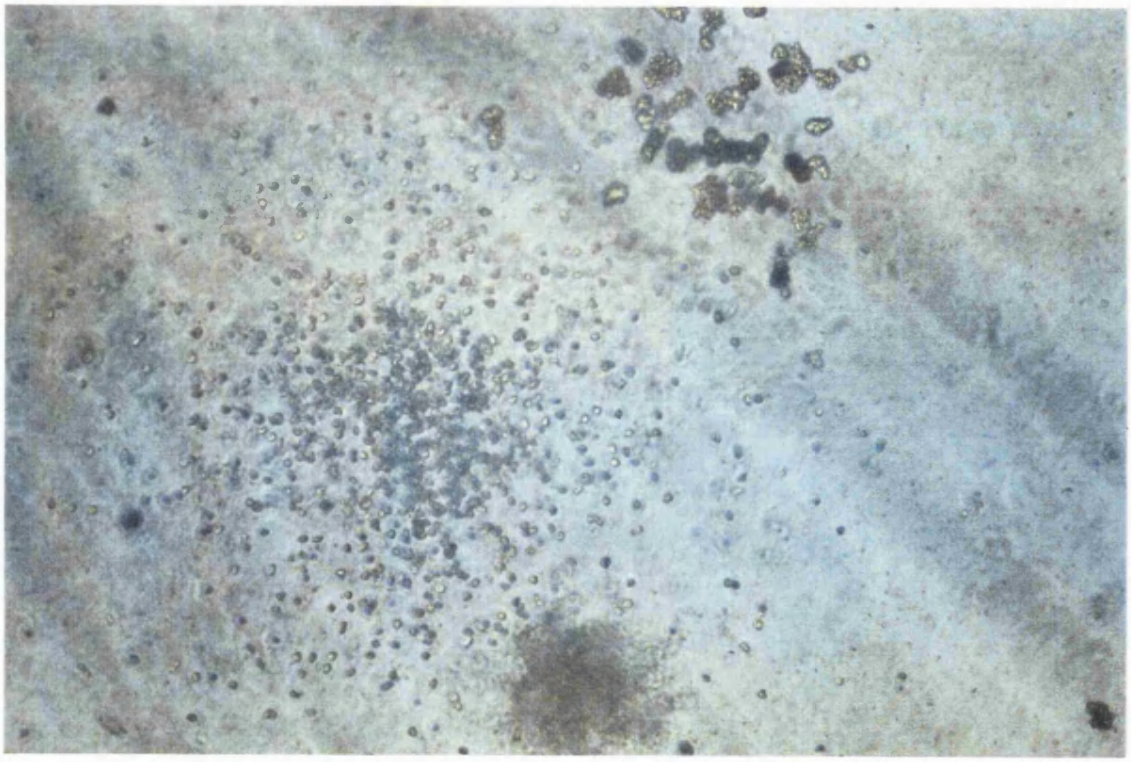

Plate 4,3.b Same colonies seen at higher magnification (x 100) (Anticlockwise from bottom: Dense small cell colony; diffuse small/large cell colony; diffuse large cell colony)

Plate 4,3.c Cluster/small colony of small cells x100 (G-CFUc) (Patient 11 Day 5)

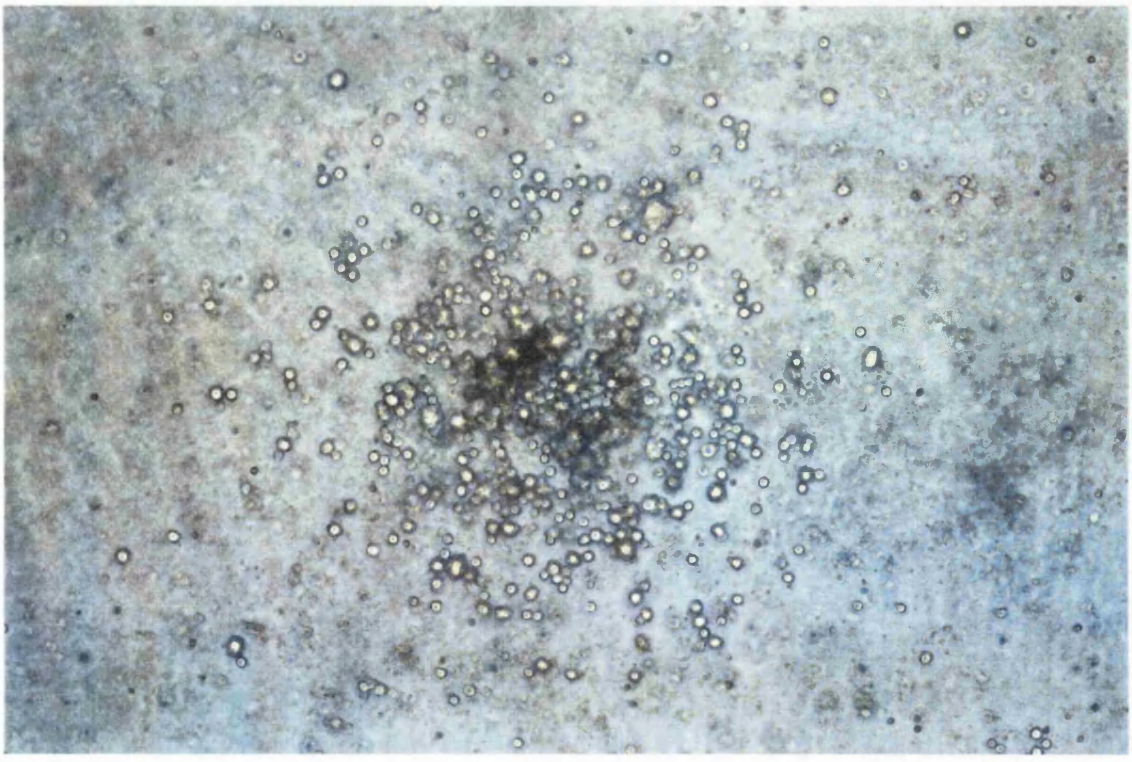

Plate 4,3.d Small colony of large cells x100 (GM/M-CFUc) (Patient 13, Day 7)

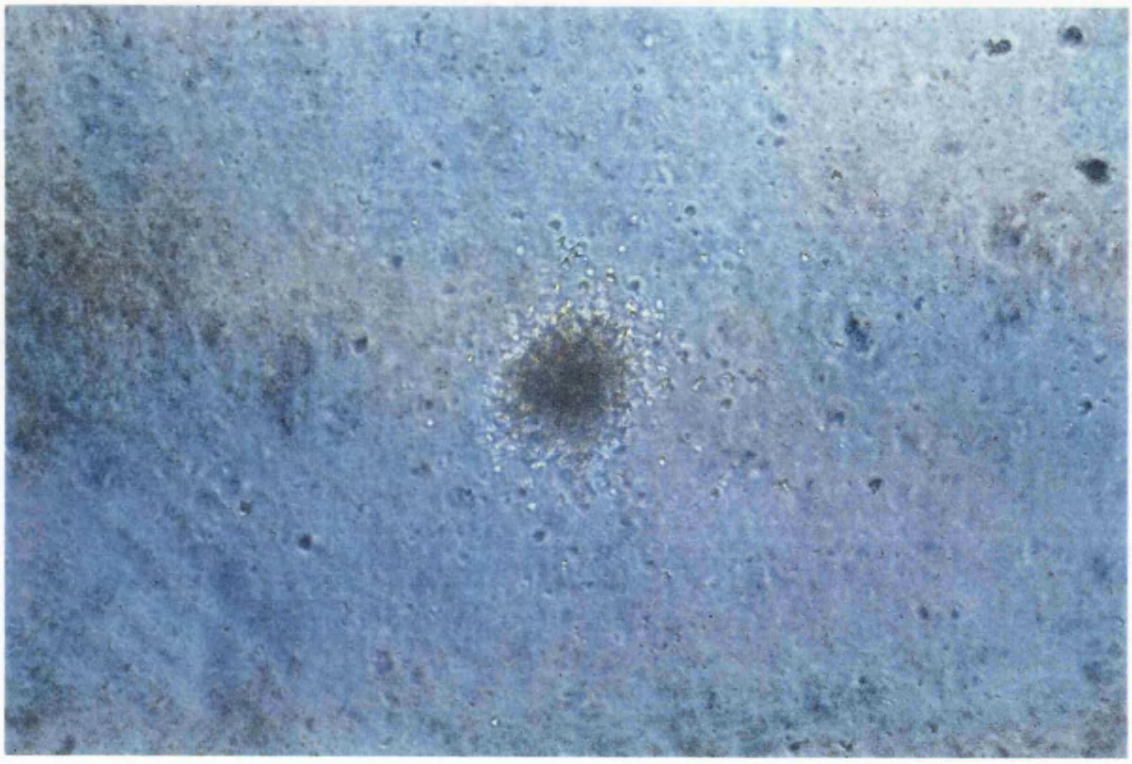

Plate 4,3.e Dense small cell colony grown in high CSA plasma x100 (G-CFUc) (Patient 11, Day 9)

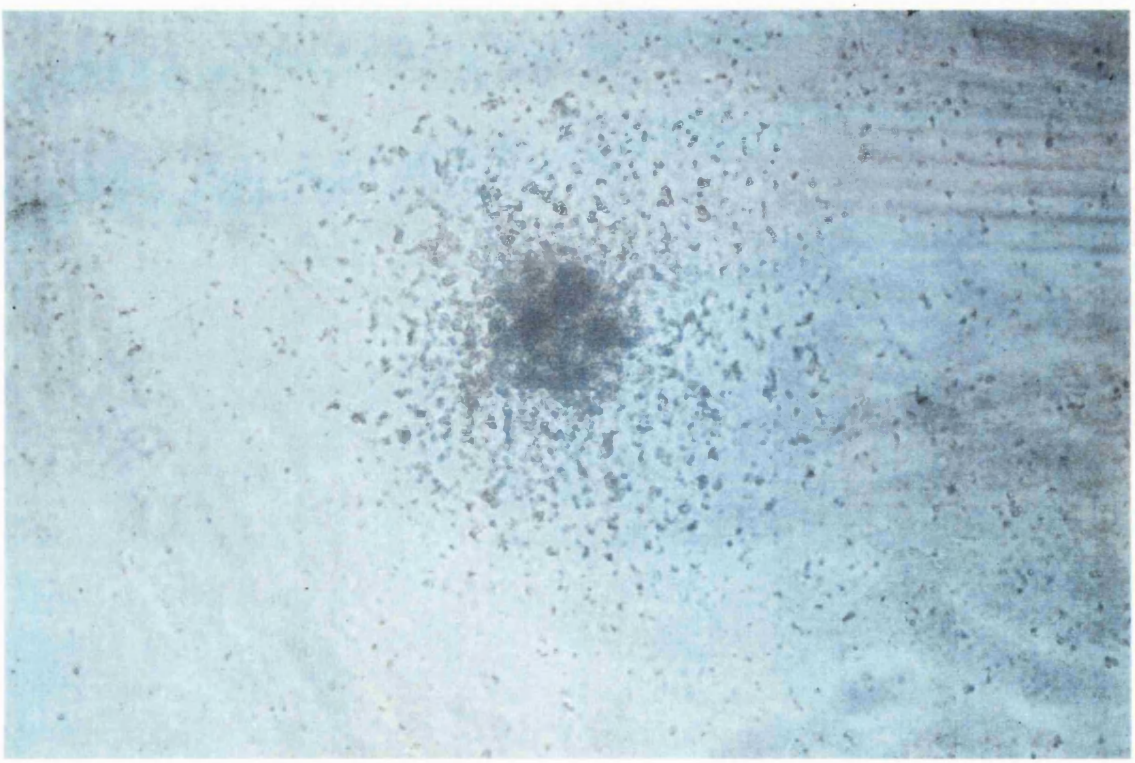

Plate 4,3.f Diffuse small cell colony from high CSA plasma. (Patient 11, Day 9)

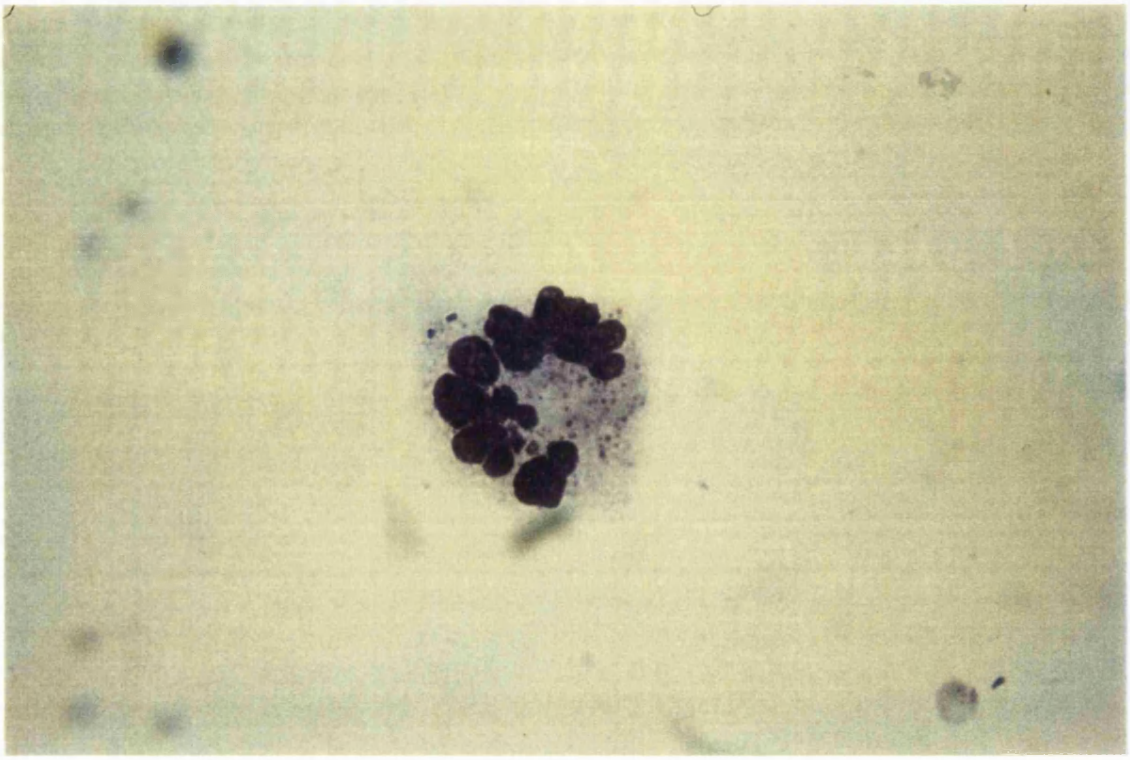

Plate 4,3.g **Multinucleated megakaryocyte grown with plasma collected after maximum CSA x100**

The effect of ABMR on CSA

In Table 4,3.D, below, recovery times for patients treated with HDM & ABMR are compared to those treated with HDM alone. Since previous HDM and age significantly prolong recovery times, a separate analysis (parentheses) has been done with the exclusion of patients over 60 years of age and those treated previously with HDM.

Table 4,3.D.

COMPARISON OF RECOVERY TIMES IN PATIENTS TREATED WITH HDM WITH AND WITHOUT ABMR

	HDM	HDM/ABMR	"p"
No Patients	7 (6)	23 (18)	
Age	50 (48.5)	45 (45)	Not Sig Not Sig
Max CSA	7.5 (9.5)	9 (9)	Not Sig Not Sig
N=0.5	32 (30)	16.5 (17)	<0.0008 (<0.0003)
N=1	32.5 (32.5)	21 (18)	0.021 (0.001)
Plt=25	31 (31)	24 (22)	0.13 (Not Sig) (0.018)
PB GM-CFUc	26 (24.5)	15 (15)	<0.0004 (0.001)

Numbers are median recovery times in days after HDM
Numbers in parentheses represent separate analysis with exclusion of patients ≥60yrs and patients previously treated with HDM.
"p"values are for comparisons using 2 tailed Mann-Whitney U tests
Not Sig - Not significant

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The exclusion of elderly and previously treated patients (figures in parentheses in Table 4,3.D) resulted in greater differences in recovery times between the two groups, particularly in respect to recovery of platelets, illustrating the effect of these variables on engraftment. Previous HDM or age did not, however, adversely affect the recovery of PB GM-CFUc in patients that received ABMR.

The time taken to reach maximal CSA was similar in patients who did or did not receive ABMR after HDM and there was no difference in the absolute levels of maximum CSA obtained compared with 5637CM (Median HDM-422% Vs HDM/ABMR-351% of 5637CM, p=0.5). These data suggest that production of CSA was independent of the graft.

Mathematical relationship between CSA and haematological recovery

Because there is a relationship between elevated levels of CSA and the recovery of circulating white blood cells, attempts were made to determine which parameters of recovery correlate most closely with CSA.

There was a significant correlation between the time taken to achieve maximum CSA and the recovery of total leucocyte count to $0.5x10^9$/litre (Pearsons r=0.54, p=0.01) but not with recovery of neutrophils to 0.5 or $1x10^9$/l (or platelets of 25 $x10^9$/l) which occurred later in each patient.

When the areas under the time curves (AUC) of CSA (between day 0 and discharge from hospital) were measured (via the ®Fig-P softwear used to draw the curves (Chapter 2,5)) there was a significant correlation between these measurements and the time taken for the re-emergence of circulating PB-GM-CFUc (Pearsons r=0.637, p=0.001).

DISCUSSION

If CSA is produced by viable haemopoietic stem cells, sufficient endogenous cells (not from the ABMR) survive the conditioning regimen to produce the observed changes in CSA, and the amount of CSA produced by the transplanted cells is too small to be detected above this endogenous background and/or occurs after a similar delay in time. Alternatively, factors responsible for CSA may be released from cells killed or damaged by the conditioning regimen. If this were the case it is unlikely that the duration of elevated CSA would so closely have reflected the period of leucopenia. This is illustrated by the patients who did not receive ABMR, who had longer periods of elevated CSA despite a smaller dose of chemotherapy, and by individual patients who had prolonged leucopenic periods and prolonged elevations of CSA such as pt 28 (Fig 4,2.5) and pt 10 (Fig 4,3.10) who also received a second infusion of cryopreserved cells on day 50 which was associated with a fall in CSA.

Although ABMR did not affect CSA directly, haematological recovery and emergence of PB GM-CFUc occurred earlier in patients who received ABMR and CSA levels fell accordingly. The contribution of ABMR was, therefore, to provide stem cells that could respond to an environment in which appropriate proliferative stimuli were present. The patients who had received previous HDM had delays in engraftment (despite the use of ABMR) but no delay in the production of CSA. This supports the hypothesis that cells which provide the proliferative component of recovery from ablative chemotherapy are not the same cells that produce CSA.

There were no instances in which PB GM-CFUc were detected during the

leucopenic nadir. The appearance of PB GM-CFUc after intensive therapy may reflect an expansion of progenitors within the bone marrow since this is likely to be a requirement of both recovery and engraftment. Neutrophil recovery did not occur in the absence of recovery of PB GM-CFUc.

Although there were significant delays in recovery of end cells and PB GM-CFUc in patients who did not receive an autograft, patients who had had previous HDM, who all received an autograft, had relatively less delay in recovery of circulating progenitors than in recovering end cells (Table 4,3.C). Assuming, therefore, that it was high numbers of committed stem cells in the autografts of these patients that resulted in the recovery of PB-GM-CFUc, these did not function as well as those in patients who had not had previous HDM and it is possible that the cumulative injury to haemopoiesis of heavy pre-treatment results, not simply in a loss of numbers of primitive progenitors, but in an impairment of proliferation passed on to more committed stem cells. Alternatively, the depleted stem cell pool in heavily pre-treated patients may be unable to provide sufficient numbers of committed progenitors (that can be measured in the circulation) to re-populate the peripheral blood compared with that in patients who have not had previous intensive therapy.

Engraftment after BMT has been studied by bone marrow aspiration and culture [Arnold et al, 1986]. At day 7 after ABMT the BM was oedematous and hypocellular and the very low numbers of BM CFUc consisted of pure granulocyte and erythrocyte colonies. Normal BM histology was restored by day 42 but the clonogenicity of the BMMNC remained low and relatively restricted to G-CFUc and erythroid colonies for over 8 weeks. Although the capacity for recovery of all circulating elements is present, the appearance of all components does not occur simultaneously. There is a process of

expansion of different populations of progenitors at different stages of engraftment to provide end cells early on followed by expansion of earlier progenitors which results in the full complement of haemopoietic cells. Control of such activity may involve an interaction of progenitors with non haemopoietic cells such as fibroblasts, endothelial cells and the other cells grouped together as "stroma". The transplantation of stroma has been found to be important after ABMT [Keating et al, 1982]. Endogenous stroma may be equally important in ABMR and is also a likely candidate for the production of CSA if haemopoietic cells are not responsible. It is likely that changes in bone marrow activity are associated with the release of different cytokines, some of these may enter the circulation and, thus, explain quantitative and qualitative changes in CSA with time during recovery.

After HDM with or without ABMR, CSA increased as total WCC decreased, and decreases in CSA occurred in association with increases in WCC. It is possible, therefore, that a simple feedback mechanism exists between one or more of the cellular components of "total WCC" and cells that produce CSA. The lack of mathematical correlation between time taken to achieve maximum CSA and parameters of recovery of neutrophils may reflect the heterogeneous group examined, since some patients were pre-treated with HDM and half received rhG-CSF. However, the correlation with area under the time curve of CSA with time taken to recover circulating PB GM-CFUc suggests that increases of CSA may be related to proliferation of stem cells.

Although neutrophil recovery did not appear to be directly related to the termination of elevated CSA, low levels of neutrophils that could not be accurately measured may have been sufficient to influence the production of CSA, possibly within

the bone marrow microenvironment. It is likely that such a feedback mechanism would be mediated by cytokines. Such cytokines might directly contribute to CSA as inhibitors or stimulators of haemopoiesis or act via other cell populations which in turn produce factors(s) that contribute to CSA.

The increase in CSA might, alternatively, relate to cell death and trauma, mediated, perhaps, by factors involved in inflammation. These could also act directly to change a balance of plasma inhibitors and stimulators of haemopoiesis or act through production of cytokines by an intermediate cell population. This hypothesis is unlikely since elevated CSA was maintained until WCC increased. Similarly, there was no evidence that the maintenance of high levels of CSA was due to continuing trauma since levels of CSA were independent of clinically apparent infective and toxic stress and there was no relationship to the occurrence of severe infection that required transfer to the intensive care unit or changes in the patients antibiotics.

In the present study CSA reached a maximum 9 days after treatment with HDM & ABMR and was maintained until the recovery of leucocytes. A similar result has been reported [Miliar et al, 1992] in leukaemic patients where CSA reached a maximum 7 days after ABMR or ABMT. This pattern was not found in 34 patients [Yamasaki et al, 1988b] who had maximum GM-CSA 21 days after receipt of ABMT. In that study, which used non-adherent cell BMMNC in the assay of CSA, plasma alone had little activity (although this peaked before day 10) and CSA was increased at 21 days when the assay contained 10% of medium conditioned by phytohemagglutinin (PHA) stimulated leucocytes. PHA conditioned medium is likely to contain lymphokines such as IL-2 and IL-6 which may interact with molecules present in plasma. It is also possible that

adherent cell depleted BMMNC may respond differently to CSA than BMMNC which retain adherent cells. The presence of adherent cells in BMMNC has been reported to differentiate between two types of serum CSA [Francis et al, 1977, Francis, 1980, Furusawa et al, 1978, Ishizaka et al, 1985], one that directly stimulates myelopoiesis and is elevated in patients with aplastic anaemia (Ishizaka et al,1985) and another that is present in normal human serum but requires the presence of adherent cells (Ishizaka et al,1985, Francis, 1980) or peripheral blood leucocytes (Francis, 1980).

The change in colony morphology that was noted using plasma collected at different times after HDM & ABMR suggests that there are qualitative as well as quantitative changes in CSA as the bone marrow regenerates. The appearance of more granulocyte colonies (G-CFUc) during the period of maximum CSA suggests that there are factors present in plasma at these times which favour granulopoiesis. The predominance of macrophage colonies (M-CFUc) later on would be consistent with the production of factors favouring macrophage differentiation.

G-CSF and M-CSF have been demonstrated in serum, by RIA, after ABMT [Janowska-Wieczorek et al, 1990], and their activities peaked at different times, days 6 and 11 respectively. When CSA (augmented by PHA stimulated leucocyte-conditioned medium) was found to peak at day 21 after ABMT in leukaemic patients (Yamasaki et al, 1988b) this activity was neutralised by the addition of antibodies to GM-CSF.

The presence of megakaryocytes in some assays of GM-CSA suggests that CSA for GM-CFUc may support the growth of Meg-CFUc, although the conditions for this may not be optimal in this assay system. Occasional megakaryocytes were detected in assays of plasma samples collected late after HDM when the colonies were no longer

dominated by granulocytes suggesting that factors stimulating megakaryocytopolesis may be present in plasma collected at this time. The two patients who had second peaks of CSA had comparatively long platelet recovery times. This may have reflected an increase in factors directed to correct a deficit in thrombopoietic capacity.

Megakaryocyte colony stimulating activity (Meg-CSA) has been described following intensive chemotherapy in several studies after ABMT and ABMR [Mazur et al, 1984, Yamasaki et al, 1988b, Fauser et al, 1988]. The time of maximum Meg-CSA after chemotherapy varied between studies. However, a consistent observation in each study was that the peaks of Meg-CSA and GM-CSA were separated in time after treatment, supporting the hypothesis that there are qualitative as well as quantitative changes in CSA during the recovery period.

The variation in timing of peaks of MEG-CSA in different studies may be due to differences in the assays employed. Mazur et al (1984) used a plasma clot assay and found peaks of MEG-CSA between 20-30 days after treatment. The assay employed by Yamasaki et al (1988) incorporated leucocyte conditioned medium and adherent cell depleted BMMNC and identified peak MEG-CSA at day 7 compared to day 21 for GM-CSA. Fauser et al (1988) used a similar assay and found peaks of MEG-CSA that varied in time depending on whether patients received ABMR, ABMT or ABMT with t-cell depletion. Thus, although sera or plasma may contain molecules that can stimulate the growth of different haemopoietic lineages, the balance between proliferative and inhibitory factors for each lineage at a given time probably controls the response of the particular target MNC population (*in-vitro*). For example, sera from patients with aplastic anaemia [Adams & Barrett, 1982] possessed the ability to directly stimulate the growth of megakaryocytes in liquid culture but required the presence of adherent cells and/or the

presence of a feeder layer containing peripheral blood leucocytes to stimulate the growth of GM-CFUc.

4,3.2 THE EFFECT OF rhG-CSF

Haematological recovery times and CSA in patients treated with rhG-CSF after HDM & ABMR were compared with a similar patient population who did not receive rhG-CSF (Chapter 4,3.1). For the purpose of comparison, patients were excluded if they were $\geq$60 years of age or had received HDM previously. Patient 22 was excluded because he received only 2 days of rhG-CSF. In this comparison 9 patients were treated with G-CSF and 8 without (Table 4,3.E below).

The details of sample collection are stated above (Chapter 4,2/4,3.1) and data are shown in Appendix II (Figs, 4,3.1-23).

The samples of blood were collected each morning before the patients daily infusion of rhG-CSF. A phase I study carried out by the manufacturers (Investigators Brochure for rhG-CSF, Chugai Pharmaceutical Co., 1988) had shown that the half life and clearance of rhG-CSF was such that no accumulation of rhG-CSF occurred and there was total clearance of the recombinant factor by 24 hours at the doses used. In a published phase I/II study of twice daily infusions of a bacterially engineered rhG-CSF [Morstyn et al, 1988) the half life of rhG-CSF was 110 minutes. The CSA described herein is, therefore, assumed to be endogenous.

Table 4.3.E

Differences in recovery between patients receiving G-CSF and placebo.

	rhG-CSF	Placebo	M-W U test
N=0.5	14	18.5	p=0.0025
N=1	16	23.5	p=0.0048
Plt=25	24	23	Not sig
PB GM-CFUc	14	15	Not sig
Max CSA	7	13	p=0.0052

Median times in days after day 0.
Counts are $x10^9/l$ for N (neutrophils) or Plt (Platelets)
Not sig = Not significant.
p values = exact 2 tail M-W U

The times at which the neutrophil count rose to 0.5 and to $1x10^9/l$ were significantly reduced in patients who received rhG-CSF, however there was no effect on the recovery of plateiets or the time at which myeloid progenitors were detected in the peripheral circulation. The maximum level of CSA in patients who received rhG-CSF occurred 7 days (range 7-9) after HDM & ABMR compared with 13 days (range 8-14) in patients who received placebo. This difference was statistically significant (Table 4,3.E).

DISCUSSION

RhG-CSF increased the rate of recovery of neutrophils and this was associated with an earlier peak in endogenous CSA.

The data show that the use of rhG-CSF is associated with an earlier rise in CSA but no difference in the levels of CSA (median maximum CSA for the groups were 358

and 350% of 5637CM p=0.5 (MW-U)). In contrast, in leukaemic patients CSA levels were higher in patients receiving a continuous infusion of rhGM-CSF compared to the leveis in a group receiving placebo [Millar et al, 1992] but the timing of the increase in CSA was not different between the two groups. Higher levels of CSA in patients receiving continuously infused rhGM-CSF (Millar et al, 1991) probably reflected the presence of rhGM-CSF in the blood samples. There was no reduction in the period of neutropaenia in that study, although there was a significant increase in the leucocyte count 13 days after transplant [Powles et al, 1990].

It is not possible to deduce whether the difference that has been shown between the effect of a short infusion of rhG-CSF and a continuous infusion of rhGM-CSF is due to differences in the biological properties of the compounds or to differences in the methods of administration. The small numbers in the present study may account for the difference in the time taken to achieve maximal CSA in the groups that did or did not receive rhG-CSF. It is also possible that the action of rhG-CSF may be partly through an interaction with endogenous cytokines.

A single short infusion of (Chugai) rhG-CSF in normal volunteers is associated with a maximum increase in WCC and neutrophil counts at 4-8 hours followed by a return to baseline levels by 24 hours. There is no accumulation of rhG-CSF when it is administered in this way (Chugai Investigators Brochure). In this study the daily leucocyte counts were made on samples taken together with the samples used for measurement of CSA. The maintenance of the elevated leucocyte counts induced by rhG-CSF may, therefore, have been attained by an interaction with endogenous activity.

The median time to achieve maximal CSA in the group of patients treated with

HDM alone (7.5 days, Table 4,3.D) was shorter than that in patients who received HDM & ABMT (although the difference was not significant) and was equivalent to that of patients treated with HDM & ABMR with rhG-CSF in whom there was loss of inter-patient variability in this time. Patients with leukaemia who received ABMT or ABMR (Millar et al, 1991) also had a median time of 7 days to achieve maximum CSA. Patients with MM who receive an autograft (at RMH), unlike the leukaemics and MM patients treated without ABMR, receive four days of high dose methyl prednisolone immediately after ABMR. It is possible that this interferes with the production of CSA and that this inhibition is overcome by the administration of rhG-CSF.

4,4 HDBu & ABMR

Patients with MM are treated with high dose busulphan when they have either relapsed rapidly after HDM, received HDM twice before or when they have inadequate renal function to clear HDM (Chapter 2,3.2). These patients were studied in order to measure the effect of a different conditioning regimen on the pattern of CSA after ABMR.

As HDBu is currently used as a second line high dose procedure, only 4 patients received HDBu & ABMR during the period of study. Their details are given in Table 4,4.A., below.

Three patients were heavily pre-treated and additionally, one of these was over 60 years of age. This patient did not survive the engraftment period. The fourth patient had poor renal function but nevertheless had a good performance status and would have been predicted to have been in a better prognostic group.

HDBu was administered over 4 days (Chapter 2,3.2e) with ABMR performed on day 6. Data showing CSA/time with WCC and PB GM-CFUc are timed from (day 0) the start of busulphan and not from the time of ABMR (Figs 4,4.1-4, page 160-1). Recovery times for these patients are given in Table 4,4.B, below.

Haematological recovery was delayed in the three patients who were heavily pre-treated when compared to that of patients that had received HDM & ABMR. In the previously untreated patient the recovery time was similar to that seen after HDM alone. Measured from the time of ABMR, this patients recovery times were comparable with those of patients receiving HDM & ABMR.

Table 4,4.A Details of patients treated with HDBu & ABMR

Pt	Age/S	Iso	DS	Previous Treatment	Ind[ctn]	HDBu/G
31	65/M	BJI	CR	HDM/ABMR → CR <18/12 C-VAMP x 6	Short Res to HDM	8/10
32	36/M		PD	HDM → CR x HDM & ABMR → CR x (interferon) C-VAMP x → NR EPIC x → NR	HDMx2	16/5[c]
33	34/M	IgG-I	PR	HDM/ABMR → CR <12/12 C-VAMP x 4 → PR → PD C-VAMP x 4 → PR Interferon X 1/12	Short Res to HDM	16/P
34	39/M		CR	C-VAMP x 5	Poor GFR	16/P

Ind[ctn] - Indication for HDBu
Short Res - Short response
GFR - Glomerular filtration rate
HDBu/G - Dose of Busulphan(mg)/G-CSF(μg/kg/day)
[c] - not in the trial - on compassionate grounds
P - Placebo
Rest of Key - see previous tables

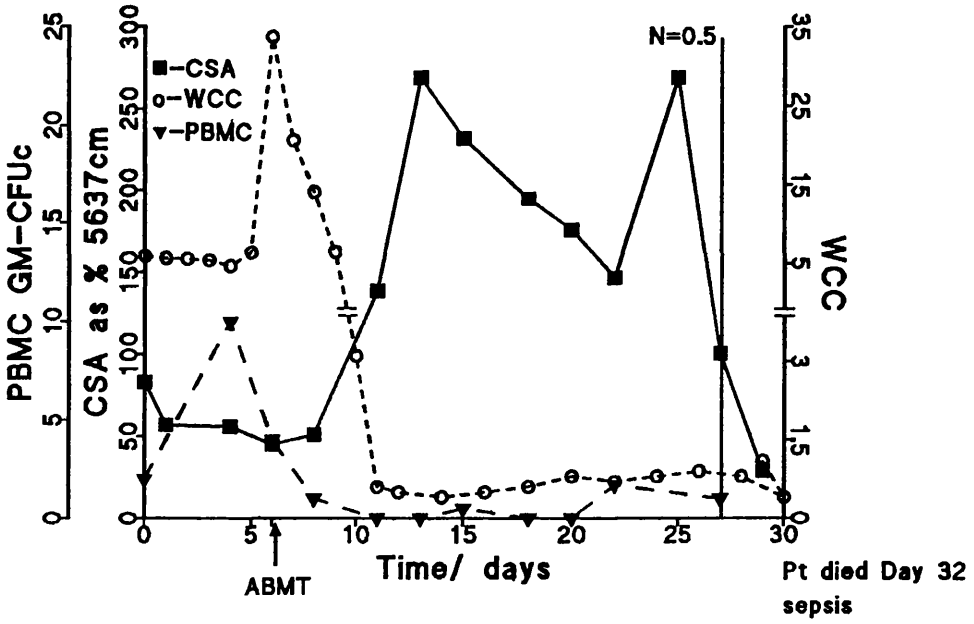
Table 4,4.B Recovery times for HDBu & ABMR Patients

Pt	Max CSA	N=0.5	N=1	Plt=25	PB CFUc
31	13	26	D	D	D
32	22	31	43	>60	34
33	14	29	33	70	33
34	21	25	28	36	20

Note - times are from day 0, ABMR given day 6

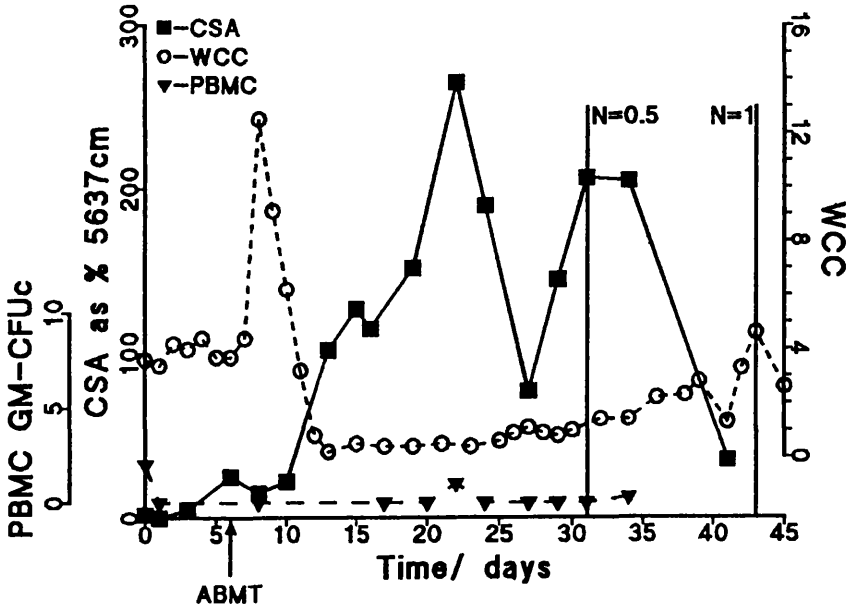
Key - See previous tables

FIG 4,4.1



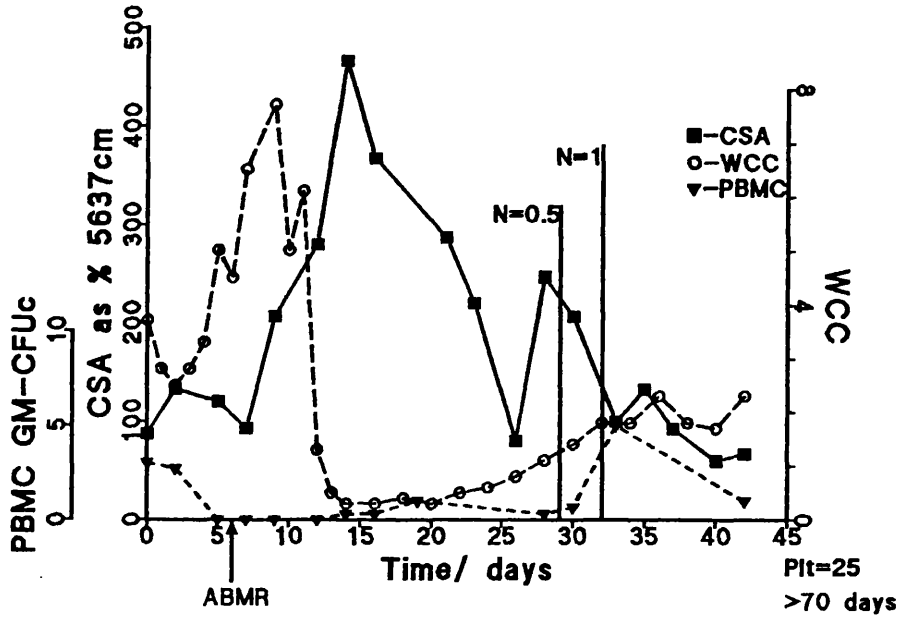
Patient 31 - HDBu days 1-4 - ABMR Day 6 - G-CSF 10μg

FIG 4,4.2



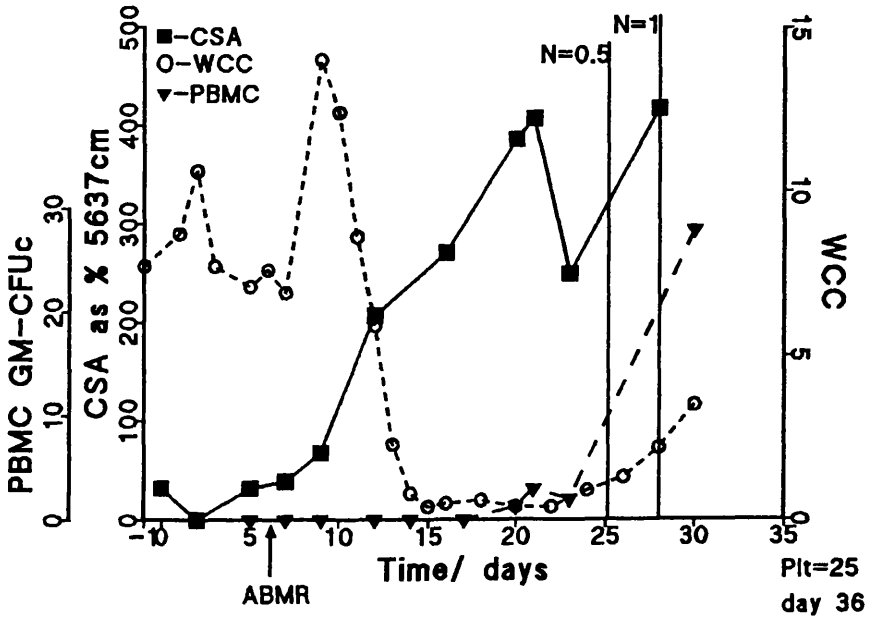
Patient 32 - HDBu Days 1-4 - ABMT Day 6 - G-CSF 5μg

FIG 4,4.3



Patient 33 - HDBu days 1-4 - ABMT Day 6- No G-CSF

FIG 4,4.4



Patient 34 -HDBu Days 1-4 - ABMR Day 6 -NO G-CSF

Before treatment CSA levels were similar to the levels in patients who received HDM with and without ABMR. Significant rises of CSA did not occur before the completion of HDBu (day 4) or ABMR (day 6) but following this the levels of CSA increased to levels equivalent to those after HDM with maximum levels obtained between days 13 and 22. There was a similiar delay (compared to treatment with HDM) before the drop in WCC, with the leucopenic nadirs attained between days 11 and 14. In each patient CSA increased as the WCC fell.

The recovery of PB GM-CFUc occurred after neutrophil recovery in the pre-treated patients but before this time in patient 34 who was not heavily pre-treated.

DISCUSSION

HDBu is more toxic to stem cells than HDM but the late haematological recovery times are probably related to previous treatment and age as well as to the effects of HDBu since patient 34 was able to recover in comparatively short time. The delay in recovery of circulating PB GM-CFUc compared with neutrophil recovery, in the pre-treated patients probably reflects the effect of accumulated stem cell toxicity from previous HDM since in patient 34 circulating GM-CFUc recovered before neutrophils, as was seen after HDM & ABMR in previously untreated patients.

The delay in the rise in CSA is likely to reflect the delay in the fall in WCC/neutrophil count that was seen in all four patients irrespective of whether they received rhG-CSF. The delay in the development of leucopenia may be related to the fact that busulphan has more effect on stem cells and toxic effects are seen in end cells after a delay in time. Additionally, the protracted period of administration may delay the accumulation of cytotoxic effects. Another possibility is that the effect of busulphan on

the tissue or cells that produce CSA may be different to that of melphalan. This is possible as these drugs have different toxicities for certain tissues, for example, the development of progressive pulmonary and, occasionally, endocardial fibrosis that has been seen after cumulative exposure to busulphan suggest that this drug may have greater toxicity for connective tissue than melphalan which is not associated with these side effects.

All of the patients had relatively long periods of raised CSA compared to HDM & ABMR patients and in all there was some evidence of a second peak of CSA in the fourth or fifth week. There are insufficient data to draw any conclusions from this about the relationship of these second peaks to neutrophil or platelet recovery.

The data from these patients show that the production of CSA is not a specific response to HDM but may be related to a decrease in bone marrow progenitors and/or mature haemopoietic cells.

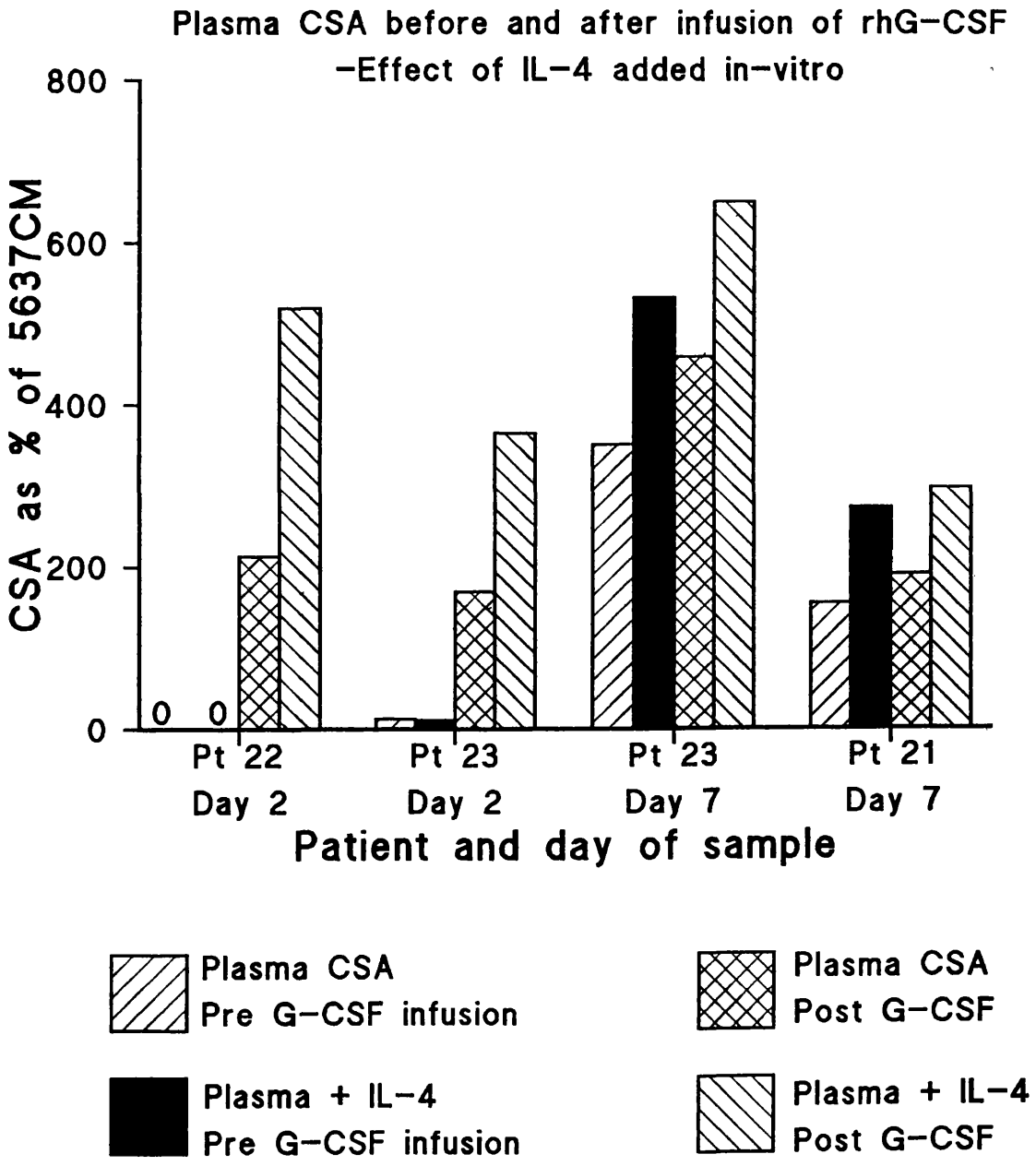
4,5 THE EFFECT OF IL-4 ON CSA IN PATIENT PLASMA AFTER HDM

IL-4 does not stimulate the proliferation of myeloid progenitor cells directly [Sonoda et al, 1990, Jansen et al, 1990] but augments the activity of G-CSF. Interleukin-4 does not enhance the activity of GM-CSF or IL-3 [Broxmeyer et al, 1988].

Preliminary Experiments

To determine whether CSA in plasma could be augmented by IL-4, which would indicate the presence of G-CSF, plasma samples were collected from 3 patients who were receiving rhG-CSF immediately before and two hours after infusion with this cytokine. Samples were collected at the time of the first infusion of rhG-CSF, on day 2 after HDM, when endogenous CSA was expected to be minimal, and/or on days 7 when endogenous activity was likely to be at a high level. Two sets of Petri dishes were prepared in triplicate each containing 300µl of plasma. 5ng of IL-4 (British Biotechnology - Chapter 2,1.5.d) was added to one set of plates before the addition of MNC (Chapter 2,2.3.b). Previous experiments showed that this concentration of IL-4 enhanced colony formation by 5ng of rhG-CSF sub-optimaliy *in-vitro* (B.C.Millar - personal communication also [Millar et al, 1992]. The results are shown in figure 4,5.1. Page 165.

Fig 4,5.1


Plasma CSA before and after infusion of rhG-CSF
-Effect of IL-4 added in-vitro
CSA as % of 5637CM
800
600
400
200
0
0
0
Pt 22
Day 2
Pt 23
Day 2
Pt 23
Day 7
Pt 21
Day 7
Patient and day of sample
Plasma CSA
Pre G-CSF infusion
Plasma CSA
Post G-CSF
Plasma + IL-4
Pre G-CSF infusion
Plasma + IL-4
Post G-CSF

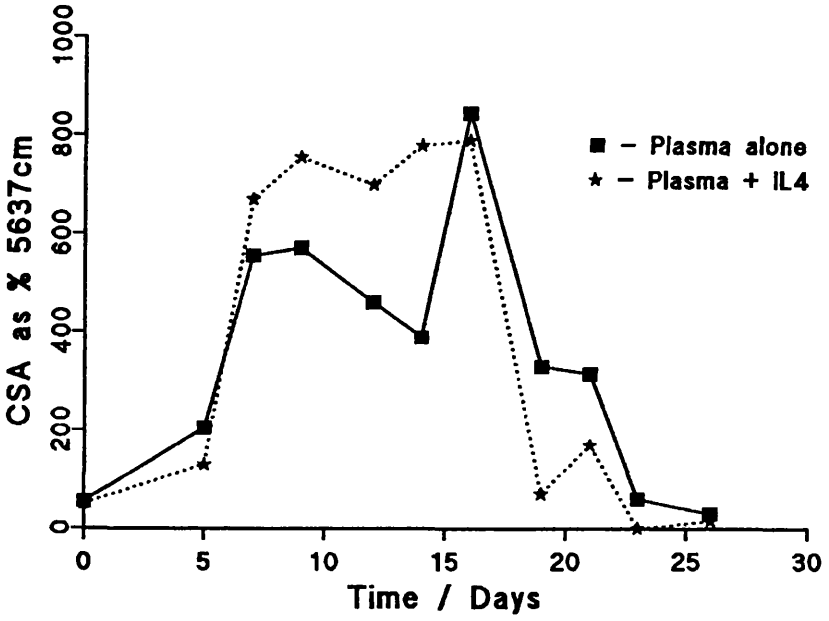
On day 2 after HDM there was negligible CSA in plasma samples collected before the infusion of rhG-CSF from either patient and there was no increase in colony numbers when rhIL-4 was added to plasma *in-vitro*. In the samples collected two hours after the G-CSF infusions activity was present which was augmented significantly by the addition of IL-4, showing that this activity was G-CSF-like. On day 7 patients 23 and 21 had significant CSA in their plasma collected before the infusion of rhG-CSF and this activity was augmented by the addition of IL-4 *in-vitro*. After the infusion of rhG-CSF both patients had increased CSA in their plasma which was augmented further by the addition of rhIL-4 *in-vitro*.

Thus, the addition of IL-4 *in-vitro* augmented the activity of rhG-CSF administered *in-vivo*. In addition IL-4 augmented CSA when rhG-CSF should have been absent from the circulation.

To investigate further the time course of this activity plasma samples were collected sequentially from 10 patients after HDM and tested *in-vitro* with and without the addition of 5ng of rhIL-4 in triplicate or duplicate Petri dishes depending on the available volume of plasma.

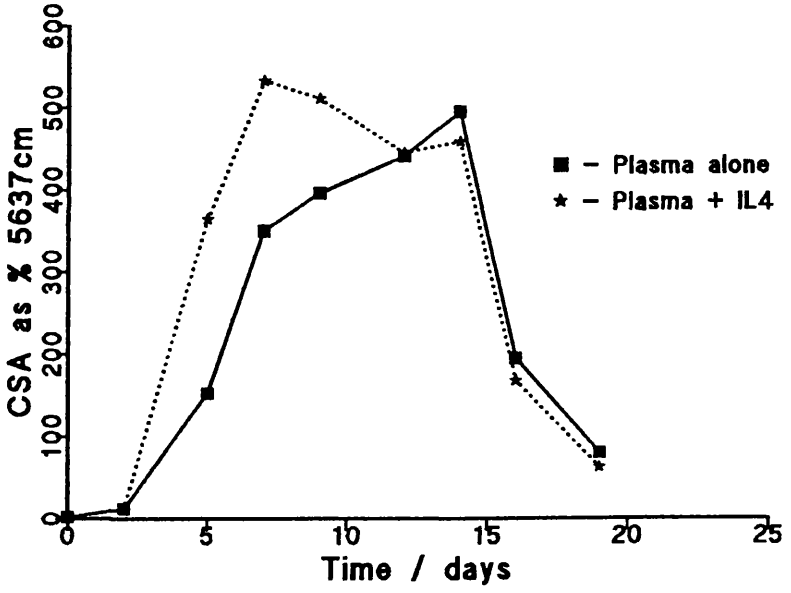
2 patients had received HDM alone, 3 had received HDM and ABMR and 5 had received HDM & ABMR with rhG-CSF. The results show the time course of CSA in plasma compared with plasma in combination with IL-4. Examples are shown on page 167 and data for all patients in Appendix II Figs 4,5.2-11.

FIG 4,5.6



CSA of Pt 19 tested against NBM
with and without the addition of IL–4 in–vitro

Fig 4,5.9



CSA of Pt 23 tested against NBM
with and without the addition of IL–4 in–vitro

In 7/10 patients IL-4 added *in-vitro* augmented the CSA of plasma after HDM, however, enhancement of colony formation by IL-4 in combination with plasma did not always mirror the activity of plasma alone. In most patients augmentation of colony formation by IL-4 occurred when CSA in plasma was a maximum. In patient 11 (Fig 4,5.4) two quite separate peaks of activity were detected, one on day 8 without the addition of IL-4 and a second at day 12 when IL-4 had been added *in-vitro*. At the time of the peak of activity without the addition of IL-4 in this patient there appeared to be an inhibitory effect of IL4 and such an inhibitory effect was seen in 5 patients either at the beginning of the time curves of CSA (e.g. Figs 4,5.3/10/11) or after the peaks of activity (e.g figs 4,5.3/4/6).

In addition to increases in colony numbers, IL-4 also influenced the morphology of colonies grown *in-vitro*. When IL-4 increased colony numbers the colonies also became larger (>200 cells) than colonies grown in aliquots of the same plasma samples without the addition of IL-4 and were composed, predominantly of small cells (Plates 4,5.a-b). These colonies consisted of granulocytes and myeloid precursors (G-CFUc).

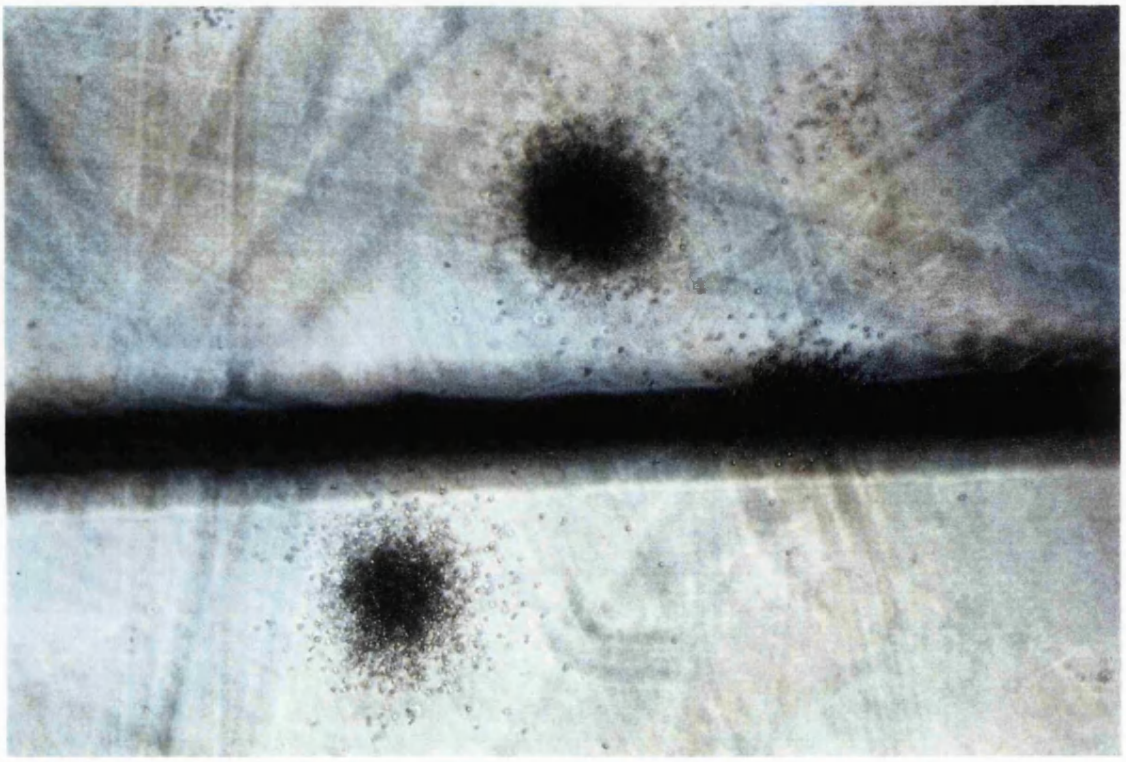


Plate 4,5.a Colonies grown in high titre CSA plasma with the addition of IL-4 *in-vitro* x40 (Compare with Plates 4,3.e-f).

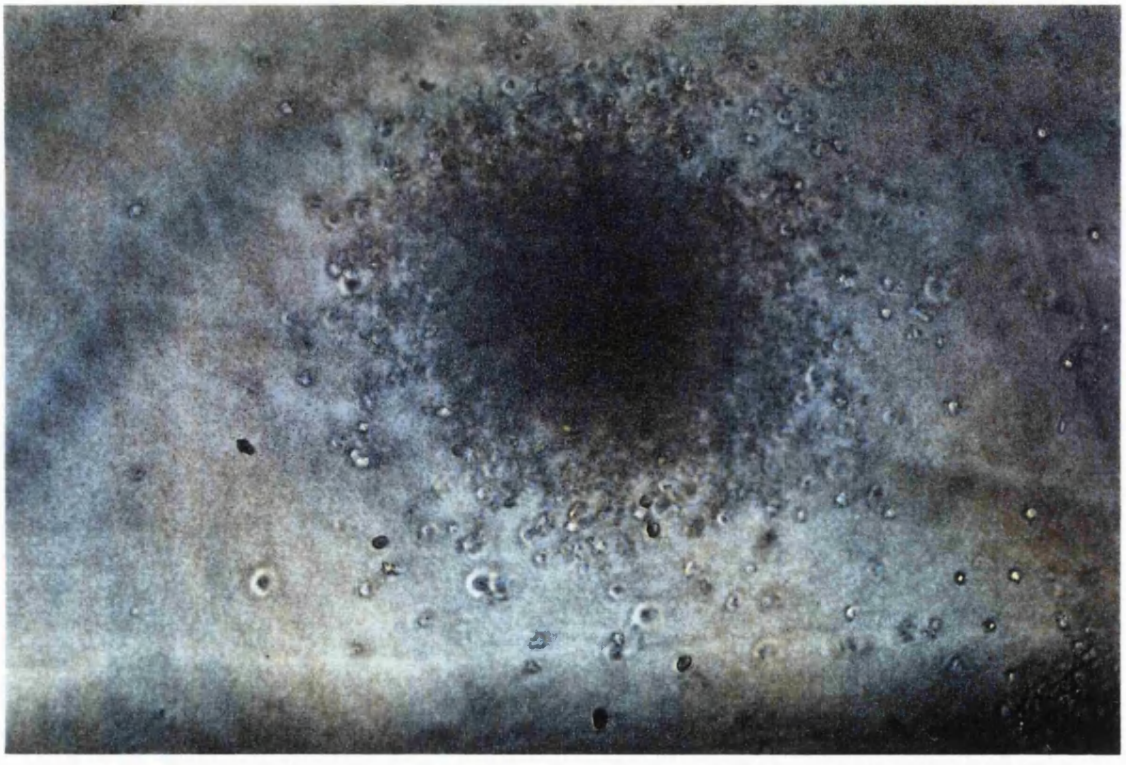


Plate 4.5.b High power view of the uppermost colony from above x100

DISCUSSION

The addition of IL-4 to plasma known to contain rhG-CSF lead to an increase in colony numbers grown *in-vitro*. IL-4 also augmented the activity of plasma with high CSA from the same patients, collected 24 hours after administration of rhG-CSF, that could not be expected to contain rhG-CSF (Chapter 4,3.b). Furthermore, IL-4 was found to augment the CSA of plasma collected from patients that had not received rhG-CSF. These observations demonstrate that IL-4 augments the activity of a factor which is produced *in-vivo* in response to treatment. Since the activity of neither IL-3 or GM-CSF are potentiated by IL-4 it seems likely that this factor is G-CSF [Broxmeyer et al, 1988].

The observation that IL-4 did not augment colony formation after HDM in some patients does not exclude the possibility that whatever factor(s) are augmented by IL-4 were still present in plasma from those patients. As plasma samples from individual patients were assayed against different donor bone marrows it is possible that in some instances the target cells may have been maximally stimulated when the appropriate factor(s) were present. For example in patients 18, 19 and 23 (Figs 4,5.5/6/9) there was augmentation of CSA by IL-4 until CSA reached a maximum. At these times IL-4 did not augment CSA. Similarly, in patient 7 activity of plasma containing IL-4 reached an equivalent activity to plasma alone around the peak of CSA without causing augmentation when there may again have been maximum stimulation of CFUc.

IL-4 did not augment CSA in plasma throughout the recovery period after HDM even in patients were there was no evidence that stem cells were maximally stimulated. In some instances IL-4 reduced colony formation by plasma. This suggests that IL-4 may have interacted with other cytokines resulting in inhibition of colony growth.

4,6 THE EFFECT OF ANTIBODY TO GM-CSF ADDED *IN-VITRO*

Antibodies to GM-CSF added *in-vitro* abolish CSA in the serum of myeloma patients [Millar et al, 1990] and in the plasma of patients after ABMT [Yamasaki et al, 1988b].

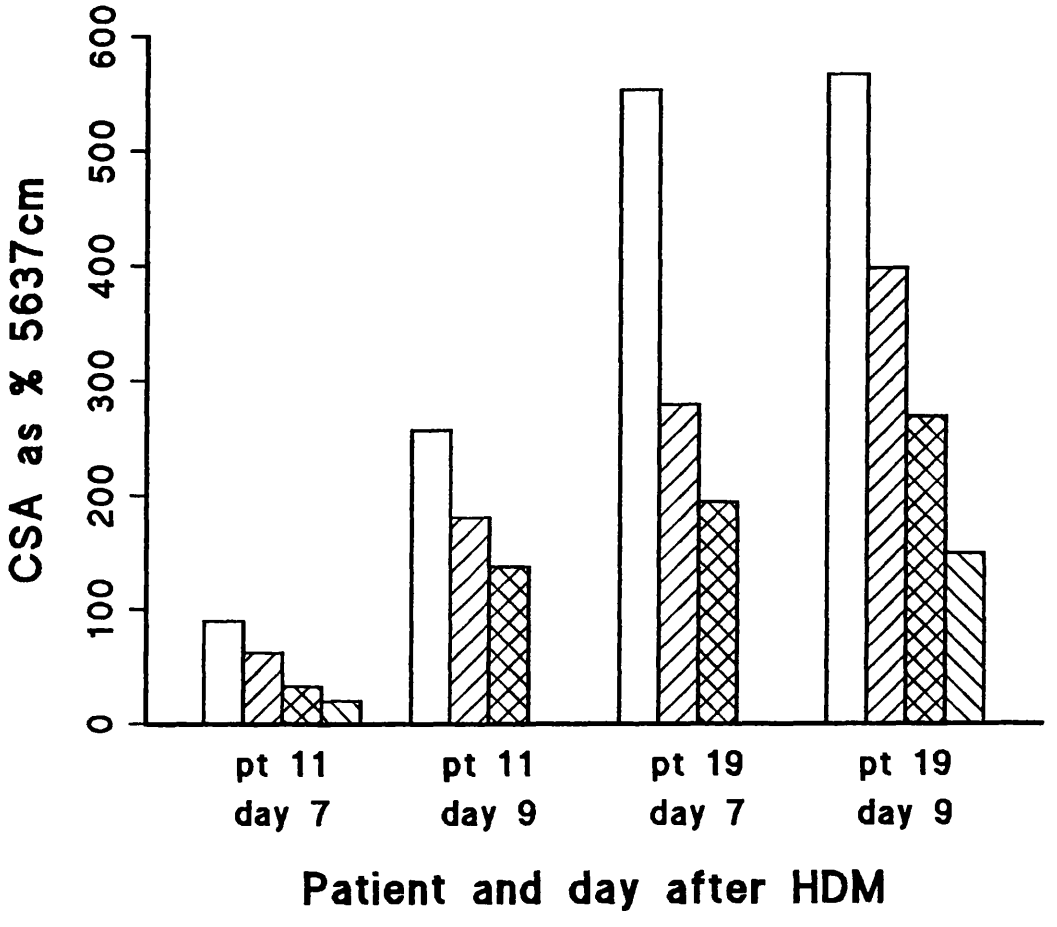
To investigate whether GM-CSF is an important contributor to endogenous CSA after high dose chemotherapy, antibody to rhGM-CSF (Genzyme, Chapter 2,1.6.a)(A-GM) was added, in increasing concentrations, to samples of plasma collected from 2 patients after treatment with HDM. Fig 4,6.1 Page 172.

Figure 4.6.1 shows that A-GM added *in-vitro* reduced the CSA of plasma in a dose dependant manner but did not inhibit the activity completely even when 20μg of antibody, sufficient to neutralise 20ng of CHO derived GM-CSF (Chapter 2,1.6), was used.

To further investigate the effect of A-GM added to plasma *in-vitro* 5ng of A-GM was added to aliquots of plasma collected sequentially after HDM from 4 patients. The data are shown in figures 4,6.2-5, Pages 173-4

The addition of A-GM to plasma *in-vitro* resulted in an a decrease of CSA throughout the period of elevated CSA in all 4 patients. This is best illustrated in patient 19, who had received rhG-CSF, (Fig 4,6.2) and patient 11, who had not, (Fig 4,6.3).

FIG 4,6.1



INHIBITION OF CSA BY ANTIBODY TO GM-CSF ADDED IN VITRO

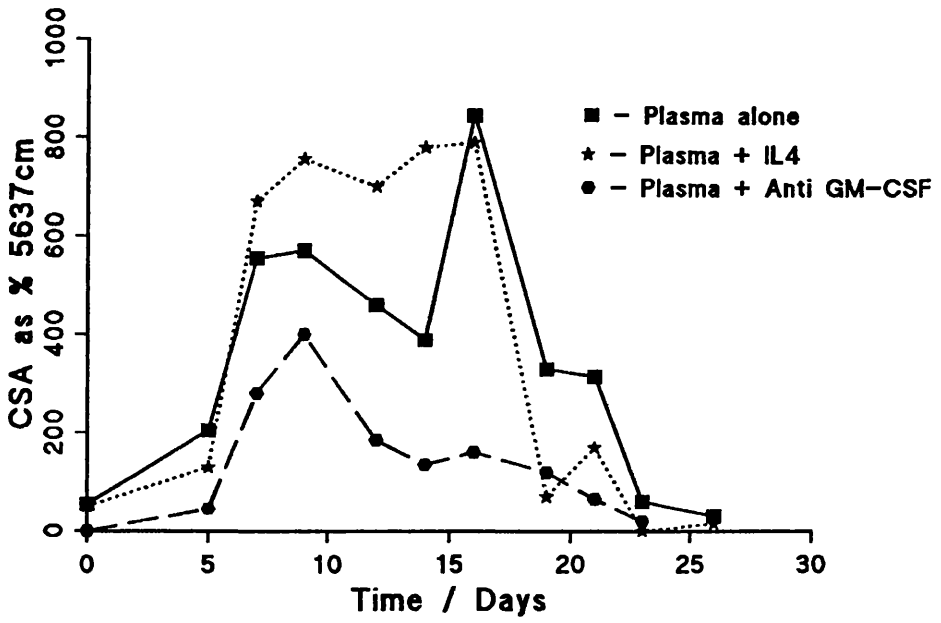
Plasma alone

Plasma+Anti GM-CSF 20μg/plate

Plasma+Anti GM-CSF 5μg/plate

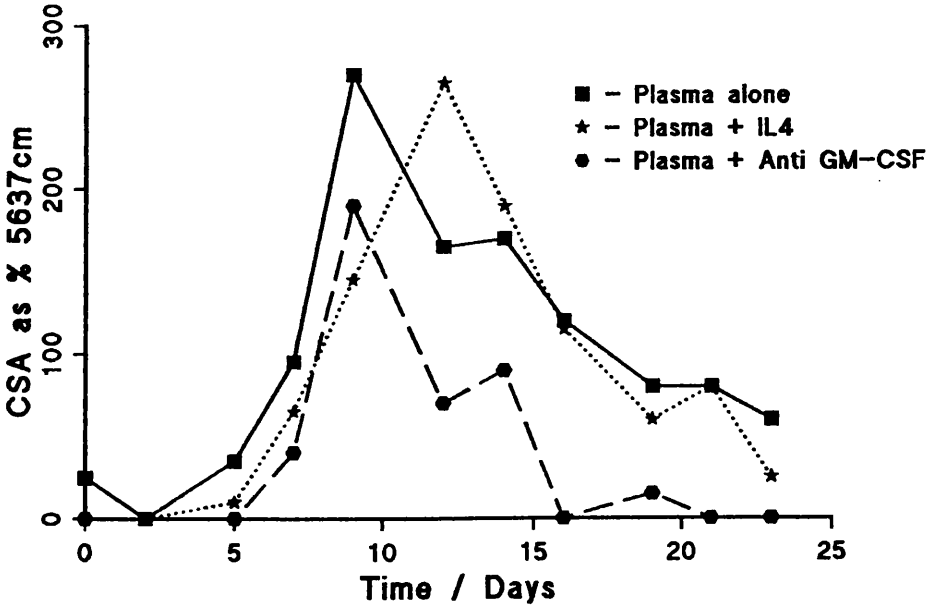
Plasma+Anti GM-CSF 10μg/plate

FIG 4,6.2



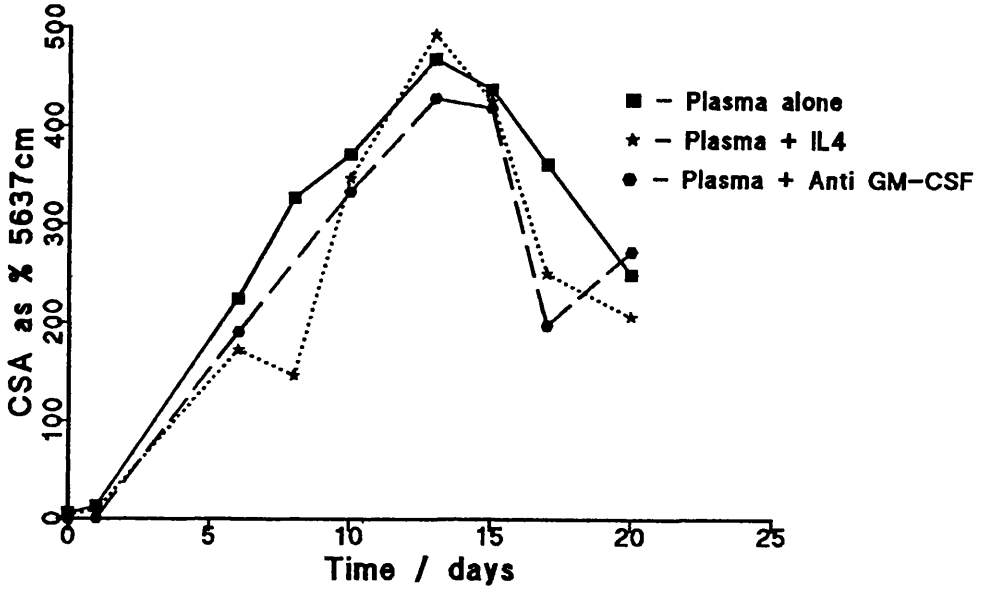
CSA of Pt 19 tested against NBM
with IL–4 or antibody to GM–CSF added in–vitro

FIG 4,6.3



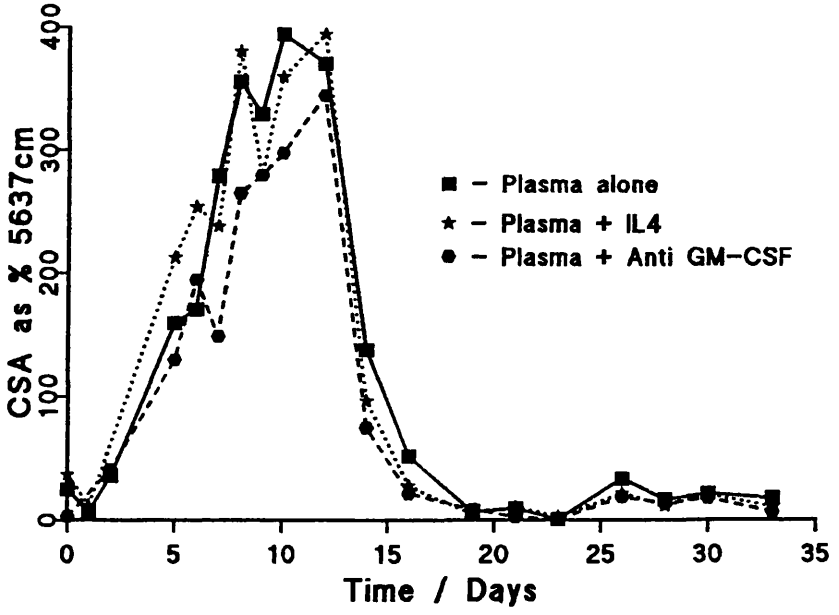
CSA of Pt 11 tested against NBM
with IL–4 or antibody to GM–CSF added in–vitro

Fig 4,6.4



CSA of Pt 7 tested agaist NBM
with IL–4 or antibody to GM–CSF added in vitro

Fig 4,6.5



CSA of Pt 6 tested against NBM
with IL–4 or antibody to GM–CSF added in–vitro

DISCUSSION

Dose response experiments using 5-20μg of A-GM showed that A-GM reduced the CSA in plasma suggesting that up to 20ng of GM-CSF might be present in 300μl of plasma.

The inhibitory effect on plasma CSA of A-GM at each time interval after HDM suggests that GM-CSF-like activity is produced throughout the recovery period whereas G-CSF-like activity is induced to a maximum and subsequently declines with time after therapy. Alternatively, A-GM may inhibit GM-CSF production by some component of the target cell population *in-vitro*. This is likely to be a specific effect of antibody to GM-CSF since antibody to M-CSF added to plasma *in-vitro* has no effect on CSA [Millar et al, 1992].

4,7 THE EFFECT OF CSA ON CD34+VE MNC

The CD34 cell surface antigen identifies a population of myeloid progenitors that have high proliferative potential [Brandt et al, 1988]. Cells that carry this antigen comprise 1-4% of bone marrow cells in man and some primates and can provide complete haemopoietic reconstitution in lethally irradiated baboons [Berenson et al, 1988]. In man such cells have been shown to be important in haemopoietic reconstitution by autologous PBSC after high dose chemotherapy [Siena et al, 1989].

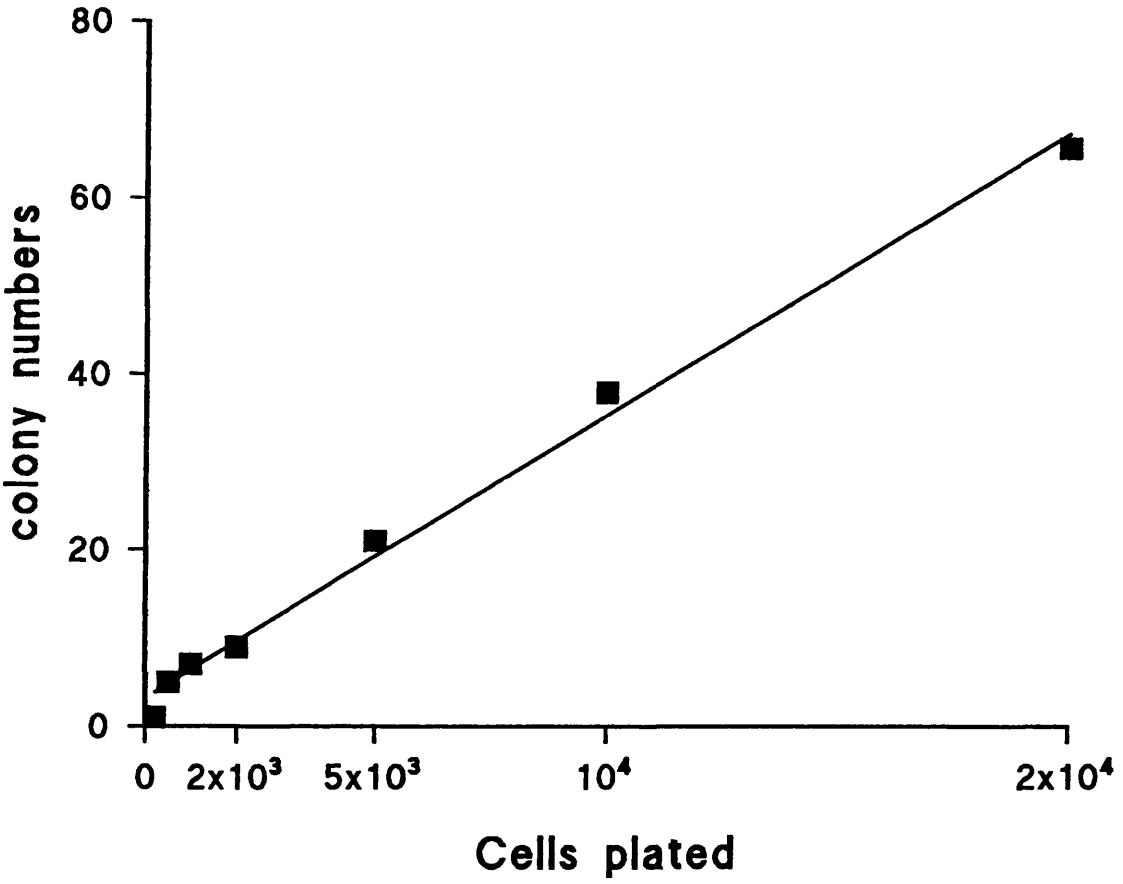
CD34+ve BMMNC were isolated from normal donor BM by positive selection with anti-HPCA-1 (Anti-CD34) as described in Chapter 2,2.2.

Preliminary experiments were done to determine the optimal cell density for clonogenic assays by plating increasing numbers of CD34+ve cells in soft agar overlayers above a soft agar underlay containing 100µl of 5637CM.

Figure 4,7.1 (Page 177) shows that between cell densities of $2x10^3$ and $2x10^4$ the relationship between the numbers of cells plated and the numbers of colonies obtained was approximately linear. The plating efficiency of CD34+ve cells was considerably higher than that of unfractionated BMMNC. In further experiments CD34+ve cells were seeded at $5x10^3$/plate.

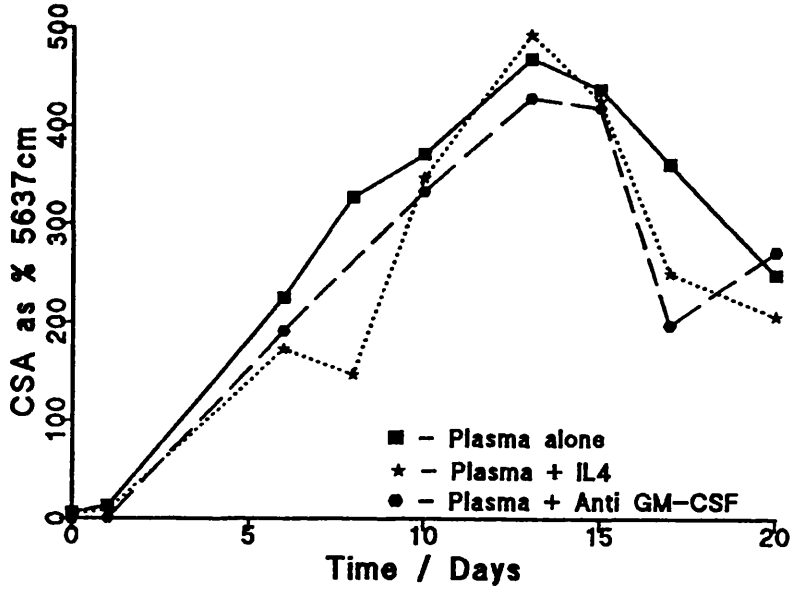
Samples of plasma taken sequentially from 3 patients were tested against unfractionated MNC and CD34+ve cells (from the same donor in 2 patients and different donors in 1 patient) with and without the addition of IL-4 *in-vitro*, in all patients, and Antibody to GM-CSF, in one patient. Fig 4,7.2, page 178 and Figs 4,7.2-4, Appendix II)

Fig 4,7.1



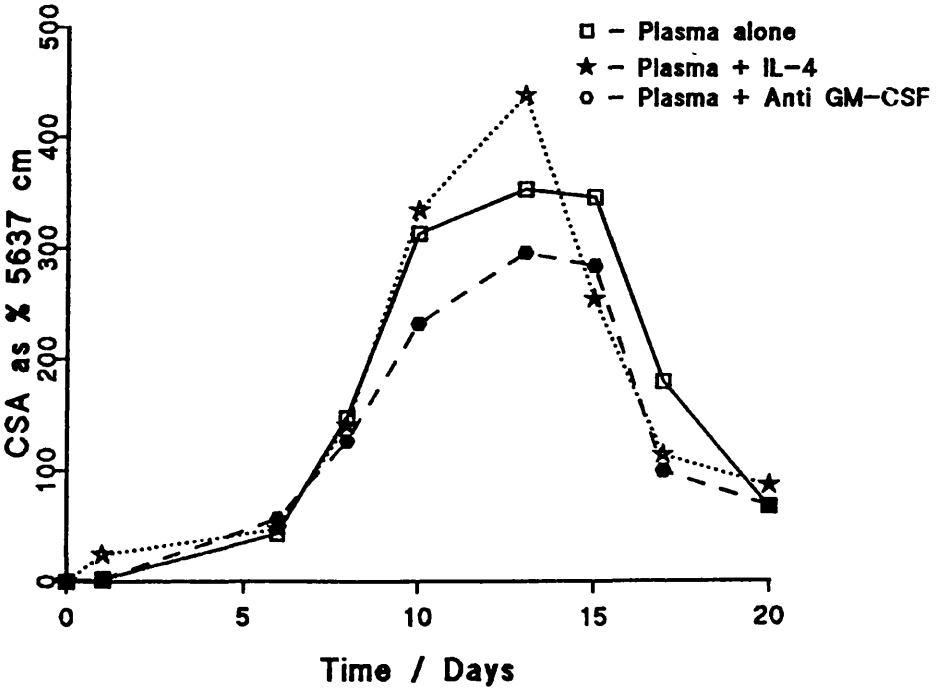
Effect of cell number plated on colony number
for CD34+ve BMMNC against 5637CM

Fig 4,7.2.A



Plasma of Pt 7 tested against unfractionated BMMNC
with/without IL–4 or Antibody to GM–CSF

FIG 4,7.2.B



Plasma of Pt 7 tested against CD 34+ve BMMNC
(same donor as above) with/without IL–4 or Anti–GM–CSF

The time course of CSA was similar when unfractionated BMMNC or CD34+ve cells were used as the target population. The effects of IL-4 and A-GM added *in-vitro* were also similar in both cell populations.

DISCUSSION

CD34+ve cells are thought to be early progenitors because of their high proliferative potential and because cells bearing this antigen give rise to the full complement of haemopoietic elements. Early progenitors respond to factors such as IL-3 and GM-CSF which can induce the proliferation of colonies comprising cells of multiple lineages. In contrast, G-CSF is thought to stimulate the proliferation of more mature elements although administration of G-CSF has been associated with stimulation of multiiineage coionies (Chapter 1,4.3.b). The data show that CD34+ve cells respond to CSA in plasma that contains G-CSF-like activity which is augmented by IL-4 in a similar fashion to that of unfractionated MNC. This suggests that these early progenitors can behave like more mature elements when exposed to an appropriate combination of factors, namely, that found in plasma during recovery from high dose chemotherapy.

In patients 7 and 29, particularly, there is a suggestion that the augmentation of CSA by IL-4 may be greater in CD-34+ve cells than unfractionated cells. It may, however, indicate that, unlike unfractionated cells, the CD-34+ve MNC were not maximally stimulated at these times and were able to respond further to an additional stimulus.

4,8 LOW TITRE CSA PLASMA

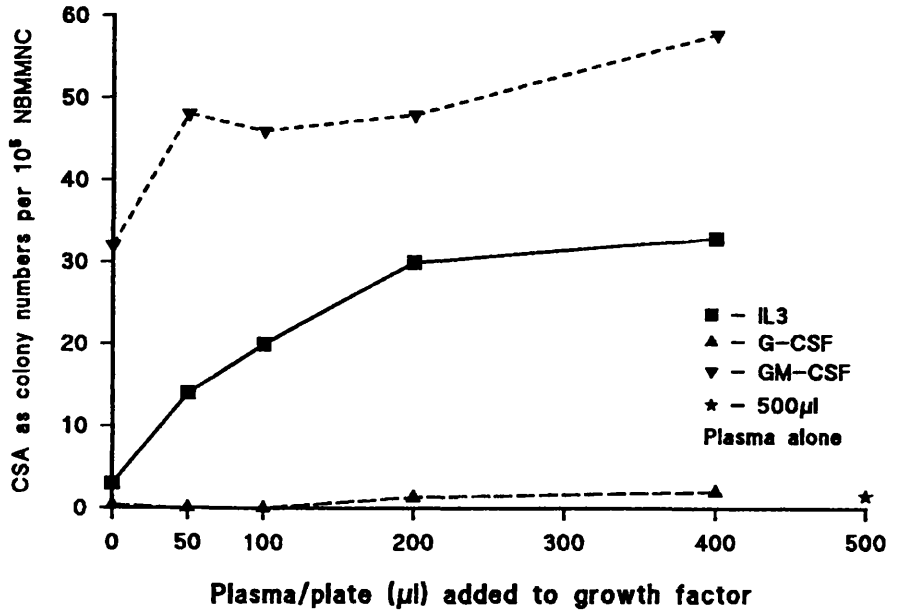
The CSA in plasma collected in the first 3 days after HDM was low and was designated "low titre plasma" (LTP). To determine whether this was due to the presence of inhibitors of haemopoiesis, known amounts of rhIL-3, rhGM-CSF and rhG-CSF were added to Petri dishes in the presence of 50-400μl of plasma pooled from patients on day 0 (Experiment 1) and days 0-2 (Expts. 2-4) after treatment. 10^5 MNC from normal donor bone marrow were added in soft agar and α-medium (Chapter 2,2.3.b/c).

The results of two such experiments using different pools of LTP and different normal BMMNC populations are shown in figures 4,8.1-2. Page 181.

In both experiments LTP failed to inhibit either GM-CSF or IL-3 but augmented the activity of these growth factors in proportion to the volume of LTP added. The normal BMMNC populations differed in the number of colonies grown, the shapes of the dose response curves for IL-3 and the fact that IL-3 induced greater colony numbers than GM-CSF in one experiment (Fig 4,8.2) but less than GM-CSF in the other (Fig 4,8.1). However, the effect of the combination of plasma with the cytokines was similar for both target cell populations in that the augmentation of both IL-3 and GM-CSF by LTP was greater than additive (as illustrated by the activity of 500μl of low titre plasma alone shown in each figure by a⋆).

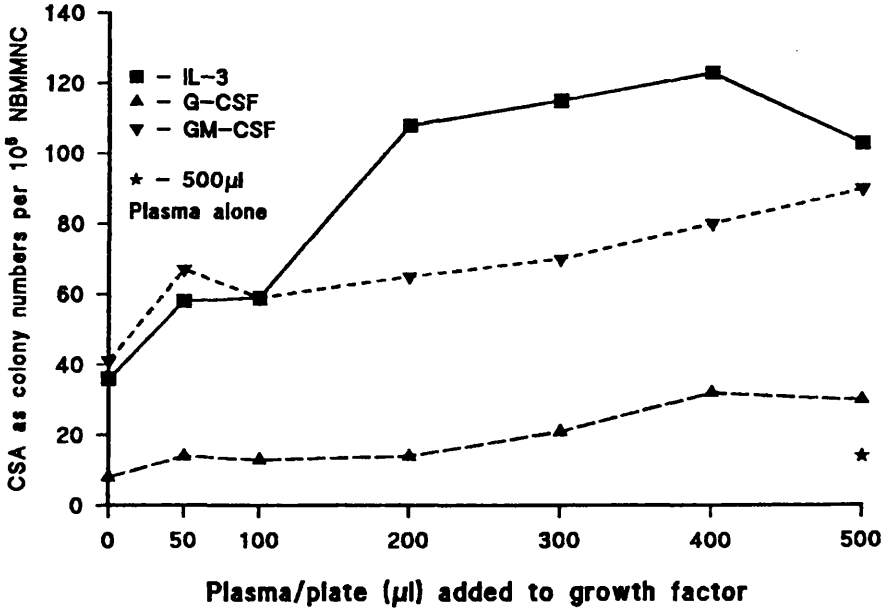
In the first experiment (Fig 4,8.1) 5ng of rhG-CSF did not stimulate colony formation by the MNC used. in the second experiment (Fig 4,8.2) the effect of plasma with 5ngG-CSF was additive.

FIG 4,8.1



Effect of addition of increasing volumes of low CSA titre plasma to IL–3 (50ng/1ml), GM–CSF (50ng/1ml) or G–CSF (5ng/1ml)

FIG 4,8.2



Effect of addition of increasing volumes of low CSA titre plasma to IL–3, GM–CSF and G–CSF as above

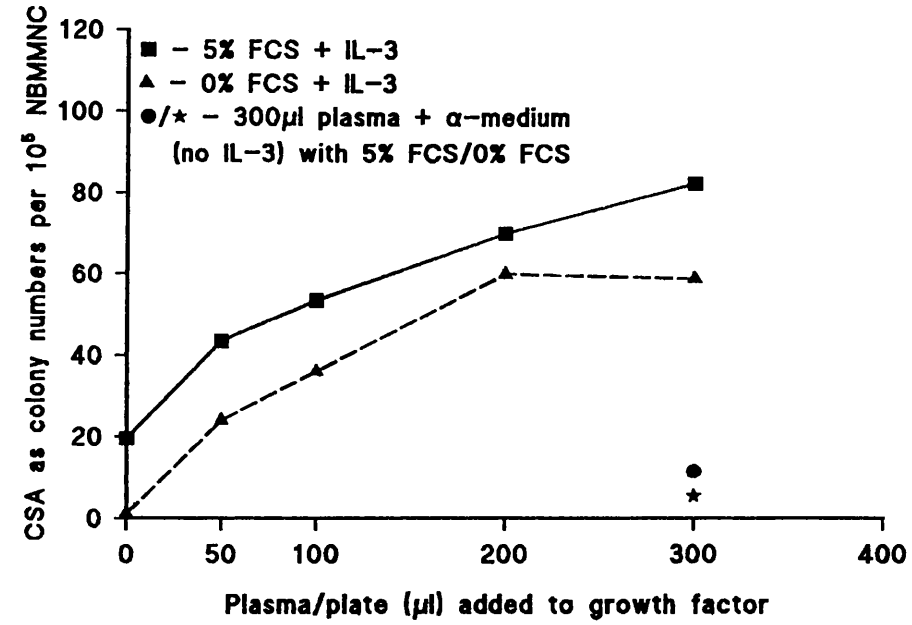
The results show that a factor(s) was present in plasma collected before and/or within two days of treatment with HDM that could augment the activity of GM-CSF or IL-3.

Although the results suggest that the enhancement of colony formation by GM-CSF and IL-3 was dependent on the concentration of plasma, a further possibility was that as the volume of plasma was increased there was a reduction of inhibitory molecules present in foetal calf serum in the culture medium used to maintain a constant sample volume of 500μl (Chapter 2,1.4).

LTP was tested in volumes of 50-300μl/Petri dish in combination with 50ng of IL-3. The volume of each sample was made up to 500μl using α-medium containing 0% or 5% FCS. IL-3 was tested alone using both media.

Figs 4,8.3 (Page 183) shows that LTP increased the colony numbers grown in combination with IL-3 in a dose dependant manner. This occurred despite a lack of intrinsic CSA. Addition of FCS to the growth medium resulted in an increase in the numbers of colonies grown at each concentration of plasma, demonstrating that FCS was not a source of inhibitory molecules in the assay.

Fig 4,8.3



Addition of low CSA titre plasma to IL–3 (50ng/1ml) in α–medium with and without 5% foetal calf serum

DISCUSSION

These experiments show that plasma collected from patients before HDM and/or up to two days after HDM contain molecules that augment the activity of IL-3 or GM-CSF but not G-CSF even though such plasma has little myelopoietic activity when used as a sole source of growth factor(s). Of the known cytokines, IL-1 and IL-6, which do not stimulate myelopoiesis directly, can enhance the efficacy of IL-3 and GM-CSF *in-vitro* [Caracciolo et al, 1989, Rennick et al, 1989 (IL-6), Zhou et al, 1988, Moore, 1991 (IL-1)] but not G-CSF.

FCS augmented the activity of recombinant growth factors added to LTP but the ability of LTP to augment the activity of IL-3 or GM-CSF was not dependant on the presence of FCS.

Changes in the concentrations of co-factors and/or inhibitors of myelopoiesis in the plasma of patients after high dose chemotherapy may be as important as the presence of growth factors and their identification could influence the timing and choice of growth factors administered to promote haemopoiesis after intensive therapy.

CHAPTER 5

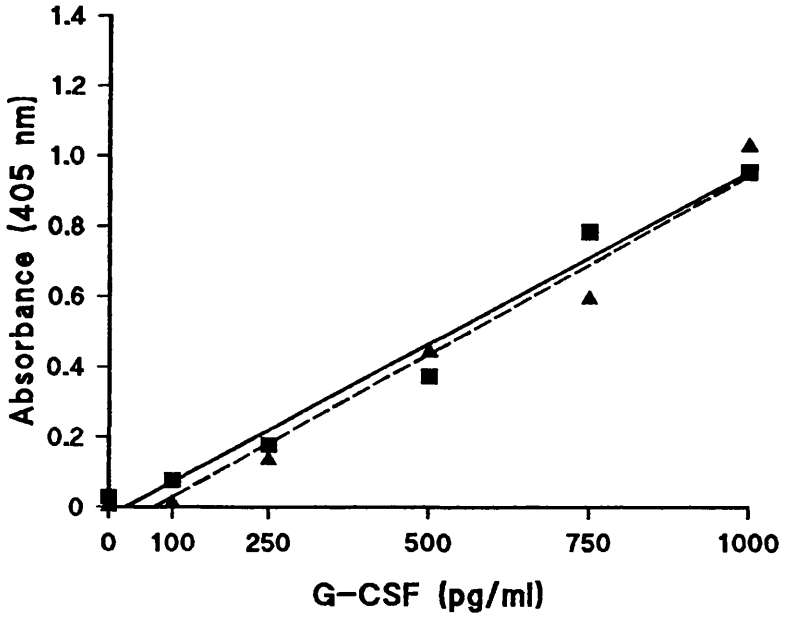
ASSAY OF PLASMA PROTEINS AND CYTOKINES

5,1 ENZYME LINKED IMMUNOASSAY OF G-CSF

Haemopoietic colony stimulating activity has been found in the plasma of patients following high dose chemotherapy with or without autologous bone marrow rescue. Addition of IL-4 to plasma *in-vitro* augmented the activity of rhG-CSF which had been administered to patients, furthermore, IL-4 augmented an activity in patient plasma that could not be attributed to rhG-CSF.

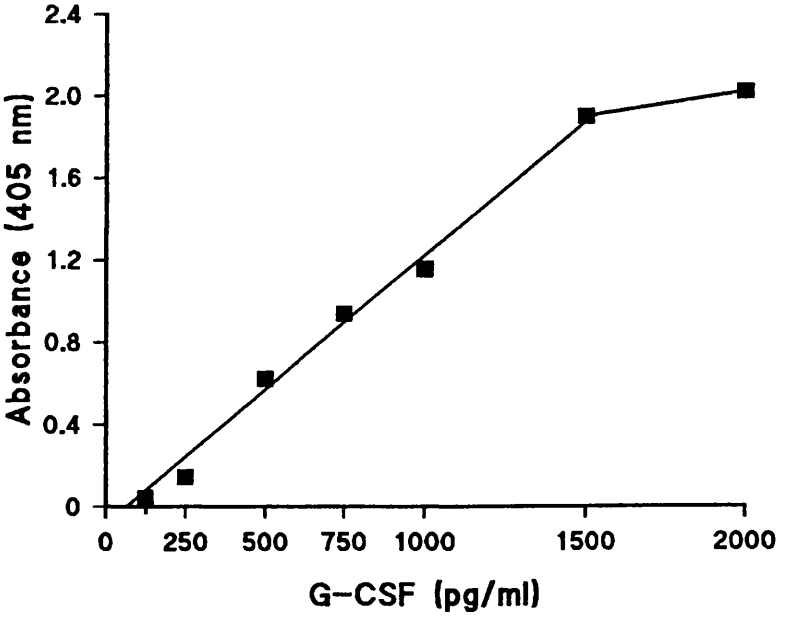
A commercially available enzyme-linked immunoassay (Chapter 2,2.5a) was used to examine plasma and serum samples for the presence of G-CSF. In each experiment standard curves were constructed to enable quantification of the activity in test samples. In two experiments standard curves were constructed with known concentrations of rhG-CSF supplied by the manufacturers. In a further experiment standard concentrations of rhG-CSF were prepared by dilution of a concentrated (2000pg/ml) standard solution (supplied by the manufacturers of the assay) in a pool of normal human serum which had been found to have no detectable G-CSF in an earlier assay. The Standard curves are shown in Figures 5,1.1-2, page 186.

FIG 5.1.1



Standard curves of G-CSF Vs Absorbance
Two experiments using supplied standards

FIG 5.1.2



Standard curve of G-CSF Vs Absorbance
Standards prepared in pooled plasma

Known concentrations of G-CSF prepared in the laboratory, in plasma (Fig 5,1.2), produced a standard curve which was similar to that indicated by the manufacturers guide and enabled the range of the assay to be extended to 1500ng/ml. The relationship between the amount of rhG-CSF and the absorbence at 405nm was essentially linear between 100 and 1500pg/ml. In contrast, there was less absorbence using standard concentrations of rhG-CSF provided by the manufacturers (Fig 5,1.2).

in preliminary experiments samples of plasma were collected immediately before and 1-2 hours after patients had received an infusion of rhG-CSF (5μg/kg) or a placebo.

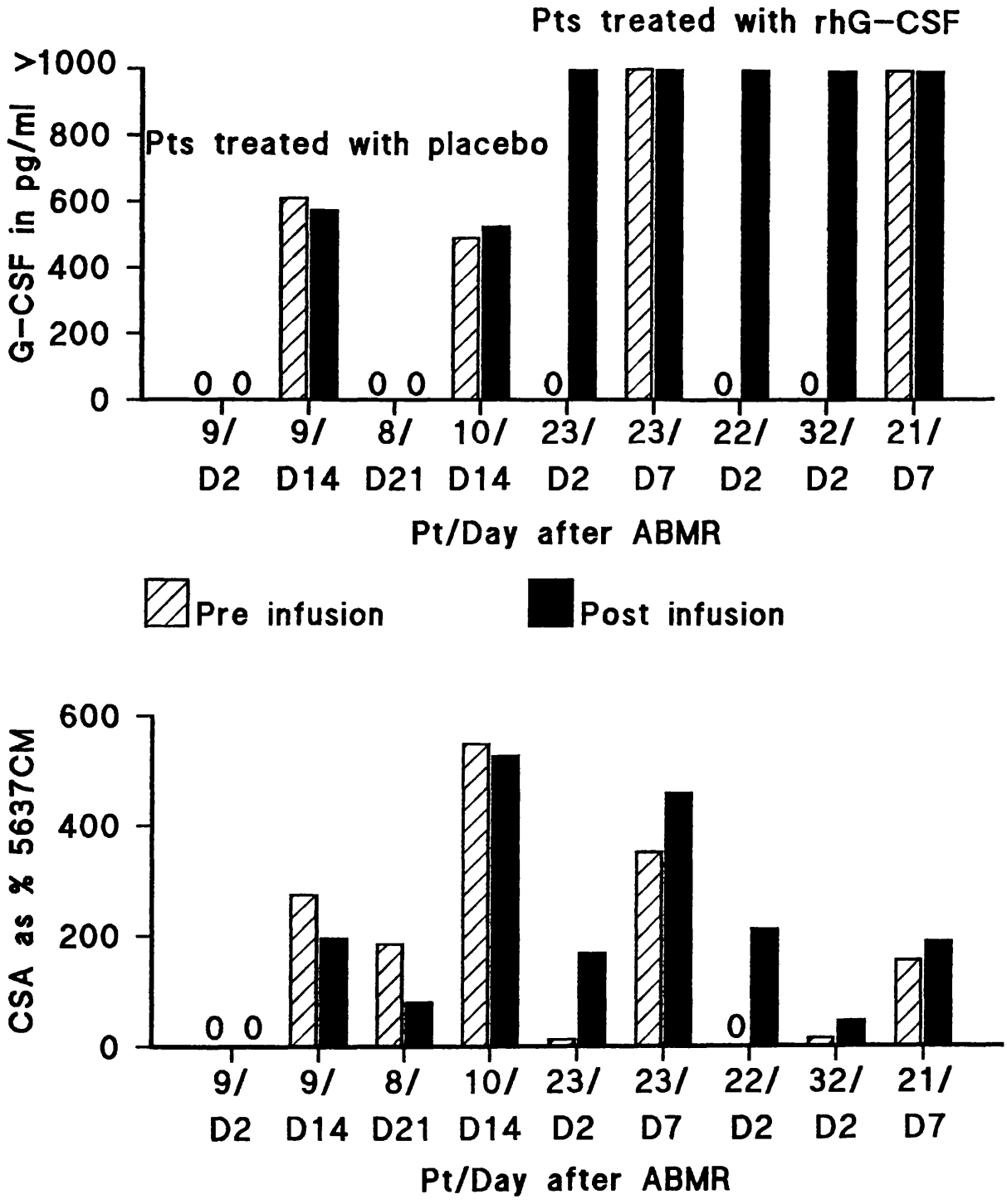
Figure 5,1.3 (Page 188) compares the levels of G-CSF in plasma before and after infusion of rhG-CSF or placebo with the levels of CSA (relative to 5637CM) that were found in the same samples (Chapters 2,2.3 and 4,2-4).

Samples collected on day 2 after ABMR before infusion of rhG-CSF or placebo contained no detectable G-CSF. In patients who received placebo there was no significant change after the infusion, whereas in those who had received rhG-CSF this was detected in the plasma. The increase in plasma G-CSF was associated with an increase in CSA.

In samples collected on days 7 and 14 after ABMR, when CSA was elevated, G-CSF was detected in plasma samples collected before the infusion of rhG-CSF or placebo in all 4 patients and CSA was present in the same plasma samples. On day 21, in patient Pt 8, CSA was detectable in the plasma but G-CSF was not.

In these experiments plasma samples were not diluted so levels of G-CSF greater than 1000pg/ml were not quantified.

Fig 5,1.3



Plasma G-CSF Pre/Post infusion
of rhG-CSF or placebo
and comparative changes in CSA

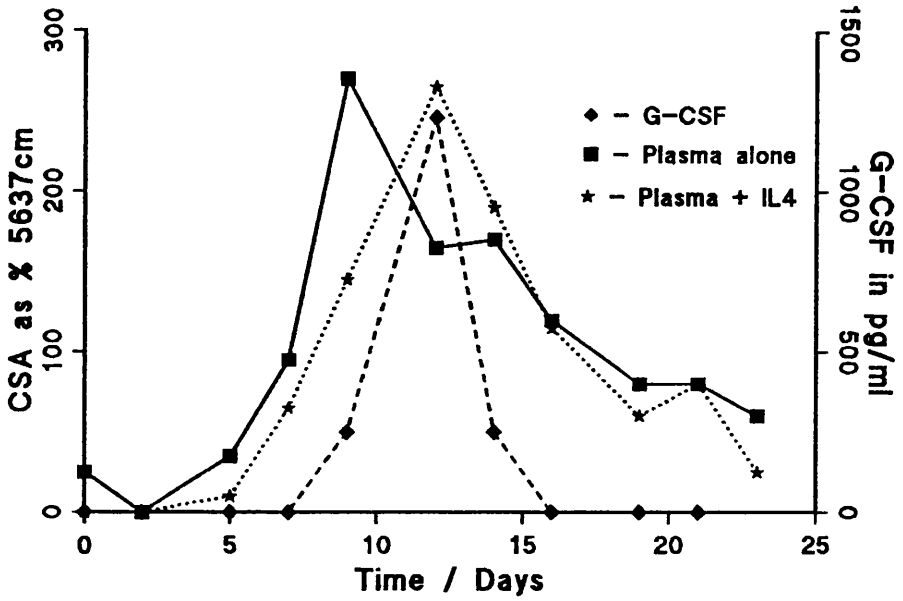
Plasma samples from 7 patients, collected sequentially after treatment, were examined for the presence of G-CSF. All of the samples had been tested for biological activity and samples from 5 of these patients had been tested for CSA with the addition of IL-4 (5ng) *in-vitro* (Chapter 4,5).

Examples of data from 2 patients are shown in Figures 5,1.5 and 5,1.7, below. Data for all patients are shown in Figures 5,1.4-10 (Appendix II). Plasma G-CSF levels are shown compared to those of CSA with and without the addition of IL-4 *in-vitro*. The plasma samples of patients 8,11,18,19 & 22 were diluted 50% in pooled normal plasma (with no G-CSF) to extend the range of the assay. In this experiment the standard curve was also extended to 1500pg (see above) so levels of up to 3ng of G-CSF could be measured in these patients. In experiments with undiluted plasma the higher limit of detection was 1ng. The lower limit of detection was between 100 - 125pg/ml.

In each patient treated with HDM & ABMR plasma G-CSF peaked at a similar time to that of CSA. The time course of G-CSF after intensive therapy was similar to that for plasma CSA in combination with IL-4 rather than plasma alone. G-CSF was detected in every sample in which IL-4 augmented the activity of plasma CSA.

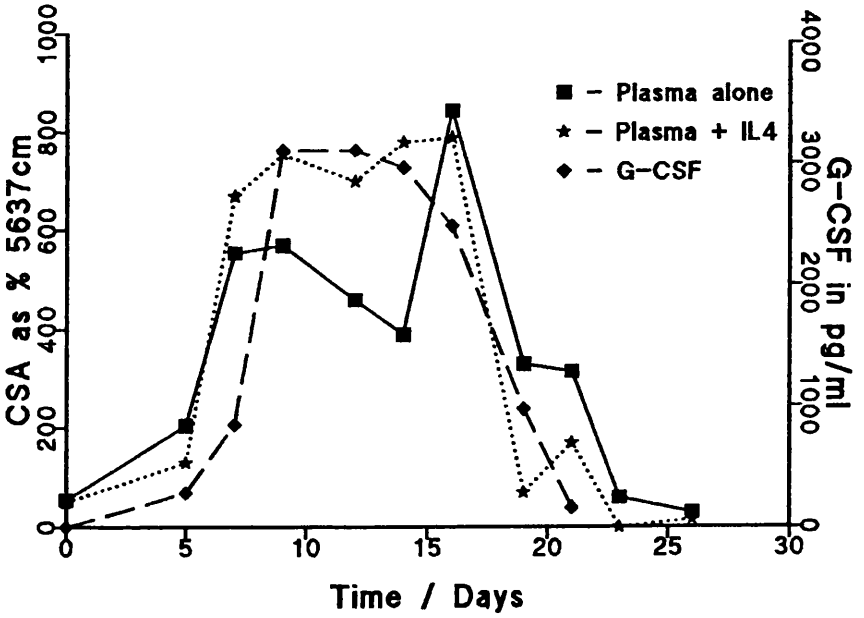
In the patient treated with HDBu (Fig 5,1.10) the peak of G-CSF occurred after that of CSA.

FIG 5,1.5



Patient 11 – Comparison of CSA +/– IL–4 with plasma G–CSF after HDM and ABMR (No rhG–CSF)

FIG 5,1.7

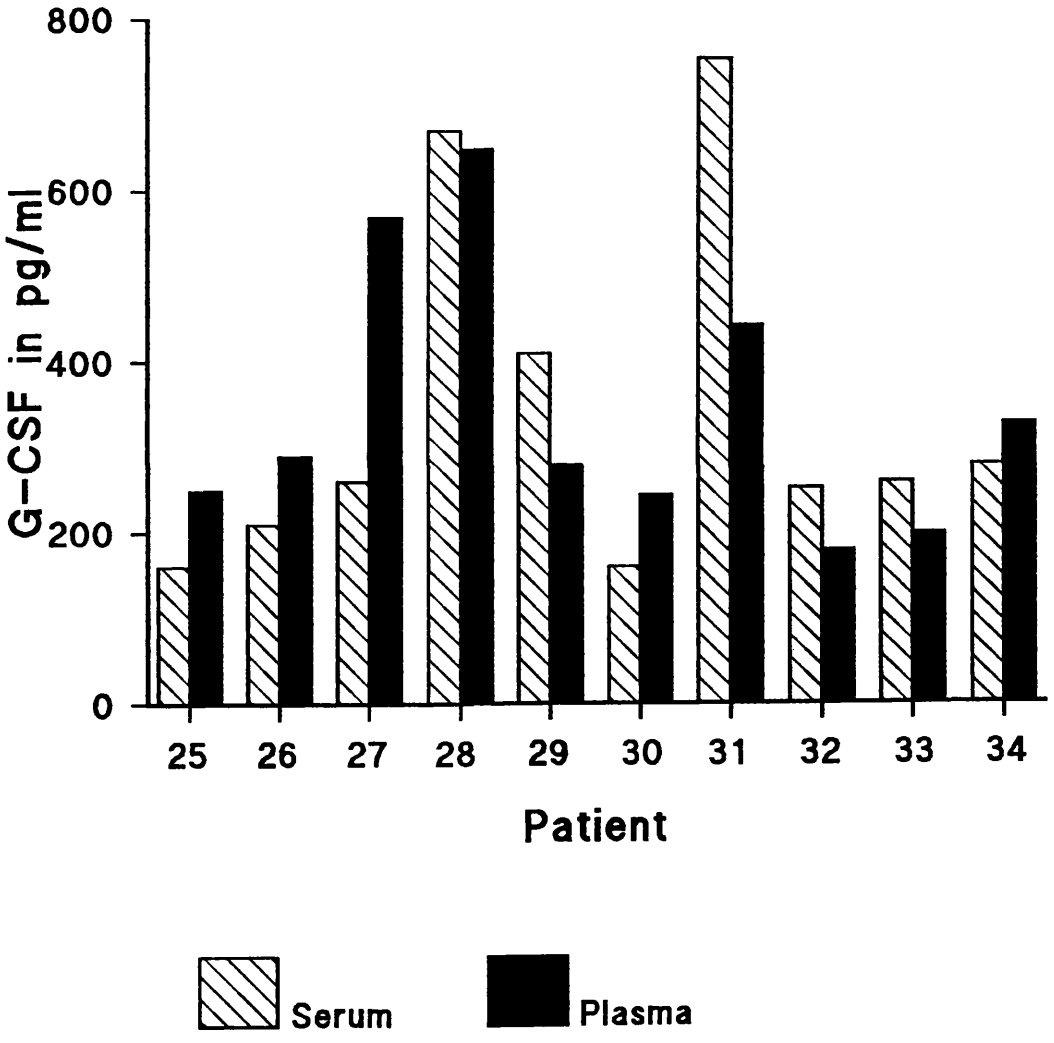


Patient 19 – Comparison of CSA +/– IL–4 with Plasma G–CSF after HDM & ABMR + rhG–CSF

Because CSA is greater in plasma than in serum (Chapter 3,2), the levels of G-CSF were compared in samples of plasma and serum collected from 10 patients. The data are shown in Figure 5,1.11, page 192.

There was no significant difference between the levels of G-CSF between plasma and serum (2 tail p=0.7, Students' paired *t*-test).

Fig 5,1.11



Comparison of G-CSF levels in plasma and serum

DISCUSSION

The close relationship between the curves of plasma G-CSF and CSA augmented by rhIL-4 (Chapter 4,5) suggest that the effect of IL-4 added *in-vitro* was due to the presence of endogenous G-CSF [Broxmeyer et al, 1988]. Furthermore, these results support the suggestion (Chapter 4,5) that failure to augment CSA by IL-4 in some patients may have been due to maximal stimulation of CFUc in the target MNC by G-CSF and other cytokines present in plasma. In patient 11 (Fig 5,1.5), for example, maximum CSA occurred on day 9 when G-CSF was detected by ELISA but IL-4 did not augment CSA. On day 12 CSA had fallen, G-CSF was present, and IL-4 augmented colony formation so that colony numbers from the target MNC were similar to those seen with day 9 plasma. Also IL-4 increased colony formation on day 14 when a low concentration of G-CSF was detected, but not on day 16 when G-CSF was absent from the plasma sample.

In a previous study serum G-CSF levels were measured at various times during induction therapy for acute leukaemia in 18 patients [Sallerfors and Oloffson, 1991] and following ABMT in 13 patients and ABMR in 8 patients [Sallerfors et al, 1991]. After ABMT or ABMR no pattern of change in G-CSF was observed with time and there was no correlation of G-CSF levels with infection although sequential samples were not collected from individual patients. However, during induction therapy for AML there was an inverse relationship between G-CSF concentration and WCC ($r = -0.495$, $p<0.001$). This association was ascribed to infective episodes, however, the documented infections occurred during the patient's leucopenic nadirs and it seems equally likely that elevated levels of G-CSF were related to low WCCs. An inverse relationship between G-CSF and

neutrophil levels was also found in patients with aplastic anaemia [Watari et al, 1989] but in that study a direct relationship between G-CSF levels and neutrophil count was found after chemotherapy in patients with lung cancer and a range of haematological malignancies. A factor that may differentiate these patients from those observed following BMT or intensive induction therapy is the development of leucopenia. The mechanisms that control G-CSF release may be adjusted or modified according to conditions *in-vivo*. For example, after a "priming" dose of cyclophosphamide (Chapter 4,1) there was a decrease in CSA but no neutropaenia, however after intensive chemotherapy which produced neutropaenia in the same patients (i.e. HDM, Chapters 4,2&3) there was a rise in CSA and endogenous G-CSF.

In a study of 18 patients with various haematological malignancies [Janowska-Wieczorek et al, 1990] serum G-CSF was found to peak at day 6 after ABMT or ABMR. This time is similar to the day 7 peak of CSA in leaukaemics observed by Millar et al [1992]. In the present study CSA and plasma G-CSF also reached their maxima at day 7 in patients treated with HDM & ABMT with rhG-CSF but the peaks of both CSA and G-CSF in patients treated with ABMT without G-CSF were delayed compared to the studies by Millar et al [1992] and Janowska-Wieczorek et al [1990].

It was suggested that the administration of high dose methyl-prednisolone (HDMP) to patients receiving ABMT might have impaired the production of CSA (Chapter 4,4) and that administration of rhG-CSF to these patients may have overcome this effect, returning the pattern of CSA to that seen in leukaemics [Millar et al, 1992] who did not receive HDMP. Since the time curves of CSA and G-CSF are similar, the putative effect of HDMP could be explained by an inhibition of production of endogenous G-CSF which might then be overcome by administration of rhG-CSF acting directly on cells that

produce G-CSF to initiate a surge of G-CSF by a positive feedback mechanism. The fact that no evidence for production of endogenous G-CSF was seen in phase I studies is not exclusive of this suggestion since phase I studies were carried out in patients with normal haematological indices and it is likely (as stated above) that responses would be different after ablative doses of chemotherapy.

Although CSA was always demonstrable in plasma that contained G-CSF, the plasma samples in which G-CSF could not be detected were not consistently lacking in CSA. For example in patients 8 and 11 CSA was present on days 16 - 21 (and beyond) but G-CSF was not (Fig 5,1.4&5). There was no effect when IL-4 was added to the plasma of patient 11 at this time. Colony morphology also indicated a qualitative change in CSA, with a change from G-CFUc to GM-CFUc as CSA declined and levels of endogenous G-CSF decreased. The same discrepancy between CSA and plasma levels of G-CSF was seen in the patient treated with HDBu (Fig 5,1.10).

These experiments suggest (Chapters 4,3 & 4,5) that there are qualitative as well as quantitative changes in CSA with time after high dose chemotherapy. Although G-CSF appears to be important during the period of maximal CSA, at later times, when WCC and neutrophils have begun to recover, other cytokines must contribute to CSA.

During recovery from ablative therapy it is likely that the recovery and/or response of different haemopoietic or non-haemopoietic cell populations is influenced by activity within other lineages and that the cytokine content of plasma (and CSA) changes as a result of the rate at which individual cell types reconstitute and repopulate. As the normal BMMNC used in the assay contain haemopoietic progenitors that can give rise to

colonies of multiple lineages, depending on the conditions in the assay, this may explain the change in colony morphology that was observed when BMMNC were grown in plasma collected at different times after intensive chemotherapy.

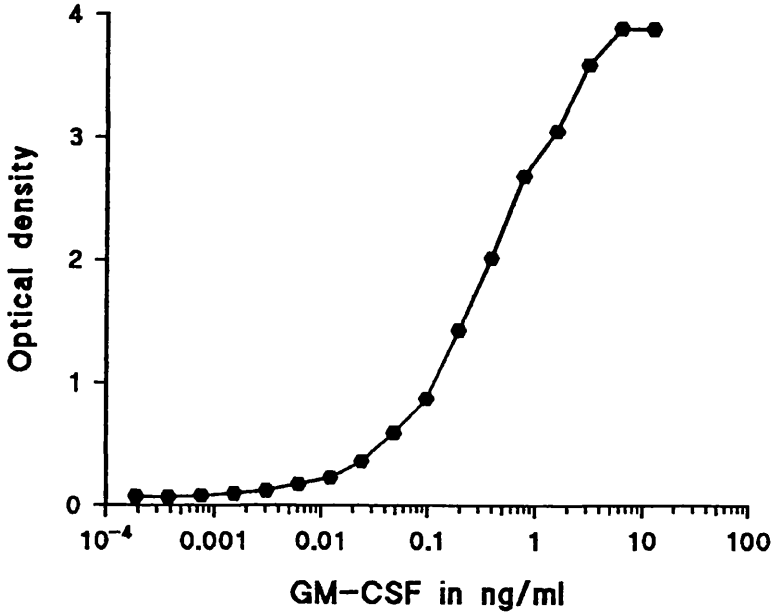
5,2 ENZYME LINKED IMMUNOASSAY OF GM-CSF

Antibodies to GM-CSF abolished CSA in serum from patients with MM [Millar et al, 1990] and also partially inhibited CSA after HDM with or without ABMR (Chapter 4,6). These data suggest that GM-CSF-like activity contributes towards CSA.

An enzyme-linked immunoassay (Chapter 2,2.5.d) developed within the Section of Medicine Laboratories at The Institute of Cancer Research (Dr S. Denham, unpublished) was used for the detection and quantification of GM-CSF in samples of plasma and serum.

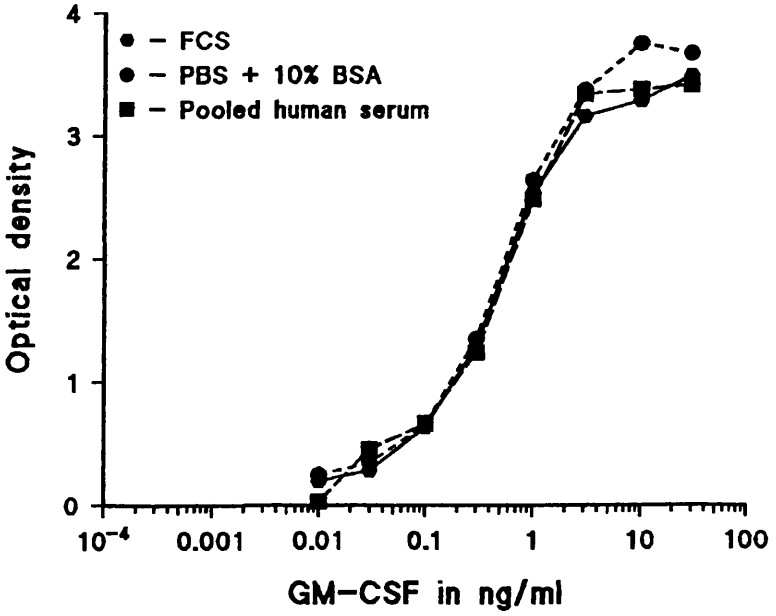
Standard curves of GM-CSF Vs absorption were prepared as described in Chapter 2,2.5.d. Known quantities of rhGM-CSF were first diluted in foetal calf serum (FCS) (Fig 5,2.1 page 198). The log/linear curve of GM-CSF Vs absorbence was 'S'-shaped but approximately linear in the range 100-3000pg/ml. There was no difference in the curve when standards were diluted in FCS, pooled human plasma (AB Neg pooled plasma - Blood Transfusion service) or PBSA with 10% bovine serum albumin (BSA) (Figure 5,2.2). Further standard curves were prepared in PBSA with 10% BSA.

Fig 5,2.1



Standard curve of GM-CSF in FCS
Vs Absorption (1)

Fig 5,2.2



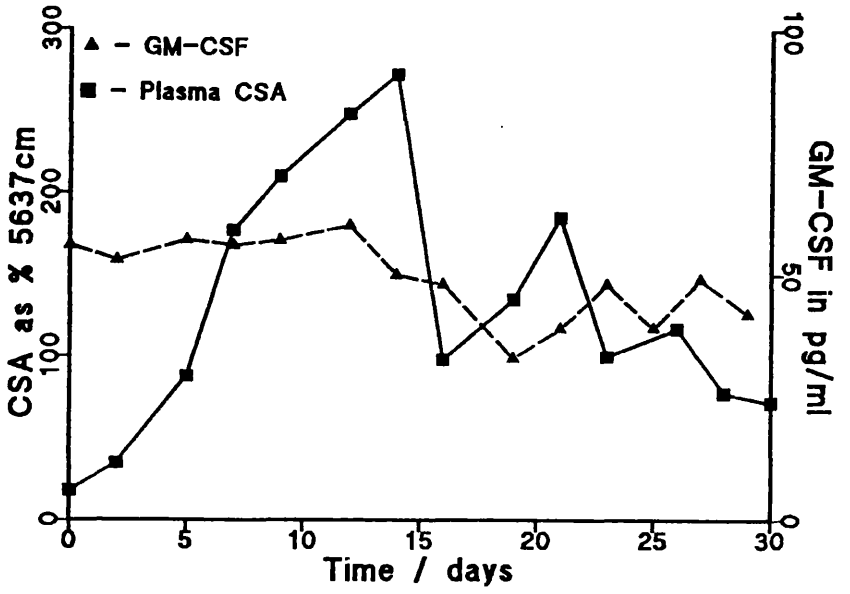
Comparison of GM-CSF standards
prepared in different media

Samples of plasma collected sequentially after HDM from 4 patients were tested for the presence of GM-CSF. In 3/4 patients low levels of GM-CSF (between 20-60pg/ml) were detected throughout the recovery time after treatment. The data are shown in figures 5,2.3-5 (pages 200-1). No GM-CSF was detected in the plasma of patient 15.

Levels of GM-CSF were similar in each patient and were near the lower limit of detection of the assay. When pure recombinant GM-CSF is used as a proliferative stimulus alone or in combination with other cytokines, concentrations of less than 5ng/ml do not result in colony formation. Thus the concentration of GM-CSF *in-vivo* is considerably less than that required to stimulate haemopoiesis *in-vitro*.
Despite the low levels of GM-CSF that were detected in plasma, CSA was inhibited by approximately 50% using antibody to GM-CSF in most samples after intensive therapy (Chapter 4,6 and Fig 5,2.4). Furthermore, the amount of colony inhibition produced by different concentrations of antibody to GM-CSF suggested that the concentration of the cytokine in plasma might be as much as 20ng/ml.

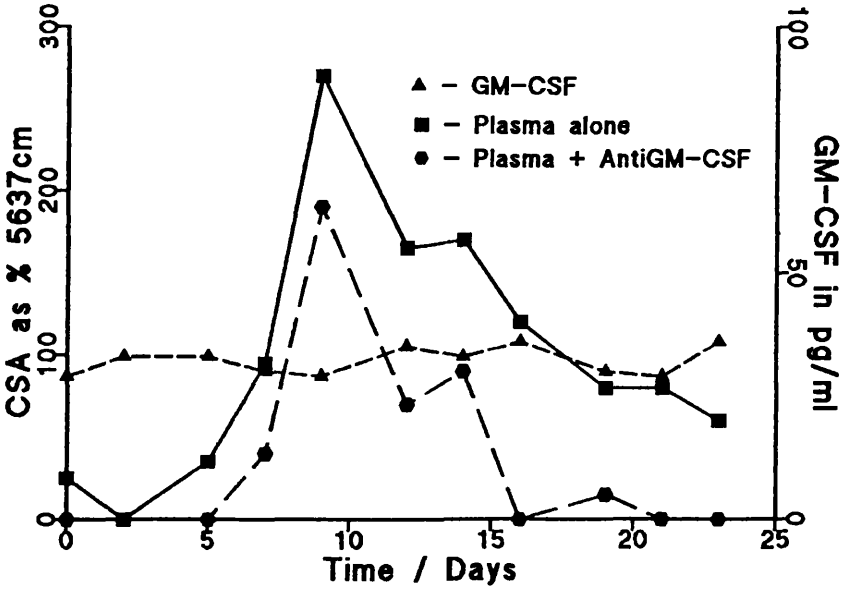
The discrepancy between the biological assay and the concentration of GM-CSF detected by ELISA could have occurred because serum proteins inhibited the binding of GM-CSF to the primary antibody used to coat the microtitre plate. To examine this hypothesis, samples of plasma from patient 29 were diluted 1:3 and 1:9 in PBSA and tested for the presence of GM-CSF using the ELISA. Sufficient plasma was not available to test all samples at each dilution. If the assay was detecting an accurate level of GM-CSF in plasma, dilution of the samples should have resulted in a proportionally constant decrease in detected GM-CSF.

Fig 5.2.3



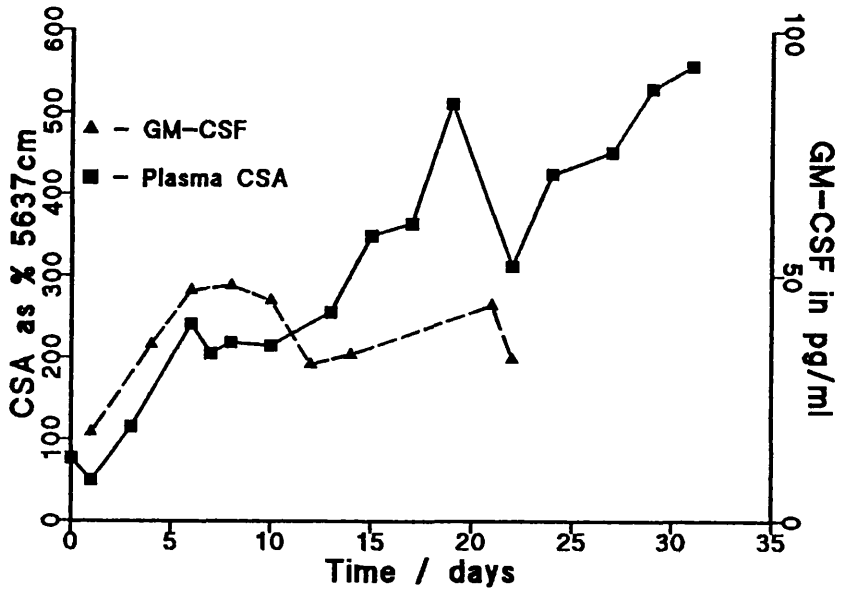
Patient 8 – Comparison of CSA with
Plasma GM–CSF after HDM and ABMR

Fig 5.2.4



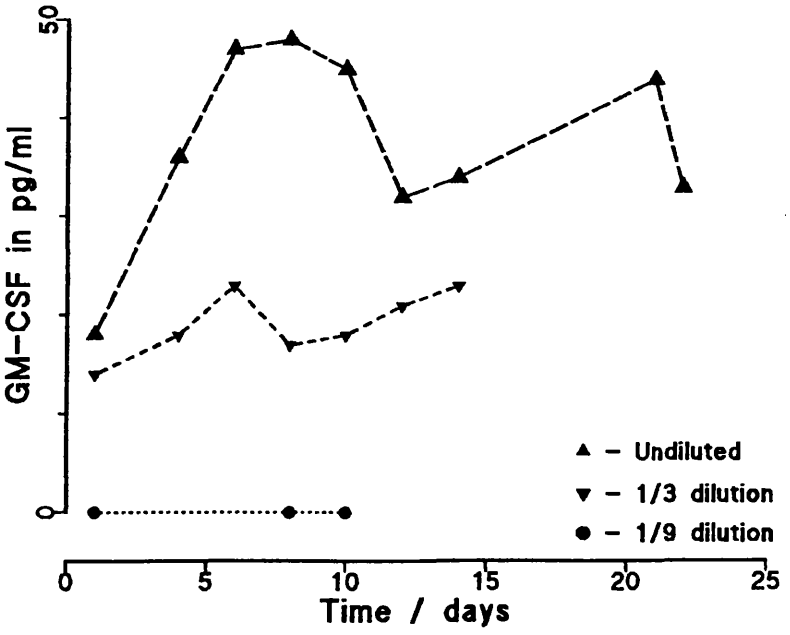
Patient 11 – Comparison of CSA +/– Anti GM–CSF
with plasma GM–CSF after HDM & ABMR

Fig 5.2.5



Patient 29 – Comparison of CSA with Plasma GM–CSF after HDM without ABMR

Fig 5.2.6



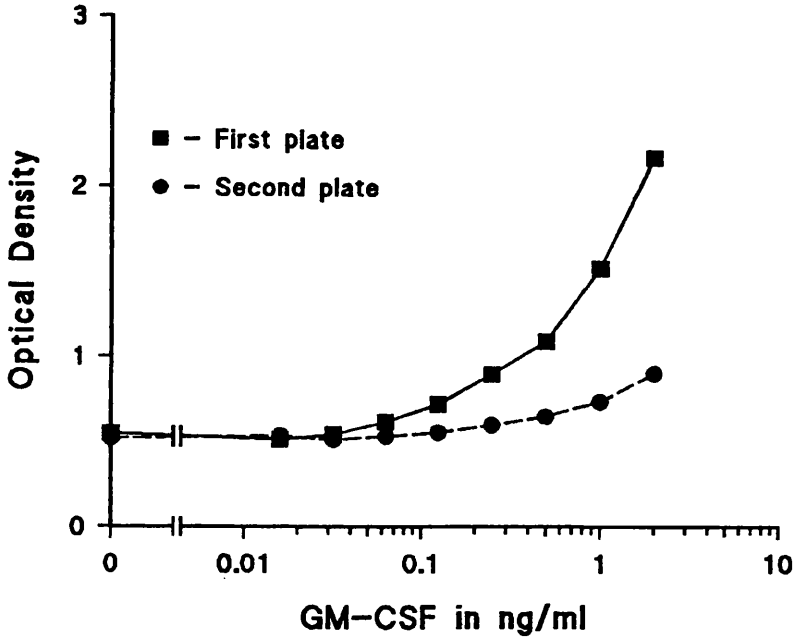
Patient 29 – GM–CSF levels after serial dilution of plasma

Conversely, data in figure 5,2.6 (Page 201) shows that the ratio of GM-CSF in μg/ml in non-diluted samples compared with samples diluted 1/3 was not constant, indicating that non-specific binding may have interfered with the assay.

To examine the possibility that the very low levels of GM-CSF that were detected were entirely due to non-specific binding of other proteins (and that no GM-CSF was present in the samples), a second experiment was conducted in which samples of plasma were incubated for two hours on a microtitre plate coated with monoclonal antibody to GM-CSF and then transferred to a second plate coated with the same antibody and incubated for a further two hours. Both plates were then processed according to the method described (Chapter 2,2.5d). Binding of GM-CSF to antibody in the first plate should reduce the amount of cytokine detected by the second plate. Whereas, similar readings on both plates would indicate non-specific binding of plasma proteins. Two standard curves for rhGM-CSF, one in PBSA and one in pooled serum were prepared (Figures 5,2.7/8, page 203). Eight samples of normal donor plasma and samples of 2 low titre CSA plasma pooled from several patients after HDM (Chapter 4,8) were also tested (Figure 5,2.9, page 204).

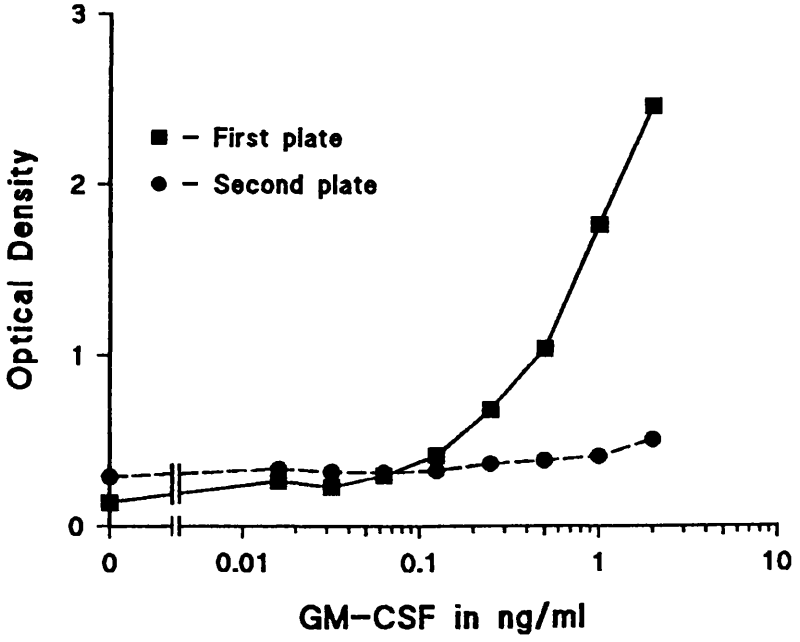
Figures 5,2.7&8 show that most rhGM-CSF was bound to the first plate. The lower limit of detection of the assay was approximately 100pg/ml and below this level there was a constant 'background' level that was not diminished by incubation on the second antibody coated plate. Samples of diluent containing no rhGM-CSF had similar background activity.

Fig 5.2.7



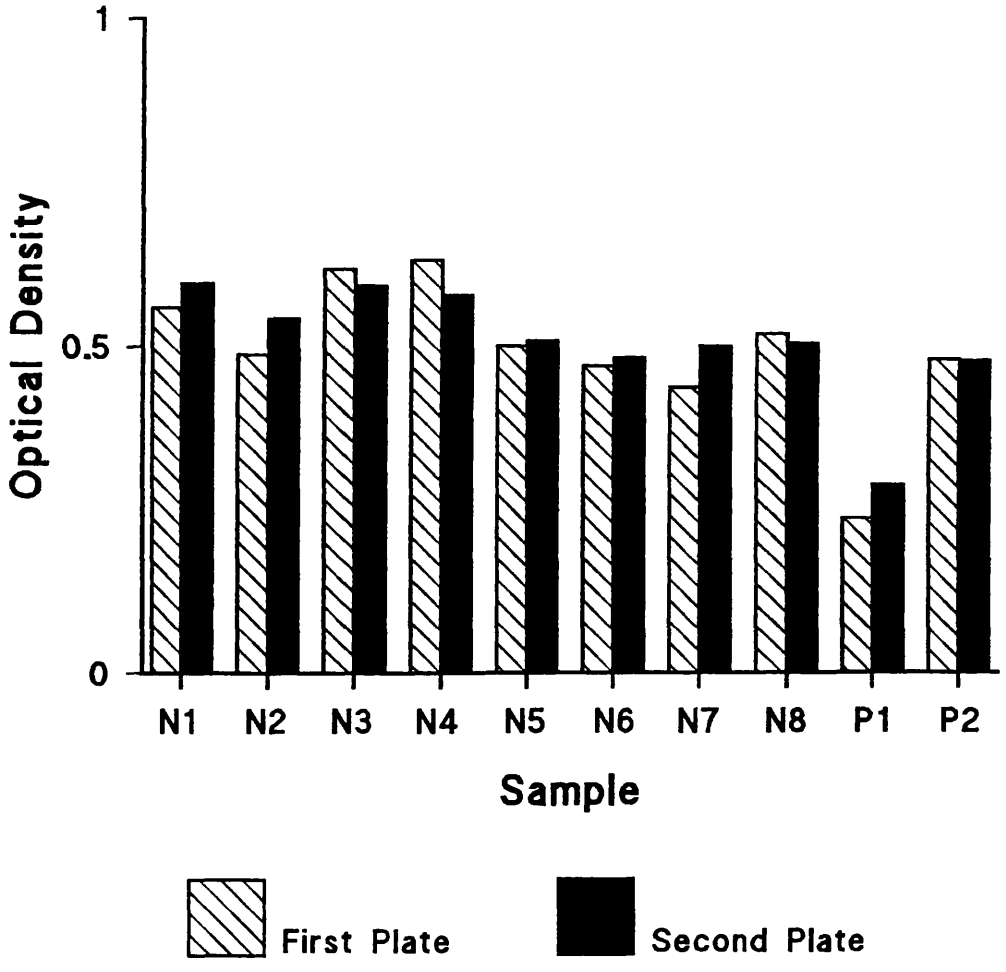
Double incubation experiment with GM–CSF ELISA
Standard curves in pooled serum

Fig 5.2.8



Double incubation experiment with GM–CSF ELISA
Standard curves in PBSA + 10% BSA

Fig 5,2.9



Optical density of normal donor plasma (N)
and pooled MM plasma (P)
after sequential incubation on ELISA plates
coated with monoclonal Anti GM-CSF

These data show that levels of GM-CSF below 100pg/ml are not detected accurately by this assay. Therefore, the apparent GM-CSF detected in plasma after HDM (between 20-60pg/ml) (Figs 5,2.3/4/5) could not be attributed to the presence of the cytokine. Figure 5,2.9. shows that apparent low levels of GM-CSF in normal plasma (N1-8) and pooled low titre CSA MM plasma (P1/P2) were not reduced by incubation on a second antibody coated piate, indicating that this activity was due to the non-specific binding of other proteins.

Since GM-CSF could not be detected in plasma after HDM with or without ABMR, the GM-CSF-like activity that stimulated colony formation and that was inhibited by antibody to GM-CSF may be due to production of this cytokine *in-vitro*.

5,3 ENZYME LINKED IMMUNOASSAY OF IL-3

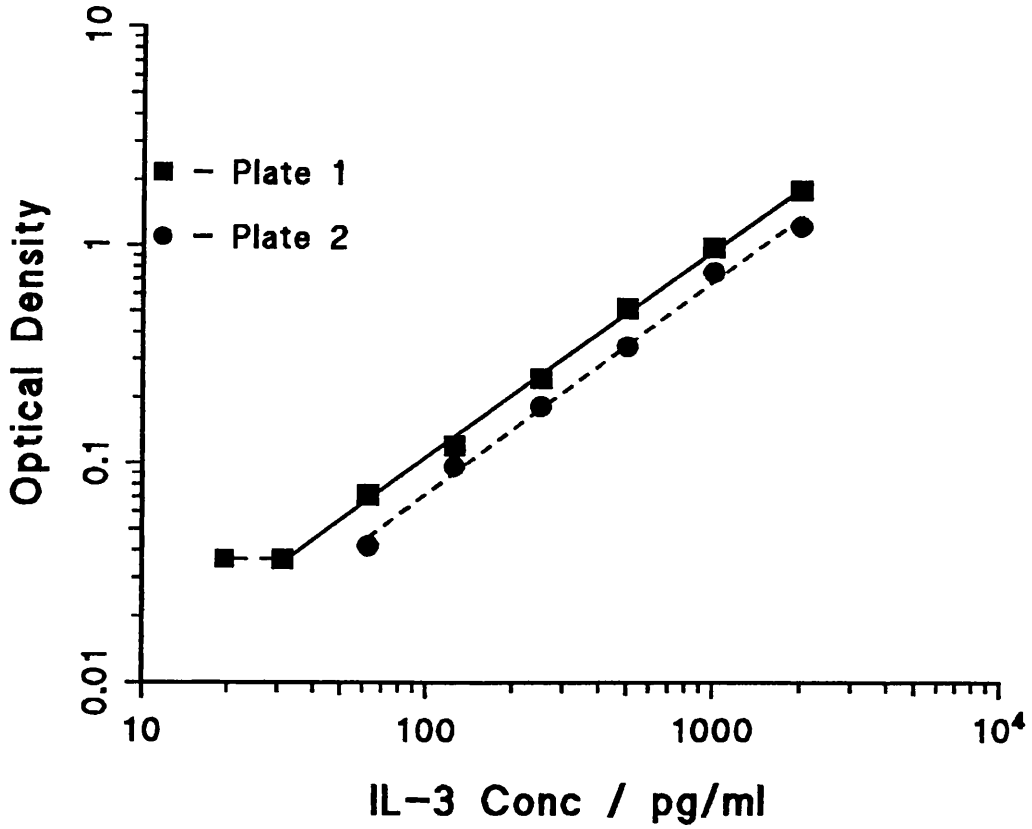
IL-3 has activity for multiple haemopoietic lineages (Chapter 1,4.3). A commercially available enzyme-linked immunoassay (Chapter 2,2.5.c) was used to test plasma, collected after HDM, for the presence of IL-3.

Assay of known concentrations of IL-3 (Fig 5,3.1 page 207) shows that the cytokine could be detected in concentrations from 30-2000pg/ml.

Plasma obtained sequentially after HDM, from 6 patients, was examined in this assay. iL-3 was not detected in any samples at levels above 30pg/ml. The patients tested were patients 7,8,20,22,23,29.. Neither patients with relatively rapid or relatively delayed engraftment had evidence of IL-3 in their plasma.

These data suggest that high levels of CSA can be present without the presence of G-CSF, GM-CSF or IL-3.

Fig 5,3.1



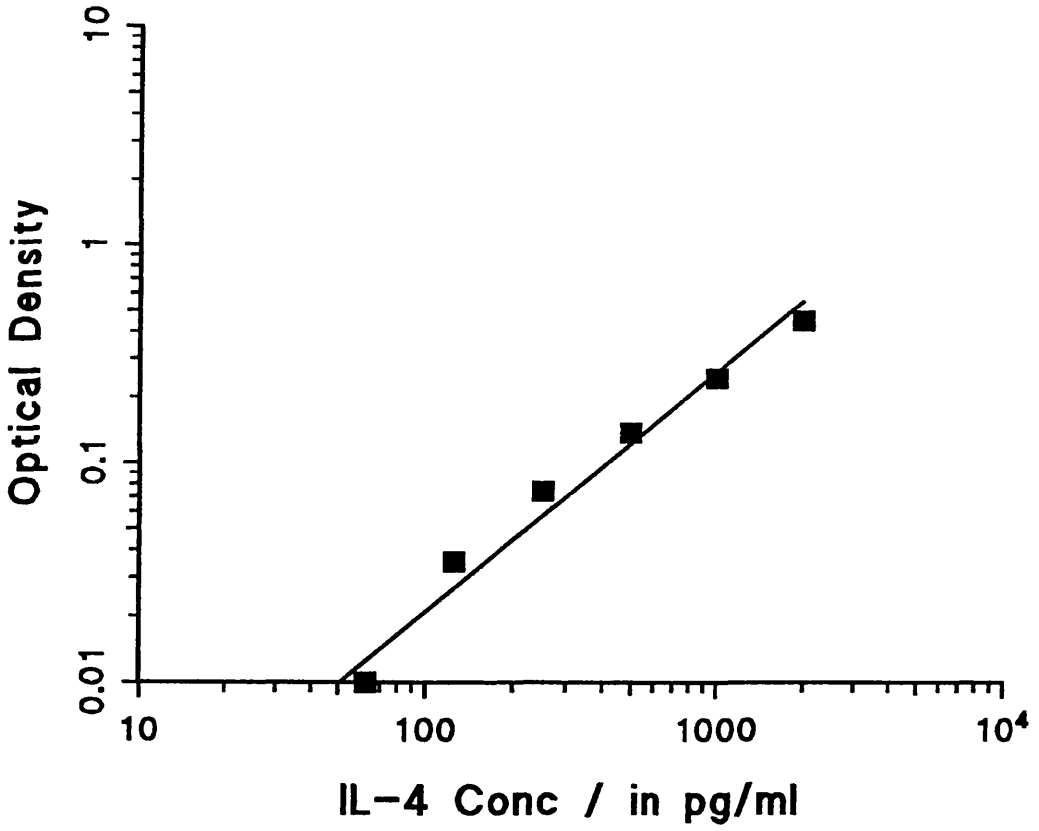
Standard curves of IL–3 Vs optical density
in two separate IL–3 assays

5,4 ENZYME LINKED IMMUNOASSAY OF IL-4

IL-4 is a cofactor in myelopoiesis and augmented the activity of endogenous G-CSF in plasma when it was added *in-vitro* (Chapter 4,5). The levels of G-CSF that were detected in plasma when CSA was elevated (Chapter 5,1) were often lower than that required to promote haemopoiesis when rhG-CSF is tested alone *in-vitro*, where concentrations of 1ng/plate (330ng/ml) are required to promote colony formation with maximum stimulation occurring with 10-20ng/plate [Millar et al, 1992]. An ELISA (Chapter 2,2.5.d) was used to examine plasma samples for the presence of endogenous IL-4.

Despite the manufacturers claim that the lower limit of detection of the assay was 3pg/ml (this proved inaccurate - Figure 5,4.1) and the mean level of IL-4 identified in the plasma of normal donors was 0.05pg/ml, IL-4 was not detected in any plasma samples from three patients (21,22 & 23). The assay was not continued because it was considered too insensitive. The data in Figure 5,4.1 show that concentrations of IL-4 in excess of 60pg/ml were necessary to produce significant changes in optical density.

Fig 5,4.1



Standard curve of IL-4 Vs optical density

5,5 ASSAYS OF IL-1 AND IL-6

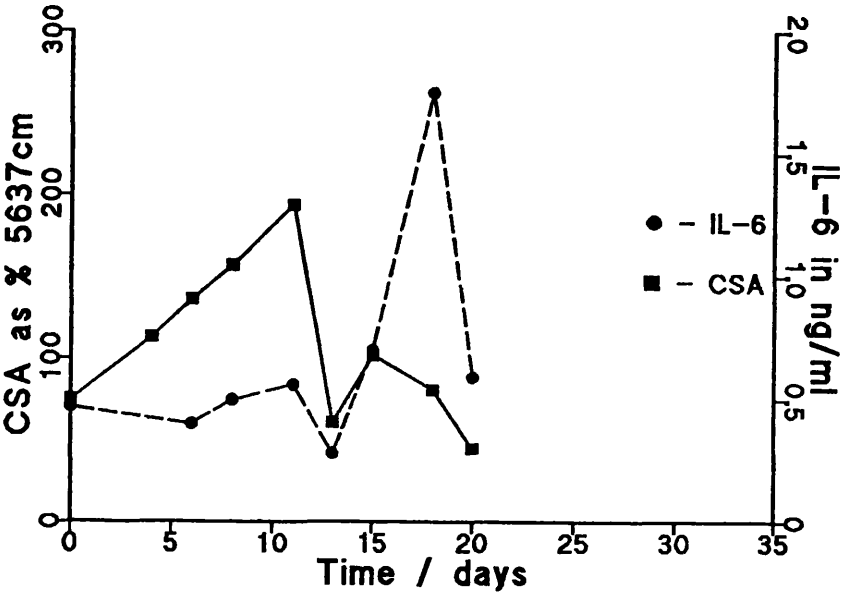
Both IL-1(α&β) and IL-6 are important cofactors in haemopoiesis. Radio-immunoassay (RIA) (Chapter 2,2.6) was used to measure levels of IL-1β in plasma (collected after HDM) from 7 patients and IL-1α In 3 patients. IL-6 was measured using a bioassay in plasma from 4 patients and using RIA in 8 patients (Chapters 2,2.6/7).

5,5.1 ASSAYS OF IL-6

In plasma from each patient IL-6 was detected at low levels throughout the recovery period after HDM (Figures 5,5.1-12, Appendix II and 5,5.9/11 below). The levels of IL-6 detected by the two assays were significantly different. The RIA detected levels of IL-6 between 0.2-0.7 ng/ml throughout the recovery period from HDM, whereas the bioassay (Patients Figures 5,5.6/7/8 & 5,5.11) suggested that the concentration of IL-6 in the plasma of patients was between 30-80pg/ml. Both assays were performed on plasma samples from patients 21 and 29. Unfortunately the RIA did not detect IL-6 at baseline levels in these patients but in three samples where IL-6 was elevated in both assays there was a log-linear relationship between the levels of IL-6 detected in each assay (Fig 5,5.13).

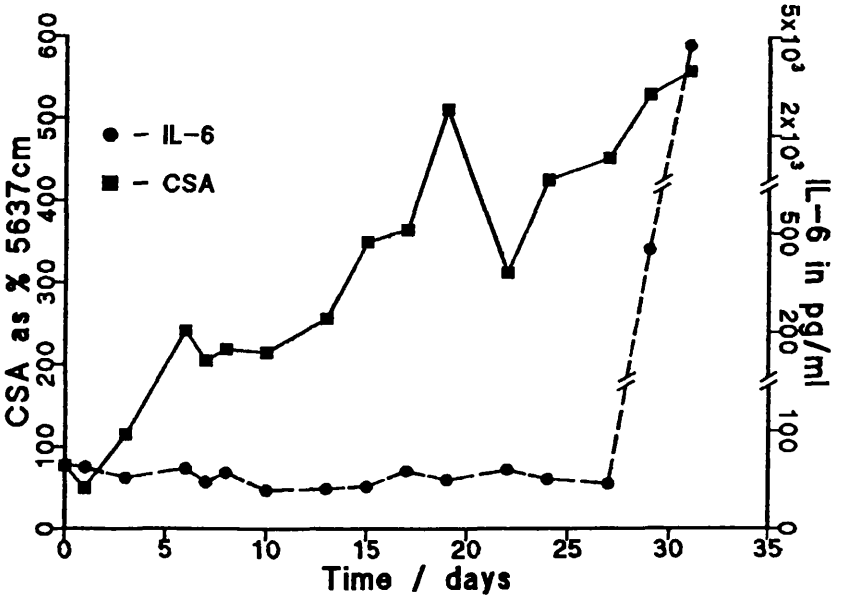
Both assays detected increases of IL-6 in patients when there was overwhelming infection. In patients 29 (Bioassay) & 25 (RIA) (Figs 5,5.11 & 9) IL-6 increased during the terminal phase of their illnesses and changes in IL-6 did not appear to be related to

Fig 5,5.9



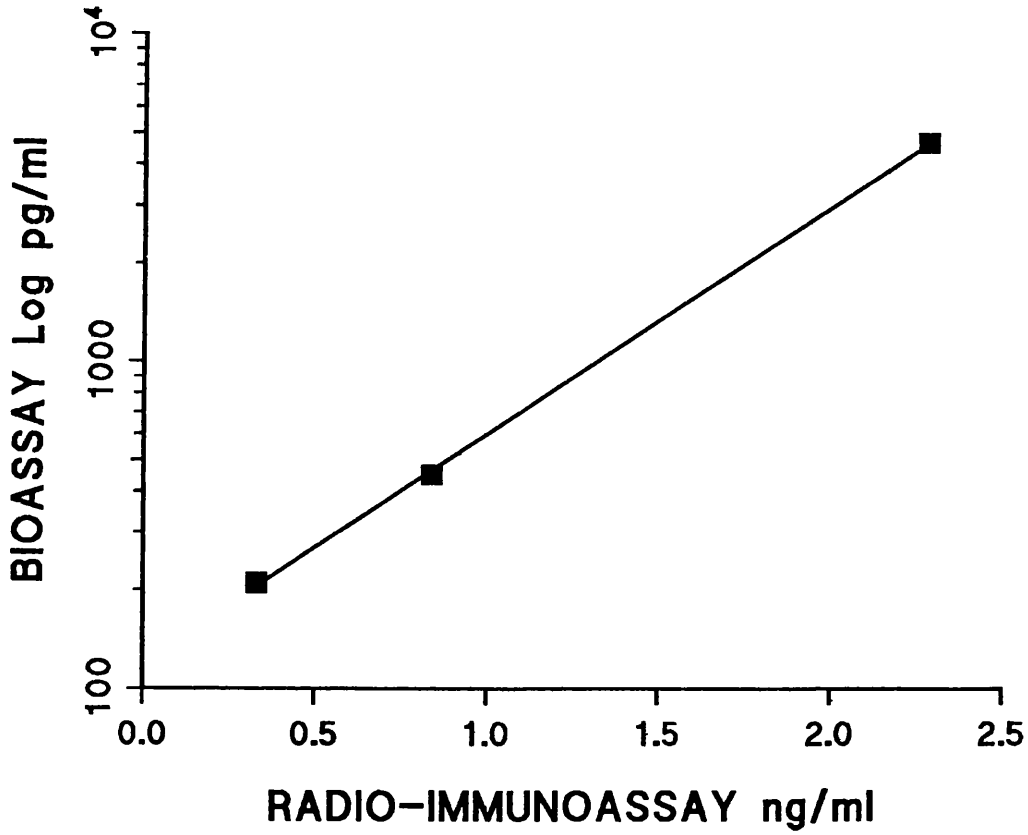
Patient 25 – Comparison of CSA with
plasma IL-6 (by RIA) after HDM without ABMR

Fig 5,5.11



Patient 29 – Comparison of CSA with
plasma IL-6 (by bioassay) after HDM without ABMR

Fig 5,5.13



Relationship between RIA and Bioassay of IL-6 in plasma samples from patients 21 & 29 r=0.99, p=0.011

changes in CSA. In patient 26 (RIA - Figure 5,5.10) IL-6 increased after engraftment and was associated with severe stress due to an oesophageal rupture leading to a mediastinitis. In the other patients there was no significant change in the level of IL-6 after HDM.

Using the RIA, mean plasma IL-6 in those patients in whom there was no significant change in levels after HDM was 0.517ng/ml (range 0.26 - 1.0)

In 4 patients plasma IL-6 was measured in samples collected before priming with cyclophosphamide (Chapter 4,1) and on day 0 before HDM. Mean IL-6 was 0.37ng/ml pre-priming and 0.405ng/ml on day 0. In these patients there was no significant difference between pre and post-priming levels of IL-6.

Disease status did not influence plasma IL-6. There was no difference in the IL-6 levels between patients in complete remission (Pts 15,17,31 Figs 5,5.4/5/12) and partial remission (PR) (12,13,14,25,26 Figs 5,5.1/2/3/9/10) whose plasma was tested with the RIA or, in patients assayed with the bioassay, between patient 29 (Fig 5,5.1) who had progressive disease and the other three patients (Figs 5,5.6/7/8) who were in PR.

DISCUSSION

The discrepancy between the concentrations of IL-6 detected in the two assays is due a difference in the recognition of IL-6. The bioassay is an extremely sensitive assay that detects biologically active IL-6. The RIA measures total IL-6 and is dependant on the binding of an antibody to a specific epitope which may not be present on biologically

active IL-6.

The mean concentration of IL-6 in the plasma of normal individuals measured by this RIA is 0.42ng/ml [Personal communication Drs P. Riches and B Millar, see also Bell et al, 1991]. Levels of IL-6 in sera from normal individuals depends on the assay used and reported "normal ranges" vary considerably. Recent studies that employed highly sensitive immunoenzymatic methods suggest that most normal donors have serum levels of IL-6 of <10pg/ml in most and <50pg/ml in all cases [Akira et al, 1993, Kurzrock et al, 1993, Scambia et al, 1993, Archimbaud et al, 1993]. Published data derived from different RIAs report maximum normal values for serum IL-6 of 0.3ng/ml [Solary et al, 1992] and 0.09ng/ml [Pettersson et al, 1992]. Comparison of the B9 bioassay has not been made with the published RIAs and ELISA assays described above. However, there was a direct correlation between the values obtained by RIA and B9 bioassay in this study.

There was no significant change in plasma IL-6 after HDM in the absence of life-threatening infection and levels of IL-6 were consistent with levels of this cytokine in the plasma of normal individuals. IL-6 did not appear to be involved in the changes in CSA that were observed after HDM although the constant levels of this cytokine may be adequate to contribute towards the CSA of plasma, since IL-6 is active *in-vitro* as a co-factor in myelopoiesis [Caracciolo et al, 1989, Montes-Borinaga et al, 1990].

Only one other study has described levels of plasma or serum IL-6 in relation to other haemopoietic cytokines after intensive therapy [Kawano et al, 1993]. In this study levels of IL-6 were measured by a commercially available ELISA (Toray-Fuji Bionics, Tokyo (Sensitivity 10pg/ml)) and levels of >50pg/ml considered significant. 5/10 children

receiving combination intensive therapy before PBSC rescue had levels of IL-6 >50pg/ml, and in these there was little change after intensive treatment, although increases in endogenous G-CSF were detected and were similar to those seen in this study.

Recent evidence suggests that IL-6 is important in megakaryocytopoiesis and thrombopoiesis in rodents, primates and in man (Chapter 1,4.2.g). In the present study there was no change in the level of plasma IL-6 after intensive therapy although each patient became profoundly thrombocytopenic and required platelet transfusions. Patients who had significantly delayed platelet recovery (>day 30 - Pts 12, 14, 29) did not have levels of IL-6 that differed significantly from those of patients with early recovery of platelets ($\leq$day20 - Pts 13, 20, 22). There was no evidence that patients with prolonged thrombocytopenia were deficient in plasma IL-6 or that early recovery of platelets was associated with a high level of IL-6.

Although plasma/serum megakaryocytopoietic activity (Meg-CSA) has been described after intensive therapy [Mazur et al, 1984, Yamasaki et al, 1988b, Fauser et al, 1988] and reaches a maximum at times distinct from CSA for granulopoiesis (Chapter 4,3), data from the present study do not suggest that this activity is related to an increasing level of IL-6. If these findings are representative of IL-6 levels after intensive therapy in other patient groups, they suggest that thrombopoietins other than IL-6 have been detected in the previous studies of Meg-CSA.

Data are now becoming available on the effect of rhIL-6 administered to man. In phase I studies [Weber et al, 1993, Olencki et ai, 1993, Aronson et al, 1993, van Gameren et al, 1993] increases in circulating platelets have been observed following

administration of rhIL-6, and also an increase in the ploidy of bone-marrow megakaryocytes [Weber et al, 1993b] suggesting an effect on megakaryocyte maturation. Studies are currently in progress to asses the effect of rhIL-6 following chemotherapy. Preliminary data show that, in individual patients, IL-6 may reduce the depth of the platelet nadir when chemotherapy treatment cycles with and without IL-6 are compared [Chang et al, 1993, Samuels et al, 1993]. Studies of rhIL-6 administered after stem cell transplants have not yet reported effects on platelet recovery, although dose escalations are continuing [Winton et al, 1993].

Administration of rhG-CSF shortens the period of neutropenia after ABMR/ABMT (Chapter 1,4.3) despite the presence of significant levels of endogenous G-CSF (Chapter 5,1). Administration of rhIL-6, an active thrombopoietin which is <u>not</u> increased constituently after intensive therapy, might augment endogenous Meg-CSA and, therefore, may represent a more physiologically appropriate and logical therapeutic manoeuvre than the administration of G-CSF to promote granulopoiesis.

Although some investigators suggest that IL-6 may be an autocrine or paracrine growth factor for myeloma [Kawano et al, 1988, Klein et al, 1989] and that levels of plasma/serum IL-6 are related to disease activity and prognosis [Klein et al, 1989, Ludwig et al, 1991, Bataille et al, 1989, Chapter 1,4.2.g], the data do not demonstrate any difference between levels of IL-6 in patients with active or inactive disease.

Serum levels of IL-6 do not provide evidence of a unique role for IL-6 in myelomatosis since raised levels of this cytokine are seen in patients with other malignancies (Chapter 1,4.2.g), in whom elevated concentrations of IL-6 reflect pyrexia and other manifestations of inflammation. Levels of IL-6 in lymphoma patients may also

be predictive of prognosis [Kurzrock et al, 1993], as is claimed for IL-6/CRP in MM (although other authors refute this [Bell et al, 1991, Brown et al, 1993b]).

These studies suggest that elevation of serum/plasma IL-6 is not a specific indication of the severity of malignant disease and may only indicate immune activity due to trauma or inflammation.

IL-6 in plasma after intensive therapy may contribute to CSA indirectly, but control of plasma IL-6 concentration is not related to the level of CSA.

5,5.2 ASSAYS OF IL-1β AND IL-1α

Interleukin-1β was detected in the plasma of each of 7 patients tested (Figs 5,5.14-20, Appendix II and Fig 5,5.17/20 below).

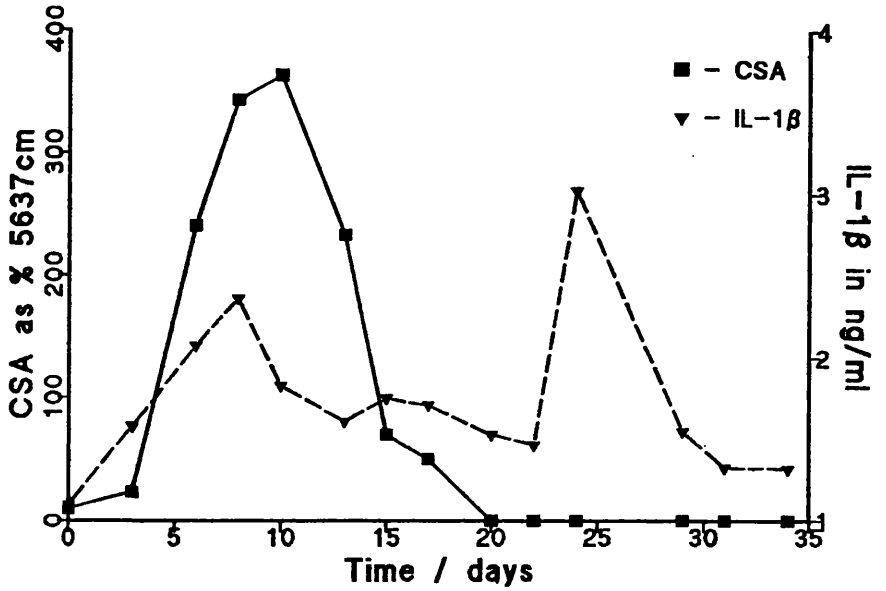
In 6/7 patients, plasma IL-1β concentration before HDM was between 1-2ng/ml. After HDM there was an increase in plasma IL-1β during the first 3-5 days after treatment in each of 5 patients who also received ABMR and rhG-CSF (Figs 5,5.15-19). This increase either preceded or occurred in conjunction with a rise in CSA. In each of these patients concentrations of IL-1β decreased as CSA reached a maximum.

There was an initial fall in plasma IL-1β in patient 11 (Fig 5,5.14) who did not receive rhG-CSF, and an abrupt fall (from 4ng/ml,to <1ng/ml between days 0-2) in patient 29 (Fig 5,5.20) (who did not receive rhG-CSF or an autograft) followed by an increase in the cytokine.

After day 5, following HDM, the IL-1β/time curve varied between patients and there was no consistent relationship between changes in plasma CSA and plasma IL-1β.

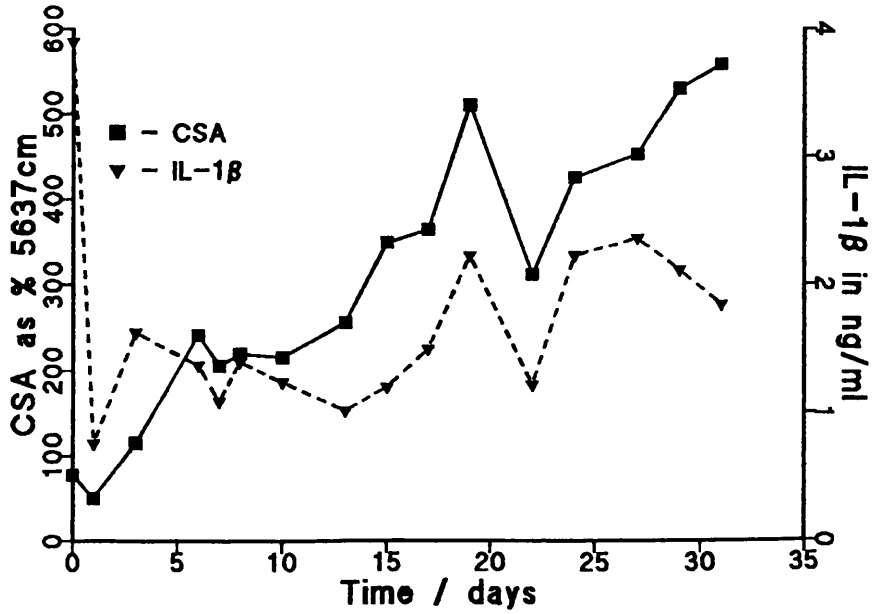
In each patient levels of IL-1β fluctuated by 1-2ng/ml during the haematological recovery period, representing, approximately, two-fold increases/decreases of baseline values. In some patients, for example patient 29 (Fig 5,5.20), levels of IL-1β appeared to be related to levels of CSA, but in this patient there was also a temporal relationship between increases in IL-1β and her terminal septic illness. Patient 20 (Fig 5,5.17) had two documented episodes of pneumonia, with bacteria cultured from bronchioalveolar lavage performed on day 8 and fungi from a lavage on day 25. IL-1β was elevated during both of these episodes. In other patients, however, there was no clear relationship

Fig 5,5.17



Patient 20 – Comparison of CSA with plasma IL–1β
after HDM & ABMR

Fig 5,5.20



Patient 29 – Comparison of CSA with plasma IL–1
after HDM without ABMR

between changes in the level of IL-1β and toxic episodes. Patient 11 (Fig 5,5.14), for example, never developed a pyrexia and was the only patient not to require a change in antibiotics, however, IL-1β increased during engraftment and continued to increase after CSA and G-CSF levels (Fig 5,1.5) had fallen.

Interleukin-1α was not detected at significant concentration in samples of plasma from the three patients tested.

DISCUSSION

Throughout the recovery period after intensive therapy variations in the levels of IL-1β amongst individual patients were no more than two-fold. The data do not indicate whether these variations are physiologically significant, or whether the increase in plasma IL-1β represents an increase in biologically active cytokine.

IL-1β is an important mediator of inflammation [Andus et al, 1988, Dinarello, 1988] and is known to stimulate the release of other cytokines *in-vitro* (Chapter 1,4.2.f). In particular, G-CSF is released in response to IL-1β *in-vitro* by bone marrow stroma in long term culture [Fibbe et al, 1988b], endothelial cells [Broudy et al, 1987] and fibroblasts [Kaushansky et al, 1988].

If the observed increases in IL-1β are significant, it may be that the increases are in response to inflammation resulting from cell death due to HDM, rather than to haemopoietic stress. In either case, it is possible that changes in serum IL-1β concentration, although, proportionally, less than that seen in G-CSF (Chapter 5,1), may

be sufficient to influence the measurement of CSA *in-vitro* or the production of CSA (and/or G-CSF) *in-vivo*.

The presence of IL-1β in plasma samples may have resulted in the release of GM-CSF *in-vitro* (Chapter 1,4.2.f), during testing of plasma for CSA, resulting in the apparent inhibition of CSA by antibody to GM-CSF (Chapter 4,6) that was observed in the absence of immunologically detectable GM-CSF in plasma (Chapter 5,2).

Furthermore, IL-1β was present in samples of plasma with low CSA and may explain, in part, the activity of low CSA titre plasma in augmenting the activity of recombinant IL-3 and GM-CSF *in-vitro* (Chapter 4,8).

The concentrations of IL-1β detected in the plasma of these patients is comparable with levels detected by this assay in patients in intensive care with or without documented sepsis ([Sheldon et al, 1993] - (median concentrations for different patient groups 0.92-1.58 μg/l)). Although IL-1β has been shown to mediate inflammation, in the study by Sheldon et al [1993] the presence of clinical or microbiologically confirmed sepsis did not influence the mean level of IL-1β in 64 patients. However, sequential changes in plasma levels of cytokines in individual patients were not examined, so the results of that study are not directly comparable with the results of the present study, in patients after HDM.

In the study by Sheldon et al [1993] IL-6 levels in the same patients did distinguish between patients with clinical sepsis and those with (only) microbiological evidence of sepsis or with no evidence of infection, indicating that IL-6 may be a more sensitive indicator of inflammation than IL-1β *in-vivo*. In that study, however, CRP was the most sensitive indicator of inflammation and IL-6 could only predict changes in plasma

levels of CRP with 50-60% efficiency.

Interleukin-1β and IL-6 were both maintained at relatively constant concentrations in plasma after HDM. Although both cytokines are active in haemopoiesis, they also have other activities within the immune system and in the acute phase response. It is likely, therefore, that their concentrations in plasma are influenced by changes in lymphopoiesis and by inflammation as well by haemopoiesis after intensive treatment with HDM.

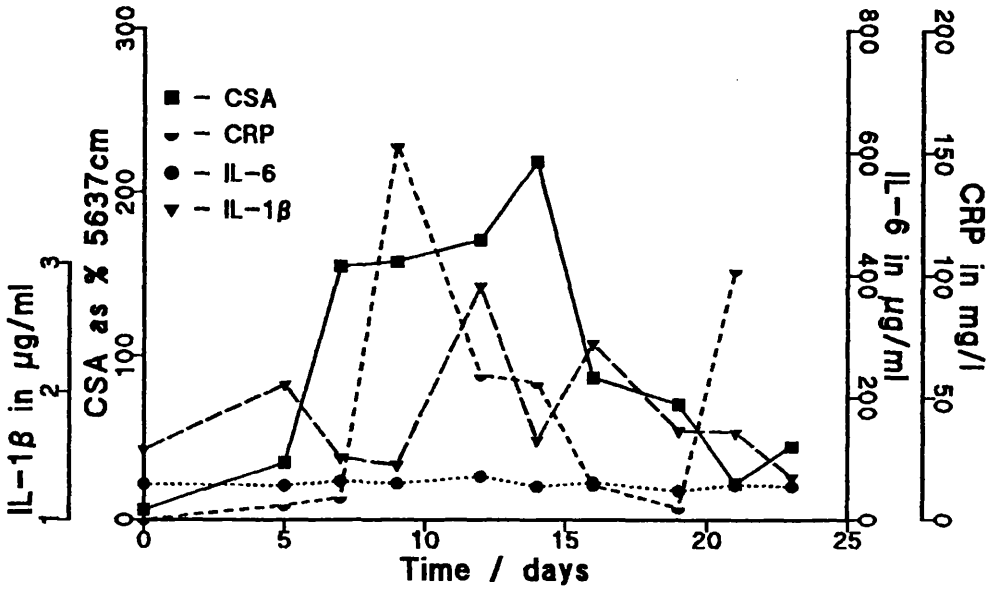
The production of CSA was unaffected by previous intensive therapy (and cumulative damage to haemopoietic stem cells) and the pattern of CSA was similar after different conditioning regimens and different doses of melphalan (Chapter 4). These data suggest that the mechanism(s) that produces CSA survives or tolerates intensive therapy and may involve the release of cytokines from non-haemopoietic tissue. Since the mechanism(s) that increases CSA in response to leucopenia and those/that that maintains plasma levels of IL-1β and IL-6 share an apparent resistance to the effects of ablative therapy it is possible that the same tissues are responsible for the production of these activities. Tissues such as bone marrow stroma, fibroblasts and endothelial cells which can produce IL-1β and IL-6 and can be induced by IL-1β to produce other cytokines, are likely candidates.

5,6 C-REACTIVE PROTEIN AND THE ACUTE PHASE RESPONSE

Elevations of CSA and G-CSF after HDM appeared to be related, in part, to an inverse relationship with total numbers of circulating leucocytes. However, IL-1β, which is a cytokine with known haemopoietic activity, was also detected at this time, although changes in its concentration in plasma seemed unrelated to changes in CSA and changes in WCC. Plasma levels of IL-6 were also increased in relation to inflamation or sepsis but did not correlate with changes in CSA. IL-6 is a mediator of inflamation and the "so-called" acute phase response [Sheldon et al, 1993]. IL-1β is also associated with production of acute phase proteins *in-vitro* although the evidence for this *in-vivo* is less clear [Sheldon et al, 1993, Whicher et al, 1991, Chapter 1,4.2.g].

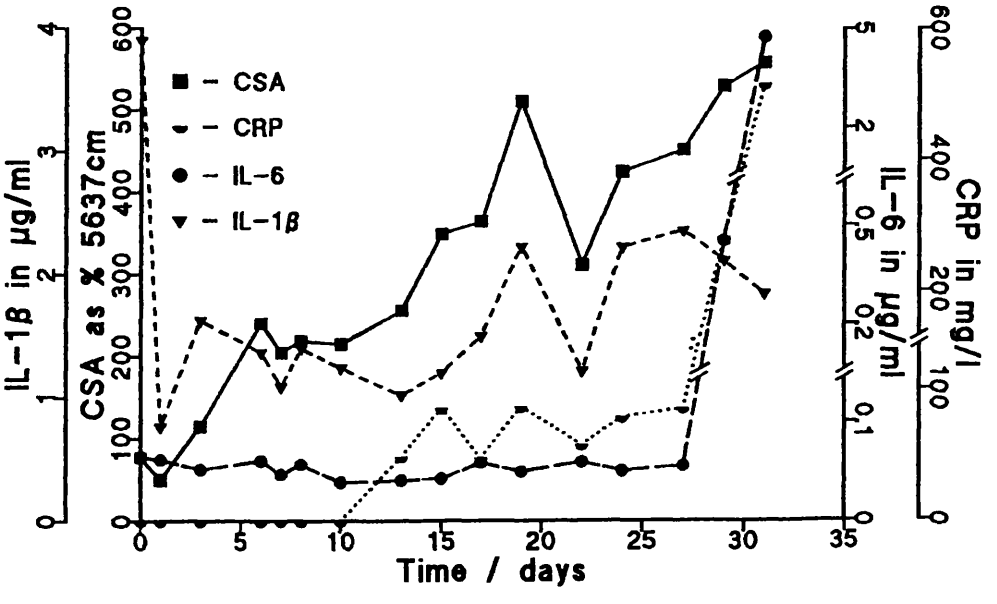
C-reactive protein was measured by rate nephelometry (Chapter 2,2.10) in plasma samples from 4 patients who had received HDM with or without ABMR. The data are shown in figures 5,6.1-4, below.

Fig 5,6.1



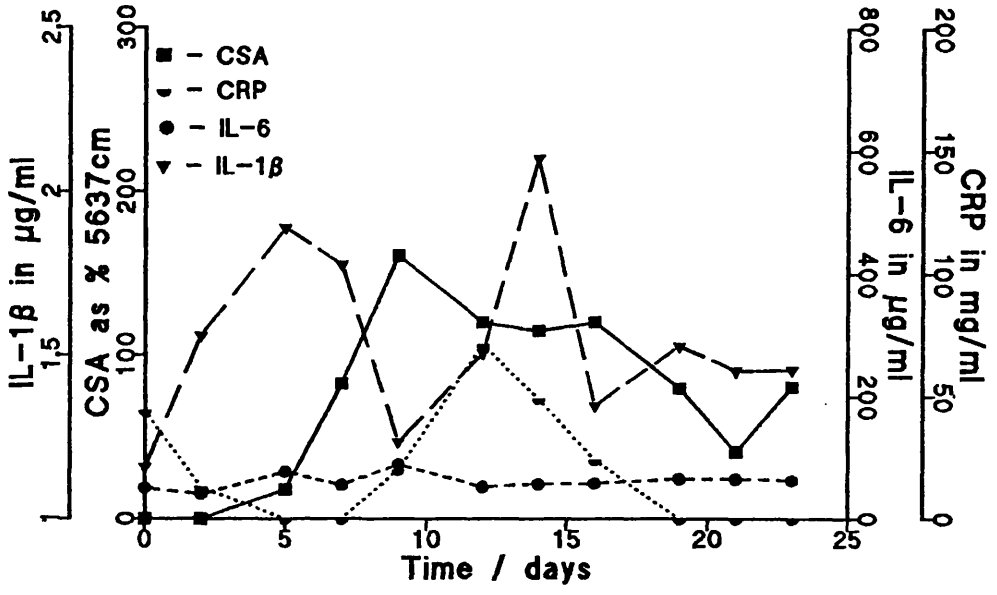
Patient 21 – Comparision of CSA with plasma IL-6, IL-1β and CRP

Fig 5,6.2



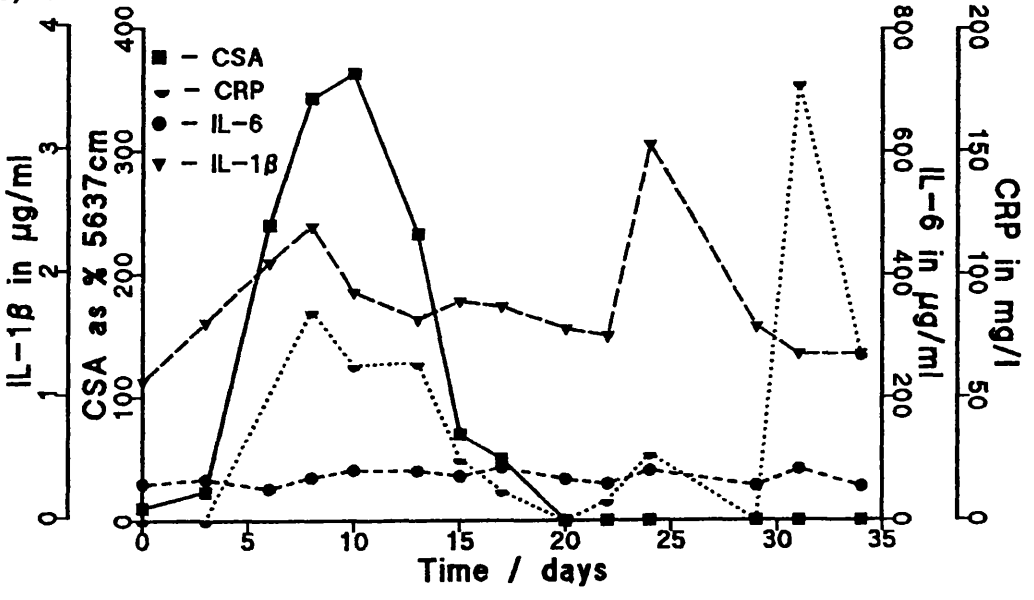
Patient 29 – Comparison of CSA with plasma IL-6, IL-1β and CRP

Fig 5,6.3



Patient 22 – Comparison of CSA with plasma IL-6, IL-1β and CRP

Fig 5,6.4



Patient 20 – Comparison of CSA with plasma IL-6, IL-1β and CRP

C-reactive protein was not detected before the administration of HDM in three patients and was low (44mg/l) in patient 22 (Figs 5,6.1-4). In patient 20 (Fig 5,6.4) CRP increased substantially and then decreased in association with changes in CSA. However, this patient had a documented bacterial pneumonia at this time and subsequent increases of CRP, although associated with a further fungal pneumonitis, were not associated with changes in CSA. In patient 29 (Fig 5,6.2) there was an increase in CRP with the development of her septic illness and a dramatic increase before death. Although CSA also increased during this period the curves of each measurement/time were not directly related. In the other two patients there was no relationship between changes in CRP and CSA and no documented infective episodes to explain the small rises in CRP that were observed.

Increases in CRP concentration occurred at the same time as an increase in IL-6 in patient 29. IL-1β also increased during this patients terminal illness. Levels of CRP and IL-1β also appeared related in patient 20.

Increases in CRP occurred in relation to documented infections but not as a result of the trauma of high dose cytotoxic chemotherapy. This was observed in the patients who received ABMR and HDMP and patient 29 who did not.

These data provide further evidence that the increases of IL-6 that were observed after high dose chemotherapy (Chapter 5,5) were derived not by changes in leucocyte numbers or haemopoietic progenitors (as is CSA and, probably, G-CSF) but as part of an inflammatory response to trauma and/or infection. The relationship between CRP and IL-1β also support the role of this cytokine in inflammation rather than haemopoiesis.

It is likely that CSA in individual patients is determined not only by proteins which

act directly to restore haemopoiesis but also by proteins which are immunoregulatory in response to infection. However, the pattern of CSA/time that has been observed (Chapter 4) can not be related to processes of inflammation, but, is determined by changes in haemopoietic cell numbers.

CHAPTER 6

CONCLUSIONS

Multiple myeloma (MM) is an incurable disease. Survival and quality of life may be improved in patients who obtain remissions and these are most frequently achieved with intensive chemotherapy with autologous stem cell rescue. Haematological toxicity and infection limits the number of such treatments that can be used in individual patients. Although recombinant myeloid colony stimulating factors such as rhG-CSF and rhGM-CSF (either after autologous bone marrow rescue (ABMR) or to facilitate peripheral blood stem cell (PBSC) collection) may limit toxicity and the risk of infection, they do not protect against the morbidity associated with thrombocytopenia seen, particularly, with repeated treatments.

It may be possible to reduce the morbidity associated with intensive therapy by the better use of recombinant cytokines. To achieve this thorough *in-vitro* testing of potentially useful growth factors is required, but there is also a need for an understanding of how these molecules contribute to endogenous activity, and how recombinant cytokines interact with endogenous activity *in-vivo*.

This thesis has concentrated on the role of endogenous haemopoietic growth factors in the recovery of normal tissue following intensive chemotherapy in patients with MM.

The data have shown that the production of plasma activity (CSA) that can support the growth of granulocyte-macrophage colonies (GM-CFUc) from normal bone marrow is a consistent response to intensive therapy in patients with multiple myeloma. These and previous data [Millar et al, 1992] show that this activity is not dependant on the patients disease, the choice of conditioning regimen, the use of bone marrow transplantation/autologous rescue or the administration of intravenous recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) or subcutaneous recombinant human granulocyte colony stimulating factor (rhG-CSF).

Prior to the administration of high dose melphalan (HDM) levels of CSA in the plasma of patients were similar to those seen previously in patients with MM and other malignancies at various times in the evolution of their diseases [Millar et al, 1990], although the patients in the present study were a more homogeneous group that had all received recent induction therapy. The levels of CSA before a "priming" dose of cyclophosphamide correlated with the number of GM-CFUc that could be cultured from the bone marrow mononuclear cells (BMMNC) of individual patients, suggesting that CSA might be related to proliferative activity.

After treatment with a low dose of cyclophosphamide there was a fall in the level of CSA but an increase in the numbers of GM-CFUc in bone marrow. Levels of CSA are, therefore, not directly dependant on numbers of GM-CFUc in bone marrow.

In contrast to the effect of intensive therapy, a less toxic injury increased the number of cells in a proliferative state with a concomitant decrease in CSA. One possible explanation for this is that the dose of cyclophosphamide used for priming, in

contrast to HDM, does not have a profound effect on the peripheral WCC. This suggests that CSA is determined by numbers of mature haemopoietic cells rather than stem cells. Since CSA decreased after cyclophosphamide, in the absence of neutropenia there may be a direct relationship between neutrophil numbers and CSA that is similar to that suggested by Watari et al [1989] between serum levels of G-CSF and neutrophil counts (Chapters 1,4.2.b & 5,1).

The administration of a small dose of chemotherapy ("priming") before ablative treatment has been shown to enhance the recovery of normal stem cells of the bone marrow and gastrointestinal tract without protecting tumour cells from subsequent treatment [Millar and McElwain, 1985, Selby et al, 1987b]. The mechanism for this is not entirely clear. In mice primed before chemotherapy [Millar et al, 1975], there was evidence that a transferable factor was involved, and prior to radiotherapy [Neta et al, 1986, Dorie et al, 1989] and chemotherapy [Castelli et al, 1988] IL-1, administered to mice had a similar effect, suggesting that this molecule might be important following priming with cytotoxic drugs. Interleukin-1 was present in plasma after priming in this study, however, sequential measurements of this cytokine were not made before and after administration of cyclophosphamide so this proposition cannot be confirmed.

In any case, since BM GM-CFUc were increased 5-9 days after treatment with cyclophosphamide, it seems likely that the effect(s) of priming is already established in haemopoietic progenitors at this time so that any direct effect of priming on CSA might not be detected after this interval.

Peripheral blood granulocyte-macrophage colony forming units (PB GM-CFUc)

were measured in each patient before priming with cyclophosphamide and numbers decreased after this treatment. Although numbers of PB GM-CFUc did not correlate with numbers of bone marrow (BM) GM-CFUc before priming they did correlate with the numbers of BM GM-CFUc that were grown 5-9 days after a priming dose of cyclophosphamide. Thus, the number of PB GM-CFUc pre-cyclophosphamide might predict the number of GM-CFUc/kg that could be harvested from bone marrow after this treatment, thereby, providing an indicator of the proliferative status of a patients BMMNC.

Following intensive therapy there was a consistent pattern of elevation of CSA in patient's plasma. CSA increased as leucocyte numbers fell and reached a maximum at the leucopenic nadir before decreasing as white cell numbers increased again. The pattern of CSA/time was not influenced by the administration of autologous stem cells although this treatment significantly reduced the perlod of neutropenia and of thrombocytopenia.

Treatment with rhG-CSF was also associated with a reduction in the period of neutropenia although it did not affect recovery of platelets. Unlike the use of ABMR, this intervention influenced the time course of CSA after treatment by reducing the time taken to achieve maximal levels of this activity and by reducing the variation of this time between patients. As rhG-CSF was not present in plasma at the time that blood samples were collected for the measurement of CSA it is likely that the effect on the time course of CSA was achieved by an interaction of the recombinant cytokine with the production of CSA *in-vivo.* Such an interaction was not seen when a continuous intravenous

infusion of GM-CSF was given to patients with leukaemia after intensive therapy [Millar et al, 1992] and in these patients there was no reduction in the period of neutropenia (although neutrophils numbers were increased after recovery), suggesting that, although both cytokines can increase the levels of circulating neutrophils, there is a mechanistic difference between the activities of these treatments. For example, it is possible that the target cells of these two cytokines differ and that although both cytokines may stimulate the proliferation of granulocyte progenitors (Chapter 1,4.2,b/c) the tissue(s) that produce endogenous growth factor activity (CSA) may respond differently to rhG-CSF and rhGM-CSF.

There was no evidence that CSA after intensive therapy resulted from the release of cytokines from dead or dying haemopoietic cells. Rather, there was an inverse relationship between levels of CSA and numbers of circulating leucocytes suggesting that CSA might be a physiological response to low numbers of circulating mature haemopoietic cells.

It is not immediately clear from the data whether CSA is related to numbers of circulating mature haemopoietic cells or to haemopoietic progenitor cell numbers or to a combination of both. The temporal relationship between CSA and leucocyte recovery after HDM suggests the former, as does the fact that administration of rhG-CSF resulted in earlier rises in CSA and earlier recovery of neutrophils.

The evidence In favour of a relationship between CSA and progenitor numbers rests on the relationship between theses two variables before priming and the fact that CSA persists after neutrophils have recovered (to levels greater than those required to initiate increases in CSA) which might suggest that CSA is determined by continuing

proliferative activity in the bone marrow. Increased BM GM-CFUc, despite recovery of granulocytes, are documented at this time after intensive therapy [Arnold et al, 1986]. However, CSA was unrelated to numbers of BM GM-CFUc after priming and the capacity to produce CSA was not influenced by treatment with a lower dose of melphalan (which might have less effect on stem cells) or by ABMR which introduces active (primed) stem cells immediately after HDM. Similarly in patients that had received previous HDM the pattern of CSA was unaffected whereas the proliferative capacity of the patients stem cells was impaired.

It seems most likely, therefore, that CSA is determined by numbers of circulating mature haemopoietic cells rather than by proliferative activity or numbers of bone marrow progenitors. As a proliferative stimulus, the efficacy of this activity is dependant on the availability of viable stem cells that have survived the conditioning regimen or have been returned in the autograft. This is consistent with the observation that ABMR, by providing a more efficient source of progenitors, resulted in earlier engraftment (when compared to patients that received a lower dose of HDM without ABMR), but did not influence the time curves of CSA.

Several findings suggest that there is a qualitative change in CSA with time after intensive therapy. There were changes in the morphology of BMMNC colonies that were grown in plasma collected at different times after therapy although the target cell population was constant for samples of plasma from each patient. These data suggest that different BMMNC were being stimulated to proliferate or that stem cell differentiation was redirected by changes in cytokine concentrations in plasma. This observation is supported by the data on activity of interleukin-4 in combination with plasma which

suggested that endogenous G-CSF was likely to be present in plasma at times when CSA was maximum but not throughout the recovery period after HDM. Subsequent measurement of G-CSF by enzyme linked immunoassay (ELISA) confirmed that this is the case. However, CSA persisted when G-CSF was not detected in plasma providing evidence that other molecules also contribute to this activity.

Interleukin-3 (IL-3) and GM-CSF, known cytokines with haemopoietic stimulatory activity, were not detected by ELISA in plasma after HDM, suggesting that they were not contributing to CSA when G-CSF was absent from plasma. A recent publication suggests that IL-3 may be present in serum after intensive therapy [Mangan et al, 1993]. However, in that study (that employed a different ELISA and a bioassay) IL-3 was not detected after day 14 following treatment and reached a maximum at similar times to those of G-CSF seen in the present study. Interleukin-3 is still not a candidate, therefore, for the activity that determines CSA when granulocytes have recovered and endogenous G-CSF decreases. Interleukin-1β and IL-6 were present in plasma and may have contributed towards the measurement of CSA *in-vitro* or the production and/or activity of CSA *in-vivo*. However, concentrations of these cytokines were low when compared to concentrations required for biological activity *in-vitro* and varied by only 2-fold in the absence of infection. It is possible that IL-1β was responsible, in part at least, for the ability of plasma with low levels of CSA to augment the activity of GM-CSF and IL-3 *in-vitro*, and it is also possible that this cytokine may have stimulated the release of GM-CSF from BMMNC *in-vitro*, explaining why colony formation was decreased by antibody to GM-CSF when this cytokine was not detected in plasma by ELISA. If these suppositions are true, these cytokines, which augment the activity of growth factors *in-*

vitro, could be important as co-factors of myelopoiesis *in-vivo* where low levels of cytokines such as GM-CSF and IL-3 may be active in the bone marrow. When tested in man *in-vivo* IL-1 caused severe fevers, hypotension, renal faiiure and severe injection site reactions, however, there were also increases in both leucocytes and platelets [Crown et al, 1990, Kitamura and Takaku, 1989, Tewari et al, 1990]. When IL-1α was used as a "priming" treatment before intensive therapy increases in endogenous G-CSF were observed before the chemotherapy was administered [Wilson et al, 1993] and earlier recovery of neutrophils were seen after treatment in patients with lymphoma.

Neither IL-1β nor IL-6 appeared to be related to changes in CSA and there was no indication that there were specific intervals after therapy at which raised levels of either cytokine might have been critical for the proliferation or maturation of particular cell types. However, both cytokines were increased in patients that were infected or dying and both were related to changes in plasma concentrations of C-reactive protein (CRP), an indicator of inflammation, which also increased in response to infections but not in response to changes in numbers of circulating haemopoietic cells. These data suggest that plasma levels of IL-1β and IL-6 are determined by inflammation, rather than by numbers of haemopoietic cells.

There was also no indication that levels of IL-6 were different in patients who were in remission or who had active myelomatosis or in patients who recovered platelets particularly early or late, suggesting that levels of this cytokine were unrelated to disease activity or to thrombopoiesis as has been suggested elsewhere.

As CSA persists beyond the time at which there is recovery of leucocytes or

neutrophils, but when platelet numbers are still low, CSA may be determined by numbers of megakaryocytes and/or circulating platelets. The colony forming assay employed in this study was not designed to detect megakaryocyte-CFUc (although occasional megakaryocytes were grown from normal bone marrow precursors) and measurement of Meg-CSA, specifically, was not attempted. However, other investigators have described activity in plasma that stimulates megakaryocytopoiesis that is maximal when granulocytes have recovered but CSA (for granulopoiesis in this study) remains increased. Although a known thrombopoietin, IL-6 is unlikely to account for increases in Meg-CSA that have been seen in other studies since its concentration, in this study, did not change in the absence of infection. The data suggest, therefore, that other thrombopoietins are present in plasma.

If CSA is involved in stimulating the proliferation of stem cells it is likely that the composition of CSA is most critical in the bone marrow. Peptide regulatory factors such as cytokines are regarded as acting predominantly in an autocrine or paracrine manner and would normally be confined in their activity to the microenvironment in which they are produced. The activity that has been measured as CSA may not reflect the balance of cytokines in the bone marrow microenvironment. In normal circumstances the stimulus for cytokine production is likely to be limited to one that produces concentrations of cytokines locally (within the bone marrow microenvironment) that are adequate to control myelopoiesis. In response to tissue injury, cytokine production by tissue in close proximity to the stem cells, at which the activity is directed, may be increased such that excess is detected in the circulation. Some cytokines such as IL-3

or GM-CSF may reach physiologically active concentrations in the bone marrow but may be metabolised rapidly *in-vivo*, such that they cannot be detected in blood plasma.

Measurement of bone marrow plasma cytokine concentrations was not attempted in this study. However, it is likely that such measurement would be difficult since aspiration of bone marrow in order to collect bone marrow plasma is likely to disrupt whatever barrier may exist between blood and bone marrow plasma and aspiration of blood will then compromise measurements of cytokines. Certainly large numbers of peripheral blood cells contaminate bone marrow aspirates when large volumes of marrow are obtained. Measurement of IL-6 in paired samples of plasma from bone marrow and blood has been studied [personal communication Dr B.C.Millar, Dr J.B.G.Bell]. Levels of IL-6 equivalent to that of peripheral blood were found in bone marrow plasma suggesting that either these samples were contaminated with peripheral blood plasma or that the concentration of this cytokine was constant in both bone marrow and peripheral blood.

Furthermore, evidence exists from *in-vitro* studies that growth factors may need to be presented to haemopoietic cells by accessory cells, such as stroma, bound to specific molecules in the extracellular matrix [Gordon et al, 1987b], such as heparan sulphate [Roberts et al, 1988], for their recognition in a biologically active form [Dexter, 1990]. Thus, the biological activity of plasma cytokines *in-vivo* may be determined by the functional integrity of bone marrow stroma.

The collection of blood samples is also a factor that may result in the measurement of cytokine levels that do not represent physiological concentrations. In this study plasma and serum were found to contain different levels of CSA which could not be attributed to changes in pH, the presence of heparin in plasma or to different

levels of G-CSF, GM-CSF or IL-6. Differing concentrations of endotoxin, and/or interaction of endotoxin or cytokines present in the blood sample, with components of the clotting cascade or thrombolytic pathways may have accounted for the difference detected. In other studies [Bell et al, 1991, Riches et al, 1992 & Riches & Gooding, 1990], cytokine levels in serum and plasma have been found to increase after collection of samples. This variable was controlled, as far as possible in this study, by separation of plasma from PB MNC within one hour of collection.

Other haemopoietic activities exist in plasma that are, as yet, not defined. Further studies of changes in the composition of plasma/serum in response to ablative chemotherapy may permit the isolation and characterisation of these molecules. Furthermore, more sensitive assays, such as the ELISA for IL-3 used by Mangan et al [1993] or the RIA now available for GM-CSF (which can detect 30pg/ml of that cytokine in serum [Mortensen et al, 1993]), may help to define the role of known cytokines in plasma.

The role of molecules such as IL-1α and IL-1β require further examination, for example, with the use of neutralising experiments with antibodies to elucidate their roles as stimulators of growth factor production from target MNC populations *in-vitro*. These experiments could be done by addition of antibodies to agar colony assays (as described for antibody to GM-CSF in the present study) but also, by extraction of the cytokine from plasma (which can be done by immune complex adsorption as in Yamasaki et al, 1988) before it is tested in clonogenic assay which may differentiate between activity present in plasma and that generated *in-vitro*.

Information may also be derived by investigation of the role of bone marrow

stroma in the support of haemopoiesis during recovery from intensive therapy. This may be possible using colony forming assays on preformed stromal layers [Gordon et al, 1985] or in long term bone marrow culture (LTBMC) [Dexter et al, 1984, Gartner and Kaplan, 1980]. Plasma collected at different times after intensive therapy might be examined in LTBMC, using established cultures, to assess changes in the production of committed progenitors or cytokines. Alternatively, plasma samples could be examined in systems where the stem cells had been ablated with cytotoxic drugs, since it has been shown [Eaves et al, 1992] that ablation of late progenitors by 4-hydroperoxycyclophosphamide does not affect the number of these cells present in culture after 8 weeks, suggesting that earlier stem cells may survive such treatment and are able to regenerate committed stem cells. This may be analogous to the survival of progenitors after intensive chemotherapy *in-vivo*.

If changes in cytokines are detected in the supernatants of LTBMC it may be possible to identify the cells that are producing these factors by the use of molecular biology techniques, such as Northern blotting to differentiate the presence of growth factor mRNA in adherent and non-adherent cell populations, or the direct examination of cells for the presence of mRNA for cytokines by in-situ hybridisation techniques.

With respect to myeloma it is critical that the role of IL-6 both in megakaryocytopoiesis and in myelomatosis is elucidated. If ongoing clinical studies demonstrate that IL-6 is a useful adjunct to intensive therapy (by promoting the early recovery of platelets), before consideration can be given to the use of IL-6 in patients with this condition, it will be essential to demonstrate that no detriment can be derived if myeloma cells are exposed to this cytokine *in-vitro* or *in-vivo*.

An understanding of the time course of expression of individual cytokines in patients after intensive therapy may permit greater exploitation of their use in clinical practice both in terms of conditioning patients in order to harvest bone marrow or peripheral stem cells and as an adjunct to intensive therapy during the recovery period. This might be achieved by administering recombinant material which has been shown to potentiate the proliferative response of progenitor cells to endogenous cytokines *in-vitro*, for example IL-4, or by scheduling a cocktail of different cytokines after treatment to mimic and amplify the time course of expression of the equivalent endogenous molecules or to counteract the effect of endogenous inhibitors.

Current studies suggest that it may be possible to promote the expansion of particular lineages such as megakaryocytes/platelets. If this can be done, for example by the administration of recombinant IL-6, without stimulating the proliferation of the malignant cells, this would be of particular relevance in attempts to increase platelet recovery in conditions such as multiple myeloma where intensive therapy with stem cell rescue has not been associated with cure, but with a better quality and duration of remission and where repeated treatments might provide further prolongation of life particularly when combined with treatments that prolong remission such as interferon.

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APPENDIX I

MISCELLANEOUS TABLES

TABLE 3,2.A

Patients in the First Comparison of Plasma and Serum

Pt	A/S	I	D.S	Previous treatment /t	Current treatment
1	41/M	IgGk	CR	HDM/ABMT /10	Nil
2	46/F	IgGk	CR	HDM/ABMT /9	Nil
3	52/M	IgGl	PD	Vampx3 /18	Nil
4	57/M	IgGk	PR	HDM /10	Interferon
5	47/M	IgGk	SD	HDM /60	Nil
6	52/M	IgGk	CR	HDM/ABMT /14	Nil
7	56/M	IgDl	CR	HDM/ABMT /9	Interferon
8	51/M	IgGk	PD	HDM /36	Nil
9	54/F	BJl	CR	CVAMPx4 /1	Nil
10	38/M	IgGl	PR	HDM/ABMT /12	Nil
11	59/M	IgGk	PR	HDM /3	Nil
12	53/M	IgGk	CR	HDM/ABMT /30	Nil
13	63/F	IgAk	PD	CVAMPx4 /1	Nil
14	70/F	IgGl	PR	CVAMPx7 C-Wklyx8 /2	Interferon
15	55/M	BJk	CR	HDM/ABMT /12	Interferon
16	55/F	IgGk	CR	HDM/ABMT /21	nil

Pt -Patient
A/S -Age/Sex
I -Isotype
D.S -Disease status
t -Time in months since last treatment
M/F -Male/Female
PR/CR -Partial/Complete Remission
PD/SD -Progressive/Stable disease
(C)-VAMP -(Cyclophosphamide)-Vincristine, Doxorubicin, Methyl-Prednisolone (VAMP regimen)
HDM -High Dose Melphalan
ABMR -Autologous Bone Marrow Rescue
C-Wkly -Weekly Cyclophosphamide

TABLE 3,2.B

Patients in the Second Comparison of Plasma and Serum

Pt	A/S	I	D.S	Previous treatment /t	Current treatment
17	47/M	IgGl	SD	HDM /28	Nil
18	59/M	IgAl	CR	HDM/ABMR /9	Nil
19	48/M	IgDk	PR	HDM/ABMR /24	Nil
20	61/M	IgAk	SD	HDM/ABMR /33	Nil
21	56/M	BJk	CR	HDM/ABMT /15	Nil
22	34/F	IgGk	CR	HDM/ABMR /33	Nil
23	46/M	IgAk	CR	HDM/ABMR /20	Nil

Key - See Table 3,2.A

TABLE 3,2.C

Patients in the Third Comparison of Plasma and Serum

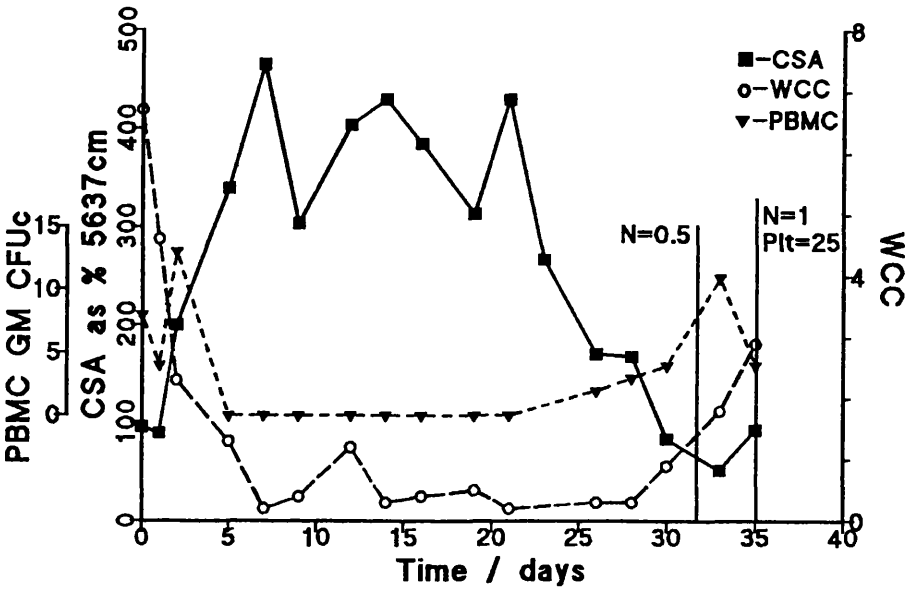
Pt	A/S	I	D.S	Previous treatment /t	Current treatment
24	55/M	IgGk	SD	HDM /60	Nil
25	53/F	IgGl	SD	HDM /90	Nil
26	65/M	IgGk	CR	HDM/ABMR /34	Nil
27	56/M	IgGk	SD	None	Nil
28	62/M	IgAk	PD	C-VAMP X4 /3	Interferon
29	59/M	IgGk	SD	HDM/ABMR /15	Interferon
30	55/F	BJk	PR	C-VAMP x3 /2	Nil
31	67/M	IgGk	PR	C-Wkly x10 /19	Interferon
32	71/M	IgAk	PR	C-Wkly x20	C-Wkly
33	59/M	IgGk	PD	HDM/ABMR /42	Nil

Key - See Table 3,2.A

APPENDIX II

MISCELLANEOUS FIGURES

FIG 4.2.1



Patient 24 – HDM Day 0 No graft No G-CSF

For this and subsequent figures:

GM CFUc PBMC = Colony numbers per $5x10^5$ PBMNC collected from the same blood sample as the plasma tested for CSA

CSA as % 5637cm = activity of 300 or 500µl of test plasma colony numbers are compared with those obtained from the same normal BMMNC stimulated by 100µl of medium conditioned by the 5637 bladder carcinoma cell line

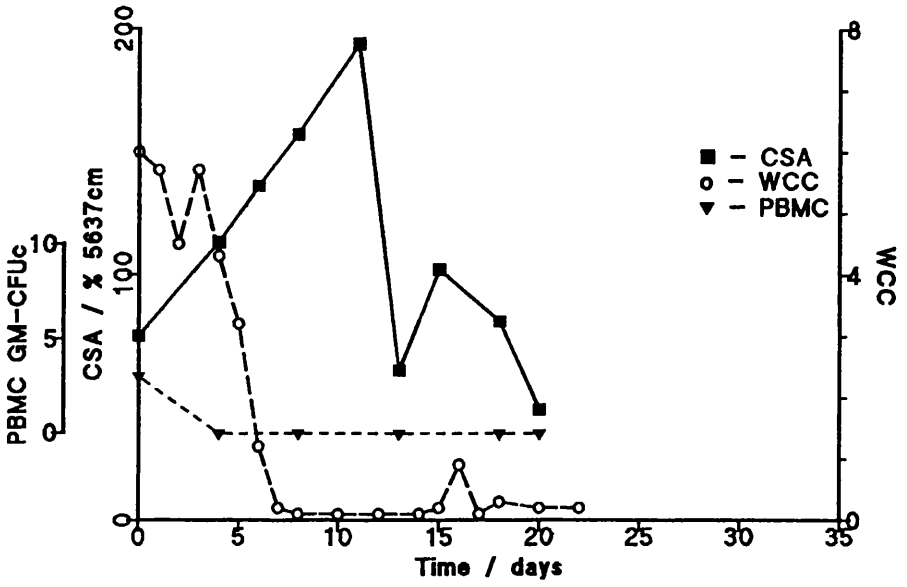
WCC = Total peripheral white cell count x 10^9/litre

N=0.5 = day on which total neutrophil count reached and remained at $0.5x10^9$/l

N=1 = day on which total neutrophil count reached and remained at $1x10^9$/l

Plt=25 = day from which platelet numbers were maintained at $25x10^9$/l without support by platelet transfusions

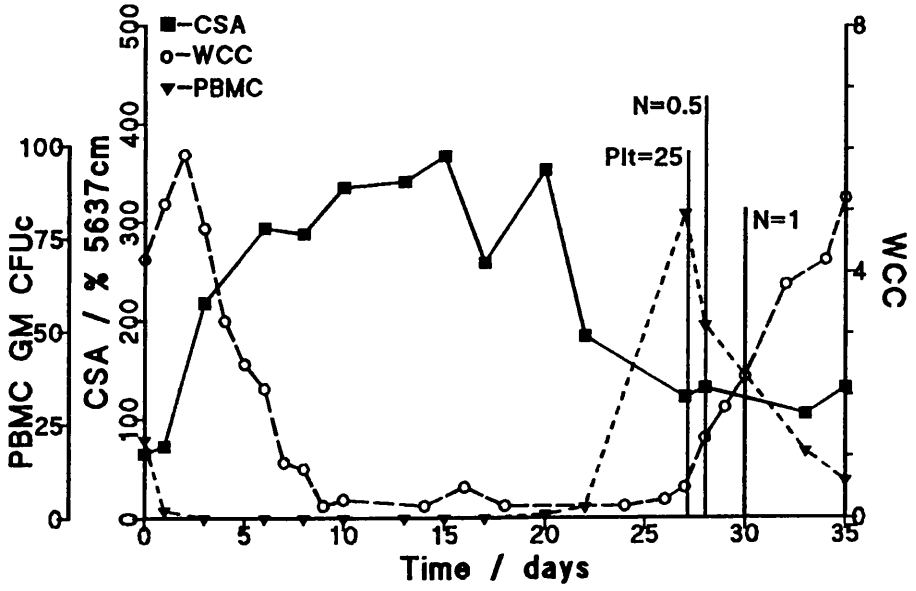
FIG 4,2.2



Pt died Day 22
(resp failure/infection)
–Pneumothorax day 1

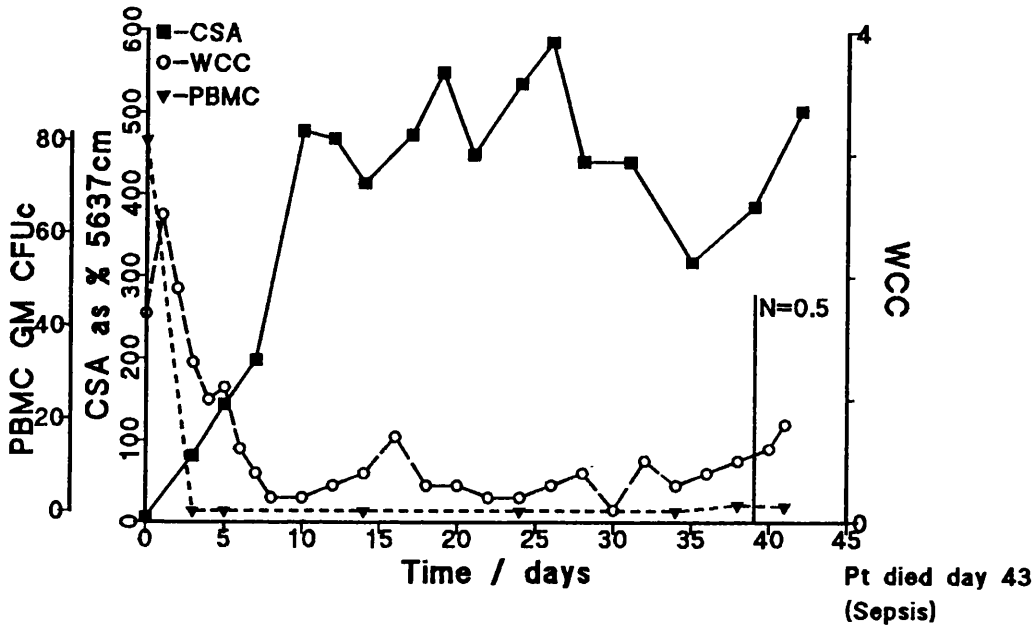
Patient 25 HDM Day 0 No graft No G–CSF

FIG 4,2.3



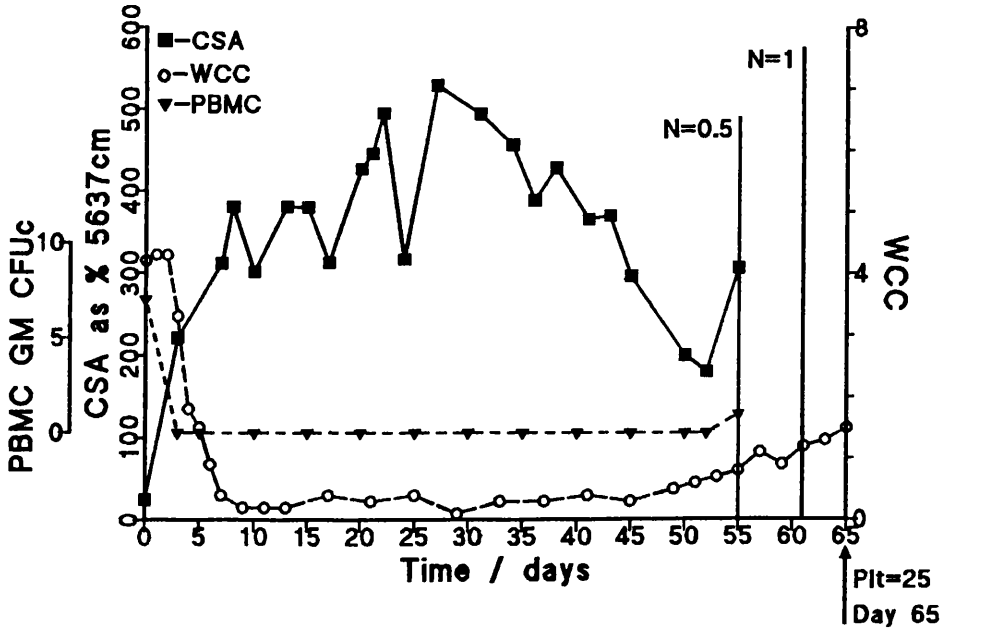
Patient 26 – HDM Day 0 No graft No G–CSF

FIG 4,2.4



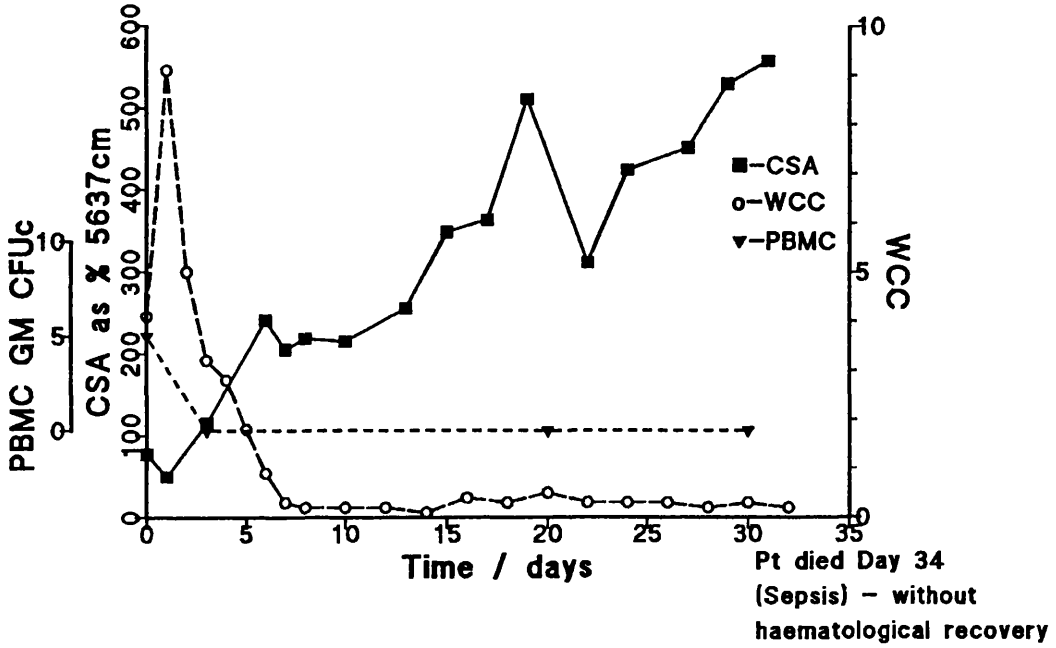
Patient 27 – HDM Day 0 No graft No G–CSF

FIG 4,2.5



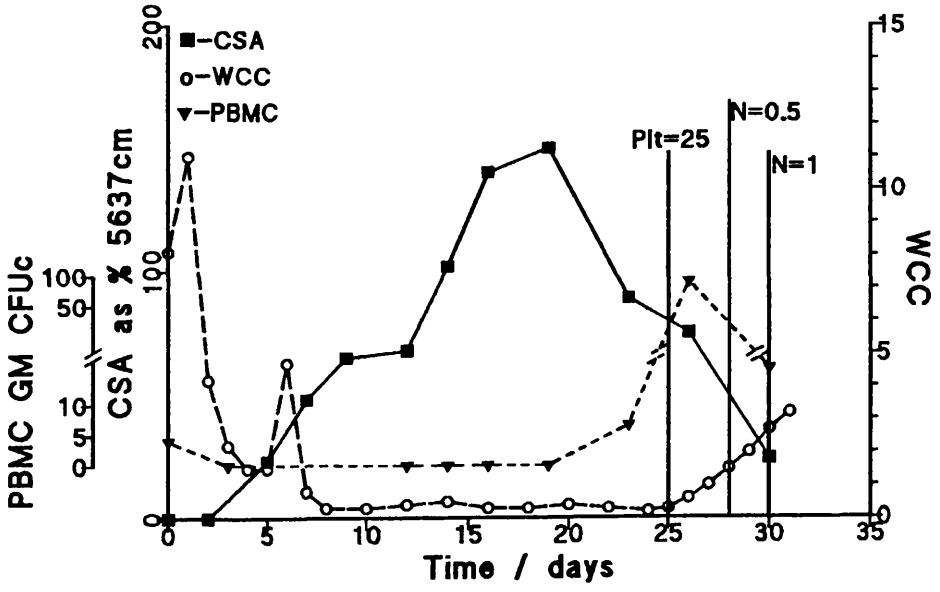
Patient 28 – HDM Day 0 No graft No G–CSF

FIG 4,2.6



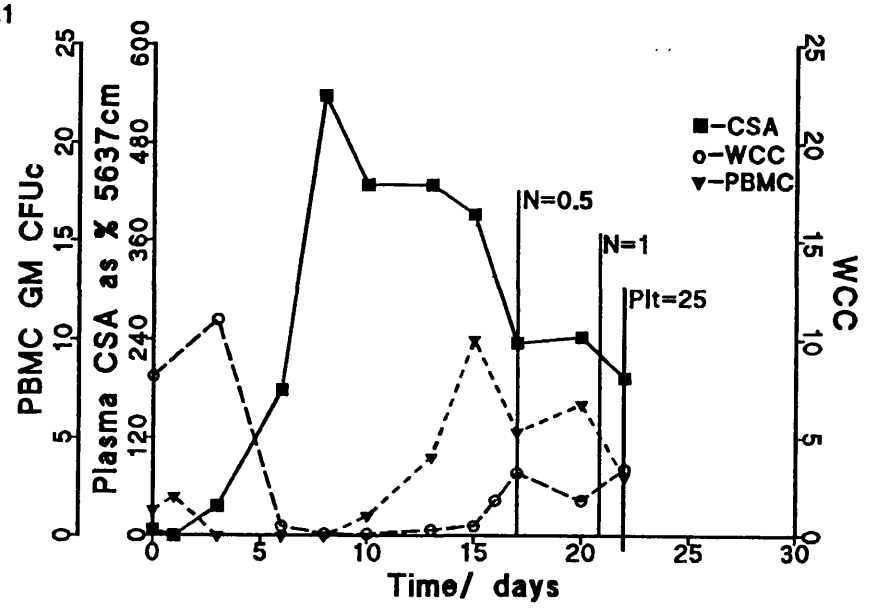
Patient 29 – HDM Day 0 No graft No G–CSF

FIG 4,2.7



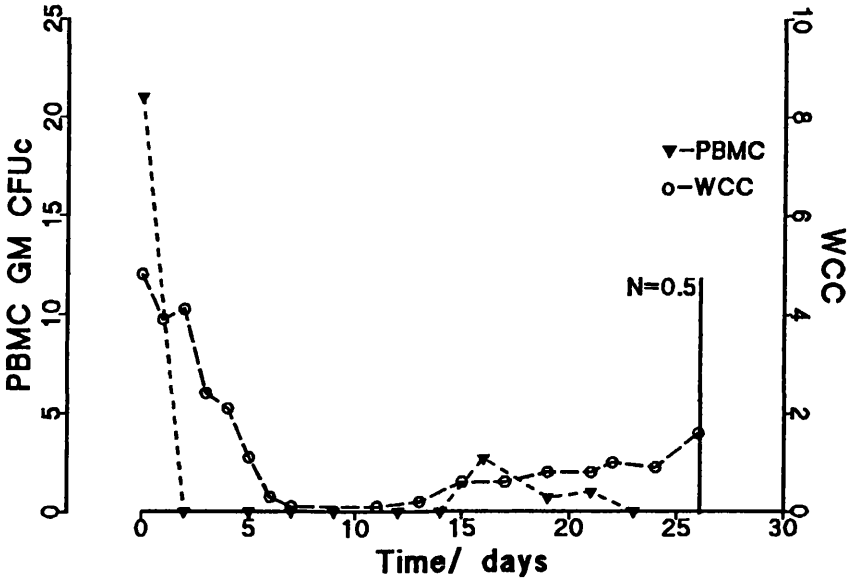
Patient 30 – HDM Day 0 No graft No G–CSF

FIG 4,3.1



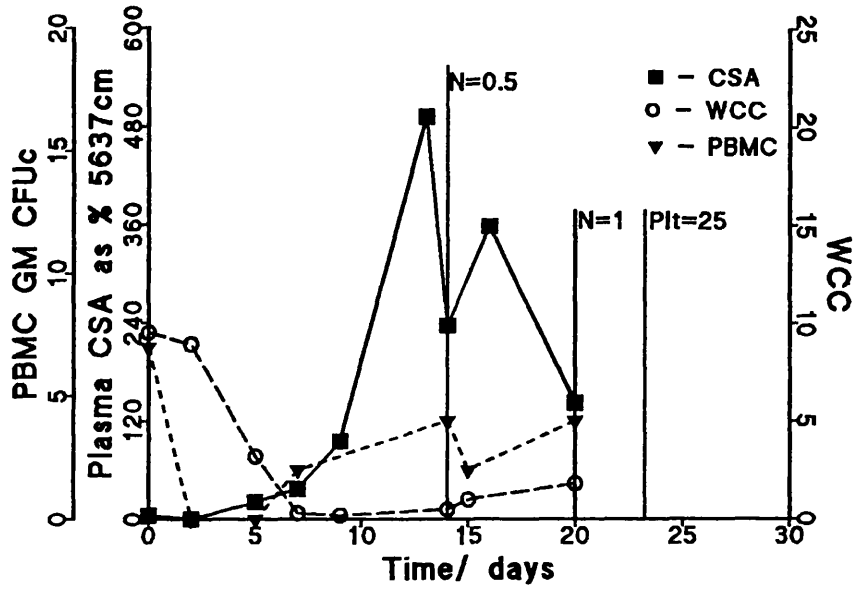
Patient 1 – HDM & ABMR Day 0 – No G–CSF

FIG 4,3.2



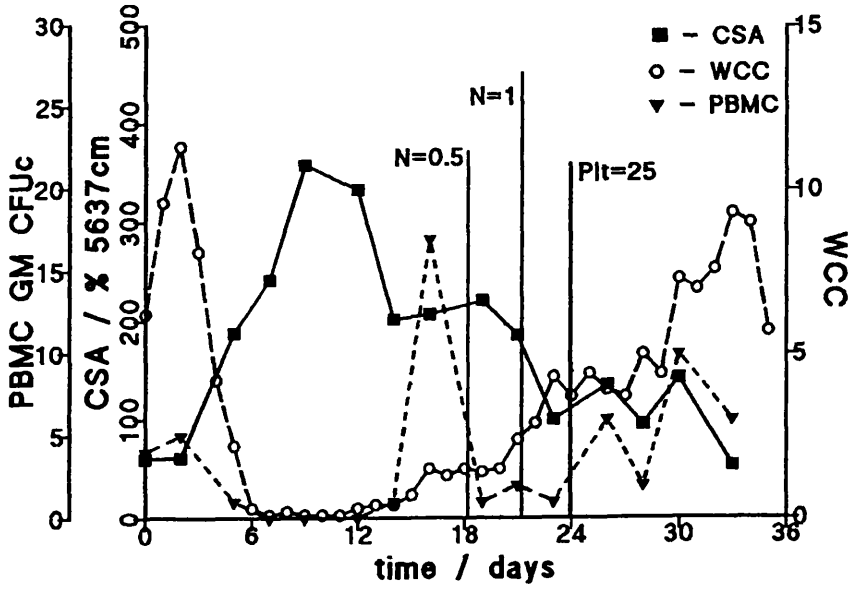
Patient 2 – HDM & ABMR Day 0 – No G–CSF
(CSA data not available – samples destroyed)

FIG 4,3.3



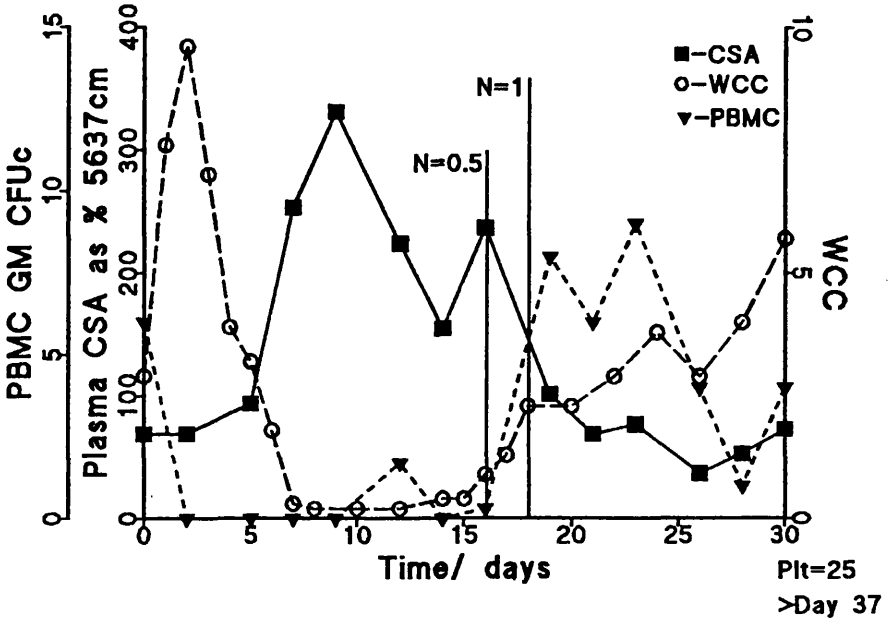
Patient 3 – HDM & BMT Day O No G–CSF

FIG 4,3.4



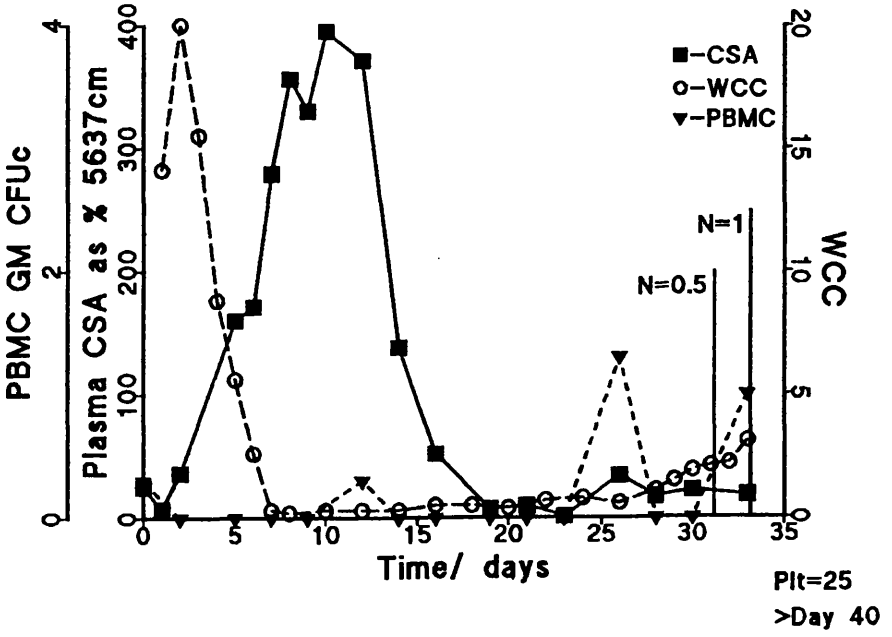
Patient 4 – HDM & ABMT Day 0 – No G CSF

FIG 4,3.5



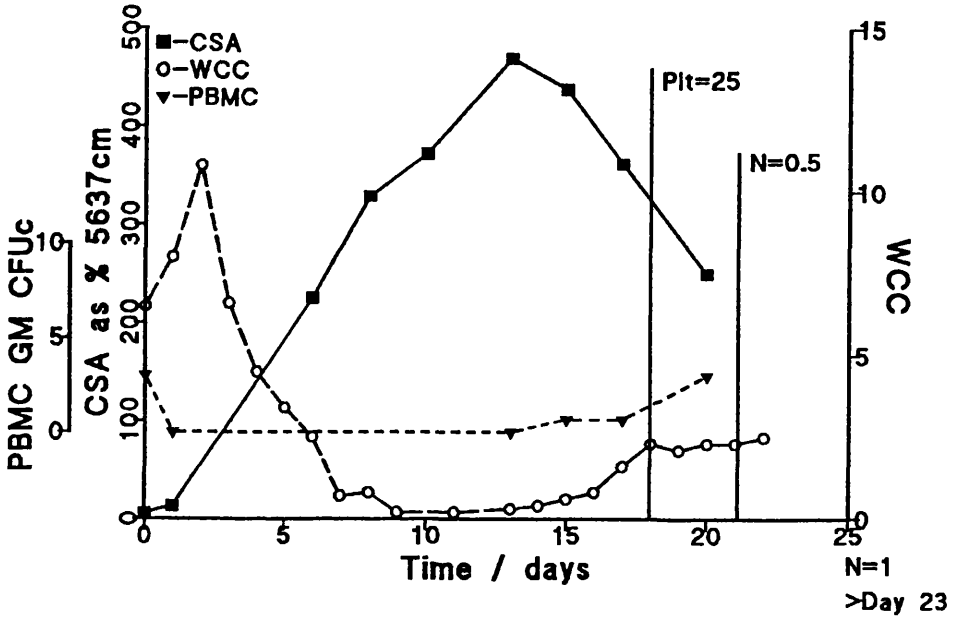
Patient 5 – HDM & ABMT Day 0 – No G-CSF

FIG 4,3.6



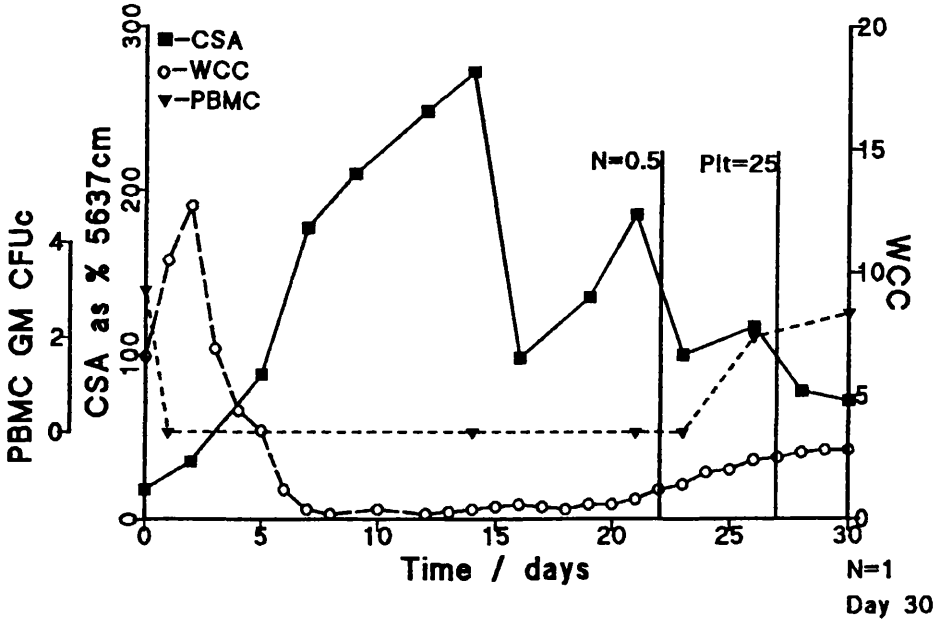
Patient 6 – HDM & ABMT Day 0 – No G-CSF

FIG 4,3.7



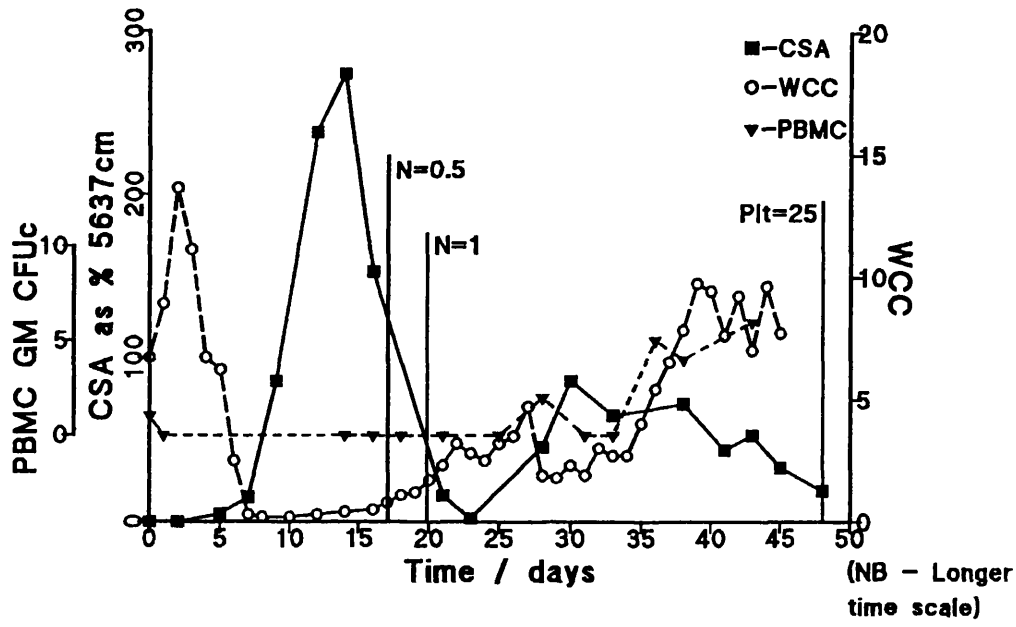
Patient 7 – HDM & ABMT Day 0 – No G–CSF

FIG 4,3.8



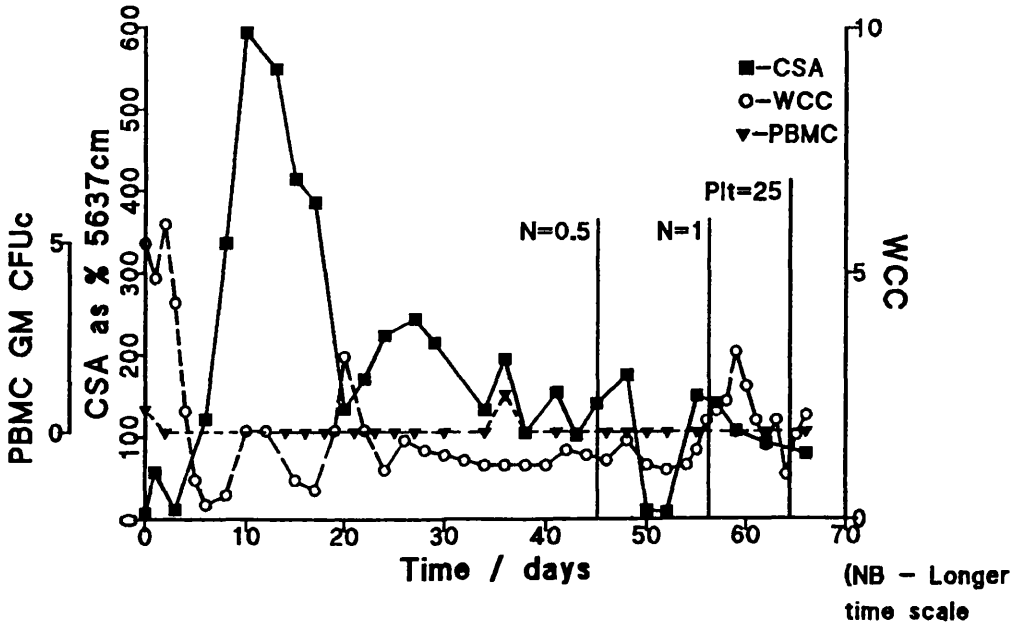
Patient 8 – HDM & ABMR Day 0 – No G–CSF

FIG 4,3.9



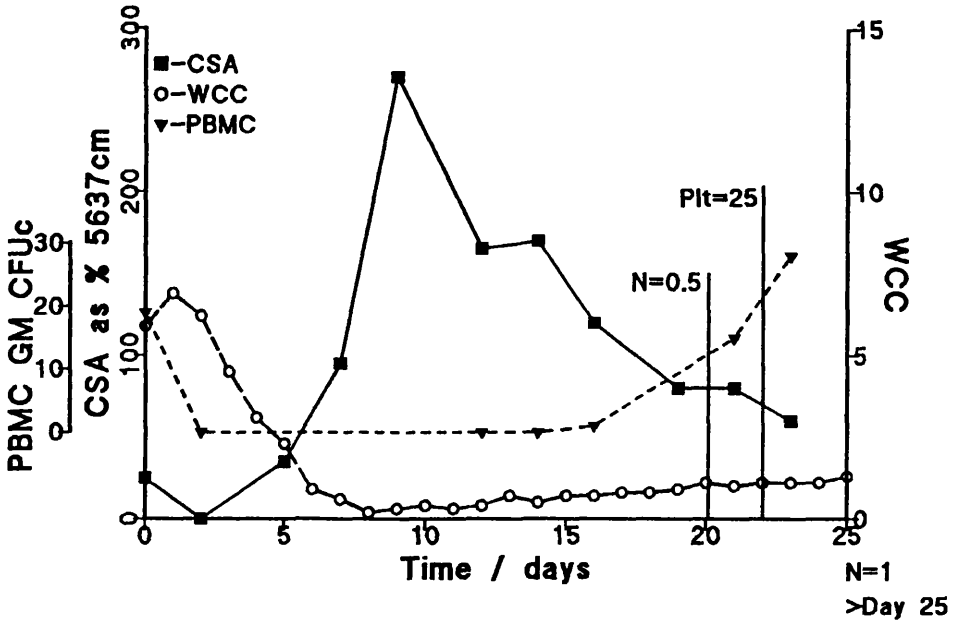
Patient 9 - HDM & ABMR Day 0 - No G-CSF

FIG 4,3.10



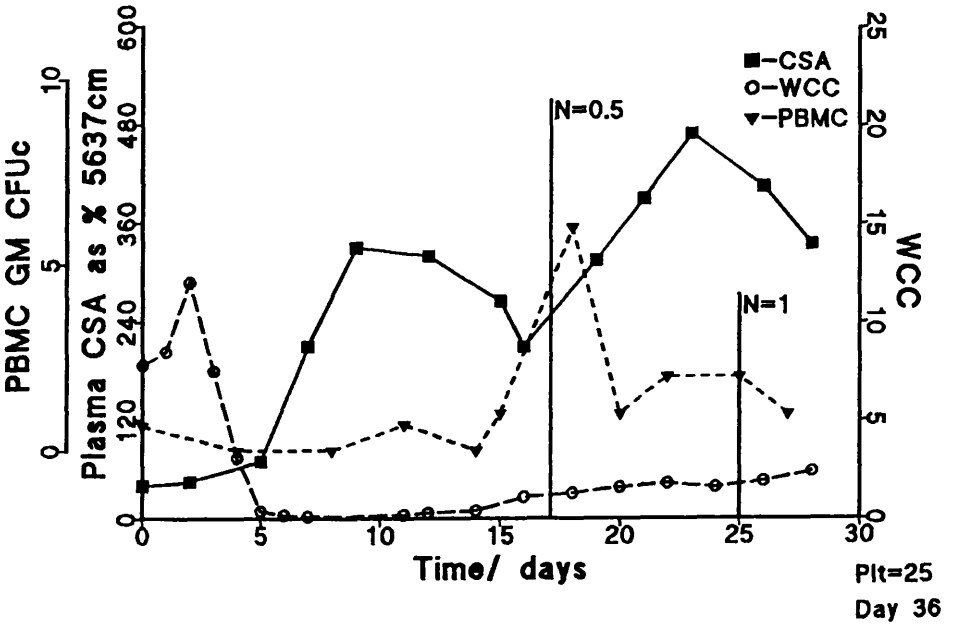
Patient 10 - HDM & ABMR (X2) Days 0 & 50 - No G-CSF

FIG 4,3.11



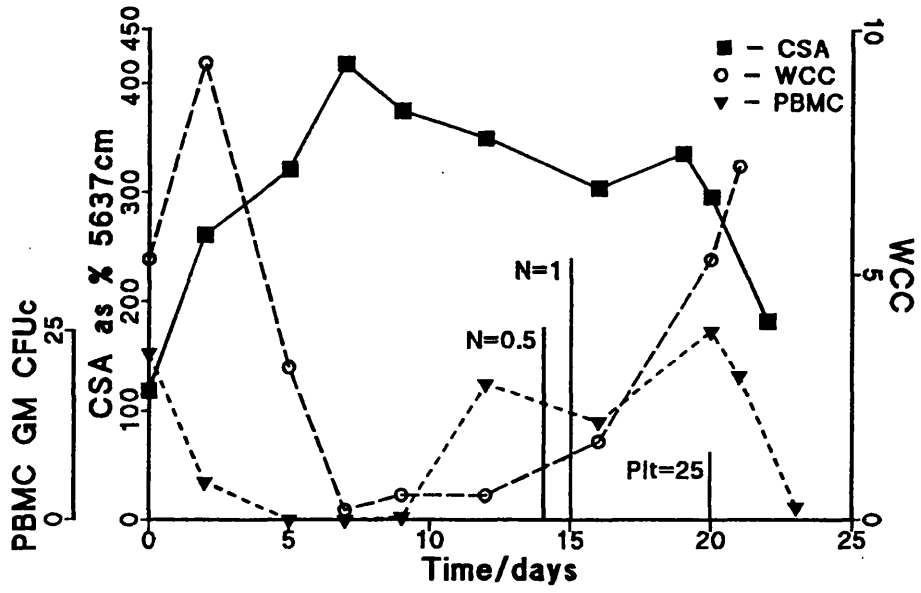
Patient 11 - HDM & ABMR Day 0 - No G-CSF

FIG 4,3.12



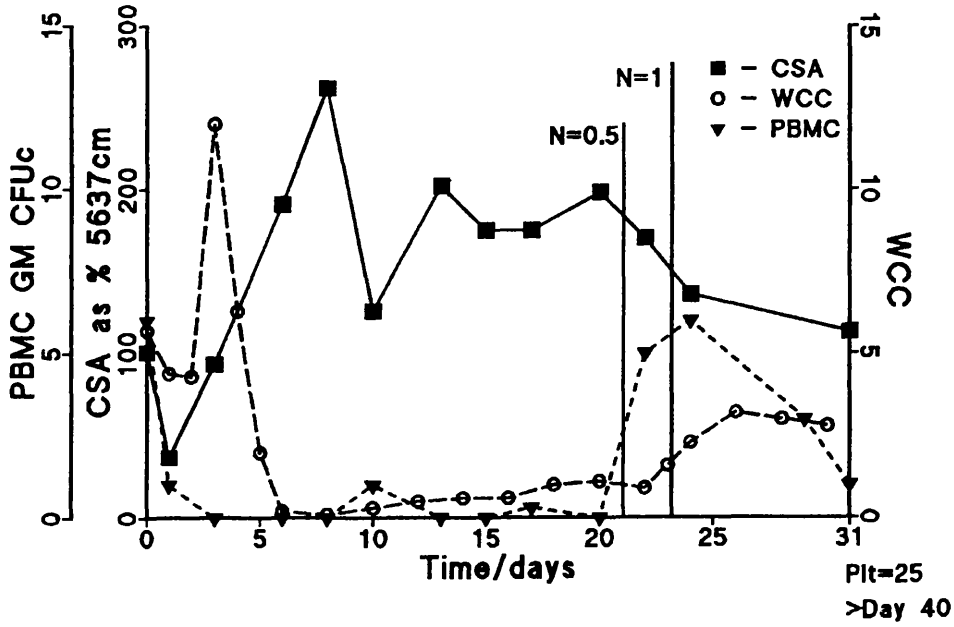
Patient 12 - HDM & ABMR Day 0 - G-CSF 2μg

FIG 4,3.13



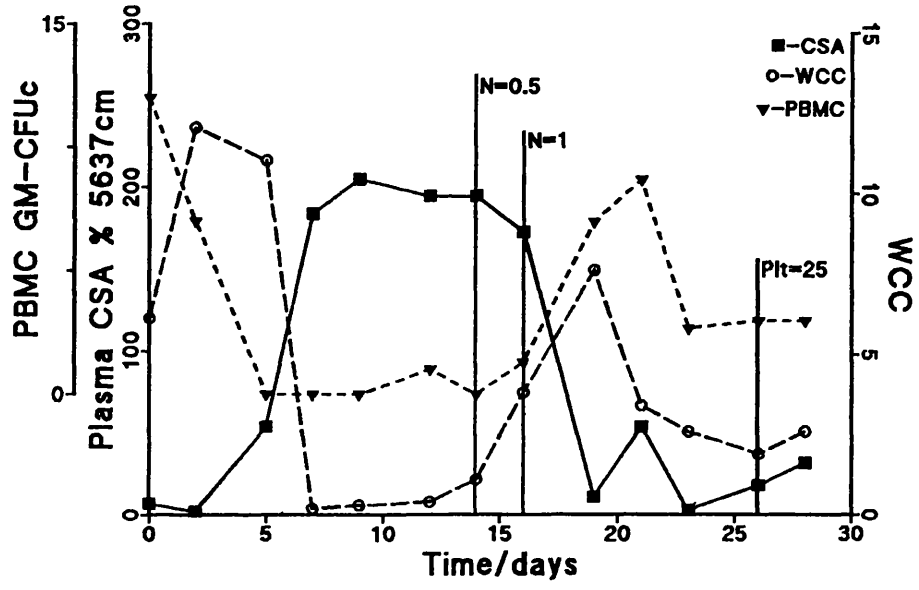
Patient 13 – HDM & ABMR Day 0 – G-CSF 2μg

FIG 4,3.14



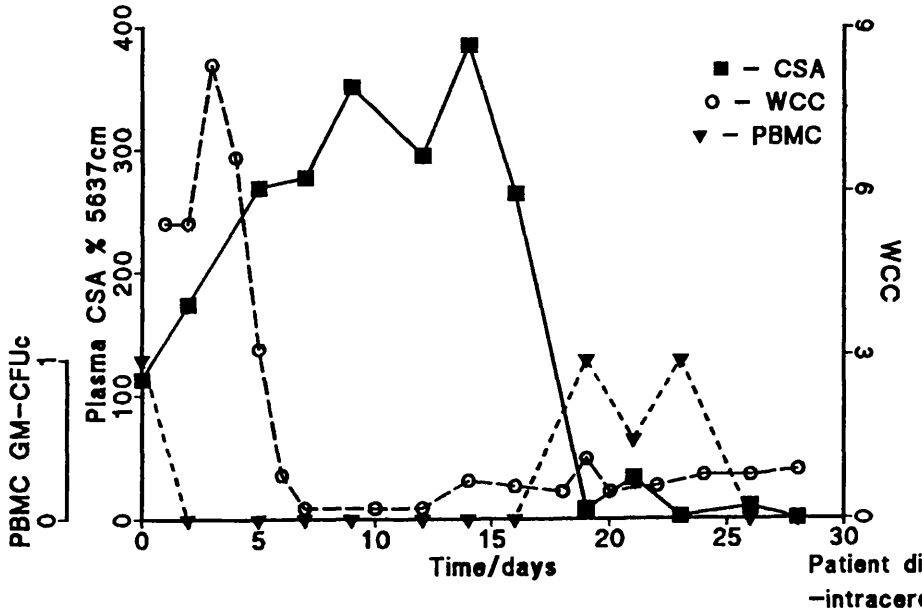
Patient 14 – HDM & ABMR Day 0 – G-CSF 20μg

FIG 4,3.15



Patient 15 – HDM & ABMR Day 0 – G-CSF 10μg

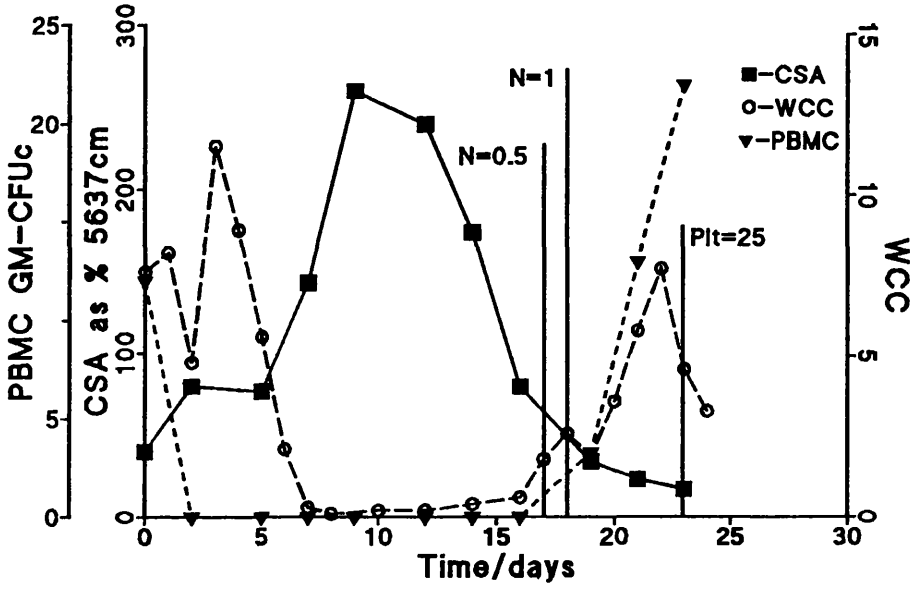
FIG 4,3.16



Patient 16 – HDM & ABMR Day 0 – G-CSF 5μg

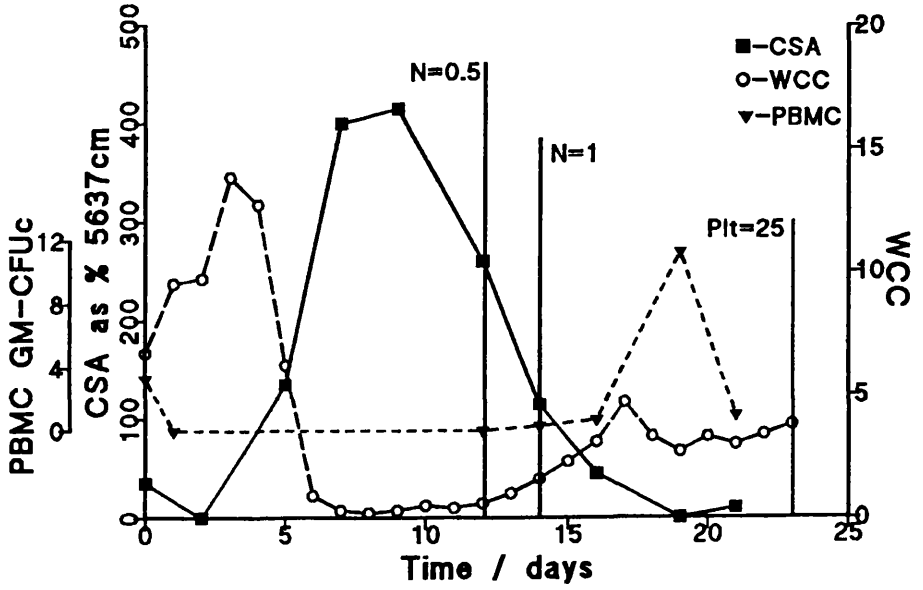
Patient died day 30
–intracerebral haemorrhage
–without engrafting

FIG 4,3.17



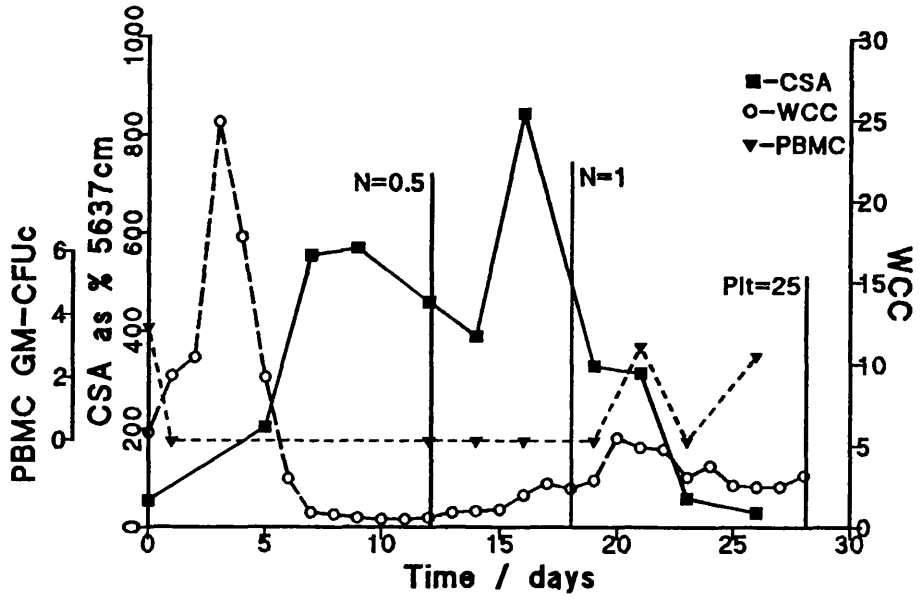
Patient 17 – HDM & ABMR Day 0 – G-CSF 2μg

FIG 4,3.18



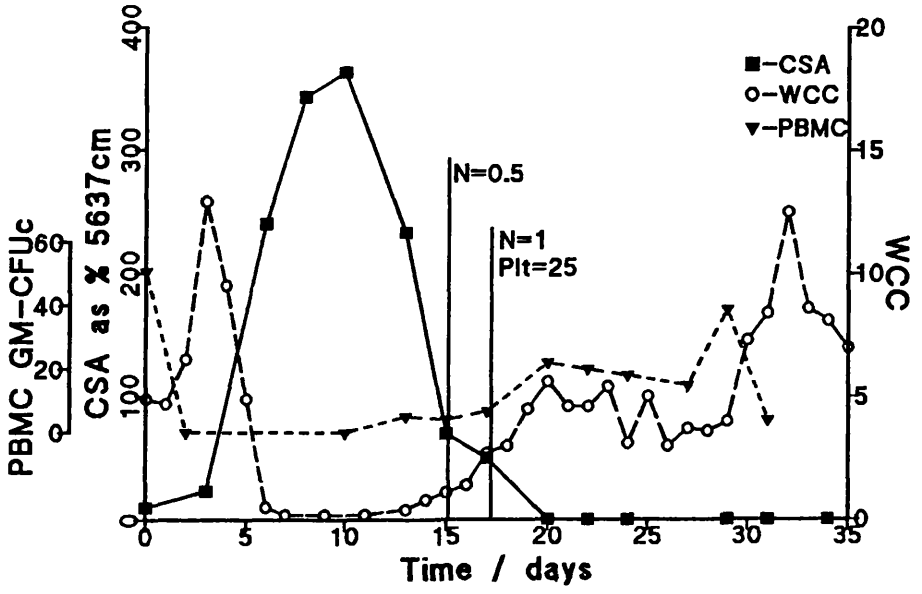
Pt 18 – HDM & ABMR Day 0 + G-CSF 5μg

FIG 4,3.19



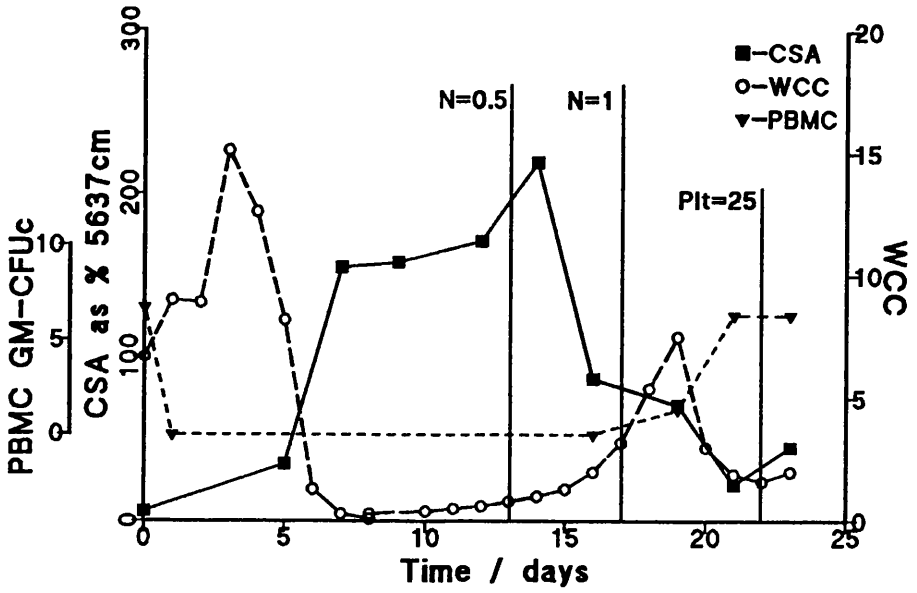
Patient 19 - HDM & ABMR Day 0 + G-CSF 5μg

FIG 4,3.20



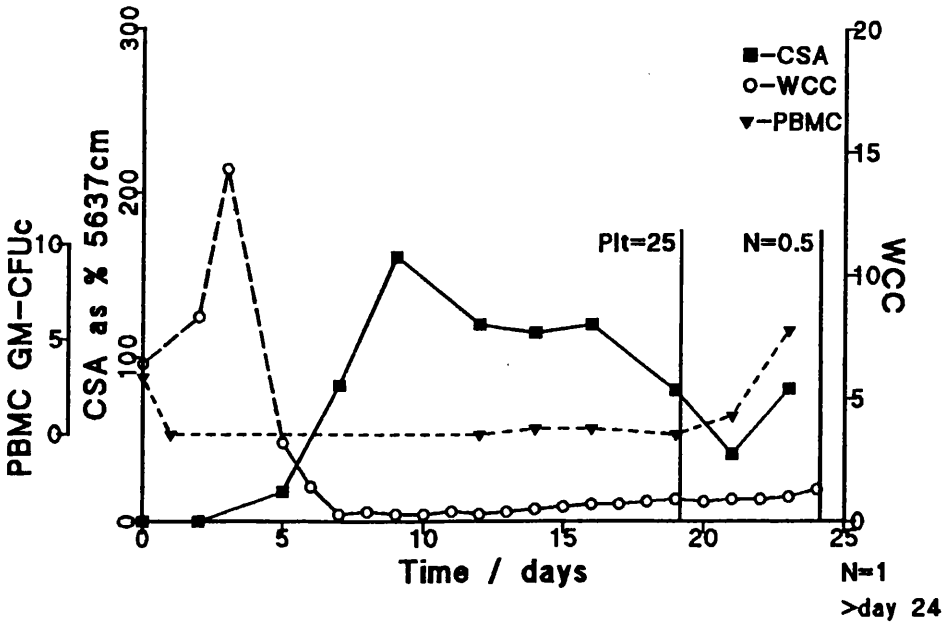
Patient 20 - HDM & ABMR Day 0 + G-CSF 5μg

FIG 4,3.21



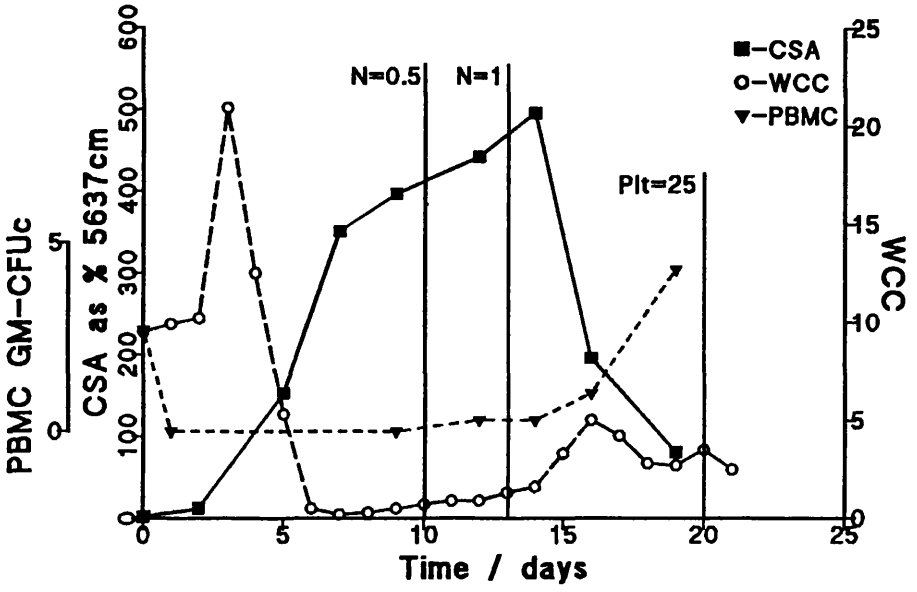
Patient 21 – HDM & ABM Day 0 + G–CSF 5μg

FIG 4,3.22



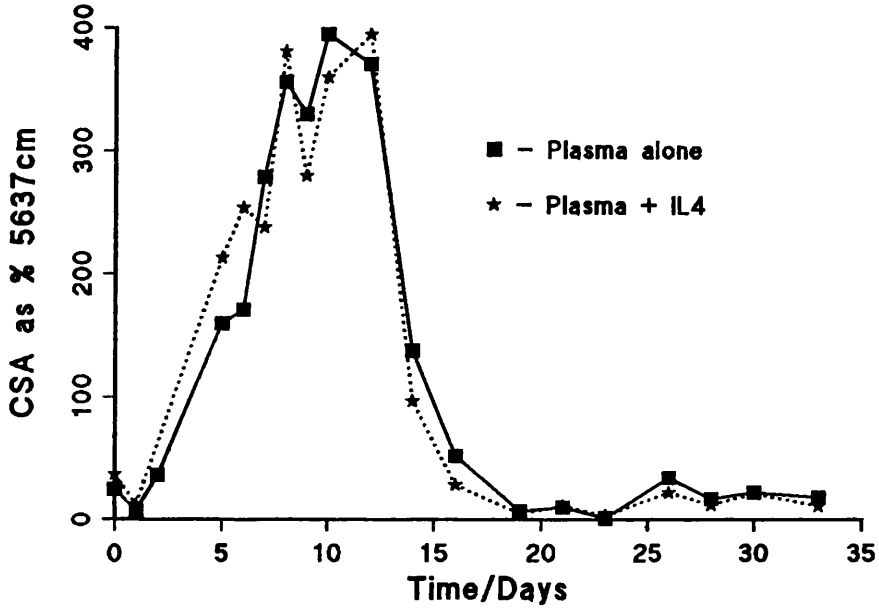
Patient 22 – HDM & ABMR Day 0 + G–CSF 5μg
G–CSF – days 2–5 only

FIG 4,3.23



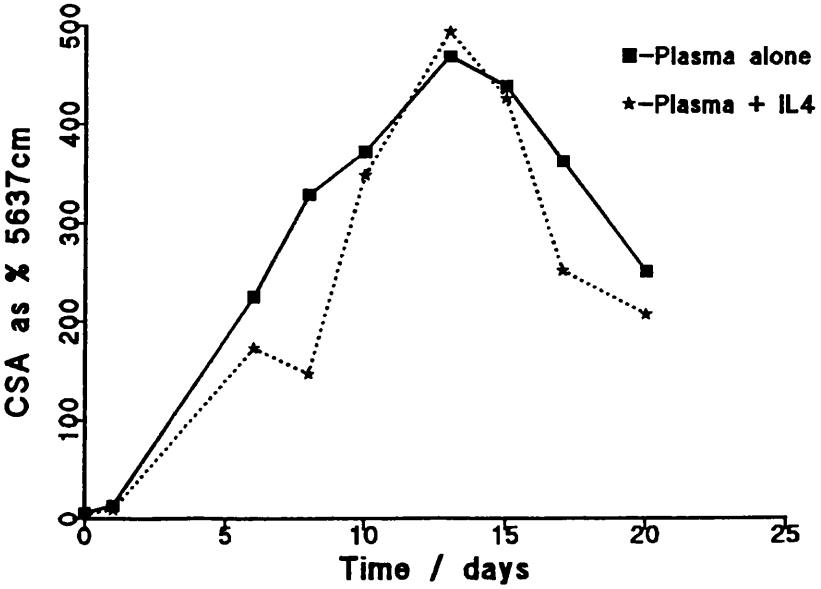
Patient 23 - HDM & ABMR Day 0 + G-CSF 5μg

Fig 4,5.2



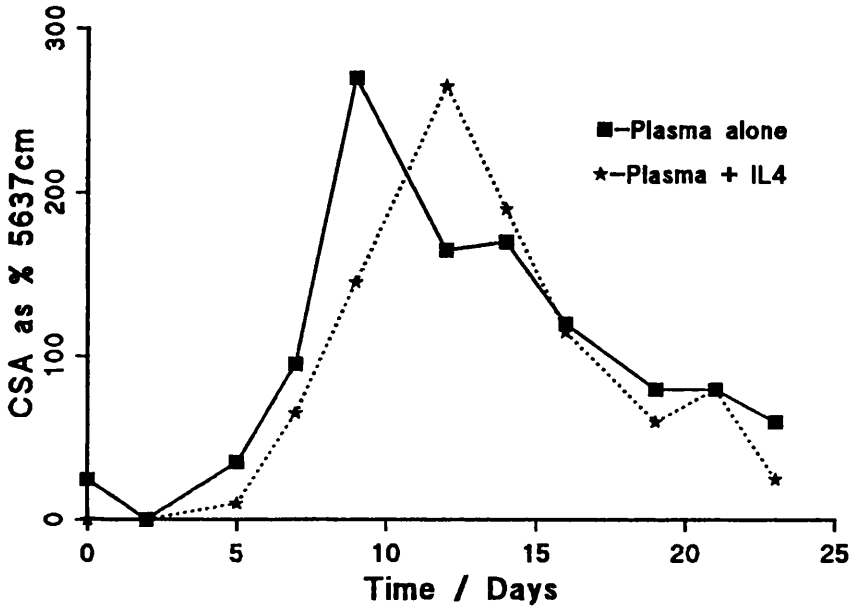
CSA of Pt 6 tested against NBM
with and without the addition of IL–4 in–vitro

Fig 4,5.3



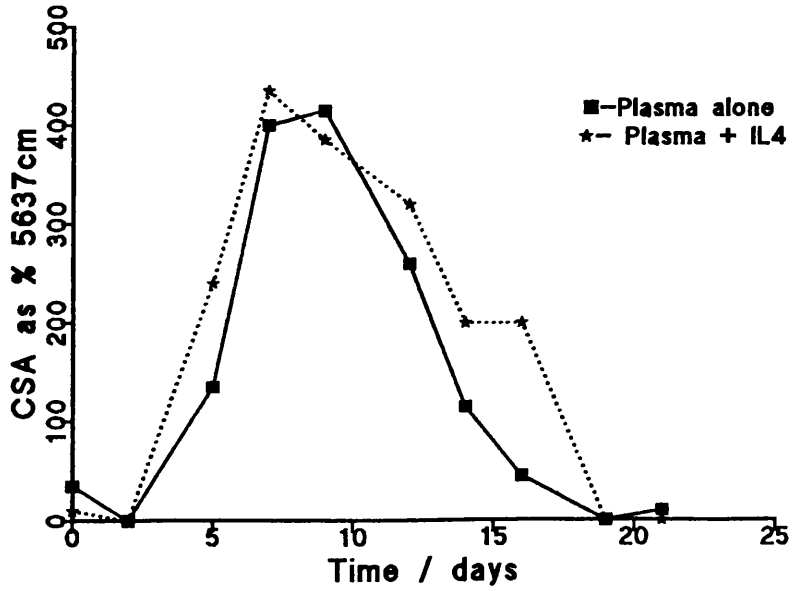
CSA of Pt 7 tested against NBM
with and without the addition of IL–4 in–vitro

FIG 4,5.4



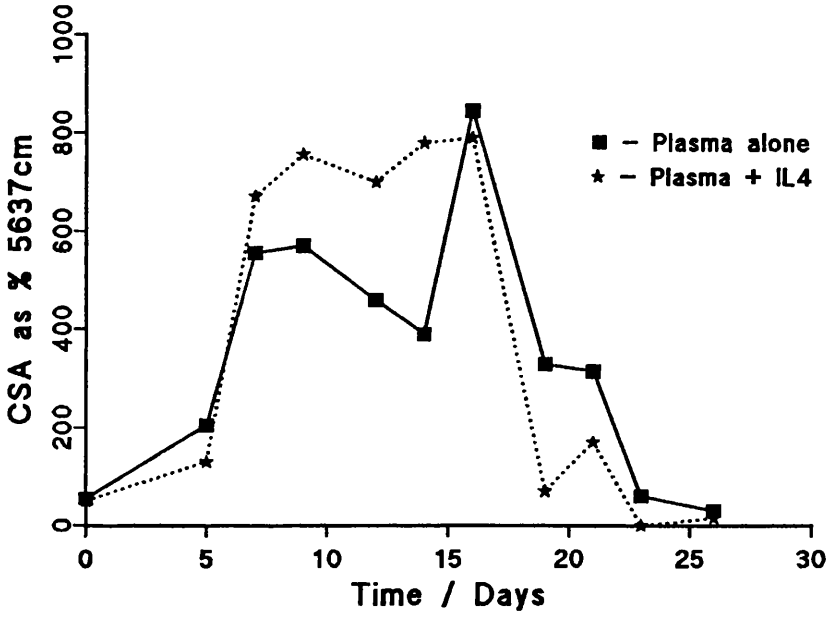
CSA of Pt 11 tested against NBM
with and without the addition of IL–4 in–vitro

Fig 4,5.5



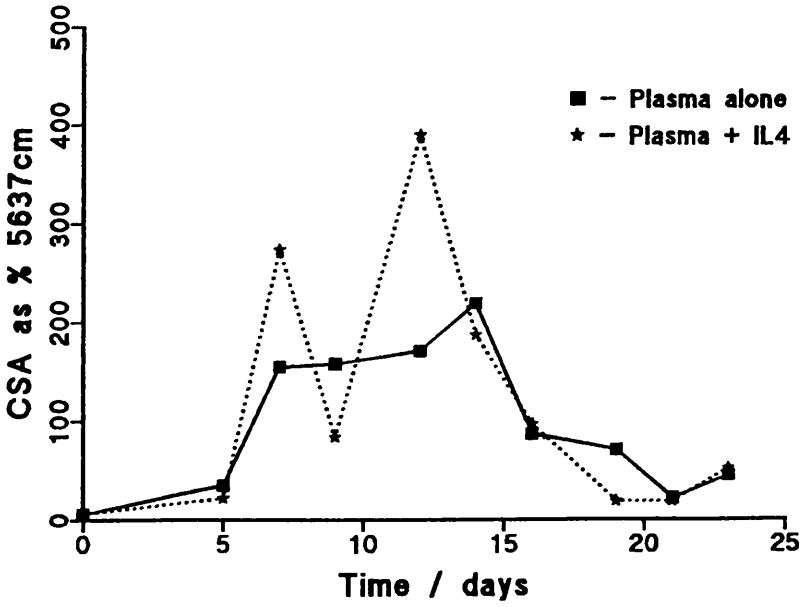
CSA of Pt 18 tested against NBM
with and without the addition of IL–4 in–vitro

FIG 4,5.6



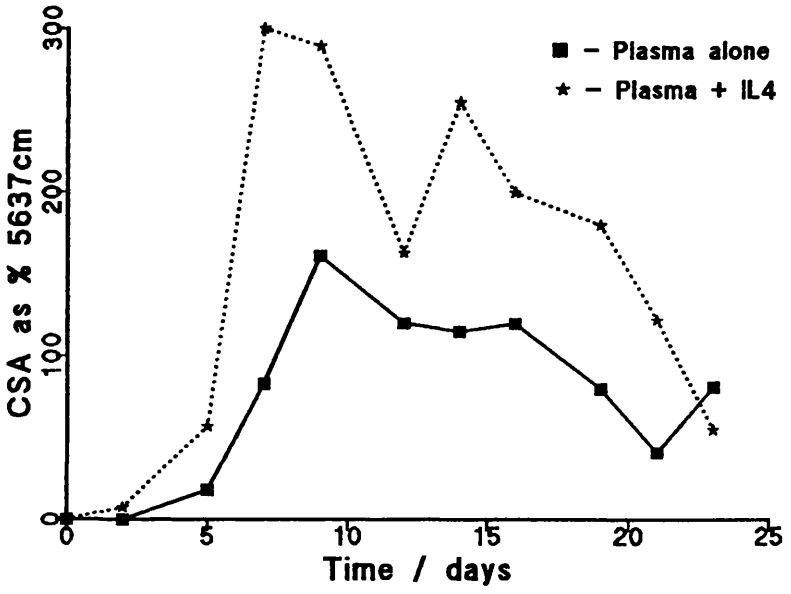
CSA of Pt 19 tested against NBM
with and without the addition of IL–4 in–vitro

Fig 4,5.7



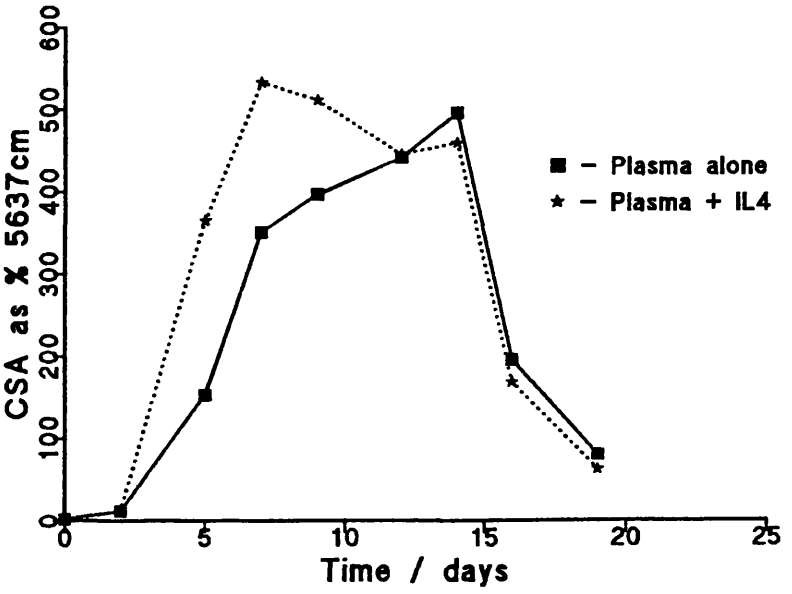
CSA of Pt 21 tested against NBM
with and without the addition of IL–4 in–vitro

Fig 4,5.8



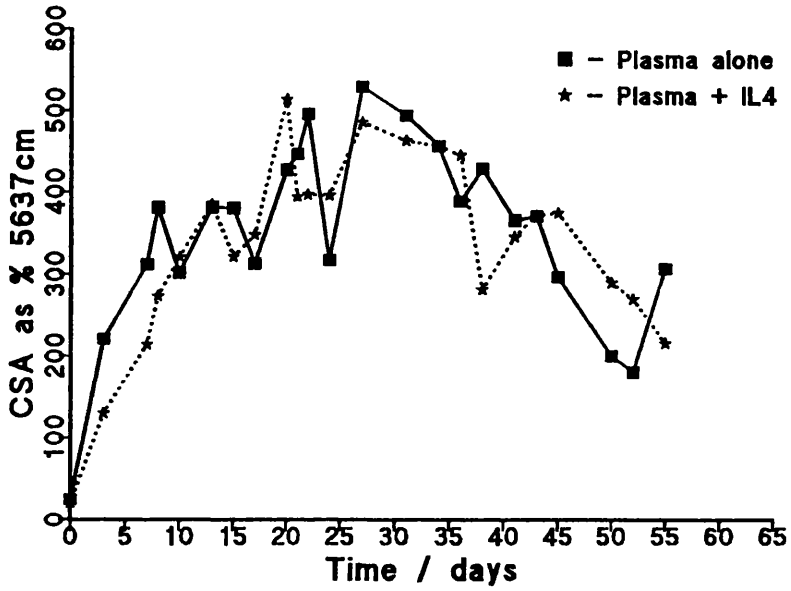
CSA of Pt 22 tested against NBM
with and without the addition of IL–4 in–vitro

Fig 4,5.9



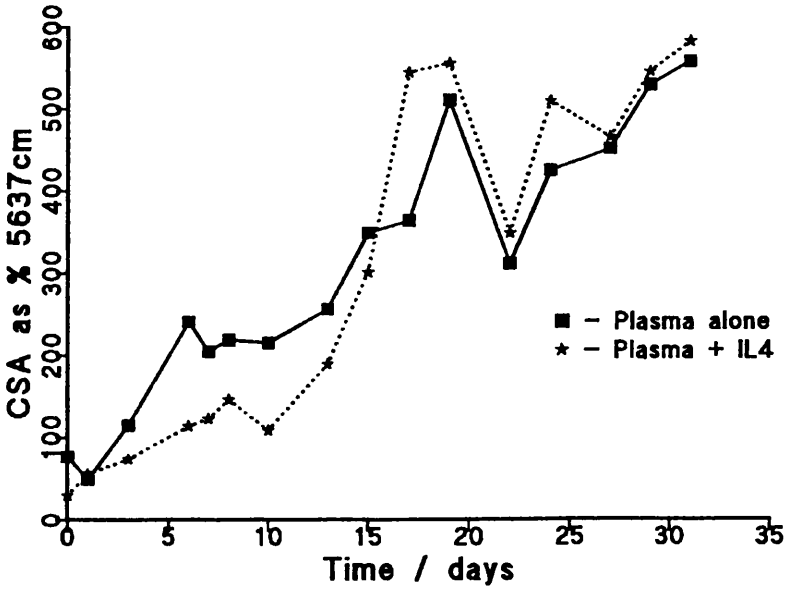
CSA of Pt 23 tested against NBM
with and without the addition of IL–4 in–vitro

Fig 4,5.10



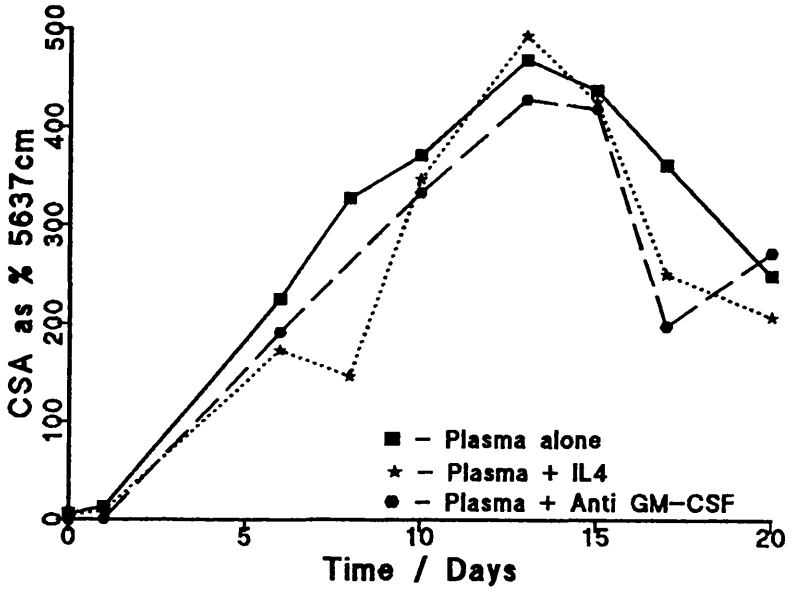
CSA of Pt 28 tested against NBM
with and without the addition of IL–4 in–vitro

Fig 4,5.11



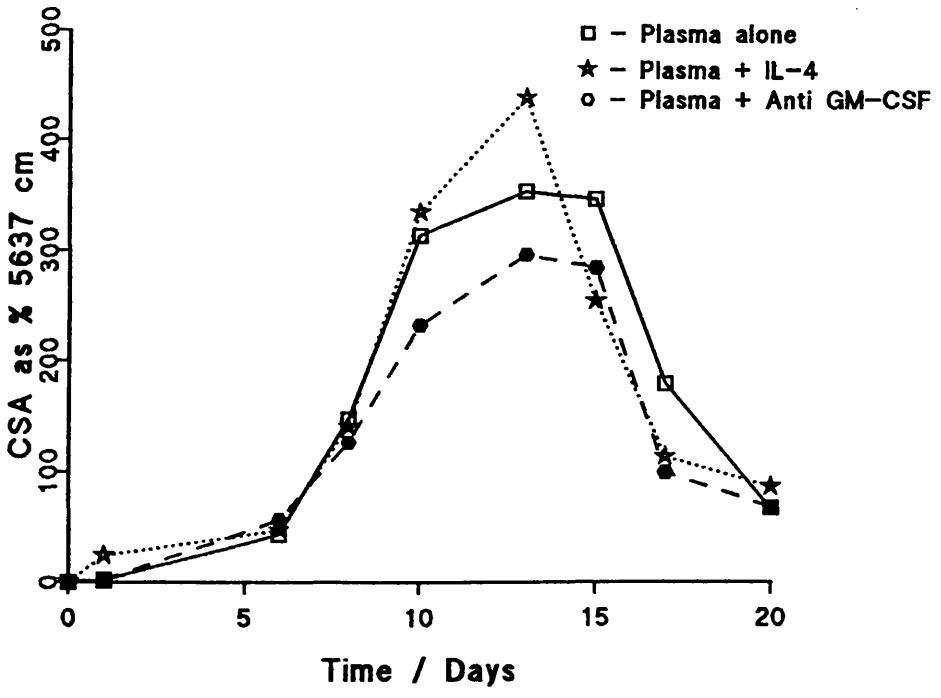
CSA of Pt 29 tested against NBM
with and without the addition of IL–4 in–vitro

Fig 4,7.2.A



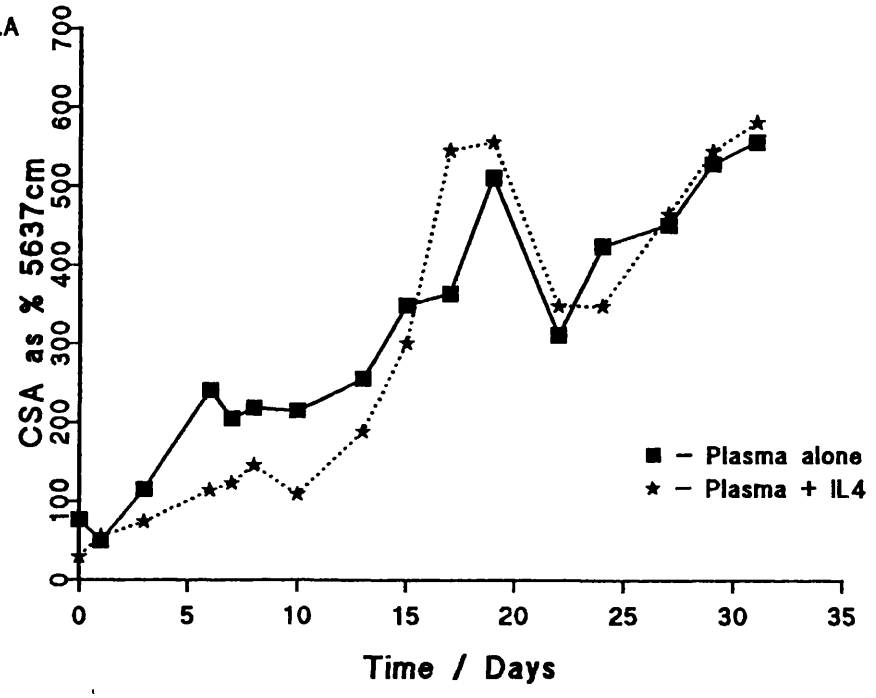
Plasma of Pt 7 tested against unfractionated BMMNC
with/without IL–4 or Antibody to GM–CSF

FIG 4,7.2.B



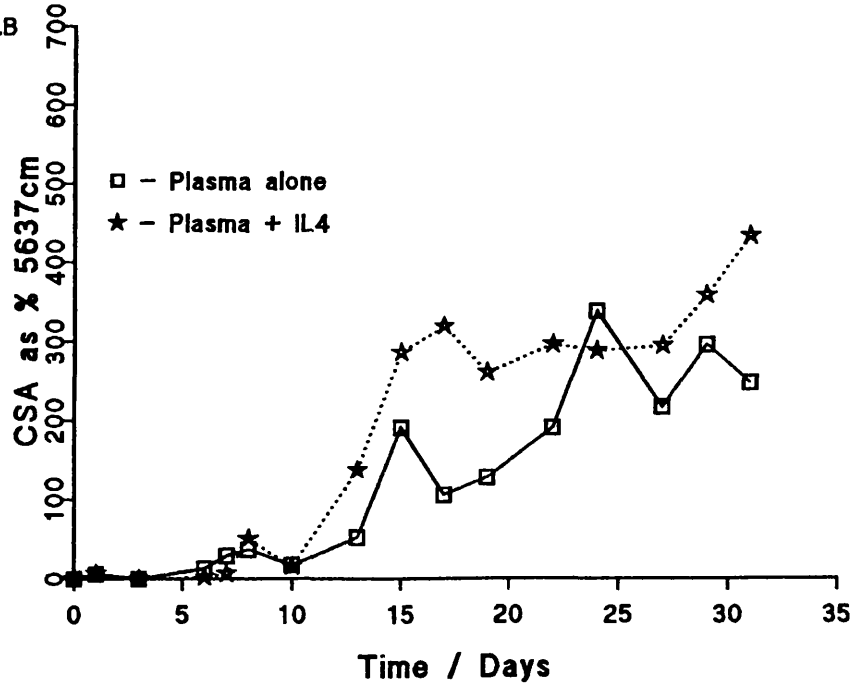
Plasma of Pt 7 tested against CD 34+ve BMMNC
(same donor as above) with/without IL–4 or Anti–GM–CSF

Fig 4,7.3.A



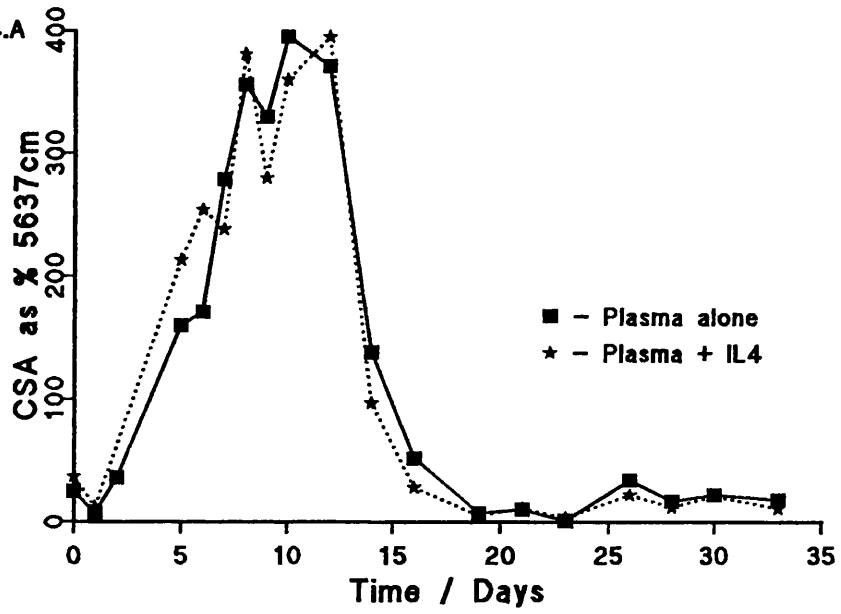
Plasma of Pt 29 tested against unfractionated BMMNC with/without the addition of IL–4

Fig 4,7.3.B



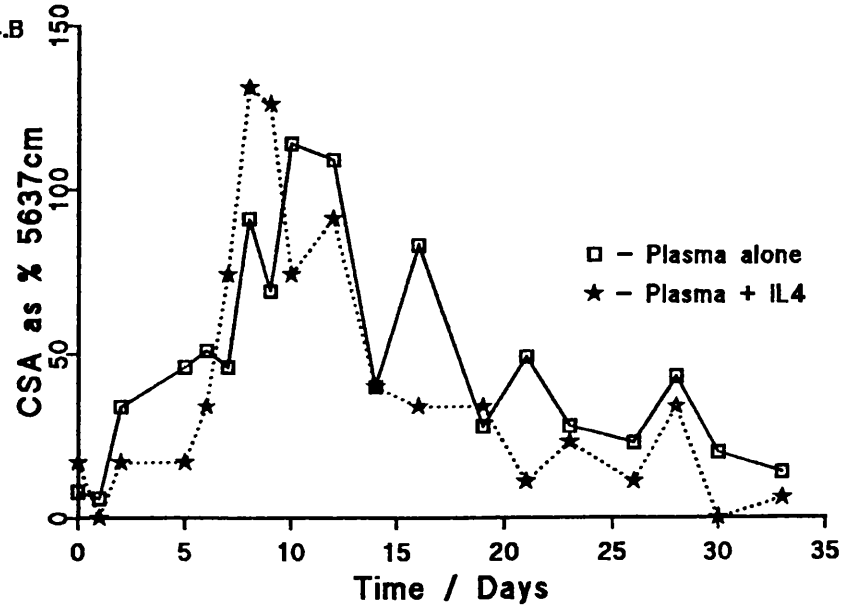
Plasma of Pt 29 tested against CD 34+ve BMMNC (same donor as above) with/without IL–4

Fig 4,7.4.A



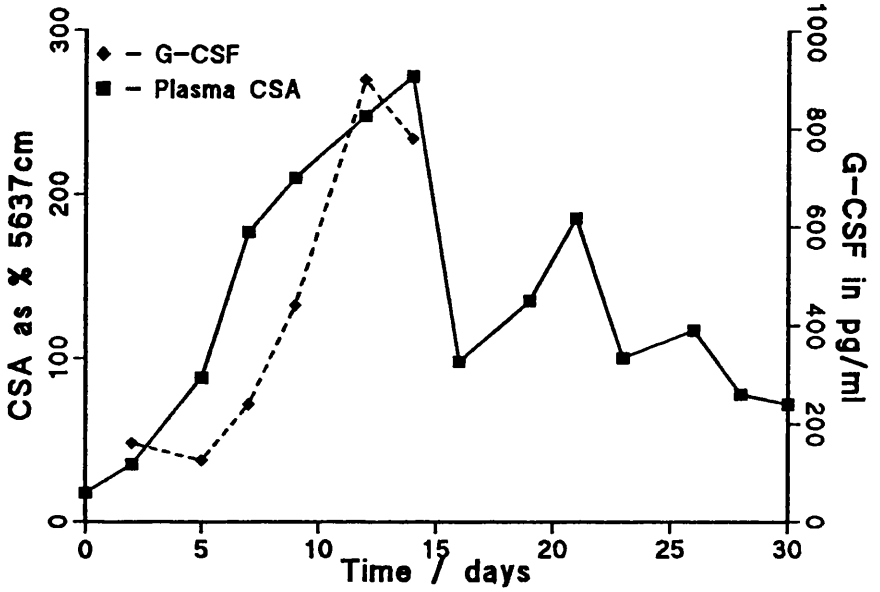
Plasma of Pt 6 tested against unfractionated BMMNC
with/without the addition of IL–4

Fig 4,7.4.B



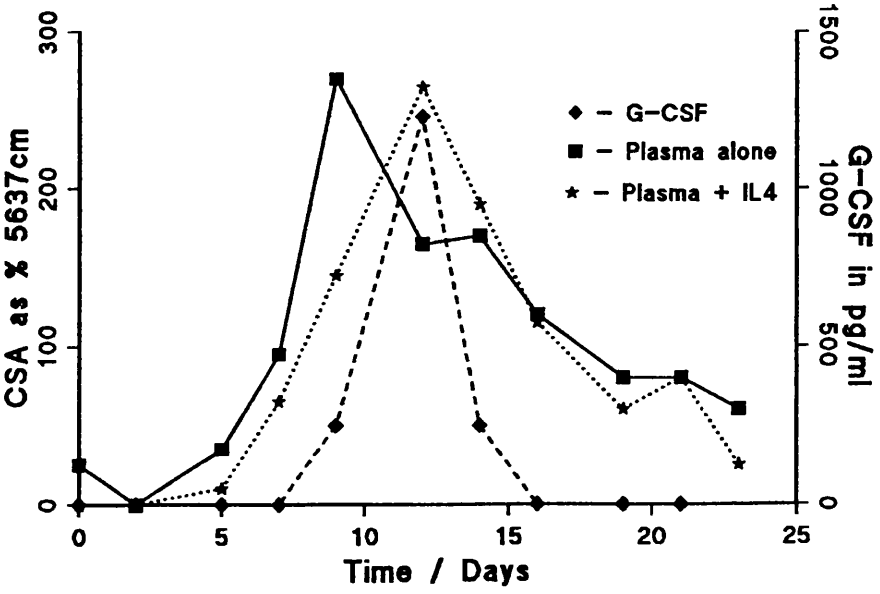
Plasma of Pt 6 against CD 34+ve BMMNC
(DIFFERENT DONOR TO ABOVE) with/without IL–4

FIG 5,1.4



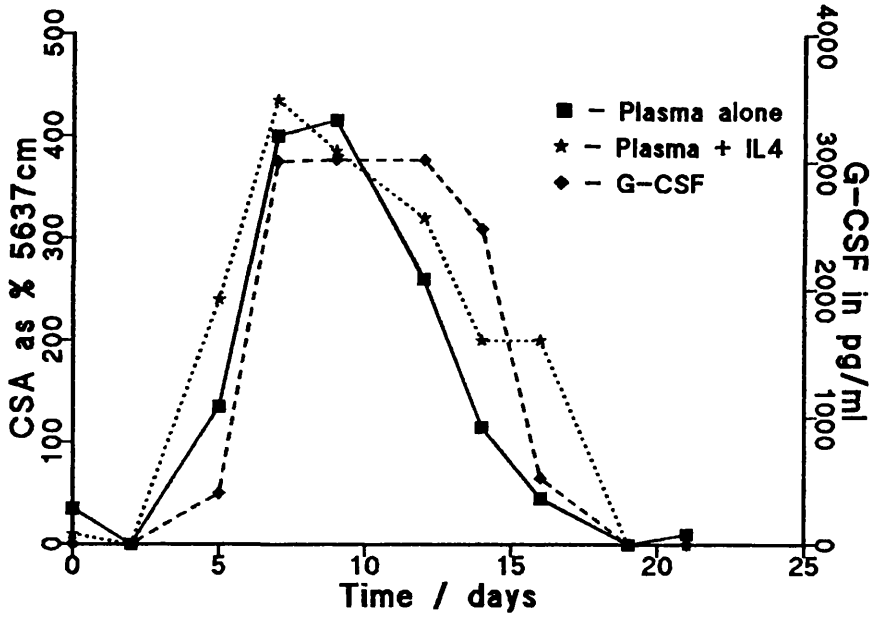
Patient 8 – Comparison of CSA with
Plasma G–CSF after HDM and ABMR (No rhG–CSF)
(NoG–CSF data after day 14)

FIG 5,1.5



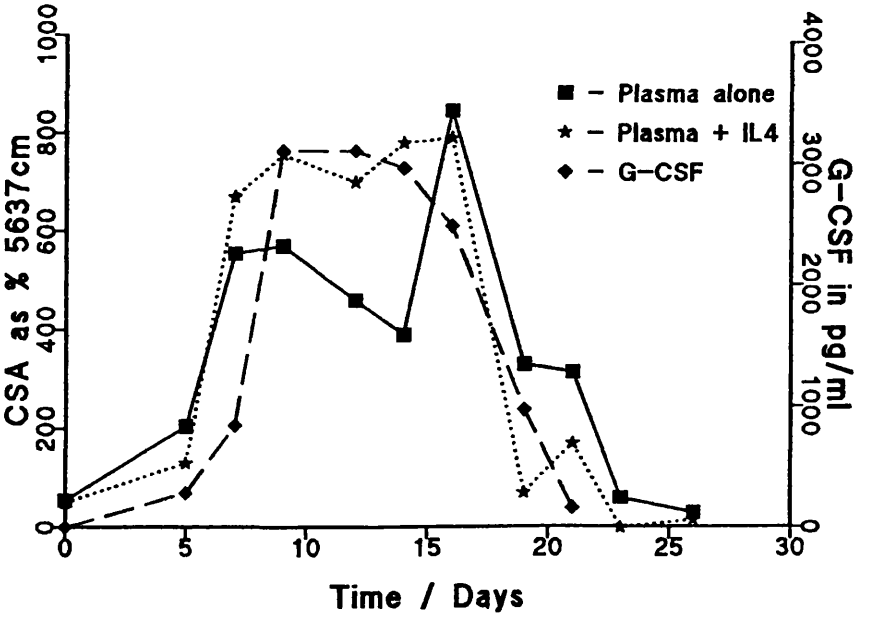
Patient 11 – Comparison of CSA +/– IL–4 with
plasma G–CSF after HDM and ABMR (No rhG–CSF)

Fig 5,1.6



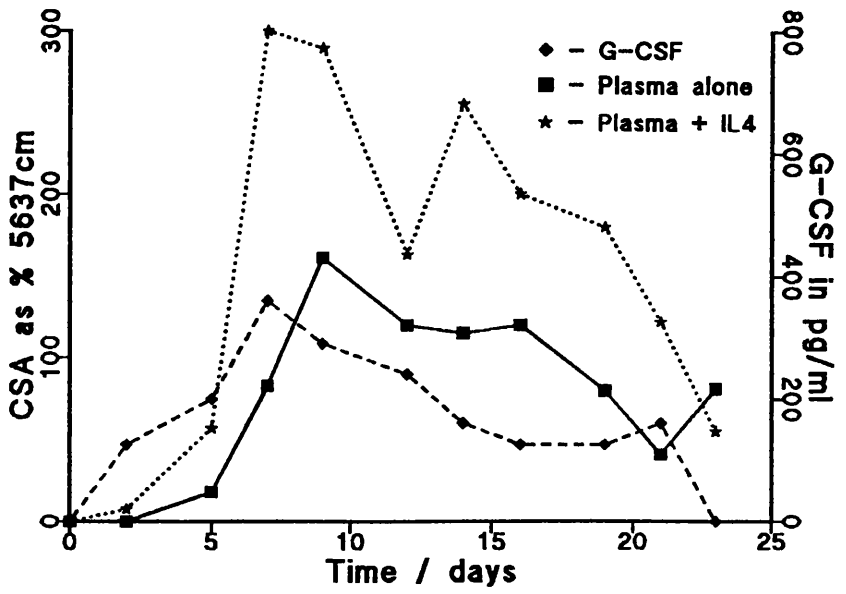
Patient 18 – Comparison of CSA +/– IL-4 with
Plasma G-CSF after HDM & ABMR with rhG-CSF

FIG 5,1.7



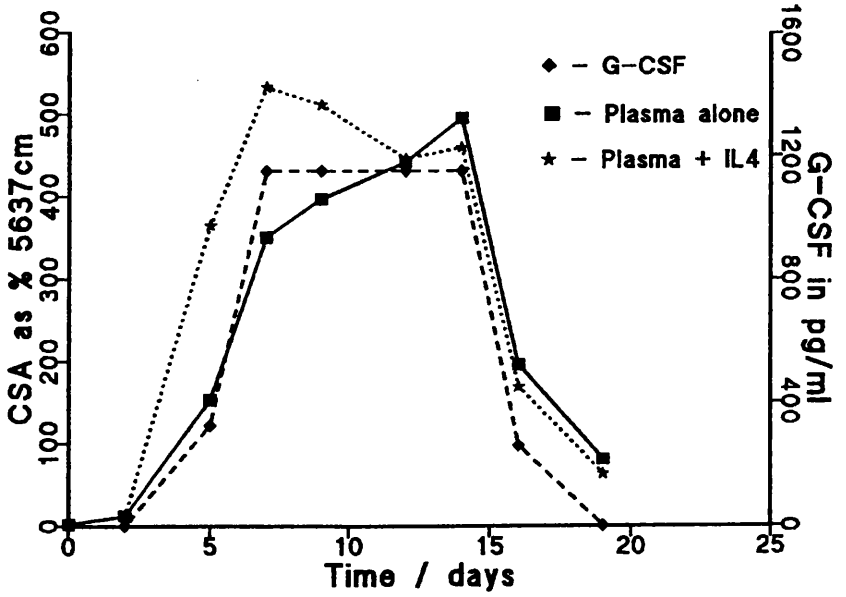
Patient 19 – Comparison of CSA +/– IL-4 with
Plasma G-CSF after HDM & ABMR + rhG-CSF

Fig 5,1.8



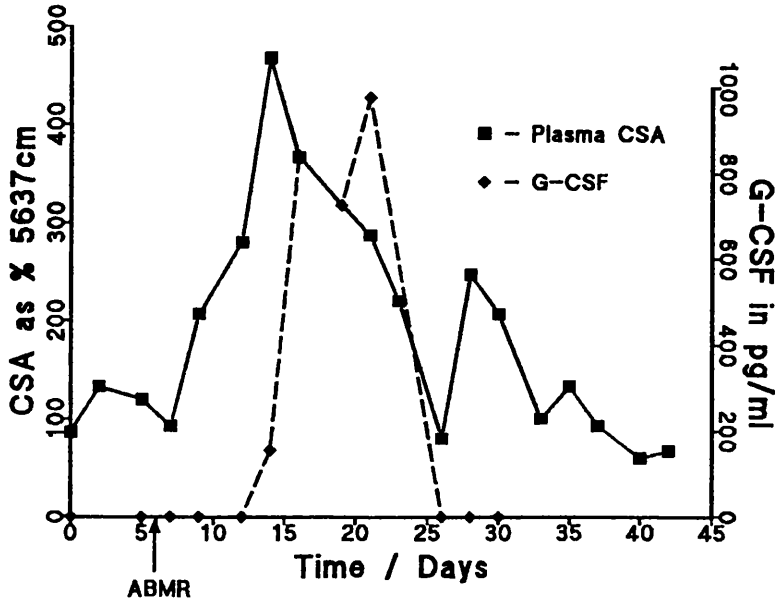
Patient 22 – Comparison of CSA +/– IL-4 with
Plasma G-CSF after HDM & ABMR + rhG-CSF

Fig 5,1.9



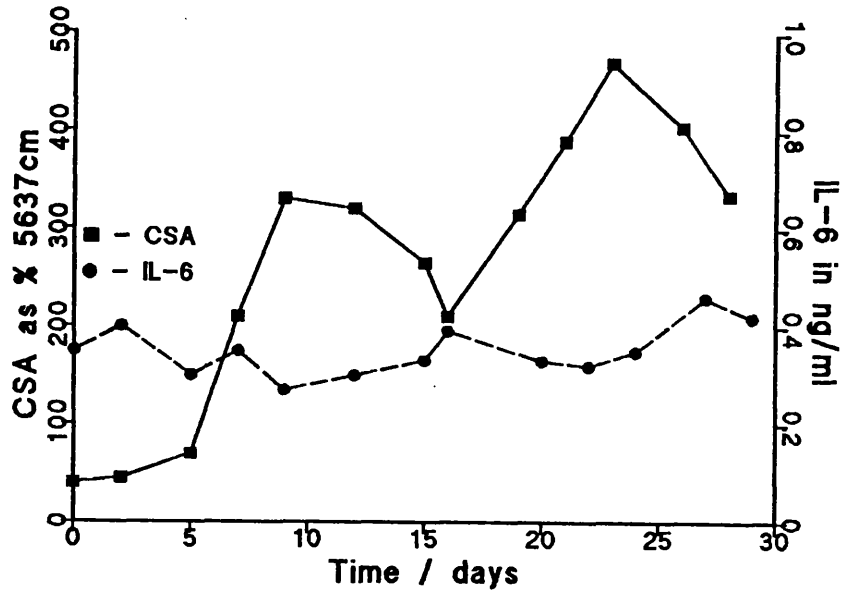
Patient 23 – Comparison of CSA +/– IL-4 with
Plasma G-CSF after HDM & ABMR + rhG-CSF

FIG 5,1.10



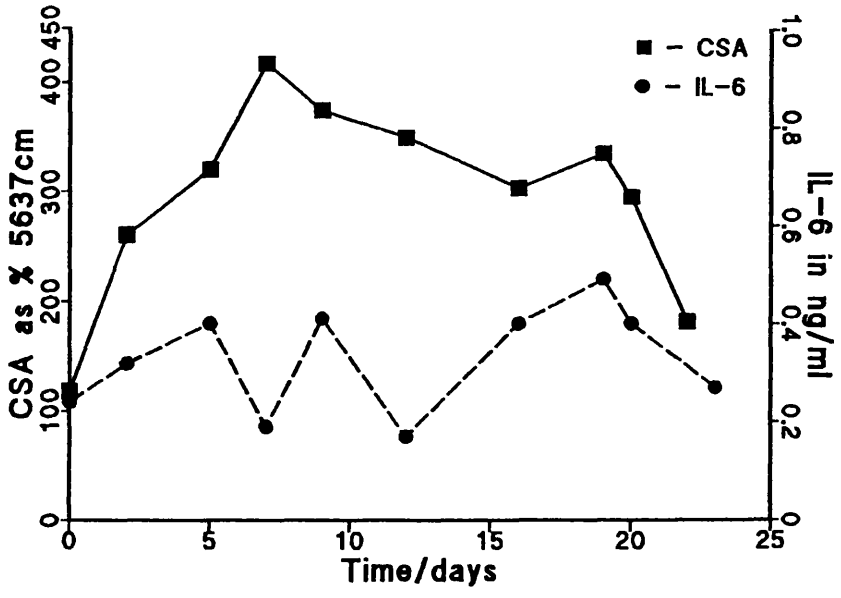
Patient 33 – Comparison of CSA with
Plasma G–CSF after HDBu & ABMR (No rhG–CSF)

FIG 5,5.1



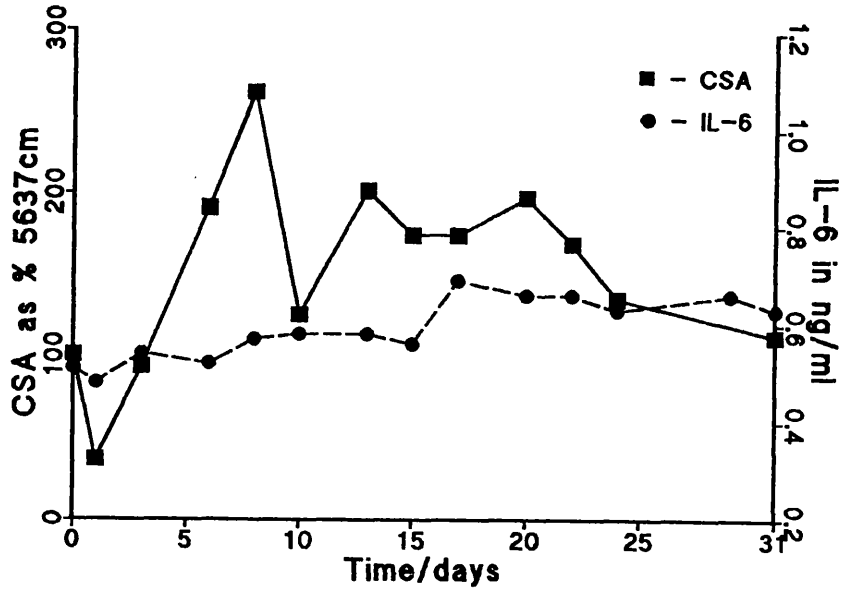
Patient 12 – Comparison of CSA with plasma IL-6 (by RIA) after HDM & ABMR

FIG 5,5.2



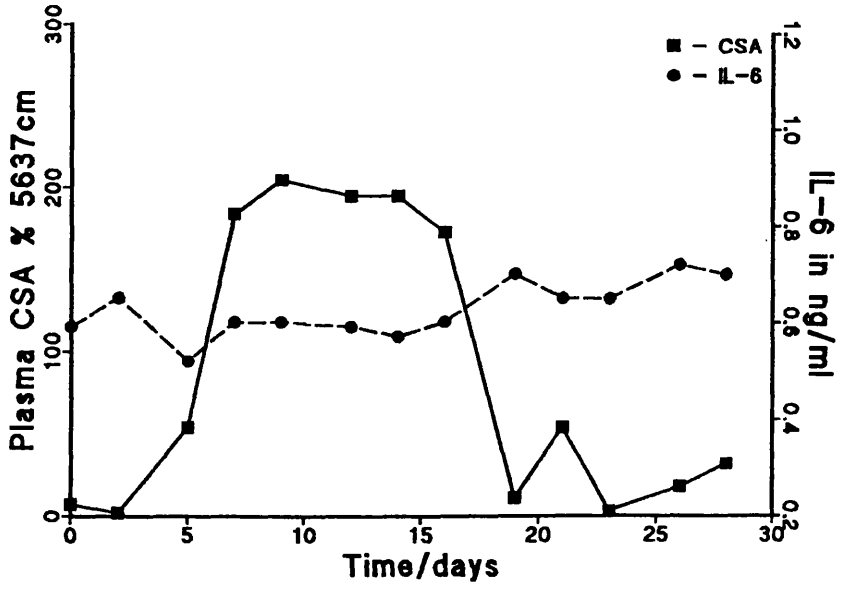
Patient 13 – Comparison of CSA and plasma IL-6 (by RIA) after HDM & ABMR

FIG 5,5.3



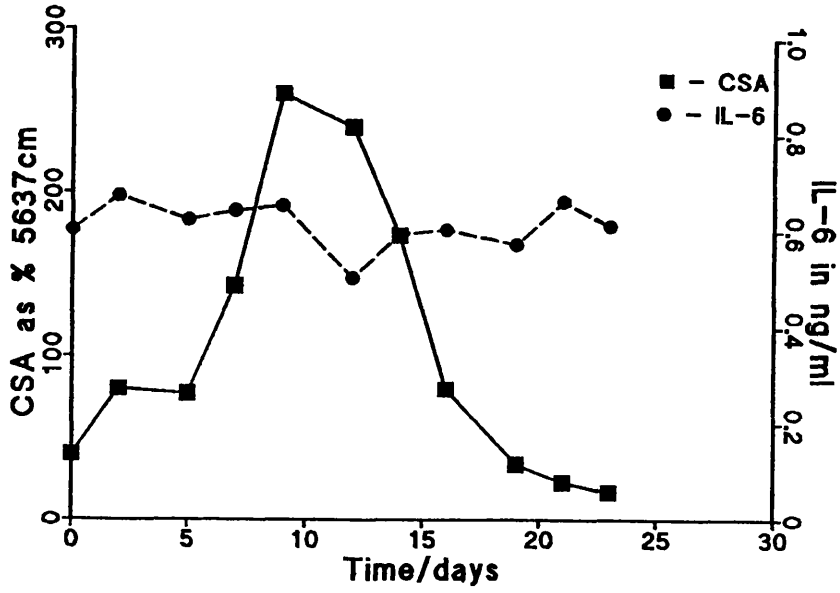
Patient 14 – Comparison of CSA and plasma IL-6 (by RIA) after HDM & ABMR

FIG 5,5.4



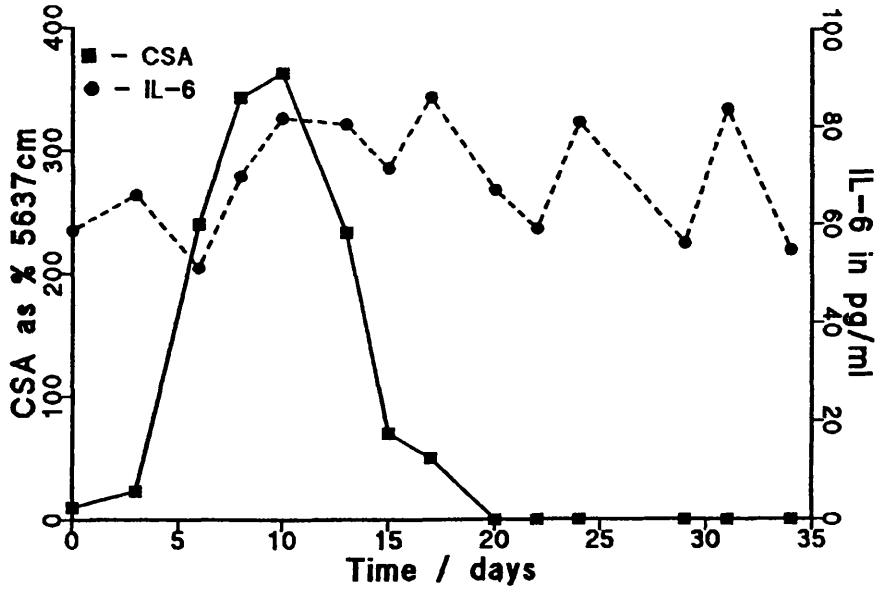
Patient 15 – Comparison of CSA and plasma IL-6 (by RIA) after HDM & ABMR

FIG 5,5.5



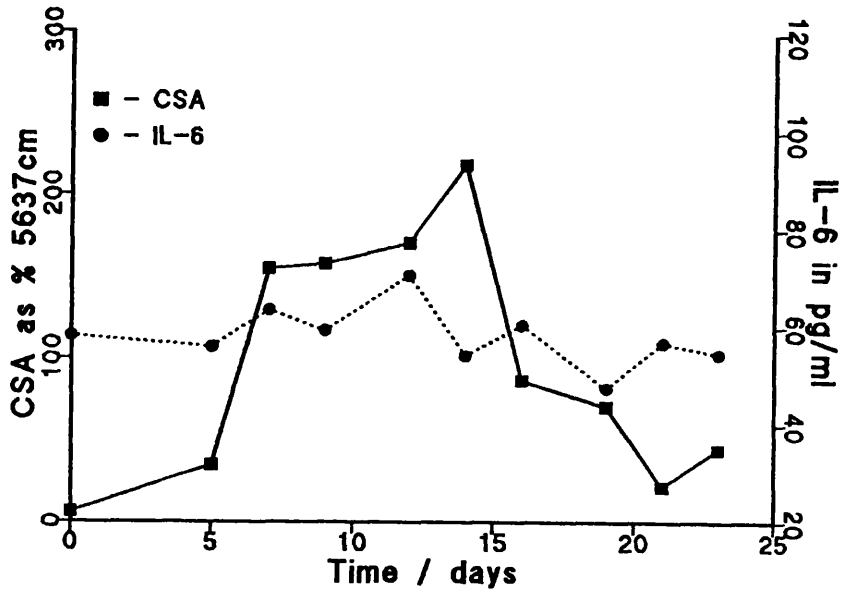
Patient 17 – Comparison of CSA with plasma IL–6 (by RIA) after HDM & ABMR

Fig 5,5.6



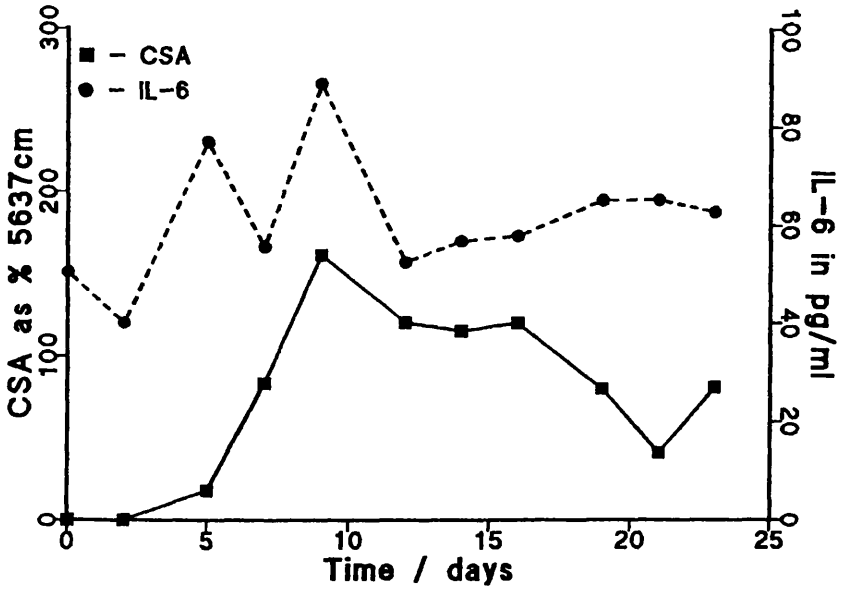
Patient 20 – Comparison of CSA with plasma IL–6 (by bioassay) after HDM & ABMR

Fig 5,5.7



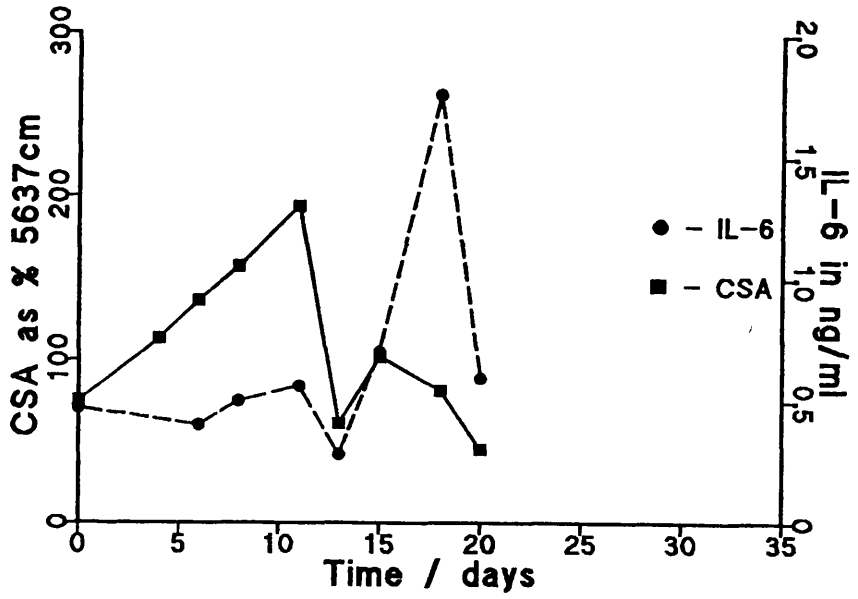
Patient 21 – Comparision of CSA with plasma IL–6 (by bioassay) after HDM & ABMR

Fig 5,5.8



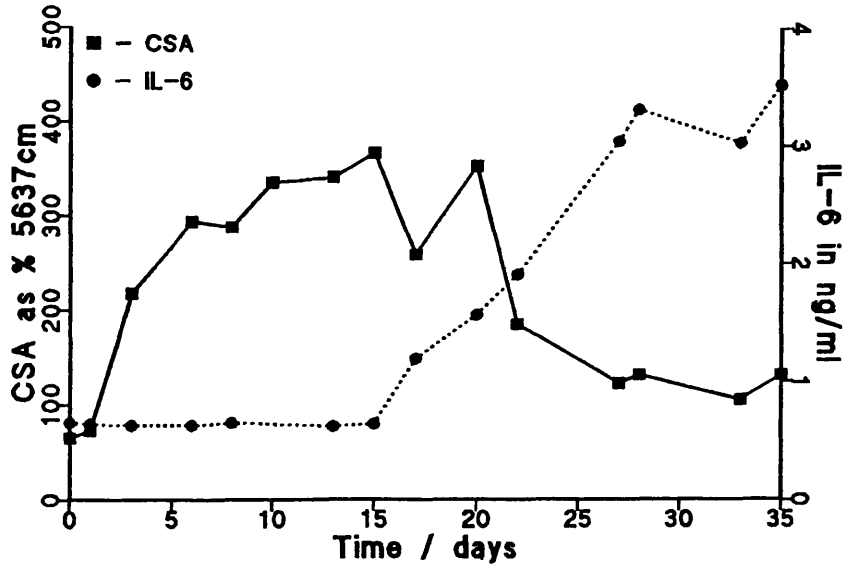
Patient 22 – Comparison of CSA with plasma IL–6 (by bioassay) after HDM & ABMR

Fig 5,5.9



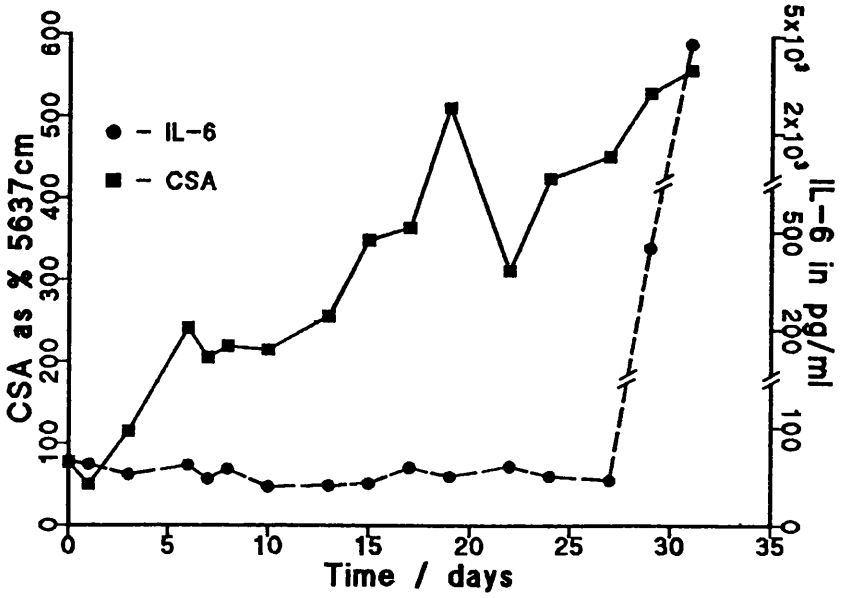
Patient 25 – Comparison of CSA with
plasma IL-6 (by RIA) after HDM without ABMR

Fig 5,5.10



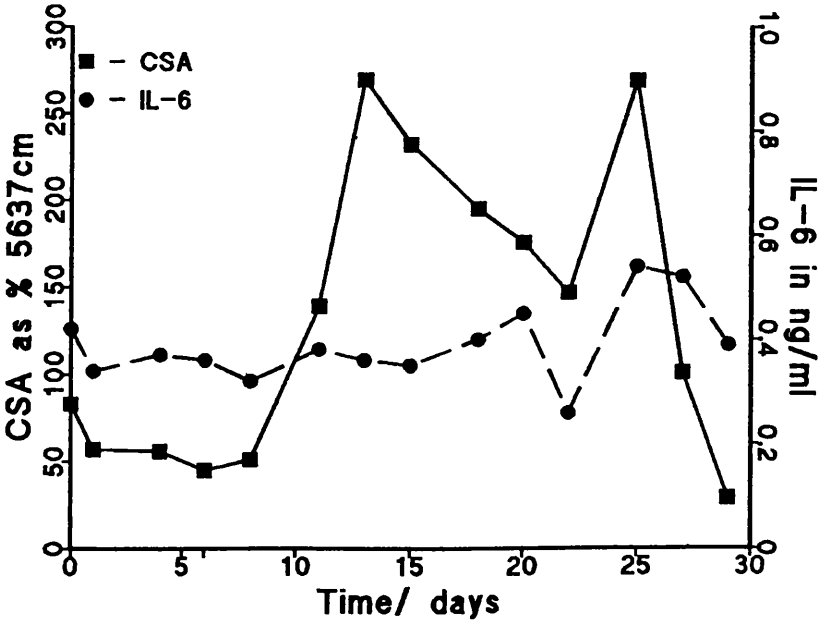
Patient 26 – Comparison of CSA with
plasma IL-6 (by RIA) after HDM without ABMR

Fig 5,5.11



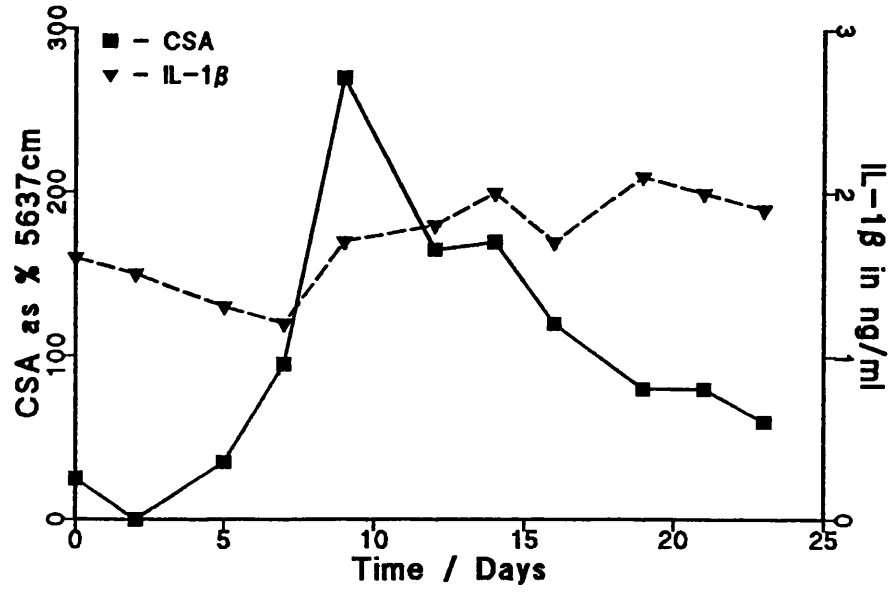
Patient 29 – Comparison of CSA with plasma IL-6 (by bioassay) after HDM without ABMR

FIG 5,5.12



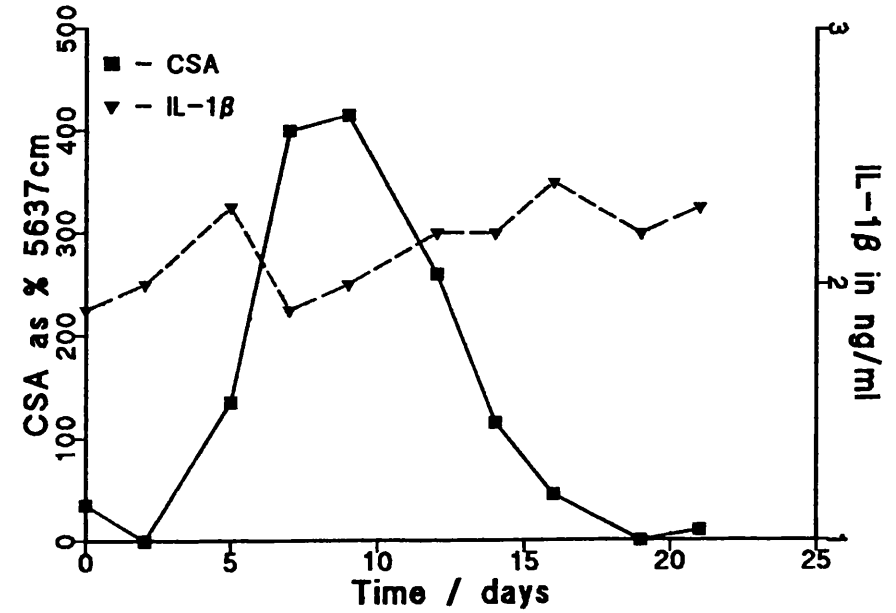
Patient 31 – Comparison of CSA with plasma IL-6 (by RIA) after HDBu (days 1-4) & ABMR (Day 6)

Fig 5,5,14



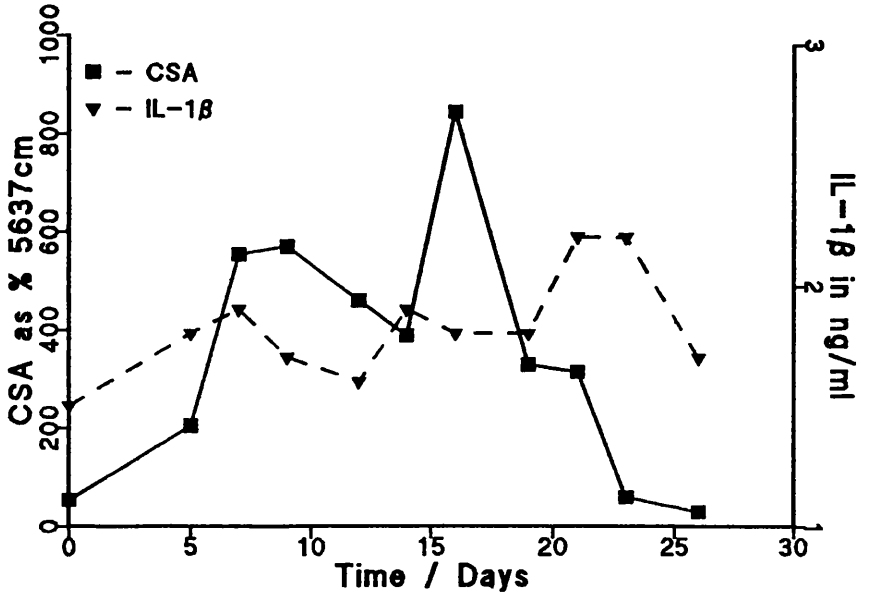
Patient 11 – Comparison of CSA with plasma IL–1β after HDM & ABMR

Fig 5,5.15



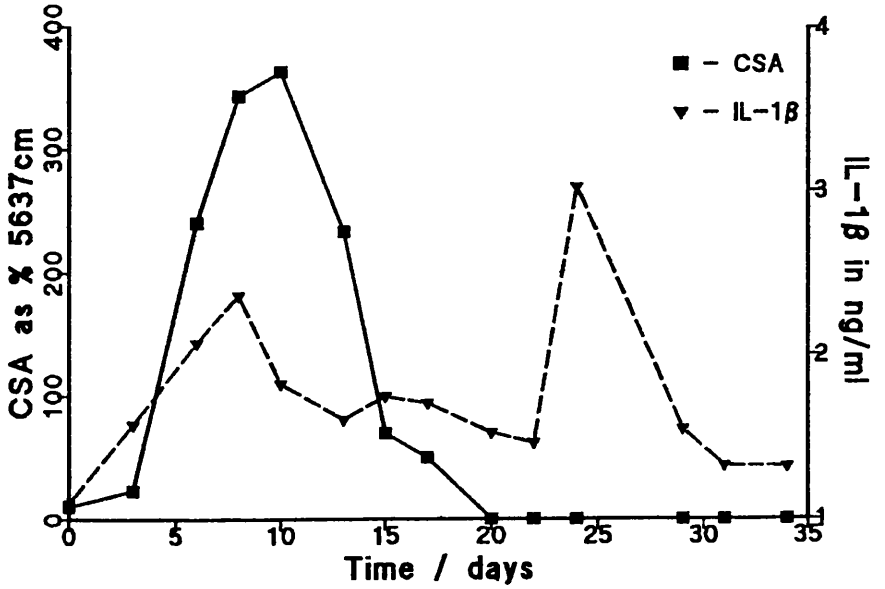
Patient 18 – Comparison of CSA with plasma IL–1β after HDM & ABMR

Fig 5,5.16



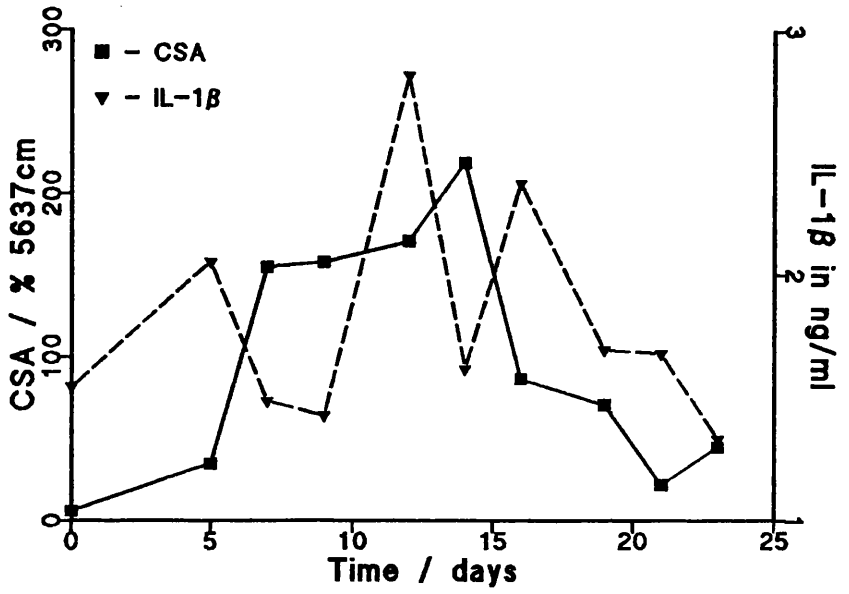
Patient 19 – Comparison of CSA with plasma IL–1β
after HDM & ABMR

Fig 5,5.17



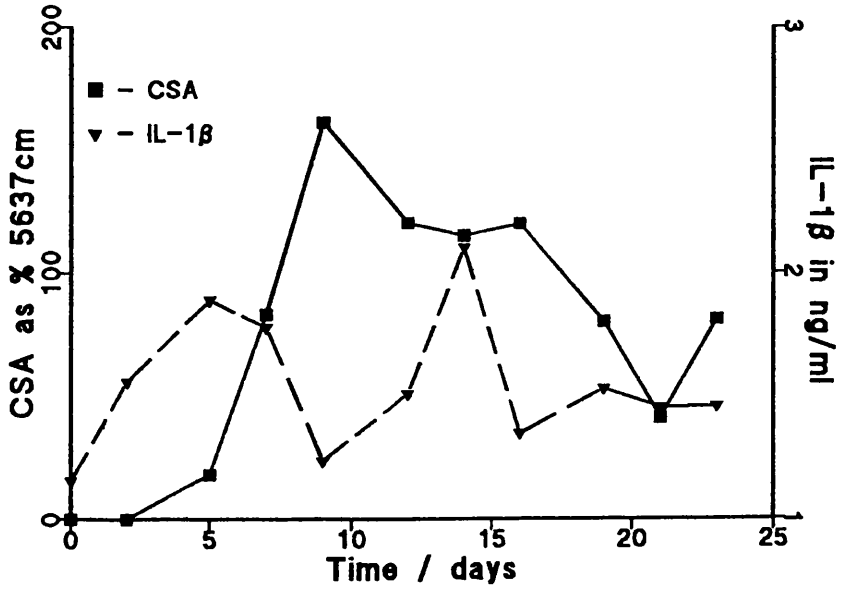
Patient 20 – Comparison of CSA with plasma IL–1β
after HDM & ABMR

Fig 5,5.18



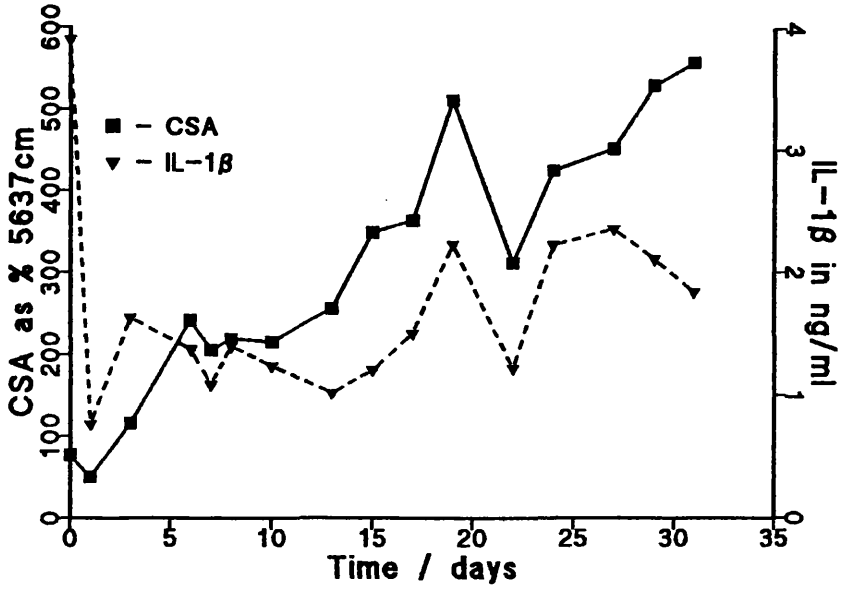
Patient 21 – Comparison of CSA with plasma IL–1β after HDM & ABMR

Fig 5,5.19



Patient 22 – Comparison of CSA with plasma IL–1β after HDM & ABMR

Fig 5,5.20



Patient 29 – Comparison of CSA with plasma IL–1 after HDM without ABMR