IN-VITRO EFFECTS OF CYTOKINES ON NORMAL AND MYELOID LEUKAEMIC HAEMOPOIESIS

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ABSTRACT

The growth of chronic myeloid leukaemic (CML) and acute myeloid leukaemic (AML) cells *in vitro* and their response to various cytokines were studied and compared to normal peripheral blood and bone marrow cells.

In normal mononuclear cells (MNC) the three cytokines [granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3)] had no appreciable difference at stimulating (colony-forming unit granulocyte-macrophage) CFU-GM-formation. By contrast, G-CSF was a more potent stimulator of CFU-CML, than GM-CSF or IL-3. Interleukin-4 (IL-4) augmented G-CSF-induced colony formation of CML chronic phase cells. Transforming growth factor β_1 (TGFβ_1) reduced G-CSF-induced CFU-CML from CML chronic phase ($p<0.005$) and blast transformation MNC, but had no effect on normal CFU-GM stimulated by G-CSF.

The growth of CML mononuclear cells *in vitro* could not be correlated to the white cell count or percentage of CD34+ cells present. The mechanisms for the inhibitory effects of TGFβ_1 on CML cells were studied using *in-situ* hybridisation techniques to demonstrate the expression of the G-CSF and IL-4 receptors. The results suggest that this response is more likely to be due to a difference in signal transduction or protein synthesis rather in transcription.

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LIST OF ABBREVIATIONS

abl	Abelson proto-oncogene
ABMR	Autologous bone marrow rescue
ABMT	Allogenic bone marrow transplant
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APES	3-aminopropyltriethoxysilane
ATRA	All trans retinoic acid
BCGF	B cell growth factor
bcr	Breakpoint cluster region
BFU-E	Erythroid burst forming unit
BM	Bone marrow
BMT	Bone marrow transplant
BSA	Bovine serum albumin
BSF	B-cell stimulating factor
cDNA	Complementary DNA
CFU-AML	Colony-forming unit acute myeloid leukaemia
CFU-C	Colony-forming unit
CFU-CML	Colony-forming unit chronic myeloid leukaemia
CFU-E	Erythroid colony-forming unit
CFU-Eo	Eosinophil colony-forming unit
CFU-GEMM	Granulocyte/erythroid/ macrophage/megakaryocyte colony-forming unit
CFU-GM	Granulocyte/macrophage colony-forming unit
CFU-MEG	Megakaryocyte colony-forming unit
CFU-mix	Mixed colony-forming unit
CFU-S	Spleen colony-forming unit
CML	Chronic myeloid leukaemia
CML CP	Chronic myeloid leukaemia in chronic phase
CML BT	Chronic myeloid leukaemia in blast transformation
CSA	Colony-stimulating activity
CSF	Colony-stimulating factor
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
Epo	Erythropoietin
FAB	French-American-British classification
FBC	Full blood count
FCS	Foetal calf serum
FSH	Follicle stimulating hormone

G-CSF	Granulocyte colony-stimulating factor
G-CSFR	Granulocyt colony-stimulating factor receptor
GM-CSF	Granulocyte-macrophagecolony-stimulating factor
GM-DF	Granulocyte-macrophage differentiation factor
GVHD	Graft-versus-host disease
HLA	Human leucocyte antigen
IFN	Interferon
IL	Interleukin
IL-4R	Interleukin-4 receptor
ISH	*In-situ* hybridisation
i.v	Intravenous
kD	kilo Daltons
LIF	Leukaemia inhibitory factor
LAK cell	Lymphokine activated killer cell
LFS	Leukaemia free survival
LPS	Lipopolysaccharide
LTC-IC	Long term culture initiating cell
m-bcr	Major breakpoint cluster region
MCGF	Mast cell growth factor
M-CSF	Macrophage colony-stimulating factor
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MIP	Macrophage inhibitory protein
MLR	Mixed lymphocyte reaction
MNC	Mononuclear cells
mRNA	Messenger RNA
MW	Molecular weight
tRNA	Transfer RNA
Multi-CSF	Multi-lineage colony-stimulating factor
NBT	Nitroblue tetrazolium
N/C ratio	Nuclear/cytoplasmic ratio
NK cell	Natural killer cell
NRK	Normal rat kidney
N/S	Non-specific
PB	Peripheral blood
PBS-A	Phosphate buffered saline-A
PDGF	Platelet-derived growth factor
PFH	Preservative free heparin
Ph	Philadelphia chromosome
RNA	Ribonucleic acid
RNAse	Ribonuclease
SCF	Stem cell factor
SCID	Severe combined immunodeficiency

SGF	Sarcoma growth factor
SSC	Saline sodium citrate
ssDNA	Single stranded DNA
TBI	Total body irradiation
TBS	Tris buffered saline
TCGF	T-cell growth factor
TGF	Transforming growth factor
Tm	Melting temperature
TNF	Tumour necrosis factor
U/A	Unable to assess
UPN	Unique patient number
UV	Ultraviolet
WBC	White blood cell count

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CHAPTER 1: INTRODUCTION

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INTRODUCTION

Blood is a suspension of differentiated, mainly non-dividing cells. It is produced by undifferentiated haemopoietic cells with a high replicative potential, belonging to the marrow and reticuloendothelial system. In recent years much time has been devoted to the study of the haemopoietic system and the factors regulating it. This has been facilitated by the relatively easy access to blood and bone marrow cells.

In-vitro, the progenitor cells of the haemopoietic system require colony stimulating factors (CSFs) to proliferate and differentiate. A significant number of these haemopoietic growth factors have now been cloned and sequenced and their effects have been well characterised. However their mechanisms of action and biological effects *in vivo* are unclear and whether they play a role in the pathogenesis of haemopoietic malignancies is unproven. Advances in recombinant DNA technology has lead to the molecular cloning of genes that encode CSFs and a series of other polypeptides released in the inflammatory response, many of which also affect haemopoietic cells. Collectively these peptides have been termed cytokines.

The aim of this thesis was to study the proliferative response of normal and leukaemic cells to various cytokines and to determine whether a combination of cytokines could be of benefit in the treatment of leukaemia. The main cytokines used were transforming growth factor β_1 (TGFβ_1),

granulocyte colony-stimulating factor (G-CSF) and interleukin-4 (IL-4). Attempts were made to elucidate the cause of the different effects that TGFβ_1 had on normal and leukaemic cells using *in situ* hybridisation techniques.

The haemopoietic system

Embryology

The three primary germ layers of the human embryo are the ectoderm, mesoderm and the endoderm. The first haemopoietic stem cells arise from the mesoderm of the yolk sac. These mesoblastic cells generate nucleated erythroblasts that synthesize "embryonic" haemoglobin during the first week of life. The production of nucleated red cells by the yolk sac declines sharply at 6 weeks and terminates by 10 weeks. From the second to the seventh month of life the liver is the primary site of haemopoiesis, supported in this role to a lesser extent by the spleen from the third to the sixth month (Lewis, 1989). The transfer of responsibility for haematopoiesis from the liver to the bone marrow begins at three months and is usually complete at birth. The liver produces a small number of haemopoietic cells for a short while after birth. A few precursor cells may persist in the spleen even in adulthood.

The human yolk sac and early embryo initially produce red cells and a small percentage of mainly pre-B lymphocytes. Megakaryocytes first appear after three months. It is only in the second half of gestation that myelopoiesis begins and granulocytes are formed (Jandl, 1989).

Sites of haemopoiesis

Bone marrow: In adults the bone marrow is the major site of haemopoiesis. The human skeleton contains approximately 600 grams of bone marrow. Over 70% of the bone marrow is located in the pelvis, vertebrae and sternum (Jandl, 1989). Progenitor cells with the ability to generate erythroid, leucocyte and megakaryocyte precursors are located within the bone marrow. Among the progenitor cells are mature blood cells and non-haemopoietic cells, all of which are supported by a reticulin framework. This reticulin framework or bone marrow stroma is an ill-defined entity that consists of endothelial cells, fibroblasts, fat cells and macrophages. The stromal cells collectively support haemopoiesis but the precise contributions of the different components are unknown (Lewis, 1989).

Spleen: The spleen is an intra-peritoneal organ located in the left hypochondrium. In healthy adults the spleen weighs between 150 and 200 grams, and measures approximately 4 x 8 x 12 cm. The spleen is normally not palpable on physical examination of healthy individuals. It consists of three compartments: the white pulp, the marginal zone and the red pulp. The white pulp consists of lymphoid follicles contained within a reticular framework. The lymphoid follicles are the sites of lymphocyte production. The marginal zone is the junctional area between the red and white pulp. It is composed of a more heterogenous population of cells but is particularly rich in monocytes. The red pulp contains both erythroid and myeloid progenitor cells (Lewis, 1989).

Lymph nodes: The lymph nodes contain lymphoid follicles which are sites of lymphopoiesis. Lymphocytes, plasma cells, macrophages and occasionally mast cells are present in lymph nodes. Granulocytes and erythroid cells are not normally found (Hess, 1987).

Peyers patches: These are lymphoid aggregates found in close association with the epithelium of the small bowel. They are similar in structure to the lymphoid follicles found in the spleen and lymph nodes and are sites of lymphopoiesis (Hess, 1987).

Thymus: The thymus is a bi-lobed lymphoepithelial gland located in the anterior and superior mediastinum. Development begins at about the eighth week of gestation. The gland continues to increase in weight until puberty after which it atrophies and is replaced mainly by fat and fibrous tissue. The main cellular components of the thymus are T-lymphocytes and epithelial cells (Hess, 1987)

Haemopoietic cell populations

The 3 main classes of mature blood cells are erythrocytes (red cells), leucocytes (white cells) and platelets. Both erythrocytes and platelets lack a nucleus, a feature that is not seen in any other cell type in the human body. They are therefore incapable of cell division. The main function of erythrocytes is O_2/CO_2 transport, and that of platelets is clotting. Leucocytes comprise of neutrophils, eosinophils, basophils, lymphocytes and monocytes. Though they have many diverse functions they are generally responsible for

aspects of humoral and cell-mediated immunity.

Haemopoietic stem cells

Mammalian blood cells have a finite life span which is considerably shorter than the life span of the organism. The maintenance of constant numbers of cells in the peripheral blood is achieved by the proliferation and differentiation of precursor cells which are located primarily in the bone marrow. These precursors are all derived from a common self-maintaining population of stem cells established during embryogenesis (Metcalf and Moore, 1971). Potten and Loeffler in 1990, defined stem cells as "capable of 1) proliferation, 2) self-maintenance, 3) production of large numbers of differentiated functional progeny, 4) regenerating the tissue after injury and 5) flexibility in the use of these options". Although several aspects of haemopoiesis are yet to be defined, some general principles have been established:- (Metcalf, 1984; Testa and Dexter, 1990; Metcalf, 1991; Wright and Lord, 1992).

- The pluripotent stem cell is the most "senior" cell in the bone marrow hierarchy and has a finite capacity for renewal.
- Once committed to a given lineage, stem cells differentiate unidirectionally, this being associated with a restriction of the cell's capacity for renewal.
- Local and systemic growth factors and inhibitors regulate the proliferation of stem cells.

- Contact with marrow stromal cells is important for stem cell proliferation.

The probability of stem cell renewal at the single cell level is thought to be a random process and hence it is not possible to predict whether an individual stem cell will self-renew or differentiate (Till et al, 1964). In contrast, at the stem cell population level self-renewal and differentiation appear to be tightly controlled so that demands for the production of differentiated cells and for the expansion of the stem cell population are met when necessary. The commitment to a particular lineage of differentiation may be stochastic (i.e. the probability of entering a particular lineage is random), predetermined or as a response to growth factors or microenvironmental cues (Gordon, 1993).

Haemopoietic tissue can therefore be broadly divided into 3 types of cell populations: multipotent progenitors or stem cells, committed progenitors and maturing/mature cells. Two functional qualities characterise a stem cell: self-renewal by self-replication, and the production of more differentiated cells. Committed progenitor cells are derived from stem cells, and are committed to cell division and differentiation along one or more maturation pathways. A system of hierarchy therefore exists with a small number of "committed" cells with a high replicative potential giving rise to cells of a particular type which possess a varying capacity to divide. The process of haemopoiesis should therefore be considered as a continuum with a series of compartments of increasing maturity, and decreasing potential for self-renewal,

which is frequently displayed as a unidirectional linear branching hierarchy.

Progenitor cells are detected by their ability to give rise to colonies of morphologically recognizable differentiation progeny in semi-solid cultures (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). The 3rd stage of haemopoiesis encompasses the bulk (approximately 95%) of the cells. It represents the proliferative amplification of differentiated cells as they mature to become fully functional blood cells. These cells are recognizable and classified by their morphological characteristics. When mature the cells leave the marrow environment via the central venous sinus and enter the peripheral circulation where they carry out appropriate functions. The differentiation pathway leading to each mature cell type is illustrated in Figure 1. In addition to giving rise to mature haemopoietic cells it now seems clear that osteoclasts (Ash et al, 1980), epidermal Langerhan cells (Katz et al, 1979), and tissue mast cells (Kitamura et al; 1977, Kitamura et al, 1981) are also derived from bone marrow cells.

In normal human bone marrow less than 1% of the stem cells are in the peripheral circulation at any one time (Gordon, 1994). Stem cells can be induced to circulate by haemopoietic growth factors and cytotoxic drugs. Brecher and Cronkite in 1951 provided evidence for circulating stem cells in mice. In 1976, Richman *et al* showed that there was an increase in the number of circulating progenitor cells during the recovery phase from chemotherapy. In 1988, it was reported that administration of haemopoietic growth factors (G-CSF and GM-CSF) resulted in mobilization of progenitor cells into the

circulation (Durstein et al, 1988; Socinski et al, 1988).

In, *in vivo* studies of murine haemopoiesis the spleen colony-forming units (CFU-S) have been widely regarded as cells most closely fitting stem cells due to their proliferative potential, self-renewal characteristics and capacity for multi-lineage differentiation (Till and McCulloch, 1961).

Spleen colony-forming unit (CFU-S) assay

The spleen colony-forming technique was developed in 1961 by Till and McCulloch. It was the first quantitative assay for measuring the numbers of haemopoietic stem cells. Mice were lethally irradiated (8-9 Gy) to destroy endogenous stem cells. They were then injected intravenously with bone marrow cells. Post mortem analysis performed between 7 and 12 days after injection of donor bone marrow cells revealed macroscopic colonies in the spleen. The colonies consisted mainly of maturing cells of erythroid, megakaryocytic and granulocytic lineages, though some undifferentiated cells were present. The numbers of spleen colony-forming units (CFU-S) can be directly related to the total number of injected cells by a seeding factor f. Thus CFU-S= CFC-S x f. The value of f is approximately 0.1.

Growth of haemopoietic progenitor cells *in vitro*

In recent years techniques have been developed to study the behaviour of haemopoietic progenitor cells in culture. The first of these *in vitro* cloning techniques was introduced by Pluznik and Sachs in 1965 and Bradley and

Metcalf in 1966. At this stage only mixed colonies consisting of granulocyte and macrophage were grown and their growth depended on the presence of a colony-stimulating activity (CSA) derived from a variety of cell or tissue sources. Variations in the culture conditions and in the source of CSA gradually extended the technique and it is now possible to demonstrate the growth of clones of the different committed precursors cells in semi-solid gels of agar or methylcellulose. Types of colony-forming units (CFU) are named according to the mature cell types arising in the colonies. Thus CFU-GEMM or CFU-mix describes mixed colonies containing granulocytic, megakaryocytic and erythroid progeny and BFU-E, CFU-GM and CFU-MEG describes progenitor cells committed to erythrocyte, granulocyte and megakaryocyte differentiation respectively. An important step forward came with the ability to grow bone marrow in long-term culture. Initially described by Dexter in 1977 and subsequently refined, the technique in its present form is capable of supporting the growth of a wide spectrum of bone marrow cells, including the stem cell population, through numerous generations over a period of many months.

Mature haemopoietic cells

There are 8 commonly recognised mature blood cell types that are derived from the pluripotent stem cell. They are neutrophils, eosinophils, basophils, monocytes, T and B lymphocytes, erythroid cells and platelets.

Neutrophil maturation and morphology

The mature neutrophil represents 60 to 70% of the total leucocyte count. The maturation of the neutrophil goes through 5 recognisable stages, from the myeloblast, to promyelocyte, myelocyte, metamyelocyte and finally to a mature segmented neutrophil.

Myeloblast: The myeloblast is the earliest identifiable precursor of the mature neutrophil. It is usually found in small numbers in the bone marrow but is absent from the peripheral blood in healthy individuals. The cell is of variable size (10-18μm) and has a large round/oval nucleus with one to five pale nucleoli, the cytoplasm is scanty and basophilic and in the more mature myeloblast may contain a few azurophilic or primary granules. Occasionally in acute leukaemia some of the primary granules coalesce to form one or more rod-like cytoplasmic inclusions known as Auer rods. These Auer rods are regarded as being diagnostic of leukaemic myeloblasts.

Promyelocyte: As the cytoplasm of the maturing myeloblast becomes more abundant a new type of granule known as the secondary granule appears. These granules stain darkly with May-Grunwald Giemsa and lie over the nucleus as well as in the cytoplasm.

Myelocyte: The myelocyte is slightly smaller than the myeloblast, but it has a more prominent cytoplasm. The nucleus is still rounded or slightly indented but nucleoli are absent. Using a May-Grunwald Giemsa stain the cytoplasm has lost some of its basophilia, it becomes more pink and contains

increasing numbers of specific granules which can be identified as neutrophilic, eosinophilic or basophilic.

Metamyelocyte: The development of nuclear indentation marks the transition from myelocyte to metamyelocyte. The nucleus becomes more kidney shaped and the cytoplasm acquires more granules. As the nucleus elongates further and bends the cell is known as a band form.

Neutrophil: The mature neutrophil is approximately 10-14μm in size. The nuclear material is divided into 3 or 4 segments which are joined by thin strands of chromatin. The mature neutrophil spends about 11 days in the marrow whilst its time in the circulation is extremely short with a half life of 6 to 8 hours. Neutrophils can adhere to and penetrate the endothelial lining of the blood vessels and are thus also found in extravascular sites. Neutrophils phagocytose and kill micro-organisms. Ingested organisms are contained within vacuoles (phagosomes) into which are released a battery of enzymes normally contained within the cytoplasmic granules. These enzymes include lysozyme, myeloperoxidase and acid hyaluronidase.

Other cells included in the granulocyte category are:

Eosinophils; these normally comprise 2 to 3% of the mature leucocytes. The eosinophil is characterised by the presence of large granules that fill the cytoplasm and stain a bright orangish-red colour with Wright's stain. Eosinophils contain about a third of the histamine found in normal blood. Eosinophils participate in hypersensitivity reactions and play a role in immunity to helminth infections (Jandl, 1989).

Basophils; are the least common of the granulocyte family, representing 0.2% of blood leucocytes. Adult forms are readily distinguished by the presence of huge metachromatic purple/black granules in the cytoplasm. They contain half the histamine found in man, nearly all of which is released during an anaphylactic reaction. Their exact function is unclear, but they appear in tissues during hypersensitivity states and anaphylactic reactions.

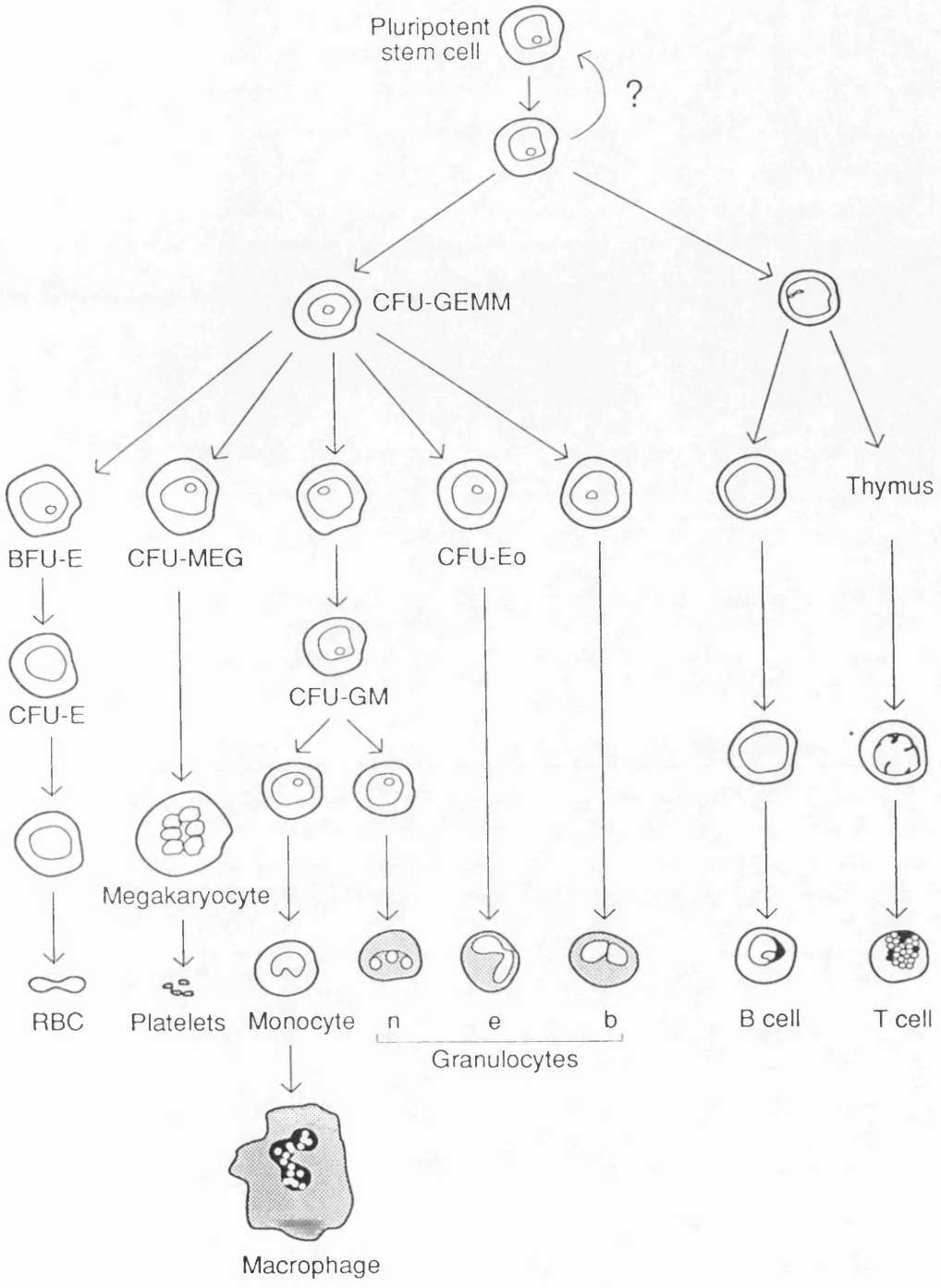
Other cells in the leucocyte compartment

B-lymphocytes: These cells possess cytoplasmic and later in their development, surface-bound immunoglobulin. They are responsible for antibody production. B-cells that are actively producing antibody are morphologically distinct and are called plasma cells.

T-lymphocytes: These cells comprise 60-85% of the circulating lymphocytes and are sub-divided on the basis of surface markers into several sub-populations with distinct functions. These include the augmentation of B-cell antibody production, suppression of both T- and B-cell responses, and also non-specific cytotoxicity.

Monocytes/macrophages: These cells are part of the mononuclear phagocyte system. Their primary function is phagocytosis of micro-organisms and the removal of damaged tissue fragments, dead cells, and inert particles produced in infected and inflammatory states. They are vital to the response of lymphocytes to antigenic stimulation. They also make soluble mediators, such as CSA with regulatory or modulatory roles and are involved in cell to cell interactions of several kinds.

Figure 1: Differentiation pathways of the haemopoietic system



The eight major mature cell types of the blood are all derived from the pluripotent stem cell compartment. From stem cells derive progenitor cells, which are committed to divide and differentiate along one or more differentiation pathways. In the presence of appropriate growth factors, these progenitors can be grown in semi-solid culture medium to form colonies and are therefore known as colony-forming units (CFU). Types of colony-forming cell are named according to the mature cell types arising in the colonies:
granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM); erythroid burst-forming unit (BFU-E);erythroid colony-forming unit (CFU-E); granulocyte-macrophage (CFU-GM);megakaryocyte (CFU-MEG);eosinophil (CFU-Eo). Other abbreviations are n, neutrophil; e, eosinophil; b; basophil.

Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is a clonal cancer arising from neoplastic transformation of the haemopoietic stem cell (Kurzrock et al, 1988). The Philadelphia (Ph) chromosome is the hallmark of the disease and is the result of a reciprocal translocation between chromosome 9 and 22. This cytogenetic aberration is seen in greater than 90% of cases of CML (Rowley, 1973).

Epidemiology

CML occurs with an annual incidence of about 1 case per 100,000 of the population (Goldman, 1989b). Approximately 650 cases per year are diagnosed in the United Kingdom. There is a slight male preponderance, the male to female ratio being 1.4:1. The diagnosis is most commonly made in the 5^{th} and 6^{th} decades of life, however the disease can affect both neonates and the elderly.

The only known predisposing cause is ionising radiation. This was clearly demonstrated in the survivors exposed to the high gamma irradiation from the atomic bombings at Hiroshima and Nagasaki. The incidence of CML in this group of patients was not age related. The leukaemia excesses have now disappeared and the leukaemia rates in the survivors today are no different to the general population of Japan (Cartwright and Staines, 1992).

Pathogenesis

In 1960, Nowell and Hungerford working in Philadelphia reported that myeloid cells from patients with CML had a deletion of a portion of the long arm of the G group of chromosomes. This was subsequently shown to involve a G group pair other than the pair implicated in Downs Syndrome (trisomy 21) and was hence designated chromosome 22. The abnormal chromosome was called the Philadelphia chromosome (Ph).

In 1973, Rowley examined banded preparations of chromosomes from patients with CML and noted that cells with the Ph chromosome had elongation of the long arm of one member of the number 9 pair of chromosomes. It later transpired that the Ph chromosome is the reciprocal translocation between chromosome 9 and 22. The resultant chromosomal fusion creates a chimeric gene composed of 5' sequences of the breakpoint cluster region (*bcr*) fused to the 3' sequences of the Abelson proto-oncogene (*abl*), (Heisterkamp et al, 1984). The rearrangement in chromosome 22, band q11 has been shown to be localised in a breakpoint cluster region (Major or m-*bcr*), (Groffen et al, 1984), which is located within the *bcr* gene. The hybrid *bcr/abl* gene formed as a result of the translocation encodes a protein kinase of p210 kD which has increased tyrosine kinase activity (Kurzrock et al, 1988; Konopka et al; 1984, Konopka et al, 1985). The precise function of the p210 protein kinase is not known but it may disturb the normal mechanisms that control haemopoiesis. A CML like disease has been observed in mice whose haemopoietic cells have been genetically engineered to express various *bcr/abl*

constructs. Thus providing further evidence that this hybrid gene plays a primary role in the pathogenesis of CML in humans (Daley et al, 1990; Elefanty et al, 1990; Kelliher et al, 1990).

Clinical features

In most patients the disease has a triphasic course ; the chronic phase, the accelerated phase and the acute phase or blast transformation.

Chronic phase

Patients in chronic phase commonly present with lethargy (due to anaemia) or abdominal discomfort (due to splenomegaly). Increased sweating and weight loss may also be common presenting complaints. Rarer symptoms include fever, spontaneous bruising, non-specific visual disturbances due to hyperviscosity and priapism. Between 20 to 30% of patients are asymptomatic when diagnosed. At diagnosis 70-85% of patients have splenomegaly, 50% have hepatomegaly and a smaller proportion have generalised lymphadenopathy.

The median duration of the chronic phase is 3.5 years, but it can range from 1 to more than 10 years (Goldman 1989b).

Accelerated phase

There is no strict definition of the accelerated phase but the following features may point to disease progression: a rapid leucocyte doubling time,

greater than 12% blasts in peripheral blood, greater than 20% blasts plus promyelocytes in marrow, more than 20% basophils plus eosinophils, anaemia or thrombocytopenia despite treatment, thrombocytosis in the absence of splenectomy, chromosomal abnormalities (in addition to the Ph chromosome), marrow failure associated with myelofibrosis and a leucocyte count resistant to treatment (Goldman, 1988).

Blast transformation

The evolution from chronic phase to blast transformation usually occurs over a period of weeks or months but on rare occasions can happen precipitously over a few days. Blast transformation is arbitrarily defined as the presence of more than 30% blasts or blasts plus promyelocytes in the peripheral blood or bone marrow. Symptoms, when present, include fever, weight loss, sweats, bone pain, pain in the splenic area, generalised lymphadenopathy and multiple subcutaneous nodules. Symptoms of hyperviscosity due to intracerebral or intrapulmonary leucostasis present less frequently. Additional cytogenetic abnormalities occur in over 80% of patients with CML approaching blast crisis (Kurzrock et al,1988) and commonly include trisomy 8, a second Ph chromosome and isochromosome 17q.

Haematological findings

The leucocyte count at diagnosis is typically between 100 x 10^9/l and 300 x 10^9/l, but can range from 20 x 10^9/l to 1000 x 10^9/l. The white cell differential

shows a full spectrum of immature and mature granulocytes with left-shifted myeloid maturation. Blasts may constitute 10 to 12% of the differential. The eosinophil and basophil count are frequently raised. The absolute lymphocyte count is normal. The amount of neutrophil alkaline phosphatase is greatly reduced or absent. Patients with a white cell count >150 x 10^9/l are usually anaemic at diagnosis. The platelet count is usually between 300 and 700 x 10^9/l but rarely is over 10^6 x 10^9/l.

The bone marrow at diagnosis is usually hypercellular, with loss of fat spaces and shows an increased myeloid population with left-shifted maturation. The number of megakaryocytes maybe increased. Erythroid activity is far less affected than myeloid maturation. Bone marrow biopsy confirms the lack of fat spaces and frequently reveals increased reticulin and diffuse fibrosis.

In patients with hepatosplenomegaly, histological examination shows infiltration of these organs by myeloid precursors and to a lesser extent megakaryocyte and erythroid precursors.

Disease progression maybe accompanied by an increased blast count, thrombocytosis, basophilia, eosinophilia, marrow fibrosis and less commonly thrombocytopenia. In 70% of cases of blast transformation the blasts have morphological and cell marker characteristics of myeloid cells, in approximately 20% the blasts are lymphoid in nature and in the remaining 5 to 10% they have mixed lymphoid and myeloid features.

Ph negative CML

Between 5 and 10% of patients with clinical and haematological features similar or identical to Ph positive CML lack the Ph chromosome.

The haematological features may initially be typical of Ph positive CML, however in some patients there maybe subtle clues that suggest Ph negativity such as a low platelet count, absence of the typical predominance of myelocytes in the leucocyte differential and the absence of an eosinophilia or basophilia. On molecular analysis of bone marrow cells some of these patients have the abnormal hybrid *bcr/abl* gene whilst in others no characteristic molecular abnormality has been defined. Initial studies suggested that patients with Ph negative CML had a worse prognosis. However more recent series where highly atypical CML's have been excluded suggest, that for patients with a form of leukaemia close to classical CML, the only difference being the absence of the Ph chromosome, the prognosis is similar to Ph positive CML with a median survival of 3.5 years (Goldman, 1989b).

Therapeutic options

Although the prognosis for newly diagnosed patients with CML has not changed substantially in the past 10 years, the number of therapeutic options has increased significantly. Patients are now treated with chemotherapy, alpha-interferon, autologous bone marrow or peripheral stem rescue or allogeneic bone marrow transplantation (ABMT).

Chemotherapy

Twenty years ago a newly diagnosed patient with CML was treated with busulphan without too much deliberation. This is no longer the case. Once the decision to treat has been reached, the choice lies between busulphan, hydroxyurea and alpha-interferon. The peripheral blood of patients with untreated CML contains large numbers of committed myeloid progenitor cells. Ideally therefore all new patients should be leukapheresed and have the buffy coat cells frozen prior to initiation of treatment. These cells could then be used to restore haemopoiesis following marrow aplasia secondary to either a failed bone marrow graft or inadvertent overtreatment with busulphan. The cells could also be used for autologous rescue, after high dose chemo-radiotherapy.

The two principal cytotoxic drugs used in treating CML are hydroxyurea and busulphan. Both drugs reverse symptoms, normalise spleen size and restore the blood count to normal. Busulphan however has a formidable list of long term side effects including interstitial pneumonitis, sterility, cutaneous pigmentation and a wasting condition. Erroneous administration can lead to severe or fatal marrow hypoplasia.

Hydroxyurea is a nucleotide reductase inhibitor. It acts on relatively mature myeloid cells, as reducing or stopping treatment leads to a rapid recurrence of leucocytosis. Hence unlike busulphan it has never been reported as causing an irreversible marrow hypoplasia (Goldman, 1990). Hydroxyurea is more likely than busulphan to cause immediate side effects

which include rashes, fevers and non-specific gastro-intestinal symptoms. Unlike busulphan, hydroxyurea needs to be given daily if the leucocyte count is to be controlled. Hydroxyurea has now superseded busulphan as one of the drugs of choice for CML because of its beneficial effect on survival (Hehlmann et al, 1993).

Alpha-interferon

Since its introduction in the early 1980's as treatment for CML, alpha-interferon has been used with increasing frequency. Not only does alpha-interferon control the spleen size and white cell count but it is also the first agent employed in treatment for CML that induces partial or even on occasion complete suppression of Ph positivity. Its exact mechanism of action is not clearly understood but it could act by suppressing more mature myeloid progenitor cells (Galvani and Cawley, 1989) or by inducing specific changes in the function or cellular composition of the marrow stroma (Dowding et al, 1991). To induce Ph negativity alpha interferon needs to be given daily, sub-cutaneously for a period of 9 to 12 months. It is reasonable to stop treatment after this period if there is no reduction in Ph positivity. Treatment with alpha-interferon also prolongs the overall median survival. The duration of survival appears to correlate with the cytogenetic response achieved, if the response is greater than 65% Ph negative metaphases the actuarial survival at 5 years is approximately 90%. Unfortunately only a small proportion of patients (about 13%) respond in this way (Kloke et al, 1993).

Autologous bone marrow rescue (ABMR)

The incurability of CML with conservative treatment, the favourable results of allogeneic transplants in the chronic phase of the disease, and the lack of suitable donors has generated considerable interest in ABMR for patients lacking a donor.

The presence of primitive haemopoietic cells in adult peripheral blood has been well recognized for 3 decades. Initial experiments showed that peripheral blood from a variety of species, including humans was capable of protecting recipients from lethal doses of whole body irradiation, by restoring blood cell formation from circulating donor cells (Goodman et al, 1962; Epstein et al, 1966). Subsequent studies lead to the demonstration and quantitation of specific progenitor populations detected by colony assays (McCreadie et al, 1971; Chervenick et al, 1971). More recently, identification of strategies for increasing the circulation of progenitors in the circulation, has heightened interest in the potential of peripheral blood harvests for clinical treatment protocols requiring haematologic rescue (Haylock et al, 1992 Gianni et al, 1989). Udomasakdi et al, in 1992a showed that peripheral blood of normal adults has a relatively small but readily detectable population of functionally defined primitive haemopoietic cells, that share properties with primitive marrow precursor cells, known as long term culture initiating cells (LTC-IC). They also showed that (Udomasakdi et al, 1992b) LTC-IC in CML patients, showed features of proliferating or activated cells. In marked contrast LTC-IC in normal donors exhibit features of a quiescent population.

Patients with CML have greatly increased numbers of pluripotent stem cells in their blood and bone marrow. These cells can be collected and cryopreserved prior to initiation of treatment and used at a latter date for autografting. Treatment involves high dose chemotherapy with or without radiation, followed by re-infusion of previously cryopreserved stem cells. Haemopoiesis in the first few months after autografting is often partially Ph negative, though the proportion of Ph positive marrow metaphases usually increases to 100% by 6 or 9 months after the autograft. One unrandomised study has shown autografting may increase survival, 56% of patients being alive at 5 years (Hoyle et al, 1994).

In 1983, Coulombel et al showed that in long-term bone marrow culture (LTBMC) Ph positive cells had a decreased survival, the survival of long-term culture initiating cells (putative stem cells) was undiminished, whereas the leukaemic cells were reduced by 30 fold. The same group, have recently shown that the *in vitro* purging procedure of LTBMC may be advantageous for those patients with CML, who have adequate numbers of long-term culture initiating cells (LTCIC) and concurrent loss of Ph positive leukaemic cells (Barnett et al,1994).

Bone marrow transplantation

In the past decade, it has become very clear that allogeneic bone marrow transplantation (BMT) can cure patients with CML. There is also general agreement, that bone marrow transplantation should be carried out whilst the patient is in chronic phase (Goldman, 1990). Thomas et al, in 1986

reported that survival for patients with CML in chronic phase, transplanted with marrow from HLA-identical siblings was better if the procedure were performed within 1 year of diagnosis. As the risk of severe or fatal graft versus host disease (GVHD) is greater in older patients, this therapeutic option is generally only available for those patients under the age of 50 years. Unfortunately the number of patients below that age with an HLA-compatible-related donor probably only constitutes 10 to 15 % of new patients.

During the past 5 years interest has been focused on the possibility of using phenotypically matched unrelated donors. Preliminary results suggest, that the incidence of GVHD and graft failure is increased in these patients when compared with those who have had a sibling transplant (Goldman, 1990). About 40% of these patients go on to be long term survivors (>5 years) as compared to the 60 to 70% survival rate in patients who have a matched sibling BMT.

Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a malignant clonal disease, arising from transformation of a haemopoietic progenitor cell, and resulting in clonal proliferation of poorly or partly differentiated myeloid cells.

It is a heterogeneous disease, differing considerably among patients with respect to cytogenetic abnormalities, cellular phenotype and response to therapy (Champlin et al, 1987).

AML does not disturb haemopoiesis or produce symptoms until a substantial burden of leukaemic cells, usually between 10^9 and 10^{12}, is present (Champlin et al, 1987).

Epidemiology

AML is predominantly a disease of adults. It accounts for approximately 80% of adult leukaemias but only 20% of childhood leukaemias. There are no major differences in incidence according to sex or race. In the United Kingdom there are approximately 2000 new cases per year.

Leukaemogenic agents

Ionising radiation

X-rays and other ionising rays were the first identifiable agents associated with the induction of leukaemia. This first became apparent in the survivors of the atomic bomb explosions in Hiroshima and Nagasaki.

It is also now well documented, that patients treated by irradiation for other malignancies (eg Hodgkin's Disease, myeloma, carcinoma of the breast

and ovary) are at an increased risk of developing AML. The combination of irradiation and alkylating agents results in an even higher incidence of secondary AML.

Chemicals

The 2 main agents suspected of being leukaemogenic are alkylating drugs and benzene (Catovsky et al, 1989).

Alkylating agents: Patients who develop AML secondary to exposure to an alkylating agent usually have a slower evolution of the disease, a lesser degree of leukaemic infiltration, but a higher incidence of chromosomal abnormalities, in particular 5q- and monosomy 5 and/or 7. The main alkylating agents implicated are nitrogen mustard, lomustine and chlorambucil.

Benzene: Benzene constitutes 6 to 8% of the content of petroleum and up to 30% of some liquid lacquers. It is known to produce chromosomal abnormalities. It has aetiologically been linked to the increased incidence of leukaemia in persons occupationally exposed to it.

Diagnosis

Patients usually present symptoms attributable to anaemia (tiredness), neutropenia (infections) and thrombocytopenia (bleeding/bruising).

The diagnosis is made by careful examination of the peripheral blood smear. The bone marrow is usually hypercellular but in 5 to 10% of cases it is hypocellular (Catovsky et al, 1989). Difficulties in diagnosis are encountered in cases presenting with a low white cell count, such as promyelocytic

leukaemia where dry aspirates maybe obtained due to rapid clot formation. If the percentage of myeloblasts in the bone marrow exceeds 30% the diagnosis is AML.

Classification

Bennett et al, in 1976 put forward a proposal for the classification of the acute leukaemias. The French-American-British (FAB) classification is now accepted internationally. A revised classification (Bennett et al, 1985) was put forward by the same group and briefly is as follows:

M0 - undifferentiated myeloblasts

M1 - myeloblasts without maturation

M2 - myeloblasts with maturation

M3 - hypergranular promyelocytes

M3 variant - hypogranular promyelocytes

M4 - myelomonocytic

M5 - monocytic; monoblastic (M5a) and promonocytic (M5b)

M6 - erythroleukaemia with >50% erythroblasts and >30% myeloblasts.

M7 - megakaryoblastic

Bennett et al (1985) suggest that the initial assessment of the bone marrow aspirate (based on a 500 cell differential) should be to establish the percentage of erythroblasts. If the percentage of erythroblasts is >50% the diagnosis is AML M6 or myelodysplastic syndrome. A diagnosis of AML M6

is made if >30% of the non-erythroid cells are myeloblasts. Cases with fewer than 50% erythroblasts and greater than 30% myeloblasts will fall into the categories AML M0 to M5 depending on the morphological nature of the blasts. Acute leukaemia with a megakaryoblastic component requires special methods for showing that the blast cells belong to the megakaryocytic lineage, for example platelet peroxidase reaction or platelet antibodies to platelet glycoprotein

Therapeutic options

The two major therapeutic options in AML are chemotherapy and BMT. The chemotherapeutic option is divided into 2 phases, remission induction and consolidation or post remission treatment. Other regimens/agents that are less commonly used are maintenance treatment and differentiation agents.

Chemotherapy

Remission induction treatment: The combination of drugs most commonly used is cytosine arabinoside and an anthracycline.There is considerable controversy regarding which drug(s) is best combined with cytosine arabinoside. The anthracyclines most commonly used are daunorubicin, doxorubicin and more recently idarubicin. Combinations of cytosine with amsacrine (Louie and Issel, 1985; Arlin et al, 1984) or mitoxantrone (Shenkenberg et al, 1986) have also been used with some success. Adding additional drugs such as 6-thioguanine or etoposide is only of

marginal benefit and does not improve survival (Foon and Gale, 1992) There are no convincing data that high dose cytosine at a dose of 1.5g-3.0g/m^2 daily for 3 days, is superior to conventional dose cytosine (100-200mg/m^2/day for 7 days) in terms of either duration of remission or survival.

Post remission treatment: It is now generally accepted that further cytoreductive treatment is required in patients who achieve remission to eradicate residual leukaemic cells and prevent relapse. Studies with the longest remissions have generally used 2 or more courses of post remission chemotherapy (Foon and Gale, 1992). Median remission duration range from 1 to 2 years, with 5 year leukaemic free survival (LFS) in 15-30% in adults and 35-60% in children (Creutzig et al, 1985; Weinstein et al, 1983). Most studies use consolidation chemotherapy similar to that used for remission induction (Foon and Gale, 1992).

Maintenance chemotherapy: This is rarely given. It involves giving cyclical courses of intra-venous or sub-cutaneous cytosine together with 6-thioguanine or daunorubicin monthly. Numerous studies have failed to demonstrate a substantial benefit in patients receiving maintenance compared to those receiving induction and consolidation chemotherapy alone (Foon and Gale, 1992).

Differentiating agents: These agents attempt to induce maturation of leukaemic cells. Agents used *in vitro* include phorbol esters, dimethysulphoxide (DMSO) and retinoids. All-trans retinoic acid (ATRA) has been used in clinical studies in patients with AML M3 with some considerable success. The

results of a recent multi-centre randomised trial comparing standard chemotherapy and ATRA to chemotherapy alone has shown improved remission rates, reduced duration of coagulopathy and longer event-free survival in the ATRA group (Fenaux et al, 1993).

Bone marrow transplantation

In AML, BMT is used primarily as consolidation treatment for patients in remission or to induce remission in patients who have relapsed or have resistant disease (Champlin et al, 1987).

BMT provides an improved anti-leukaemic effect compared with chemotherapy alone. The major limitation of BMT is the transplant-related mortality usually due to pneumonitis or GVHD. The International Bone Marrow Transplant Registry (IBMTR, 1989) analyzed data from 704 recipients of HLA-identical sibling transplants for AML in first complete remission. The 5 year leukaemia-free survival (LFS) was 48% and the leukaemia-relapse rate was 20%. Improved LFS was associated with younger age and lower white cell count at diagnosis. These results are similar to those reported after chemotherapy. Similarly there is no convincing data to show that autografts prolong LFS. Controversy therefore reigns as to whether chemotherapy, autografting or allogeneic BMT is the more effective treatment for AML in first remission. The most effective strategy maybe to reserve autografts, HLA-identical-sibling or unrelated BMT's for people failing chemotherapy (Foon and Gale, 1992). Substantial improvements in the results of treatment of AML

require the development of new and more effective chemotherapeutic agents and innovative measures to overcome the major complications of BMT.

Cytokines

Introduction

Cytokines are a group of hormone-like polypeptides of 60-160 amino acids that are released in inflammatory responses. Unlike classical endocrine hormones, cytokines usually exert their effects on cells in the immediate vicinity of their source, acting in a paracrine or autocrine fashion. The term "cytokine" encompasses growth factors, colony-stimulating factors and interleukins. With few exceptions, cytokines are released only following trauma or invasion by microorganisms, and their major function is to mobilize the defence and repair response of the body.

Over the past decade the following growth factors have been identified cloned and recombinant forms produced:

Name	*Molecular weight*	*Gene site*
Colony-stimulating factor-1	45-90,000	5q
Erythropoietin (Epo)	39,000	7q
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	18 - 30,000	5q
Granulocyte colony-stimulating-factor (G-CSF)	20,000	17q
Macrophage colony-stimulating-factor (M-CSF)	70 - 90,000 45 - 50,000	5q
Interleukin-3 (also known as multipotent CSF)	15 - 30,000	5q

Name	*Mol. wt*	*Gene site*
α-interferon	19-26,000	9
β-interferon	20,000	9
γ-interferon	17,000	12
Interleukin-1	17,000	2q
Interleukin-2	14-16,000	2q
Interleukin-4	16 - 20,000	2q
Interleukin-5	46,000	5q
Interleukin-6	19 - 21,000	7p
Interleukin-7	22-25,000	8q
Interleukin-8	8,000	4q
Interleukin-9	40,000	5q
Interleukin-10	17-21,000	
Interleukin-11	23,000	19q
Platelet derived growth factor I (PDGF I)	31,000	7
Platelet derived growth factor II (PDGF II)	28,000	7
TGFα	5-20,000	2
TGFβ_1	25,000	19q
Tumour necrosis factor (TNF)	17-50,000	6
Stem cell factor	30,000	
Macrophage inflammatory protein-1α	8,000	

The growth factors mentioned above are glycoproteins, with polypeptide chains of similar length. The carbohydrate component of the molecule is not involved in its biological function but may protect the molecule from degradation and help to increase its half life *in vivo* (Metcalf, 1989b). The common characteristics of growth factors include a high biological activity but low tissue concentrations under basal conditions and a rapid increase in levels in response to inducing signals such as infections, blood loss or antigenic stimulation. Unlike classic polypeptide hormones they are produced by many different cell types including fibroblasts, endothelial cells and stromal cells.

Studies on the molecular control of neutrophilic granulocytes, monocyte and macrophage formation *in vitro* identified 4 glycoproteins capable of stimulating the formation of maturing colonies of these cells. The generic name colony-stimulating factor (CSF) was given to this group of regulators. A prefix indicates the main cell type stimulated by low concentrations of the CSF. Thus G-CSF is mainly a stimulus for granulocyte formation and GM-CSF for granulocyte and monocytes/macrophages. G-CSF, GM-CSF and IL-3 are single chain polypeptides, whereas M-CSF is a dimer of two identical chains.

In humans the gene for GM-CSF, M-CSF, the M-CSF receptor (equivalent to the proto-oncogene *c-fms*), IL-4 and IL-5 are grouped together on the long arm of chromosome 5 and their transcription may be co-ordinated (Barlow et al, 1987; Young et al, 1988).

Inhibitors of haemopoiesis

The study of molecules that inhibit haemopoiesis has lagged behind the study of stimulators of haemopoiesis. This may be because researchers tend to be cautious in their interpretation of inhibition which may be due to non-physiological toxic agents. Axelrad, 1990 defined negative regulators as growth inhibitory proteins or peptides of non-toxic nature that act within minutes or hours in a reversible manner and prevent stem cells from entering or being in S phase of the cell cycle. Molecules that conform to this definition are MIP-1α and inhibin (Zipori and Honigwachs-Sha'anani, 1992). Cytokines such as TNF and interferons however are excluded by the above definition. The line drawn between "inhibitors" versus "stimulators" has further been blurred by the discovery of molecules like TGFβ which is known to have both stimulatory and inhibitory effects depending on the target cell and growth conditions.

Granulocyte colony-stimulating-factor

Humans on average produce approximately 120×10^9 granulocytes per day, this number maybe increased 10 fold under stress due to an infection (Demetri and Griffen, 1991). G-CSF regulates the proliferation of neutrophils but may also play a role in their distribution within the body and their response to inflammatory stimuli (Demetri and Griffen, 1991).

G-CSF was identified by Burgess and Metcalf in 1980, from work done on a murine myelocytic cell line. In 1985, Nicola and Metcalf, identified a human analogue of the murine G-CSF. On the basis of biological activity and

receptor-binding studies human G-CSF was shown to be the same as CSF-β, a stimulatory agent previously identified in human placenta-conditioned medium. (Nicola et al, 1985a). At the same time Welte et al purified to homogeneity a substance named human pluripotent colony-stimulating-factor also known as pluripoietin (Platzer et al, 1985). This was a colony-stimulating-factor that was secreted into the culture medium by the 5637 human bladder carcinoma cell line. Stromal cells, macrophages, fibroblasts, endothelial cells and the human bladder carcinoma cell line 5637 all produce G-CSF (Clark and Kamen, 1989).

In 1986, Souza cloned the cDNA encoding human G-CSF from the 5637 cell line. The clone they derived encoded a protein of 174 amino acids. Nagata et al in 1986, cloned the cDNA for G-CSF using the squamous cell carcinoma cell line CHU2. The cDNA they obtained encoded a larger protein of 177 amino acids. This protein has less stimulatory activity than the smaller protein. Okabe et al, 1990, suggested that the amino acid terminus may play a role in the functional activity of G-CSF. Subsequently Nagata et al, 1986b cloned the cDNA encoding for the smaller protein from the same CHU2 cell line.

The chromosomal location of the G-CSF gene to chromosome 17 at 17q11-12 raised speculation about whether the G-CSF gene was involved in the breakpoint of the t(15;17) translocation characteristic of acute promyelocytic leukaemia. The G-CSF gene however is located proximal to this breakpoint, and is not rearranged in the malignant clone that gives rise to

AML M3 (Simmers et al, 1987).

The murine G-CSF gene is highly homologous with the human gene, with 69% nucleic acid sequence homology in both coding and non-coding regions and a 73% sequence homology in the amino acid sequence. Both the proliferation and differentiation-inducing activities of the murine and human G-CSF molecules cross species boundaries, unlike IL-3 and GM-CSF which are species specific. Nicola and Metcalf in 1985a, first described the existence of a high affinity receptor on granulocytes for G-CSF. Receptor numbers increase with leucocyte maturation, mature neutrophils having 2 to 3 times more receptors than mature metamyelocytes. However even neutrophils express a relatively low number of G-CSF receptors, approximately 50 to 500 receptors per cell.

G-CSF receptors have also been described on a variety of other cells including human myeloid leukaemia cells, human placenta and vascular endothelium and human small cell cancer. The functional significance of G-CSF receptors on most non-haemopoietic cells remains unclear. In contrast G-CSF has been shown to act as a potent proliferative stimulus for some types of myeloid leukaemia. G-CSF receptors on myeloid leukaemic cells appear to have a similar affinity to receptors found on normal granulocytes (Budel et al, 1989). This has meant that there is a general reluctance to use G-CSF in acute myeloid leukaemia.

The primary effects of G-CSF on normal haemopoietic cells are on cells of the granulocytic lineage. *In-vitro* G-CSF stimulates proliferation and

differentiation of neutrophil colony-forming cells. G-CSF acts on a relatively mature progenitor population that is primarily committed to neutrophil differentiation. Cultures of bone marrow progenitors enriched for the relatively primitive population expressing the CD34 antigen show minimal response to G-CSF when added alone (Demetri and Griffen, 1991).

The interaction of G-CSF with other growth factors in inducing proliferation of progenitor cells is complex. G-CSF appears to behave in a synergistic fashion with both GM-CSF and IL-3 (Vellenga et al, 1987a). Interleukin-4 however interacts in a more complex manner. Several studies have shown that IL-4 can augment G-CSF-induced proliferation of neutrophil colonies *in vitro*. Unlike GM-CSF or IL-3, IL-4 alone does not stimulate myelopoiesis. It also appears that IL-4 must be present early in cultures to have an enhancing effect. It could therefore be suggested that IL-4 sensitises committed cells to the effects of G-CSF (Sonoda et al, 1990).

The G-CSF receptor and the IL-4 receptor have 50% sequence homology (Fukunaga et al, 1990). There are possibly therefore similarities in signal transduction triggered by these 2 receptor-ligand systems.

Human myeloid leukaemia cells in culture exhibit a heterogenous response to G-CSF in combination with other cytokines. This reflects the variability inherent in the biology of the myeloid leukaemias (Kelleher et al, 1987; Young et al, 1988; Nara et al, 1987). In some AML samples, IL-4 may augment G-CSF-induced proliferation of myeloblasts (Vellenga et al, 1990). A large proportion of the blasts from patients with AML fail to express G-

CSF receptors. This is consistent with the observation that only about 50% of cases of AML proliferate when stimulated by G-CSF *in vitro* (Vellenga et al, 1987a). There has been no clear relation between the number of G-CSF receptors on myeloblasts and their ability to proliferate *in vitro* when stimulated by exogenous G-CSF.

Granulocyte macrophage colony-stimulating factor

GM-CSF is a glycoprotein that stimulates the proliferation and differentiation of neutrophil, eosinophil and monocyte precursors *in vitro*. It is produced by monocytes, endothelial cells, fibroblasts and lymphocytes. The cDNA for human GM-CSF has been cloned (Wang et al, 1985). The cDNA encodes for the 144 amino acid precursor containing a 17 amino acid signal peptide. The 127 active amino acid product has 2 potential N-glycosylation sites and is homologous with the murine sequence. GM-CSF, unlike G-CSF is species specific in its activity.

The biological activities of GM-CSF are mediated through binding to a high affinity receptor. In the mouse the receptors are found on cells of the myelomonocytic lineage and both high and low affinity sites have been described (Walker et al, 1985a; Walker et al, 1985b). In man it appears that GM-CSF binds to a single class of receptor and that there are between 50 and 1000 receptors per cell. The receptors are found on neutrophils, macrophages and eosinophils. Like G-CSF the number of receptors increase with maturation of the cell (Park et el, 1989). GM-CSF receptors have been

described on leukaemic cells and, similar to G-CSF receptors on leukaemic cells, their numbers do not predict response to stimulation. Binding of GM-CSF results in internalisation of the receptor/ligand complex, however the receptors are recycled and appear again at the surface.

In clonogenic assays using human bone marrow mononuclear cells GM-CSF promotes growth of mixed colonies of granulocytes, macrophages/monocytes and eosinophils. With the addition of erythropoietin GM-CSF stimulates BFU-E and mixed colonies (CFU-GEMM). Megakaryocyte colonies are also produced but in lower numbers than with IL-3. 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 et al, 1990).

Interleukin-3

IL-3 was originally derived from the murine myelocytic cell line WEHI 3 (Ihle et al, 1983) and was called colony-forming unit stimulating activity (CFU-stimulating activity).

A human analogue to murine IL-3 has been cloned (Otsuka et al, 1988). Homology at the DNA level between murine and human IL-3 is approximately 45%, compared to 29% homology at the amino acid level. The human mRNA yields a 15-25 kD translation product.

Unlike G-CSF and GM-CSF receptors, the receptors for IL-3 are at their highest frequency on primitive cells, decreasing in number with increasing

maturation. Receptors have been identified on myeloid and some pre-B cell precursors but not on the majority of lymphocytes or on cells of erythroid lineage. The IL-3 receptor has also been described on AML cells and normal monocytes (Budel et al, 1990).

With the exception of the WEHI 3 cell line and perhaps epidermal cells IL-3 is produced exclusively by activated T-lymphocytes (Luger et al, 1985).

Reported activities of IL-3 include stimulating the differentiation of neutrophils, macrophages, megakaryocytes and mast cells (Ihle et al, 1983) and stimulating the proliferation of mast cells (Ihle et al, 1981). Recently it has also been shown that IL-3 enhances IL-2-dependant growth of human T lymphocytes (Santoli et al, 1988; Schneider et el, 1988), induces IgG secretion of IL-2-activated B lymphocytes (Tadmori et al, 1989) and also potentiates the activities of eosinophils, basophils and monocytes (Hauck-Frendsch et al, 1987; Cannistra et al, 1988). IL-3 is less effective in promoting CFU-GM 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 erythropoietin is required for erythrocyte maturation.

Stem cell factor

Stem cell factor (SCF) is a normal stromal cell derived cytokine which stimulates mast cells and is a ligand for the oncogene *c-kit* (Anderson et al, 1990). It is also known by the names *c-kit* ligand, mast cell growth factor, and steel locus factor. It has been called Steel locus factor because mice with

mutations at the *Steel* locus, Sl^d exhibit defects in haemopoiesis, coat colour and fertility (Flanagan et al, 1991). Stem cell factor differs from IL-3, GM-CSF and G-CSF in that alone, it stimulates the growth of haemopoietic stem and progenitors only weakly and is relatively ineffective as a colony-stimulating factor (Moore, 1991; Metcalf, 1991). However, SCF exhibits a strong synergistic action on stem and progenitor cells in the presence of other haemopoietins like IL-3, G-CSF, GM-CSF or erythropoietin (Metcalf, 1991; Moore, 1991). SCF also synergizes with cytokines such as IL-1, IL-4, IL-6 and IL-7 that have little or no colony-stimulating activity or direct ability to stimulate the division of stem cells (Schrader, 1992).

The mechanisms causing the release of stem cells from the bone marrow are largely unexplained. One factor may be the action of cytokines like IL-3 and GM-CSF which down-regulate the expression of *c-kit*, the receptor for SCF. The cell bound, transmembrane form of SCF is expressed on stromal cells and can act as a cell-adhesion molecule. The binding of *c-kit* expressing haemopoietic cells to stromal cells may lead to disengagement of stem cells from their bone marrow microenvironment (Schrader, 1992).

Interleukin-4

Interleukin-4 (IL-4) was first described in 1982 by William Paul and called B-cell growth factor (Paul and Ohara, 1987). It has also been called B cell-stimulatory factor (BSF-1), T-cell growth factor-2 (TCGF-2) and mast cell-growth factor (MCGF-2). When the cDNA for IL-4 was isolated and cloned

it became clear that the molecule had diverse effects on B cells together with effects on lymphoid and non-lymphoid cells. It was then given interleukin status and called interleukin-4.

IL-4 is a small glycoprotein with a molecular weight of 15 to 19 kD. The cDNA for human IL-4 has been cloned and shown to code for a protein of 153 amino acids which is cleaved between the glycine at position 24 and the histidine at position 25 to yield an active protein of 129 amino acids (Gallard, 1991). Human and murine clones share a 50% homology at the amino acid sequence. IL-4 however, is species specific in respect to its receptor binding and biological effects. There are 2 potential N-linked glycosylation sites, but glycosylation does not seem to be required for activity. The gene for IL-4 is located on chromosome 5q23-31 (Paul, 1991)

IL-4 is produced by activated T lymphocytes. It is also produced by some B-cell lymphomas, B cells from patients with systemic lupus erythematosus and bone marrow stromal cell lines.

The cDNA encoding for the IL-4 receptor was cloned in 1990. More recently several lines of investigation have suggested a second IL-4 receptor (Gallard, 1991). The cDNA for this receptor has not been isolated. IL-4 was initially described as a co-stimulator of DNA synthesis for B-cells stimulated with anti-IgM antibodies. Since then a wide range of biological functions have been attributed to IL-4 including increasing MHC class expression in resting B-cells and enhancing the production of IgG_1 and IgE isotypes following lipopolysaccharide (LPS) stimulation. IL-4 also acts to increase the viability

and growth of normal resting T-cells and some T-cell lines, as well as to co-stimulate growth in some mast cell lines. IL-4 may also act as a autocrine growth factor for antigen specific T-cells in combination with IL-1 and enhance antigen presentation in bone marrow-derived macrophages (Kupper et al, 1987; Zlotnik et al, 1987). It augments the development of cytotoxic T-lymphocytes from resting murine cells and also induces lymphokine-activated killer (LAK) activity (Trenn et al, 1988; Peace et al, 1988). In contrast to these findings, it has been reported that IL-4 may actually inhibit human IL-2 induced LAK cells *in vitro*. (Gerosa et al, 1988). IL-4 also enhances the proliferation of CFU-GM, BFU-E and CFU-Meg in response to G-CSF, Epo and IL-1 respectively (Peschel et al, 1987).

Transforming growth factor β_1

Transforming growth factors (TGFα and TGFβ) belong to a family of polypeptide factors that share certain structures and functional characteristics. In 1978 DeLarco and Todaro reported that murine 3T3 cells transformed by a sarcoma virus produced polypeptide growth factors called sarcoma growth factors (SGFs). These polypeptides were secreted into the extracellular medium and had the unusual property of being able to cause anchorage-independent growth of normal rat kidney cells, clone 49F (NRK-49F). The transforming activity of SGFs on NRK cells was phenotypic, on removal of SGF from the NRK cells, they reverted back to their normal morphology and growth properties. Since then similar transforming activity has been reported

in the conditioned medium of several neoplastic cell lines and a variety of murine cells transformed by a sarcoma virus. These transforming activities were also characterised by their ability to compete with epidermal growth factor (EGF) for binding to its receptor.

In 1980 Roberts extracted the transforming peptides from the tumour cells and the term "transforming growth factor" (TGF) was coined. Using the NRK 49F cell as an assay 2 principal types of TGFs were isolated and defined. Peptides that competed with EGF for receptor binding but did not require additional EGF for promotion of growth of cells in soft agar were called TGF-α and peptides that did not compete with EGF for receptor binding but did require EGF for promotion of growth in soft agar were called TGF-β. TGF-α and TGF-β have been purified to homogeneity, cloned and sequenced. They represent distinct families of peptides; their amino acid sequences, cell membrane receptors and mRNAs are unique. They share only a common nomenclature based on their discovery as synergistic effectors of the transformation of NRK fibroblasts. More recently a series of independent findings has shown other factors that are structurally and functionally related to TGF-β. They include inhibins which are heterodimeric proteins of gonadal origin that act on the pituitary to suppress follicle-stimulating hormone (FSH) secretion; activins which are dimeric peptides also of gonadal origin and are potent stimulators of pituitary FSH secretion; Mullerian inhibitory substance which is a homodimeric testicular glycoprotein that causes regression of the Mullerian duct during development of the male embryo and finally the

product of the decapentaplegic gene complex which is involved in pattern formation and dorsal-ventral specification during Drosophila development (Roberts and Sporn, 1988).

Bone represents the largest reservoir of TGFβ. Platelets are the most concentrated source of TGFβ with a yield of approximately 2-3mg TGFβ/kg wet weight of human platelets. Soft tissues such as kidneys and placenta have a much lower concentration of TGFβ (Roberts and Sporn, 1988).

The cDNA sequence for this group of growth factors indicate that the mature proteins are synthesized from the carboxyl-terminal half of larger precursors and that they share homologies to each other within this region. Common to several members of this family of polypeptides is their apparent involvement in developmental processes such as embryogenesis and in tissue repair.

There are at least 5 different isoforms of TGFβ coded for by different genes on separate chromosomes. Three of the isotypes have been well characterised. TGFβ_1 is a disulphide-linked homodimer consisting of 2 β_1 subunits, TGFβ_2 is a disulphide-linked homodimer consisting of 2 β_2 subunits, TGF$\beta_{1,2}$ is a disulphide-linked heterodimer consisting of one β_1 and one β_2 subunit. The β_1 and β_2 subunits are closely related and share 70% aminoacid sequence homology in their N terminal halves. TGFβ_1 aminoacid sequences are highly conserved and are identical in man, monkeys, cows, pigs and chickens (Sporn and Roberts, 1989).

The cDNA encoding TGFβ_1 was isolated from a library derived from

human placenta (Derynck et al, 1985). The gene was subsequently localised to the long arm of human chromosome 19 and to murine chromosome 7 (Fujii et al, 1986). The human TGFβ_1 cDNA encodes a precursor polypeptide of 391 amino acids (Derynck et al, 1985). A monomer of 112 amino acids is cleaved from the C-terminal end of the parent molecule. The TGFβ_1 precursor contains 3 potential N-glycosylation sites, none of which are located in the TGFβ_1 polypeptide. The 112 amino acid sequence contains 9 cysteine residues and forms a 25 kD dimer, which under reducing conditions yields a single 12.5 kD band.

However TGFβ receptors have been found in virtually all tissues and cell types examined. The number of receptors per cell ranges from 600 on human tonsillar T-lymphocytes to about 80,000 in Swiss 3T3 cells (Roberts and Sporn, 1988). Three TGFβ receptors have been described, Class I, II and III with molecular weights of 53-65, 73-95 and 250-350 kD respectively. The Class III receptor is also known as TGFβ-binding proteoglygan betaglygan. Both the Class I and II receptor glycoproteins display a 10-fold higher affinity for TGFβ_1, than for TGFβ_2. The function of these 2 receptors nevertheless remains unknown. The human Class II receptor has recently been cloned (Massague, 1992). The third receptor, the 280 kD glycoprotein exists as part of a larger receptor complex. This receptor does not appear to discriminate between TGFβ_1 and TGFβ_2 and it has been implicated in the mediation of certain functions including inhibition of epithelial cell proliferation and adipogenesis and induction of fibronectin and collagen expression.

In-vitro effects of TGFβ_1

TGFβ_1 is highly pleiotropic and has a multiple range of actions on almost all cell types. It can stimulate proliferation in some cells whilst being a potent inhibitor of proliferation in others. Its main *in-vitro* effects are as follows:-

1. Stimulates proliferation of fibroblasts and enriches secretion of extracellular matrix proteins.

2. Stimulates expression of type II collagen in immature precursor cells; inhibits type II collagen expression in mature cells; stimulates proteoglygan synthesis.

3. All the isoforms except for TGFβ_2 have an inhibitory effect on the growth of endothelial cells.

4. TGFβ_1 has a mitogenic effect on osteoblast proliferation and it also regulates synthesis of matrix proteins.

5. Inhibits differentiation and expression of creatinine kinase gene.

6. TGFβ_1 exerts a mainly inhibitory effect on the growth of both normal B- and T- lymphocytes. In B lymphocytes it inhibits secretion of IgG and IgM from B lymphocytes and enhances secretion of IgA.

7. TGFβ_1 has a chemotactic effect on both neutrophils and monocytes. In addition it causes monocytes to secrete TNF, TGF-α, fibroblast growth factor, IL-1 and TGFβ_1 itself.

8. TGFβ_1 inhibits growth of normal bone marrow cells stimulated by IL-3 and GM-CSF. In addition it has an anti-proliferative effect on megakaryocytes.

9. Inhibits growth of hepatocytes and keratinocytes.

10. Inhibits differentiation of adipocytes.

11. Inhibits steroid formation in adrenocortical cells and Leydig cells and enhances FSH activity in granulosa cells (see Roberts and Sporn, 1992).

In-vivo biological effects of TGFβ_1

The action TGFβ_1 on a target tissue depends on many parameters including the state of differentiation and the presence of other peptide regulators. The plasma half-life of active TGFβ_1 is 2-3 minutes (Roberts and Sporn, 1992). It is degraded by the liver, kidney, lungs and spleen. By contrast the plasma half-life of latent TGFβ_1 (the form secreted by most cells and released by platelets) is >100 minutes (Wakefield et al, 1990). TGFβ_1 has a role in the following physiological and pathological processes (from Robert and Sporn, 1992; Sporn and Roberts, 1989).

1. Embryogenesis: Immunocytochemistry studies have shown that TGFβ_1 has an important role in mammalian embryogenesis particularly during the remodelling that occurs during the formation of vertebrae, limb buds, teeth, facial bones and the cardiac valves. High levels of TGFβ_1 have been found in potential sites of malformation such as the palate and inter-ventricular septum of the heart and it is therefore likely that TGFβ_1 is an important modulator during normal human embryogenesis.

2. Inflammatory response and wound healing: There is now considerable information on the role of TGFβ_1 in inflammation and wound healing.

Platelets are the most concentrated source of TGFβ and it is stored in platelet α-granules. The process of platelet degranulation releases TGFβ at the site of a wound. $TGF\beta_1$ attracts monocytes, macrophages and fibroblasts by chemotaxis to the site of the wound. Sub-cutaneous injections of TGFβ can stimulate formation of granulation tissue and enhance wound healing (Roberts et al, 1986; Mustoe et al, 1986). TGFβ plays an important role in bone formation and fracture healing, by stimulating the synthesis and secretion of matrix proteins, and chondrogenesis.

3. Immune function: TGFβ is a potent suppressor of lymphocyte proliferation and function. It is 10,000 to 100,000 times more potent than cyclosporin (Sporn and Roberts, 1989). TGFβ inhibits proliferation of T-lymphocytes stimulated by IL-1 or IL-2, proliferation and antibody production by B-lymphocytes and cytotoxic T-lymphocyte and lymphocyte-activated killer cell activity. It also depresses cytolytic activity of natural killer cells.

In vivo effects of $TGF\beta_1$ on haemopoiesis

Direct intravenous injection of $TGF\beta_1$ into the femoral artery of a mouse (to avoid first pass metabolism) inhibited constitutive and IL-3-driven bone marrow growth. The inhibition was selective in that 100% of CFU-GEMM was inhibited while only 50% of the more mature committed colony-forming cells were affected (Goey et al, 1989). This inhibition was time and dose-dependent with the maximum effect observed at 24 hours and at a dose of 5μg/mouse. Carlino et al, 1990 studied the effects of daily sub-cutaneous TGFβ on a variety of murine haematological parameters. After 14 days there

was a decrease in mature erythroid and platelet numbers, together with an increase in the white cell count in the peripheral blood. Increased granulopoiesis was observed in the spleen and the bone marrow while there was no change in the circulating neutrophil counts. The fact that the neutrophil count remains normal throughout TGFβ treatment despite a strong inhibitory effect on primitive stem cell proliferation suggests that TGFβ is able to stimulate neutrophil production.

Macrophage inflammatory protein-1α

MIP-1α, an 8 Kd glycoprotein is another negative regulatory protein of haemopoiesis. The expression of MIP-1α appears to be limited to cells of the mononuclear phagocyte system, mast cells and lymphocytes. Broxmeyer et al, in 1990 showed that MIP-1α has a direct suppressive activity on immature progenitor cells but a myelopoietic enhancing activity for more mature progenitors. It is inhibitory to CFU-A and CFU-S (Wright and Pragnell, 1992). The CFU-A assay detects the human counterpart of the murine colony-forming unit. These cells form large mixed lineage colonies in agar culture. They have a low replating capacity for macroscopic colony formation but a high replating efficiency for producing small colonies of neutrophils and macrophages. They appear to occupy a position in the haemopoietic hierarchy that is intermediate between the CFU-GEMM and CFU-GM (Gordon, 1993). MIP-1α can block the recruitment of CFU-S into cycle and this effect can be exploited to protect the stem cell compartment during the administration of

chemotherapy (Lord et al, 1992; Dunlop et al, 1992). MIP-1α enhances the proliferation of more committed progenitor granulocyte macrophage-colony forming cells in response to GM-CSF (Clements et al, 1992)

Regulation of haemopoiesis

Haemopoiesis involves a series of complex cellular events in which a small number of stem cells continuously need to generate a large population of more mature cells. This is achieved by the use of regulatory molecules that can be humoral or cell-associated. At least 40 such regulators which have a effect on haemopoiesis have been identified, in addition there are a variety of factors in the early stages of characterisation. A current estimate of possible combinations is 2.2178877^{48} (Quesenberry et al, 1993). It is important to note that no two haemopoietic growth factors stimulate exactly the same type of colony formation as judged by colony number or by the lineage and maturation of the cells making up the colony (Metcalf, 1993). There may however be a great deal of similarity, for example G-CSF, IL-3, GM-CSF, SCF, MCF and IL-6 all stimulate neutrophil granulocyte colony-formation. Hence there is, a degree of overlap between the actions of the different growth factors. Whilst self-renewal and differentiation of stem cells and progenitors appear to be a stochastic (that is random) process, survival and proliferation of the cells is regulated by cytokines. The growth factors may be divided into 3 categories:- (Ogawa, 1993)

(1) Late-acting lineage specific factors such as erythropoietin, M-CSF and

IL-5.

(2) Intermediate-acting lineage-nonspecific factors such as IL-3, IL-4, GM-CSF. IL-4 has both stimulatory and inhibitory effects on haemopoiesis (Peschel et al, 1987; Broxmeyer et al, 1988).

(3) Factors affecting kinetics of cell cycle dormant primitive progenitors. Leary et al, (1990 and 1992) found that IL-6, G-CSF, IL-11, SCF and IL-12 act synergistically with IL-3. In addition to these factors leukaemia inhibitory factor was found to augment proliferation of human progenitor cells. It has been proposed that part of the synergistic effect of these factors is to shorten duration of G_0 of the primitive progenitors (Ogawa, 1993). More recent studies have provided biochemical explanations, IL-6 and G-CSF share structural homology (Hirano et al, 1986), receptors for IL-6, LIF and IL-11 share signal transducing protein IL-6 gp 130 (Gearing et al, 1992; Yin et al, 1992). IL-12 is a heterodimer consisting of 35Kd and 40Kd proteins, each sharing homology with IL-6 and its receptor respectively (Merberg et al, 1992). Thus there may be a biochemical reason for the functional duplication of the synergistic growth factors.

The action of individual growth factors is not restricted to cells of a single lineage, and for example erythropoietin which has an effect on mature erythroid cells probably acts on megakaryocyte precursors (Spivak, 1986). A further important feature of the haemopoietic growth factors is that in combination their response can be enhanced. When two or more growth factors act on the same progenitor cell and induce increased colony size this

process is called "synergy". In the second process known as "recruitment" the combined actions of two or more growth factors allow increased numbers of progenitor cells to proliferate either because distinct subsets of progenitor cells exist that respond exclusively to one factor or because some progenitors require simultaneous stimulation by two or more factors before being able to respond (Metcalf, 1993).

Stem cell factor when acting alone maintains the survival of the stem cells but does not induce cell division (Li et al, 1992). Not all combinations of growth factors leads to an enhancement of response, GM-CSF with M-CSF acting on murine cells leads to a decrease in the formation of macrophages than that obtained with M-CSF alone (Gliniak et al, 1990).

Some of these combinations of growth factors have successfully been used in the mobilisation of peripheral blood progenitor cells, GM-CSF and IL-3 (Ganser et al, 1992, Brugger et al, 1992).

To randomly ascertain whether a particular combination of growth factors is effective would strain the financial resources of most research units, hence more *in vitro* data is needed if unexpected favourable combinations are not to be overlooked.

Clinical uses of growth factors

Clinical studies with G-CSF and GM-CSF commenced in 1986 and their potential clinical uses are listed below:

1. reduction of the period of neutropenia following chemotherapy

2. prophylaxis against infection

3. treatment of neutropenia due to other causes e.g. cyclical neutropenia, glycogen storage diseases, immune neutropenia

4. following autologous bone marrow rescue or allogenic bone marrow transplantation

5. peripheral stem cell mobilisation

6. as an adjunct to antibiotic therapy

7. in conjunction with anti-retroviral drugs in the acquired immunodeficiency syndrome (AIDS)

8. in the treatment of aplastic anaemia and the myelodysplastic syndromes

9. in combination with other cytokines and growth factors

IL-3 has been used with limited success in the treatment of MDS and aplastic anaemia and Diamond-Blackfan syndrome (Davis and Morstyn, 1992). IL-3 in addition to improving erythropoiesis, and increasing the neutrophil count also has a beneficial effect on thrombopoiesis in some patients. Toxicity due to IL-3 include fever, nausea and fatigue. The concerns regarding its clinical use relate to the possible stimulation of leukaemic blasts and side effects secondary to the stimulation of mast cells, although the latter has not been noted in clinical trials to date.

In vivo animal studies using G-CSF

Studies in mice (Shimamura et al, 1987) and monkeys (Welte et al,

1987) have demonstrated that G-CSF can induce a neutrophilia in normal animals and accelerate neutrophil recovery after 5-flurouracil, cyclophosphamide or total body irradiation (TBI). Lord et al (1989 and 1991) have shown by tritiated thymidine labelling studies that granulopoiesis induced by G-CSF affects all stages of neutrophil maturation but particularly the myeloblast stage. There was no change observed in the half life of the circulating neutrophils, nor was any organ sequestration noted.

Clinical studies using G-CSF

In 1965 Bodey et al reported that the percentage of days spent with infection in patients with acute leukaemia treated by chemotherapy increased when the absolute neutrophil count fell to below 1.0×10^9/l. The duration of neutropenia was the most important factor in predicting risk of infection and patients in whom the neutropenic period had exceeded 3 weeks had a 60% chance of developing a severe infection.

Towards the end of the last decade an important landmark in treating febrile neutropenic patients was the introduction of the *in vivo* use of G-CSF and GM-CSF. An underlying concern in the use of these agents in treating chemotherapy related neutropenia of leukaemia was the possibility that they could stimulate/re-activate the leukaemic clone. Negrin et al, in 1990, treated 18 patients with MDS with G-CSF. Three of the patients developed AML whilst on therapy. Thus the role of G-CSF in MDS is not well defined. Ohno et al, 1990, carried out a prospective randomised study to determine the safety

and efficacy of recombinant G-CSF after intensive chemotherapy in 108 patients with relapsed or refractory acute leukaemia (67 with AML, 30 with ALL, 9 with CML in BT and 2 with transformation of MDS). I.V. G-CSF (at a dose of 200μG/square metre of body-surface area per day was begun 2 days after the chemotherapy and continued until the neutrophil count rose above 1500/cubic mm. Treatment with G-CSF significantly accelerated neutrophil recovery ($p<0.001$) and furthermore the rate of relapse was similar in both groups. To date, there are no reports citing a definite link between the use of G-CSF and leukaemic relapse or disease transformation.

Phase I studies have shown that G-CSF is effective in increasing the neutrophil count in a dose dependent fashion, whether given by continuous intravenous infusion, short intravenous infusion or sub-cutaneous infusion (Bronchud et al, 1987). G-CSF has few side effects. The main adverse effects are bone pain in 20% of patients, elevation of alkaline phosphatase and lactic dehydrogenase, elevation of serum urate and splenic enlargement in children with chronic neutropenia (Davis and Morstyn, 1992).

Peripheral stem cell mobilisation using G-CSF

Sheridan et al, 1990, demonstrated that G-CSF is effective at mobilising progenitor cells into the peripheral blood where they may be collected using leukapheresis. When infused together with autologous bone marrow cells following high dose chemo-radiotherapy neutrophil recovery follows similar kinetics to that of autologous bone marrow rescue plus G-CSF, although

recovery may be slightly accelerated. The platelet recovery however is greatly accelerated with patients achieving platelet counts of >20 x 10^9/l at a median of 9 days, and >50 x 10^9/l at a median of 14 days. The mechanism of G-CSF action in releasing early progenitors into the circulation is not clear and these "stem cells" have not been well characterised. The circulating cells mobilised by G-CSF are similar to normal bone marrow in their capacity to generate CFU-GM in culture and these cells may therefore be an alternative to bone marrow cells after marrow ablation for allogeneic transplantation.

In vivo animal studies using GM-CSF

In mice GM-CSF given intra-peritoneally has the effect of increasing granulopoiesis and the accumulation of mature neutrophils and monocytes has been observed in the liver and spleen (Metcalf et al, 1987). Human GM-CSF given to non-human primates causes a marked leucocytosis which is maintained for up to 28 days, with increases in neutrophils, eosinophils monocytes and lymphocytes (Donahue et al, 1986). In mice given recombinant murine GM-CSF following melphalan, the period of neutropenia was shortened and there was a reduction in mortality during neutropenia (Douer et al, 1987).

Clinical studies using GM-CSF

Early studies with GM-CSF were hampered by the species specificity of this growth factor. Phase I studies have confirmed that GM-CSF administered

by various routes results in leucocytosis which is predominantly comprised of neutrophils as well as eosinophils and monocytes (Antman et al, 1988; Lieschke et al, 1990). Sub-cutaneous or continuous i.v. infusions of GM-CSF are more potent and preferable to short i.v. infusions of GM-CSF (Lieschke et al, 1990). A "first dose" effect was recognised with intravenous GM-CSF (Lieschke et al, 1989). Within 20 minutes of the first dose many patients develop symptoms of flushing, sweating, nausea, vomiting, back pain, involuntary leg spasms and dyspnoea, with hypotension, tachycardia and hypoxia being observed. These reactions were seen more often in patients receiving short infusions. These responses were not seen in subsequent doses in the same course of GM-CSF but were seen at the start of following courses. Other side effects observed at high doses in phase I studies include thromboses, pleural and pericardial effusions, inflammation and oedema. At lower doses skin rashes, arthralgia, myalgia, lethargy, anorexia, malaise and nausea (Lieschke et al, 1989; Davies and Morstyn, 1992). Intravenous dosage is associated with a higher incidence of first dose reactions than the equivalent sub-cutaneous dose (Lieschke et al, 1989).

All clinical studies using GM-CSF in the setting of myelotoxic chemotherapy have shown that it shortens the duration of the neutropenic period. Some studies have shown clinical benefit, being fewer days with fever or on antibiotics (Herrmann et al, 1990). The use of GM-CSF in aplastic anaemia, MDS and AML are not well defined. After ABMR and ABMT, GM-CSF reduces the period of neutropenia (Gianni et al, 1989; Powles et al, 1990)

but there have been no significant reductions in infection related mortalities.

Peripheral stem cell mobilisation using GM-CSF

During phase I studies it was noticed that GM-CSF administration increases circulating haemopoietic progenitor cells in the peripheral blood (Siena et al, 1989; Villeval et al, 1990). These cells are capable of restoring haemopoiesis after high dose chemotherapy (Haas et al, 1990) and lead to early platelet, as well as myeloid, engraftment (Nemunaitis et al, 1988; Gianni et al, 1989). When ABMR is combined with peripheral stem cell stimulated by GM-CSF, engraftment is more rapid and platelet recovery is also accelerated (Gianni et al, 1989). Seven patients with large granular cell lymphoma were treated with cyclophosphamide followed by TBI and melphalan. Neutrophil counts of 1 x 10^9/l or more were achieved at a mean of 9.9 days (range 8-13) and platelet counts of more than 1 x 10^9/l were reached at a mean of 13.6 days (range 13-21). The method of stimulating peripheral stem cells is important. The objective is to obtain as many progenitor cells as possible from the peripheral blood and the optimal conditions for achieving this are not yet determined. It is probably important to have minimally pretreated patients who are more likely to have good marrow reserve. Some of these studies use cyclophosphamide which is known to stimulate the mobilisation of peripheral stem cells (To et al, 1990) and to have a relatively sparing cytotoxic effect on stem cells (Smith et al, 1983). The mean CFU-GM was 43.88 x 10^4 versus 6.16 x 10^4 per kg body weight per leukapheresis in the

cyclophosphamide only group.

Circulating levels of haemopoietic growth factors in serum

Amount of colony stimulating activity (CSA) in the serum increases substantially after high dose chemoradiotherapy (Millar et al, 1992). They studied 62 patients after intensive treatment for haematological malignancies. In 44/62 patients maximum CSA occurred at 7 days. Despite this early peak in CSA there was no correlation between the time at which CSA activity was maximum and the return of the neutrophil count to 0.5 x 10^9/l. In a previous study, (Millar et al, 1990), showed that CSA activity in serum of patients treated with high dose chemoradiotherapy for multiple myeloma could be neutralised with anti-bodies to GM-CSF. Other workers have shown that G-CSF, GM-CSF (Watari et al, 1989) and megakaryocyte CSA (Fauser et al, 1988) are present in serum post transplantation.

Watari et al using an enzyme immunoassay specific for human G-CSF (which estimates serum G-CSF concentrations greater than 30pg/ml) showed that 49/56 (83%) of normal donors had serum G-CSF levels below the sensitivity of the assay. Conversely 82% of patients with aplastic anaemia, 50% of patients with MDS, 42% of patients with AML and 33% of patients with CML had serum G-CSF levels ranging from 46pg/ml to >2000pg/ml. The serum G-CSF level rose after chemotherapy or BMT in 3 patients with leukaemia.

Aims of the thesis

Haemopoiesis is controlled by appropriate combinations of positive and negative regulators. Although much information has been accumulated concerning positive regulators such as colony-stimulating factors, the role of negative regulators remains controversial. Molecules which have been identified as potential negative regulators include tumour necrosis factor (TNF), TGF-β, MIP-1α and the interferons.

The production of TGFß$_1$ by haemopoietic cells suggests that it may play an important role in regulating haemopoiesis. In murine bone marrow cells TGFß$_1$ inhibits colony formation induced by IL-3 but not by GM-CSF (Keller et al, 1988). In human haemopoiesis TGFß$_1$ inhibits CFU-GM production by IL-3 and GM-CSF and megakaryocyte development by IL-3 but has no effect on G-CSF-induced cell proliferation from normal bone marrow cells *in vitro* (Sing et al, 1988; Han et al, 1992).

In this study the effects of TGFß$_1$ on the G-CSF-induced proliferation of normal, CML and AML cells were studied alone or in combination with IL-4 which is known to enhance the number and size of colonies induced by G-CSF (Broxmeyer et al, 1988). The purpose being to ascertain if the effects of TGFß$_1$ on normal and myeloid leukaemic cells were different and to determine if this differential effect could be exploited in future treatment strategies of CML. *In-situ* hybridisation studies were done to determine if the effects of TGFß$_1$ on normal and myeloid leukaemic cells were mediated via receptor expression

CD34+ is a heavily glycosylated surface antigen which is preferentially expressed on haemopoietic stem/progenitor cells and is progressively lost as cells differentiate. The expression of CD34+ on early haemopoietic cells has been used in transplantation studies and these cells may be the target of gene therapy protocols. Immunophenotypic studies were done to characterise surface antigen markers on CML and AML cells, to determine whether an increased CD34 positivity (signifying a greater number of early progenitors) correlates with increased CFU-C *in vitro*. The effects of G-CSF, IL-4 and TGFβ_1 on CD34+ enriched cells were compared to their effects on unmanipulated normal, AML and CML mononuclear cells, to determine if these cytokines have a similar effect on more primitive progenitor cells.

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

Patient samples

Bone marrow: Samples were collected with the consent of the patients either at their out-patient visit or during their bone marrow harvest. Samples were aspirated from one or other of the iliac crests. When samples were unobtainable from the iliac crest they were aspirated from the sternum. Five to ten ml of lignocaine (Lignocaine 2%, Antigen Ltd, U.K) was injected into the skin, and the subcutaneous tissue and periosteum infiltrated with the local anaesthetic. A Salah bone marrow aspirate needle was introduced into the marrow cavity. Two to five ml of marrow was aspirated into a sterile plastic syringe (Gillette, U.K.) and then transferred into a sterile universal container (Sterilin Ltd, U.K.) containing 0.1ml preservative free heparin (PFH), (1000 units/ml Monoparin, CP Pharmaceutical Ltd, Wrexham, U.K).

Peripheral blood samples: Samples were collected with the consent of the patient either during their visit to the out-patient clinic or when they were undergoing leukapheresis. During leukapheresis white cells were separated from red cells and platelets using a continuous-flow blood cell separator (Cobe Spectra, U.S.A). Ten to twenty ml of venous blood was collected into universal containers containing PFH.

Details of patients including age, sex, disease and previous treatment are given in the appendix (Tables I and II).

Normal donor samples

Samples of blood and bone marrow from volunteers and normal donors for allogeneic bone marrow transplantation were collected with their consent using the method detailed above.

Cell lines

HL60: This cell line was derived from the peripheral blood of a patient with acute promyelocytic leukaemia (Collins et al, 1977). Suspension cultures of HL60 cells were maintained in exponential growth in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 7.5% foetal calf serum (FCS- Flow Laboratories), penicillin 1000u/ml and streptomycin 100μg/ml (Penicillin-streptomycin solution, Gibco, Scotland), 20mM Hepes buffer (Gibco), 2mM glutamine (Gibco) and 0.1% $NaHCO_3$ (7.5% solution, Gibco). Cells were seeded at 2 x 10^5/ml in 40ml of the supplemented RPMI 1640 medium in 80cm^2 tissue culture flasks (Nunc, Denmark), incubated at 37^{0}C and passaged every 3-4 days. At each passage a cell count and viability were determined using a haemocytometer (Improved Neubauer, Weber Scientific International, U.K). Cell viability was estimated using trypan blue exclusion. 0.1ml of the cell suspension was mixed with an equal volume of a 0.25% solution of trypan blue dye (Gibco Ltd, U.K). The mixture was placed in the counting chamber of a haemocytometer under 200x magnification. Viable cells exclude the dye, whereas dead cells are stained blue. Viability was estimated by counting the number of cells which excluded the dye in a total

of 200 cells.

Culture media

Alpha-modification of Eagles minimal essential medium (α-medium): The alpha-modification of Eagles medium (Flow Laboratories, U.K) was used in all clonogenic assays. It was supplied as a powder and reconstituted in distilled water with the addition of extra vitamins (100ml Eagles minimal essential medium) to produce a concentrated stock solution (6.25x concentrated α-stock) which was sterilised by passage through a 0.2μl filter and 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 made up at double concentration (2xα). 100ml aliquots of 2xα were prepared in sterile glass measuring cylinders by mixing the following sterile stock solutions to 32ml of α-stock and completing the volume with sterile distilled water;

Foetal calf serum (FCS) (Flow Laboratories)	10ml
Sodium bicarbonate (7.5%- Gibco)	6ml
10% bovine serum albumin (BSA) (Sigma)	20ml
Transferrin 10mg/ml (Sigma)	1ml
L-ascorbic acid 1ml of 100mg/20ml (BDH, Poole, U.K)	1ml
Gentamycin 80mg/2ml (Cidomycin, Roussel, U.K)	0.1ml
L-glutamine 200mM (Gibco, U.K)	5ml

2xα-medium was poured into sterile screw top bottles and stored at 4^0C. The constituents of alpha medium and RPMI 1640 are given in the appendix (Table III and IV).

5637 conditioned medium

The 5637 cell line was kindly supplied by Dr R.E Stanley at the Albert Einstein College of Medicine, U.S.A. Serum-free 5637 CM was prepared by growing 5637 cells in RPMI 1640 medium supplemented with 2.4mM L-glutamine, 100units/ml penicillin, 100µg/ml streptomycin, 3.63 ng/ml hydrocortisone, 5µg/ml insulin, 100µg/ml transferrin, 2.72ng/ml β-oestradiol and 10.4ng/ml sodium selenite (all supplied by Sigma). 5637 CM contains a variety of growth factors which include haemopoietin-1, G-CSF, IL-6, IL-1, GM-CSF and stem cell factor.

Recombinant human growth factors and lymphokines

Recombinant human granulocyte colony-stimulating factor (rhG-CSF): rhG-CSF manufactured by Chugai was a gift from Chugai Pharmaceutical Co. Ltd, Japan. The Chugai product 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. rhG-CSF was supplied as a sterile lyophilised powder, free of pyrogens in vials of 100µg. It was reconstituted in 2ml of sterile water to produce a clear solution of Ph 6.5-8.0. Aliquots were diluted in phosphate buffered saline A (PBSA) containing 0.1% bovine serum albumin

in sterile plastic Bijou bottles and stored at -20^{0}C until required.

Recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF): rhGM-CSF was supplied by Sandoz, U.K. It was derived from Chinese hamster ovary cells. The product is 95% pure and is species specific. It was supplied as a lyophilised powder at a concentration of 216 μg/vial. It was diluted in 108ml of PBSA and 0.1%BSA, to give a final concentration of 2μg/ml. Aliquots of the stock solution were frozen at -20^{0}C until used.

Recombinant human interleukin-3 (rhIL-3): rhIL-3 was supplied by Sandoz, Leeds, U.K. It was expressed in yeast from cDNA cloned from activated human lymphocytes. It differs from natural human IL-3 by an intentional substitution of aspartic acid for asparagine at positions 15 and 70 to reduce N-linked glycosylation. The molecular weight was 15kD and the stated activity was 1.5 x 10^6 i.u./mg. It was supplied as a frozen solution containing 4μg/ml. The stock solution was diluted in aliquots of 1μg/ml and stored frozen at -20^{0}C for later use.

Recombinant human interleukin-4 (rhIL-4): rhIL-4 was supplied by British Bio-technology Ltd, Abingdon, U.K It was expressed in E.Coli from a designer gene (BBG 15). The sequence differed from that of natural human IL-4 by the presence of a N-terminal methionine. Activity measured by ^{3}H-thymidine incorporation into T-lymphocytes was 1-3 x 10^6units/mg. It was supplied in vials containing 5μg as a lyophilised powder and reconstituted and stored as above.

Transforming growth factor β_1 (TGFβ_1): TGFβ_1 was a gift from Professor

M Dexter, The Paterson Institute, Manchester, U.K. It had a purity of 95% and specific activity of $1x10^6$ units/mg. It was supplied in vials of 20μg/ml. It was diluted down to 1μg/ml and stored frozen at -20^0C for later use.

Monoclonal and polyclonal anti-bodies

Monoclonal mouse anti-human progenitor cell antigen (Anti-HPCA), (anti-CD34): The purified antibody was obtained from Beckton Dickinson, Oxford, U.K. CD34 is a human progenitor cell associated antigen with a molecular weight of 115kD. The CD34-positive compartment of normal human bone marrow (approximately 1% of nucleated cells) is comprised of unipotent and multipotent progenitor cells (CFU-E, BFU-E, CFU-GEMM, CFU-GM, CFU-blasts) as well as all terminal deoxynucletidyl transferase (TdT) positive cells. It is not found on normal peripheral blood lymphocytes, monocytes, granulocytes or platelets.

Monoclonal mouse anti-human Leu-17 (anti-CD38): The purified antibody was obtained from Beckton Dickinson, Oxford, U.K. CD38 is a human lymphocyte antigen of molecular weight 45kD. It is present in low density on most normal human natural killer (NK) cells, most B-cells, subsets of CD8+ and CD4+ T cells and monocytes. CD38 is found in high density on plasma cells, mitogen-activated T cells and several B and T cell lines. Approximately 40% of bone marrow cells (including myeloblasts and promyelocytes are positive.

Monoclonal mouse anti-human CD33: The purified antibody was

obtained from Serotec, U.K. The CD33 antigen has a molecular weight of 70kD. The antibody is restricted to cells of the myeloid lineage. It binds strongly to monocytes and to the majority of acute myeloid leukaemia progenitor cells. Approximately 25% of normal bone marrow MNC are CD33 positive.

Polyclonal rabbit anti-human IL-4 antibody: The purified antibody was supplied by Genzyme, Herts, U.K. The antigen used to generate the antibody was recombinant human interleukin-4. The antibody was aliquoted and stored at -20^{0}C.

Oligonucleotide probes

The DNA probes for the G-CSF receptor and IL-4 receptor were supplied from British Bio-technology, Oxford, U.K. The probes were single stranded oligonucleotides which were chemically synthesised and based on the anti-sense sequence. They were designed to detect the 5', central and 3' region of the receptor. The 5' end of each oligomer was labelled with digoxigenin. The sequences of the probes are given below:

G-CSF receptor

Top of receptor 5'- 5GT TGT GAG GTT CAT GAG GCA GGA GA -3'

Middle of receptor 5'- 5CT CCA GGT CTT GTT GCT ATT GCT CG -3'

End of receptor 5'- 5GA GTG GAG TCA CAG CGG AGA TAG - 3'

IL-4 receptor

Top of receptor 5'- 5CC AGG TGG TGT TAT AGC ACT GAG CC -3'

Middle of receptor 5'- 5GC GTC TCT GTG CAA GTC AGG TTG TC -3'

End of receptor 5'- 5AT GAG CAC CTC TAG GCA ATG ACC AC -3'

In addition, a cocktail of 3, 25 mer sense probes to the IL-4R was used as a negative control. The mRNA sequence for the G-CSF and IL-4 receptors are shown in the appendix (Figure 2 and 3)

Laboratory methods

Cell culture: The work was carried out in a class II microbiological safety cabinet (Envair, U.K Ltd).

Separation of blood and bone marrow mononuclear cells: The volume of blood/bone marrow was measured and then diluted with an equal quantity of PBSA. Aliquots (4-5ml) of the diluted blood/bone marrow were layered onto 10ml of Ficoll-Hypaque (Lymphoprep, density 1.077; Nycomed, Norway) and separated at 128*g* by centrifugation (IEC-CENTRA 7-International Equipment Company, U.S.A) for 20 minutes. Using this technique most red cells and mature granulocytes are forced to the bottom of the tube, leaving mononuclear cells (including blast cells) at the interphase (Boyum, 1968). The leucocytes lighter than 1.077g/ml were collected from the interphase and diluted with 15ml of PBSA. The cells were washed by further centrifugation at 450*g* for 10 minutes. The supernatant was discarded and the mononuclear cells resuspended in single strength α-medium. 40µl of this cell suspension was diluted in 20ml Isoton solution (Coulter Electronics, Luton, U.K). The residual red cells were lysed with 6 drops of Zapo-globin (Coulter

Electronics), and the white cells counted using a Coulter counter (Coulter Electronics).

Cryopreserved samples: Blood and bone marrow samples from patients with AML and CML were collected into PFH as previously detailed. The samples were either taken at leukapheresis prior to treatment or were the initial diagnostic samples. The cell suspension was diluted with TC199 growth medium (Wellcome, U.K) containing 5% dimethylsulphoxide (DMSO, Fisons plc, U.K) and was then dispensed into 2ml sterile glass ampoules. These were heat-sealed and then immediately cooled using a liquid nitrogen gas-phased programmed freezer (Planar Ltd, Model R201) to a temperature of -130^{0}C. All the ampoules were finally stored in the gas phase of liquid nitrogen at below -150^{0}C. When required an ampoule of frozen cells was thawed, and its contents transferred into a universal container. The suspension was diluted in RPMI 1640 and centrifuged 128*g* for 10 minutes. After centrifugation the cell pellet was resuspended in medium and the mononuclear cells counted as above.

Assay of CFU-GM, CFU-CML and CFU-AML from peripheral blood and bone marrow mononuclear cells: A modification of the two-layer soft agar technique developed by Bradley and Metcalf in 1966 was used (Bradley and Metcalf, 1966). Each assay was carried out in triplicate in 35mm sterile Petri dishes (Nunc, Denmark) to which aliquots of cytokines were added. The first agar layer was prepared using a concentration of 1% agar (Agar noble, Difco, U.S.A) in distilled water. The agar was sterilised by heating in an

autoclave (Ensign-Rodwell, U.K) at 120^{0}C for 15 minutes. The agar was melted using a microwave oven (Finess 500) and was maintained at 56-60^{0}C on a hot plate. This temperature range was sufficient to maintain the fluidity of the agar without damaging the components of the medium or killing the cells. Volumes of cytokines no greater than 100μl were added, and then a 1ml, 1:1 mixture of the 1% agar and 2xα-medium was dispensed into the Petri dishes, and the underlayers allowed to cool and solidify at room temperature. The overlay contained the normal donor or leukaemic mononuclear cells in a 0.5ml of a 1:1 mixture of 0.66% agar (prepared as above) and 2xα-medium. The number of peripheral blood mononuclear cells incorporated in the overlay was between 10^4 and 5 x 10^5/plate and the concentration of bone marrow mononuclear cells was between 10^5 and 5 x 10^5/plate. Petri dishes were placed in plastic boxes, gassed with a combination of 5%CO_2, 10%O_2 and 85%N_2, sealed and incubated for 12-14 days at 37^{0}C. Distilled water (5ml) was added to each box for humidification (to prevent drying of the agar).

Colony morphology and numbers: Colonies were counted using an inverted microscope (Nikon, Japan). A colony was defined as more than 50 cells and a cluster as between 10-50 cells. Individual colonies were removed from the agar overlay using a fine pasteur pipette. The cells were diluted in a few drops of PBSA and cytocentrifuged (Shandon, U.K) at 500rpm for 5 minutes. The slides were air dried, fixed in 99.8% methanol (BDH) and stained with May-

Grunwald-Giemsa (Gurr, BDH). The cells were examined under a light microscope (Axioscope, Zeiss, W.Germany).

Chromosome analysis: Chromosome analysis was carried out by Mr J. Swansbury (Dept. of Cytogenetics, The Royal Marsden Hospital, Sutton, U.K.). Briefly the technique used was as follows; the mitotic divisions were arrested in metaphase by destroying the spindle with colcemid (1μg/ml; Gibco, U.K) overnight. The cells were treated with a hypotonic solution of potassium chloride (0.075M) to help spreading of the chromosomes. The fixed cells were spread on slides and air-dried. To induce banding patterns the slides were immersed in salt solution [2xSSC; NaCl (17.53g) and sodium citrate (8.82g) made up to 1 litre of aqueous solution] at 60^0C followed by a brief exposure to trypsin (2.5% diluted 1:50 PBS), (Williams et al,1984). The slides were stained and analysed on a Cytoscan automated analysis system (Applied Imaging, U.K.).

Detection of CD33, 34 and CD38 antigens: The proportion of the cells carrying these antigens was estimated by incubating 10^6 MNC with 10μl of each antibody at 4^0C for 20 minutes. The cells were washed in PBSA containing 1% bovine serum albumin (PBSA/0.1%BSA) and then exposed to 5μl fluorescein-conjugated rabbit anti-mouse immunoglobulins (Dako Ltd, U.K) for a further 20 minutes at 4^0C. The cells were washed as above and

examined by fluorescence microscopy.

Detection of IL-4 receptor: Ten μl of the rabbit anti-human IL-4 polyclonal antibody, (Genzyme, Herts, U.K) was added to 10^6 mononuclear cells and the cells incubated at 4^0C for 20 minutes. The cells were washed in PBS-A/0.1%BSA and then exposed to 50μl of biotinylated sheep anti-rabbit IgG (Serotec, U.K), and incubated at 4^0C for 20 minutes. The cells were washed in PBSA/0.1%BSA and exposed subsequently to 10μl of Streptavidin bound R-phycoerythrin (Serotec, U.K). After further incubation for 20 minutes at 4^0C, the cells were washed with PBSA/0.1%BSA and examined under fluorescence microscopy.

Enrichment of CD34+ cells: CD34+ cells were prepared from peripheral blood and bone marrow mononuclear cells by exposing them to 100μl anti-CD34/2 x 10^7 MNC at 4^0C for 20 minutes. After washing with PBSA they were exposed to magnetic beads coated with sheep anti-mouse IgG (Dynal, Oslo, Norway) at 4^0C for a further 20 minutes. The bead:cell ratio was approximately 3:1. The cells were washed with PBSA and the beads removed by magnet. The cells attached to the magnetic beads were incubated overnight in single strength α-medium which contained normal human serum (1:1v/v) and 5% 5637-CM. The overnight incubation allowed the cells to detach from the beads because of regeneration of the cell membrane. The beads were removed by magnet and an aliquot of the cell suspension was diluted 1:1 with trypan blue (0.25% final concentration). A viable cell count was performed

using a haemocytometer. A second aliquot of cells was re-examined for CD34 positivity as detailed above. Using this method 60-65% of the CD34+ cells were recovered. Of the recovered cells 90-95% were CD34 positive. The yield of CD34+ cells from marrow samples was 1-2% of the population of MNC. Cultures were plated in triplicate as described above. The concentration of cells plated per dish varied from 5×10^3 to 5×10^4/dish.

In-situ hybridisation

In-situ hybridisation was first described in 1969 (John et al, 1969; Gall and Pardue, 1969) for the detection of multiple copy ribosomal RNA genes. Since then the technique has become well established for the detection and isolation of both DNA and mRNA within cells and tissue sections (Warford, 1988). Two developments in particular have contributed to the accessibility of this technique. Firstly the availability of synthetic oligonucleotide probes and secondly the use of non-radioactive detection methods (Guitteny et al, 1988; Hankin and Lloyd, 1989; Larsson, 1989; Pringle et al, 1990).

The following methods were employed to reduce contamination of tissue sections, solutions and equipment with ribonuclease (RNAse). RNAse is ubiquitous and is not destroyed by autoclaving, therefore special precautions must be taken to exclude it from experimental procedures.

a. Disposable gloves were worn at all times when preparing solutions

and handling materials and apparatus.

b. Whenever possible sterile disposable plasticware which is essentially RNAse-free was used.

c. All glassware was baked at 180^{0}C overnight and was designated for *in-situ* hybridisation only.

d. Slides, cytocentrifuge tubes and holders were rinsed in water treated with diethyl pyrocarbonate (DEPC) which was prepared as detailed below.

e. All prepared equipment was covered and stored away from dust.

Preparation of hybridisation chamber: The plastic lid of a sandwich box measuring 17cm x 11.5cm and 2 x 5ml plastic pipettes were used. The pipettes were snapped in two and fitted snugly into the plastic lid and glued using chloroform. Filter paper (fibre-free Postlip paper, Hollingsworth, U.K) was layered onto the bottom of the lid. The filter paper was moistened with DEPC-treated water and the chamber sealed with plastic tape whenever used.

Preparation of solutions:-

DEPC-treated water: DEPC is a suspected carcinogen and was handled wearing gloves in a fume hood. A 10%(v/v) solution of DEPC (Sigma, U.S.A) in absolute ethanol (BDH, U.K) was prepared in a glass bottle. Fifteen ml of this solution was added to 1485ml of distilled water in a 2 litre screw top

bottle. The final concentration of the DEPC was 0.1%. The bottle was shaken and left to stand overnight in a fume cupboard. It was autoclaved the following morning at 120^{0}C for 15 minutes. The autoclaving destroys any residual DEPC.

0.2% para-formaldehdye fixative solution: 0.2g of para-formaldehyde (BDH,U.K) was added to 100ml of DEPC-treated water and the solution dissolved by heating in a microwave (low) for 15 minutes. The bottle was covered in silver foil and allowed to cool by placing at 4^{0}C for 1 hour.

20xTris-buffered saline (TBS): The following were added to 800ml of DEPC-treated water, 160g NaCl (Rose chemicals, U.K.) 7.6g KCl (BDH, U.K.) and 60g Tris (Sigma, U.S.A.). The Ph was adjusted to 7.6 by adding approximately 25ml of concentrated HCl.

Modified Tris-buffered saline (TBS)/Triton: 5ml of 20xTBS (Sigma, U.S.A), 2mM $MgCl_2$ (BDH, U.K), 0.1g BSA (Sigma, U.S.A) and 0.2ml Triton (Sigma, U.S.A) were added to 95ml of DEPC-treated water. The solution was aliquoted into 2 bottles.

TBS wash: To 95ml of DEPC-treated water were added 5ml of 20XTBS and 0.1g BSA. The solution was aliquoted into 2 glass bottles.

0.6M NaCl in 30% formamide: 6.96g NaCl and 60ml formamide (BDH Laboratory supplies, U.K) were added to 140ml of DEPC-treated water. This solution was used for both the pre-hybridisation and hybridisation reactions and was stored at -20^{0}C.

Post-hybridisation washes: Following hybridisation non-specifically bound probe is washed out whilst retaining the specifically bound probe. This is achieved by using three 37^{0}C washes (see below; a-c) of a fixed formamide concentration containing decreasing salt concentrations and therefore increasing stringency.

a. 4xSaline sodium citrate (SSC)/30% formamide: DEPC-treated water:20xSSC (Sigma):formamide (5:2:3 by volume)

b. 2xSSC/30% formamide: DEPC-treated water:20xSSC:formamide (6:1:3 by volume)

c. 0.2xSSC/30%formamide: DEPC-treated water:20xSSC:formamide (69:1:30 by volume)

The bottles were swirled, the necks covered with foil and then placed in a 37^{0}C incubator for an hour to warm.

Controls for *in-situ* hybridisation:

No probe: The hybridisation solution was applied without the addition of the oligonucleotide probes. This provided an assessment of any non-specific binding.

RNAse pre-treatment: In order to demonstrate that the target nucleic acid is RNA, some of the cell sections were incubated with RNAse before application of the probe. All mRNA within the section will be degraded by this control and any resultant labelling is non-specific. Care was taken not to

contaminate the positive controls with RNAse.

HL60 cells: Preliminary experiments showed that these cells expressed the mRNA for the IL-4 receptor and they were therefore used as a positive control.

Actin probe: (British Bio-technology, U.K.) Actin is present in all animal cells and tissue and the probe was used as a positive control.

In-situ hybridisation looking at mRNA expression has not been carried out on bone marrow or peripheral blood MNC. Several difficulties were encountered which will be detailed later. In an attempt to overcome these difficulties 3 different methods were used.

Method 1

In-situ hybridisation can be divided into 5 stages, fixation, addition of RNAse positive controls, pre-hybridisation, hybridisation, post-hybridisation and the revealing reaction. The technique was employed to study the expression of the mRNA for the IL-4 and G-CSF receptors in blood and bone marrow samples from normal donors and leukaemic patients.

Preparation of slides: Slides were prepared by cytocentrifugation as described previously using two drops of a cell suspension containing 10^6 cells/ml for each slide. The slides used were coated with the adhesive, 3-aminopropyltriethoxysilane (APES; Maddox and Jenkins, 1987). The slides,

cytocentrifuge tubes and holders were all washed in DEPC-treated water prior to use. When slides were stored, they were kept at -70^{0}C. They were thawed at room temperature, washed in DEPC-treated water and dried in a 37^{0}C incubator overnight.

Fixation: The process of fixation should crosslink the cell matrix, preserving the RNA intact and in its original location A thorough investigation of fixatives used for *in-situ* hybridisation has been performed by Singer and co-workers (Singer et al, 1986; Lawrence and Singer, 1985). Their work showed that good RNA retention was given by paraformaldehyde and glutaraldehyde but that ethanol:acetic acid and Carnoy's fixative caused significant loss of both RNA and cellular morphology. Their work however was done on tissue sections. On cytospins of cell samples the most satisfactory fixation was fixing for 10 minutes in a 1:1 solution of acetone and methanol. The slides were then transferred to a Hallendahl jar (Raymond Lamb, U.K.) containing the cooled 0.2% paraformaldehyde solution. Using this method, the cellular architecture and membranes were retained. Acetone/methanol, ethanol, ethanol/ 0.2%paraformaldehyde or a higher concentration of paraformaldehyde all caused loss of cellular architecture.

Addition of proteinase: In paraffin embedded tissue sections digestion with a proteolytic enzyme is necessary to expose the target mRNA in the section to the incoming probe. However in cell preparations used in this study even a small concentration of proteinase (1μl/mg) completely destroyed

cellular architecture. It was therefore not used.

Addition of RNAse: Half the slides were placed in a Hallendahl jar containing 100µl/ml of RNAse and 2xSSC with 10mM $MgCl_2$. The remaining slides were placed in a Hallendahl jar containing only the 2xSSC and $MgCl_2$. The slides were incubated at 37^0C for 1 hour. The slides were washed with PBS/DEPC-treated water. The "no RNAse" slides were washed first to avoid contamination of the other slides with RNAse. The slides were drained onto postlip paper.

Pre-hybridisation reaction: Before the hybridisation it is conventional to block any non-specific binding sites in the cell section by pre-hybridising in the absence of the probe. The pre-hybridisation solution contains 0.6M NaCl, 30% (v/v) formamide and 150µl/ml salmon sperm DNA (Sigma, U.S.A). 300µl of the pre-hybridisation solution was placed on each slide, the filter paper at the bottom of the hybridisation chamber was moistened with DEPC-treated water, the box sealed and placed in a 37^0C incubator for 1 hour.

Hybridisation reaction: During hybridisation the oligonucleotide probes anneal by complementary base pairing with the target mRNA. The specificity of hybridisation is controlled by the stringency used (stringency refers to the degree to which reaction conditions favour the dissociation of nucleic acid duplexes) a low salt concentration and high temperature constitute high stringency and high specificity. The addition of formamide to the hybridisation

at a given salt concentration and temperature increases the stringency still further. The inclusion of formamide for *in-situ* hybridisation maintains a high stringency but allows the use of lower temperatures which helps to preserve tissue morphology. 15µl of each probe (0.1µg/1µl) was added to the hybridisation solution to a final oligonucleotide concentration of 0.2µg/ml. The pre-hybridisation solution was drained from all the slides except the "no probe" controls. 100µl of the hybridisation solution was placed on the slides (except onto the "no probe" controls). The slides were covered with a 18mm x 50mm coverslip (Chance Propper Ltd, U.K) and placed in the moistened hybridisation chamber. The chamber was sealed and placed in a 37^{0}C incubator for 16-17 hours.

Post-hybridisation washes: Following hybridisation, non-specifically bound probe is washed out whilst retaining the specifically bound hybrids. This is achieved by using three washes at 37^{0}C of a fixed formamide concentration containing decreasing concentrations of salt. The first wash containing 4xSSC/30% formamide provides stringency almost identical to those of the hybridisation reaction. It would therefore wash away any excess or loosely bound probe. Two further washes of increasing stringency containing 2xSS/30% formamide and 0.2xSSC/30% formamide are then used to ensure that any non-specific loosely bound probe is washed away. The slides were removed from the hybridisation chamber and placed for 5 minutes, at 37^{0}C in a pre-warmed Hallendahl jar containing the 4xSSC/30% formamide. At the

end of the 5 minutes the wash solution was discarded and a fresh 50ml of the pre-warmed 4xSSC/30% formamide poured into the Hallendahl jar and the slides incubated for a further 5 minutes at 37^0C. This process was repeated using the 2xSSC/30% formamide and the 0.2xSSC/30% formamide. When the post-hybridisation solutions were not being used they were kept in the incubator at 37^0C and not allowed to cool. After the final wash the slides were transferred to a Hallendahl jar containing modified TBS/Triton. They were rinsed in this solution at room temperature for 15 minutes. The slides were drained onto postlip paper and allowed to dry.

Revealing reaction: The purpose of the revealing reaction is to detect the target/probe complex. The method is a sandwich technique, the biotinylated antibody binds to digoxigenin on the probe. Streptavidin bound horseradish peroxidase is then added which binds to the target/probe complex. In the presence of 3'3' diaminobenzidine an insoluble brown precipitate is formed on the addition of H_2O_2, the reaction being catalysed by peroxidase. The details are given below:-

To block endogenous peroxidase the slides were placed in a jar containing 3%H_2O_2 (FSA Laboratory supplies, U.K) for 10 minutes at room temperature, after which they were washed in TBS/0.1%BSA. 100µl of the anti-digoxigenin-POD, Fab fragments, which binds to digoxigenin labelled compounds (150u/ml Boehringer Mannheim Biochemica, W Germany) was added to 3.2ml of a solution containing Tris-HCl 100mmol/l and 150mmol/l

NaCl (pH 7.5) plus 0.4ml foetal calf serum. This quantity of solution was sufficient for 12 slides. 300µl of this solution was placed on each slide. The slides were placed inside the hybridisation chamber and left at room temperature for 30 minutes and then were drained onto postlip paper and washed twice in TBS/0.1%BSA for 5 minutes. The slides were then transferred to a jar containing 68g Imidazole (Sigma, U.S.A) in 50ml water and 4µl of H_2O_2 plus 50mg 3,3'-diaminobenzidine (Sigma, U.S.A) in 50ml of Tris-HCl pH 7.6 and left at room temperature for 10 minutes. If a brown colour change was not observed in the positive controls they were left for a longer period of time (30 minutes), or more H_2O_2 was added. The slides were counter stained with Mayer's Hemotoxylin solution (Sigma, U.S.A) for 2 minutes and mounted using DePeX (BDH Ltd, U.K).

Using the above method the following problems were encountered:

(1) Loss of cellular architecture due the paraformaldehyde and formamide.

(2) Non-specific positive staining with the sense probes and negative staining with the actin probe.

In an attempt to overcome these problems the following methods were adopted:

Method II

To overcome the problems encountered with Method I, the following changes were made to the methodology.

1. The slides were fixed in a 1:1 mixture of acetone and methanol and then

rinsed in PBSA.

2. The composition of the prehybridisation solution was changed to - 5.0ml deionised formamide (Sigma, U.S.A), 2.0ml 20xSSC (Sigma, U.S.A), 0.2ml of 50x Denhardt's solution (Sigma, U.S.A), 0.5ml of salmon sperm DNA (10mg/ml, Sigma, U.S.A), 0.25ml of yeast tRNA (10mg/ml) and 2.0ml of dextran sulphate (Sigma, U.S.A). 500 μl of the above solution was added to each slide. The slides were incubated in a humidified chamber for 1 hour at room temperature.

3. Hybridisation - the slides were rinsed in 2xSSC and 40μl of the prehybridisation solution containing a 1/50 dilution of the probes were placed on the slides. Hybridisation was allowed to occur in a humidified chamber at 37^0C overnight.

4. Post-Hybridisation - the post-hybridisation washes consisted of the following steps

a. The slides were incubated in 2xSSC for 1 hour at room temperature

b. 1xSSC for 1 hour at room temperature

c. 0.5xSSC for 30 minutes at 37^0C

d. 0.5xSSC for 30 minutes at room temperature

5. The revealing reaction was carried out as detailed in Method I.

This method (Crabbe et al, 1992) was employed to detect mRNA expression in chondrocytes. Peripheral blood and bone marrow MNC are more fragile than chondrocytes and using this method problems with non-

specific background staining together with loss of cellular architecture, were encountered. A dot blot analysis revealed that the salmon sperm bound to the anti-digoxigenin-POD, Fab fragment. A further method was therefore was devised which omitted both the formamide and the salmon sperm.

Method III

1. Fixation - the slides were fixed in acetone/methanol.

2. Addition of RNAse - the slides were incubated in 500μg/ml RNAse in 2xSSC and 0.1% triton for 30 minutes at 37^{0}C. The slides were washed in PBS followed by DEPC treated water.

3. Hybridisation - the hybridisation solution consisted of 8μl of 5mM of $MgCl_2$ (BDH, U.K) 26μl of annealing buffer [50mM Tris (Sigma, U.S.A), 250mM KCl (Aldrich Chemical Company, Inc, U.S.A) in DEPC water pH 8.3] and 2μl of each probe (0.1μg/μl) giving a total volume of 40μl/slide. The slides were incubated at 60^{0}C for 2 minutes using a thermal cycler (Hybaid, Teddington, U.K)

4. Post hybridisation - the slides were washed sequentially in the following solutions

   a. 4xSSC for 10 minutes at 37^{0}C

   b. 2xSSC for 10 minutes at 37^{0}C

   C. 1xTBS/triton for 15 minutes at room temperature.

5. Revealing reaction - was carried out as detailed in Method I. In order to give a greater contrast in colour (peroxidase stains brown) than that obtained

with Mayer's Haematoxylin, light green (Raymond Lamb, U.K) counter stain was used.

Using this method the cellular architecture was in most instances not destroyed. Furthermore there was no non-specific back-ground staining. The addition of the RNAse whilst reducing the signal also caused deterioration in cellular integrity and detail. The most disturbing problem with this technique was that whilst the "no probe, no antibody" gave a negative result the "no probe, plus antibody" which should have given a negative result on some occasions gave a positive result (UPN 52 and 53). Neutrophils contain peroxidase which has an important role in its anti-microbial activity. It may be that in these patients high levels of endogenous peroxidase were present (after incubation with 3% H_20_2) permitting non-specific deposition of chromogen following addition of substrate.

Statistical analysis

Statistical analyses were performed using paired, non-parametric Sign Rank Wilcoxon tests from a computer programme from Epistat (Epistat software, U.S.A).

CHAPTER 3

EFFECT OF CYTOKINES ON PROGENITOR CELL GROWTH

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Introduction:

Early stages of haemopoietic cell development can be quantitated and characterised using *in vitro* colony assays. Clonogenic assays are now available for all types of committed granulopoietic, erythroid and megakaryopoietic progenitor cells. Colony assays are carried out in semi-solid medium and individual colonies can be identified and distinguished by their size, morphology and cytogenetic analysis.

Growth of CML cells *in vitro*:

In patients with CML the abnormal leukaemic clone expands and usually replaces the normal haemopoietic system. This results in a marked increase in the number of granulocytes in the circulation. Colonies generated in assays of chronic phase CML peripheral blood or bone marrow specimens are morphologically indistinguishable from colonies generated by normal progenitors because the mature blood cells produced by the neoplastic clone are not grossly altered (Eaves, 1992). The total number of clonogenic progenitors is increased in CML patients with untreated disease and this has been correlated exponentially with the white cell count (Eaves,1992). In normal individuals the more primitive types of clonogenic cells in the marrow, and all circulating clonogenic progenitors are quiescent populations. In CML these populations are in continuous turnover (Eaves and Eaves,1987). At present it is not known how this deregulation of control is achieved *in vivo*.

Otsuka et al, 1991) showed by Northern blot analysis that there was no evidence of increased expression of G-CSF, GM-CSF, IL1-α, IL1-β, IL-3, IL-6 or tumour necrosis factor-α (TNF-α) in CML blood samples compared with normal donor samples. Furthermore they showed there was no evidence of a decreased production of putative inhibitors of haemopoietic progenitor cells, i.e. TGFβ and macrophage inflammatory protein-1α (MIP-1α) by CML cells compared to normal cells.

In long term cultures the most primitive progenitor cells are known as long term culture initiating cells (LTC-IC). The LTC-IC are so named because of their ability to generate clonogenic cell progeny that are detectable after a minimum of 5 weeks incubation on competent fibroblast feeder layers. LTC-IC are multipotent cells and are the earliest progenitors that can be detected by *in vitro* cultures. In patients with CML both normal and Ph positive LTC-IC are present. Ph positive LTC-IC are present in elevated concentrations in blood but the number of Ph positive LTC-IC in the marrow of chronic phase patients is usually very low. In LTC the Ph positive LTC-IC decrease in number in contrast to the normal LTC-IC which are usually well maintained (Eaves, 1992). This is the rationale for using these cells for autografting.

Growth of AML *in vitro*

Haemopoiesis in AML has some features in common with that of normal haemopoiesis in that there is a hierarchical organisation of leukaemic cells with a small population of stem cells which maintain the leukaemic clone.

The requirements of these cells for proliferation have been studied both in clonogenic assays in semi-solid media and liquid culture.

Clonogenic assays with AML cells have demonstrated that leukaemic blast progenitors proliferate *in vitro* in the presence of colony-stimulating factors (CSF) including GM-CSF, G-CSF and IL-3 (Delwel et al 1988; Hoang et al, 1986; Vellenga et al; 1987a, Miyauchi et al; 1987 and Delwel et al; 1987). GM-CSF and IL-3 have been found to have an equivalent activity in stimulating AML blasts which is usually greater than that seen with G-CSF (Kelleher et al, 1987). The response of AML blasts to these growth factors have shown a marked patient to patient variation which is in keeping with the heterogenous nature of the disease (Vellenga et al, 1987b; Kelleher et al,1987). This heterogeneity of *in vitro* growth has not been correlated with the morphological FAB sub-type of the disease (Russell, 1992). The effect of a single growth factor has frequently been sub-optimal and combinations of factors such as G-CSF with GM-CSF have been synergistic in stimulating proliferation (Vellenga et al, 1987a; Kelleher et al, 1987). Other growth factors implicated in the *in vitro* growth of AML include IL-1 and IL-6. IL-1 synergises with GM-CSF and G-CSF in stimulating the proliferation of some AML cells (Hoang et al, 1988a). Similarly IL-6 which augments IL-3-dependent proliferation of normal bone marrow mononuclear cells (Ikebuchi et al, 1987) synergises with GM-CSF in stimulating AML cells from some patients *in vitro* (Hoang et al, 1986).

TNF-α has both stimulatory and inhibitory effects on the growth of

AML cells *in vitro*. It synergises with GM-CSF and IL-3 to stimulate proliferation (Hoang et al, 1989), but inhibits the stimulating effect of G-CSF on AML blasts (Hoang et al, 1989; Salem et al, 1990) by down-regulating the G-CSF receptor (Elbaz et al, 1991). The effects of TNF-α on AML progenitors parallels its effects on normal bone marrow mononuclear cells (Caux et al, 1990).

Autocrine growth of AML cells

In 1986 Young and Griffin reported 2 cases where the autonomous growth of AML blasts was associated with the presence of GM-CSF transcripts on Northern blot analysis. Since then many reports have confirmed these findings and have shown that transcripts for other cytokines including G-CSF, M-CSF, IL-1, IL-6, TNF-α as well as GM-CSF are frequently detected on AML blasts (Oster et al, 1989; Griffen et al, 1987; Young et al, 1988; Kaufman et al, 1988; van der Schoot et al, 1989). Young et al were unable to correlate the expression of GM-CSF transcripts with autonomous growth in culture (Young et al, 1987; Young et al, 1988). In contrast other workers have been able to correlate CSF production with partial or total autonomous growth (Reilly et al, 1989; Bradbury et al, 1992). This discrepancy probably reflects the differences in culture and assay methods used to detect CSF production. In some but not all AML blasts that produce GM-CSF autonomous growth can be inhibited with neutralising anti-bodies to GM-CSF (Young and Griffin, 1986; Reilly et al, 1989, Murohashi et al, 1989; Rodriguez-Cimadevilla et al, 1990) or with antisense oligonucleotides to GM-

CSF (Rogers et al, 1991).

Classification of the autonomous growth of AML in vitro

Reilly et al, in 1989 have proposed a classification of AML growth properties based upon the presence or absence of autonomous growth characteristics in methylcellulose. Four major groups of AML blasts were identified:

Group 1 blasts - failed to grow in the presence or absence of 5637 conditioned medium (5637 CM)

Group 2 blasts - were totally dependent on the presence of 5637 CM for their growth

Group 3 blasts - exhibited a variable degree of autonomous growth but could be further stimulated by growth factors present in 5637 CM

Group 4 blasts - exhibited totally autonomous growth

In a series of 25 patients that they studied autonomous growth was observed in 70% of cases (Reilly et al, 1989). Autonomous growth of AML is associated with lower remission and survival rates (Lowenberg et al, 1993). It should be noted that 5637 CM contains a variety of haemopoietic growth factors which include haemopoietin-1, G-CSF, IL-6, IL-1, GM-CSF and stem cell factor.

Interleukin-1 in AML

In normal haemopoiesis IL-1 can induce the production of a number of cytokines including GM-CSF, G-CSF, M-CSF, IL-6 and IL-1 from multiple

cell types including bone marrow stromal cells (Bagby, 1989). Some AML blasts can proliferate in response to IL-1 alone and GM-CSF is synergistic with IL-1 (Hoang et al, 1988a; Delwel et al, 1989). In addition some AML blasts express IL-1 transcripts and secrete IL-1α and IL-1β (Griffen et al, 1987; Cozzolino et al, 1989; Sakai et al, 1987). There is also evidence to suggest that IL-1 is an autocrine growth factor in AML (Bradbury et al, 1992).

Experiments

Effect of G-CSF, GM-CSF and IL-3 on normal, CML and AML cells

Objective

The aim of these experiments was to determine which cytokine (G-CSF, GM-CSF or IL-3) was most effective at stimulating the growth of colonies from normal, CML or AML cells *in vitro*.

Results:

Preliminary experiments were done to determine the optimum concentration of G-CSF, GM-CSF and IL-3 necessary to yield maximum colony numbers. Only 4/15 normal peripheral blood and 8/12 normal bone marrow mononuclear cells grew *in vitro*. The number of cells plated varied from 10^4 to 10^6/plate. As autostimulation of cells can occur, higher number of cells were not plated. Dose response curves in these patients were not possible because low numbers of mononuclear cells from normal PB and BM were obtained. Preliminary experiments done by other workers in the laboratory showed that in six normal donor bone marrow mononuclear cells, 15ng G-CSF, 50ng GM-CSF and 50ng IL-3 gave maximum colony numbers. Increasing the concentrations of the cytokines did not increase colony numbers further (B Millar and J B G Bell, personal communication).

When leukaemic mononuclear cells were plated, the CFU-GM numbers obtained was variable. Low numbers of CFU-GM were obtained in all dose-response curves attempted (12 in total) except in the 3 illustrated in Figure 4. These 3 patients are not representative of the patients studied, UPN 25 was

Ph negative; UPN 26 and 35 were in lymphoid and myeloid blast transformation respectively. From this limited data 50ng of GM-CSF gave a plateau in response in all 3 patients, 50ng of IL-3 in UPN 25 and 35, and the amount of G-CSF to give a plateau in response varied in the 3 patients. Because there was some variation in dose response of the leukaemic cells to growth factors (see Fig 4) the concentrations of G-CSF, GM-CSF and IL-3 chosen were based on data from normal donor BM mononuclear cells. The following concentration of cytokines and cells were used in the clonogenic assays:-

Peripheral blood mononuclear cells = $5x10^5$/plate*

Bone marrow mononuclear cells = 10^5/plate*

Concentration of G-CSF used = 15ng/plate

Concentration of GM-CSF used = 50ng/plate

Concentration of IL-3 used = 50ng/plate

* - if the CFU-C obtained were too high in the leukaemic samples (>1000/plate), cells were plated at a lower concentration.

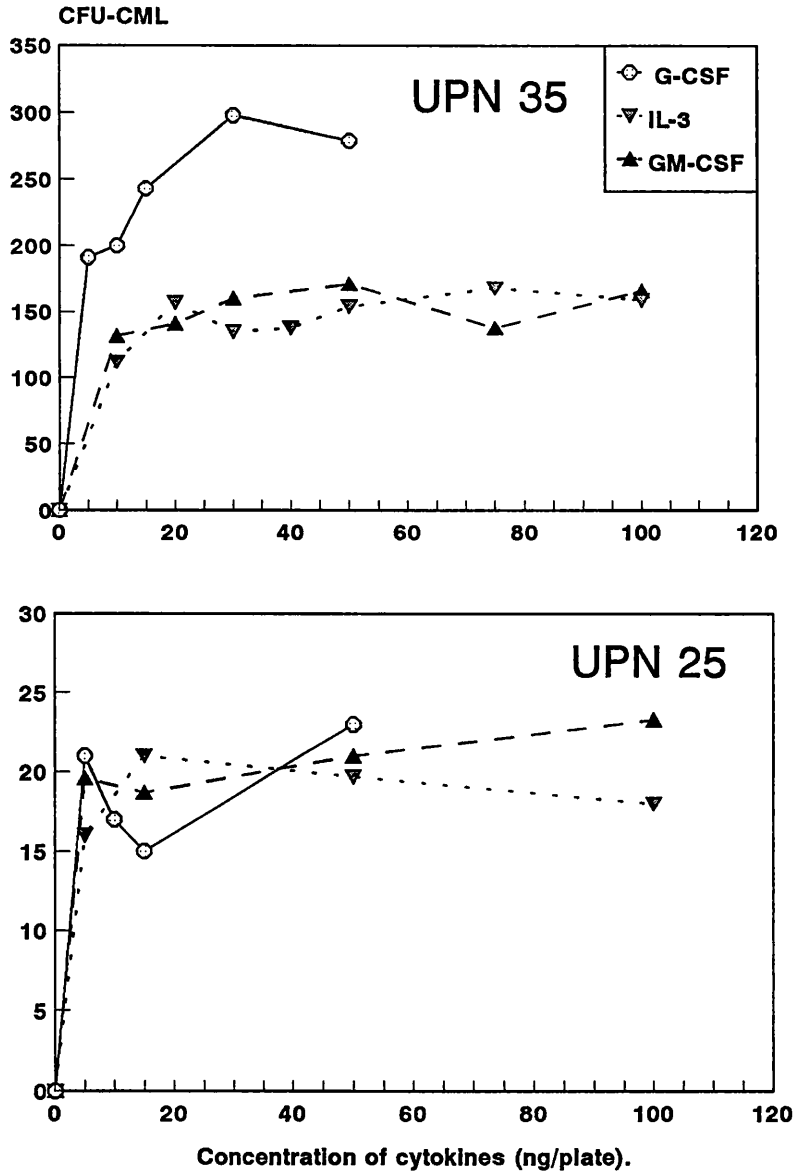
Legend to Figure 4

The figures shows the dose response curves of the 3 colony-stimulating factors used and the CFU-CML numbers obtained. The symbols indicating the different cytokines are shown in figure of UPN 35. The underlying diagnosis and number of cells plated are as follows:-

UPN 25, Ph negative CML, cells plated = $5x10^5$/plate

UPN 26, CML in lymphoid blast transformation, cells plated = $4x10^5$/plate

UPN 35, CML in myeloid blast transformation, cells plated = 10^4/plate


CFU-CML
UPN 35
G-CSF
IL-3
GM-CSF
350
300
250
200
150
100
50
0
0
20
40
60
80
100
120
UPN 25
30
25
20
15
10
5
0
0
20
40
60
80
100
120
Concentration of cytokines (ng/plate).

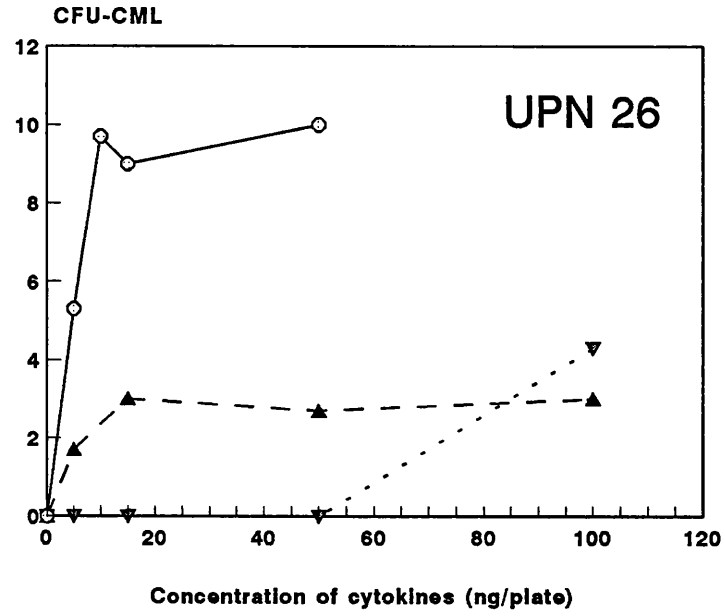

CFU-CML
UPN 26
12
10
8
6
4
2
0
0
20
40
60
80
100
120
Concentration of cytokines (ng/plate)

Figure 4

The effects of G-CSF, GM-CSF and IL-3 on normal bone marrow CFU-GM are shown in **Table V**, below.

UNIQUE PATIENT NUMBER	G-CSF 15ng	GM-CSF 50ng	IL-3 50ng
1	5.3	7.3	5.0
2	13.3	3.3	1.7
3	2.3	0.0	0.0
6	6.6	4.3	6.6
7	4.3	2.3	3.6
10	24.7	31.3	18.7
12	19.0	68.3	59.3
13	5.7	4.7	2.0

The cells were plated at 10^5/plate. In each instance the mean colony numbers (of triplicate samples) are shown. The mean number of CFU-GM per plate, obtained with G-CSF was 10.2 (range 2.3-24.7); with GM-CSF was 15.2 (range 0-68.3) and with IL-3, 12.0 (range 0-59.3). Colony numbers obtained in all instances were extremely low.

The effects of G-CSF, GM-CSF and IL-3 on normal peripheral blood CFU-GM are shown in **Table VI**, below.

UNIQUE PATIENT NUMBER	G-CSF 15ng	GM-CSF 50ng	IL-3 50ng
4	6.0	5.0	14.0
5	4.6	6.3	7.7
8	1.3	1.6	1.6
18	4.0	3.7	7.3

The cells were plated at $5x10^5$/plate. Cytokine concentrations used are shown in the table. Mean colony numbers (of triplicate samples) are shown.

Of 12 normal bone marrow and 15 normal PB samples, only 8 and 4 grew *in vitro* respectively. The number of colonies obtained in all instances were small.

The effect of G-CSF, GM-CSF and IL-3 on peripheral blood CFU-CML are shown in **Table VII**, below. Cells plated were $5x10^5$/plate, in each instance the mean colony numbers obtained (of 3 plates) are shown. The concentrations of cytokines used are given in the table. All samples were obtained from CML patients in chronic phase, * =Ph negative CML in chronic phase.

UNIQUE PATIENT NUMBER	G-CSF 15ng	GM-CSF 50ng	IL-3 50ng
21	8.3	3.0	2.6
22	52.7	55.3	35.7
23	23.0	6.0	4.7
24	30.0	13.0	9.0
25*	15.3	21.0	19.7
30*	45.0	42.0	32.0
31	202.0	65.0	60.0
37	90.7	67.3	43.0
38	67.0	79.0	63.0
39	0.0	0.0	0.0
54	0.0	0.0	0.0
55	73.0	25.3	19.3
56	0.0	0.0	0.0

Table VIIa below, shows the effect of G-CSF, GM-CSF and IL-3 on CML blast transformation MNC. UPN 26$ was in lymphoid BT, the others were in myeloid BT.Cells from UPN 26 and 27 were plated at $5x10^5$/plate, UPN 34 and 35 were plated at $2x10^4$/plate and UPN 54b at $2x10^5$/plate. The concentrations of cytokines used were G-CSF 15ng, GM-CSF 50ng, IL-3 50ng and $TGF\beta_1$ 5ng.

UNIQUE PATIENT NUMBER	G-CSF 15ng	GM-CSF 50ng	IL-3 50ng
26$	11.0	4.0	<1.0
27	311.0	119.0	135.0
34	310	15.3	12.0
35	278.0	342.0	55.0
54b	334.3	493.0	837.0

The mean CFU-CML obtained with CML CP samples (n=13) using G-CSF were 46.69(range 8.3-202); with GM-CSF 28.99 (range 3.0-79) and with IL-3 22.23 (range 2.6-135). There was a difference between using G-CSF and IL-3 as stimulators of CFU-CML (p=0.05). This difference does not take into account the different concentrations of cytokines used.

The four patients in myeloid blast transformation had satisfactory *in vitro* growth with G-CSF, 3/4 also had good *in vitro* growth with GM-CSF and IL-3. As the cells were plated at different concentrations the only conclusion that can be deduced from this data is that G-CSF, GM-CSF and IL-3 induced satisfactory CFU-CML from CML blast transformation MNC.

Table VIII, below shows the effect of G-CSF, GM-CSF and IL-3 on CFU-AML, the legend to the table is given below.

UNIQUE PATIENT NUMBER	FAB SUB-TYPE	G-CSF 15ng	GM-CSF 50ng	IL-3 50ng
44	M1	26.3	0.0	0.0
44b*	M1	2.7	0.0	0.0
47	M2	21.0	104.0	115.0
49	M4	25.3	230.0	ND

UPN 44, 47 and 49 were peripheral blood samples. UPN 44 was plated at $5x10^5$/plate and UPN 47 & 49 were plated at $5x10^4$/plate. UPN 44b was obtained from bone marrow and was plated at 10^5/plate. ND - not done

Of a total of 18 AML samples studied only 6 grew CFU-AML *in vitro*, 4/6 were plated with G-CSF, GM-CSF and IL-3 and 2/6 grew when stimulated by 5637 CM. Of the 4 samples, 4/4 grew when stimulated with G-CSF, 2/4 grew *in vitro* with GM-CSF and 1/3 grew *in vitro* with IL-3. **None of the CML or AML grew *in vitro* in the absence of growth factors or 5637 CM.**

Effect of TGFβ_1 on normal, CML and AML cells stimulated with either G-CSF, GM-CSF or IL-3

TGFβ is a highly stable peptide which has a multiple range of activities. It is produced by almost all cells and most normal cells have receptors for TGFβ (Sporn and Roberts, 1989). The production of TGFβ by haemopoietic cells suggests that it may play an important role in regulating haemopoiesis. In human haemopoiesis TGFβ inhibits CFU-GM production by IL-3 and GM-CSF and megakaryocyte development by IL-3 (Sing et al, 1988a, Han et al, 1992) but has no effect on G-CSF-induced cell proliferation from normal bone marrow cells *in vitro* (Sing et al, 1988a). TGFβ inhibits the G-CSF-directed proliferation of CML cells *in vitro* (Sing et al, 1988a, Aglietta et al, 1989). The inhibitory effects of TGFβ on human haemopoiesis are augmented by TNF-α and α-interferon *in vitro* (Sing et al, 1988b). TGFβ has also been reported as having an inhibitory effect on CFU-AML *in vitro* (Rogers et al, 1992, Shirakawa et al, 1992). In contrast TGFβ has no inhibitory effects on 2^{ry} AML cells (Shirakawa et al, 1992).

Objective

Experiments were carried out to compare the effects of TGFβ_1 on the proliferation of normal, CML and AML cells stimulated with either G-CSF, GM-CSF or IL-3.

Results

The effects of TGFβ_1 on normal BM and PB MNC stimulated with either G-CSF, GM-CSF or IL-3 are shown in **Table X and XI below and overleaf.**

Table X shows the effect of TGFβ on G-CSF, GM-CSF and IL-3-induced proliferation of normal BM MNC. The concentration of G-CSF, GM-CSF and IL-3 were 15ng/plate, 50ng/plate and 50ng/plate respectively. The dose of TGFβ was 5ng/plate. Cells were plated at 10^5/plate. Mean, of triplicate CFU-GM numbers, are shown.

UPN	G-CSF	G-CSF + TGFβ	GM-CSF	GM-CSF + TGFβ	IL-3	IL-3 + TGFβ
1	5.3	0.0	7.3	3.0	5.0	0.0
2	13.3	14.7	3.3	0.0	1.7	0.0
3	2.3	2.3	0.0	0.0	0.0	0.0
6	6.6	6.6	4.3	3.0	6.6	<1.0
7	4.3	2.3	2.3	1.6	3.6	2.0
10	24.7	44.3	31.3	15.0	18.7	4.0
12	19.0	28.7	68.3	ND	59.3	ND
13	5.7	12.0	4.7	<1.0	2.0	<1.0

Table XI below, shows the effect of TGFβ on G-CSF, GM-CSF or IL-3-induced proliferation of normal peripheral blood MNC. Cells were plated at $5x10^5$/plate. The concentration of cytokines used was G-CSF 15ng, GM-CSF 50ng, IL-3 50ng and TGFβ_1 5ng. Mean colony numbers (of triplicate samples) are shown in each instance.

UPN	G-CSF	G-CSF+ TGFβ	GM-CSF	GM-CSF+ TGFβ	IL-3	IL-3+ TGFβ
4	6	5.7	5.0	10.3	14.0	10.0
5	4.6	4.3	6.3	2.0	7.7	2.6
8	1.3	2.0	1.6	<1.0	1.6	<1.0
18	4.0	2.0	3.7	<1.0	7.3	2.0

In normal BM and PB samples, TGFβ_1 had no consistent effect on CFU-GM stimulated by GM-CSF or IL-3 or G-CSF. As colony numbers were low it is not justifiable to do statistical analysis on this data.

The effect of TGFβ_1 on CML chronic phase peripheral blood MNC (Figure 5) and CML blast transformation peripheral blood MNC (Figure 6) are shown overleaf. Comparison between samples containing TGFβ_1 and each of the growth factors were normalised to colony numbers obtained with single growth factors alone, for each patient [For example in UPN colony numbers obtained with G-CSF (n=30) was arbitrarily taken as 1; the colony numbers obtained with G-CSF and TGFß$_1$ (n=8.0) were normalized to the value obtained with G-CSF i.e. 0.26]. Actual colony numbers are given in Table XII and XIIa, also shown overleaf. In addition Table XIII shows CFU-CML numbers obtained with CML bone marrow MNC in 4 patients (not illustrated).

In the CML chronic phase samples TGFβ_1 reduced CFU-CML all 12

samples where G-CSF-induced CFU-CML were obtained ($p<0.005$). See Tables XII (overleaf). TGFβ_1 also reduced GM-CSF and IL-3-induced CFU-CML (mean colony numbers were reduced from 26.0 to 11.7, and 28.9 to 12.9 respectively). Thus in the CML chronic phase samples, TGFβ_1 had an inhibitory effect when either G-CSF, GM-CSF or IL-3 was used as the stimulus. Similarly in patients in myeloid blast transformation, TGFβ_1 inhibited G-CSF-induced CFU-CML in 4/4 samples, and GM-CSF- and IL-3-induced CFU-CML in 3/4 samples.

Figure 5: Effect of TGFß on CML CP MNC stimulated by G-CSF, GM-CSF and IL-3

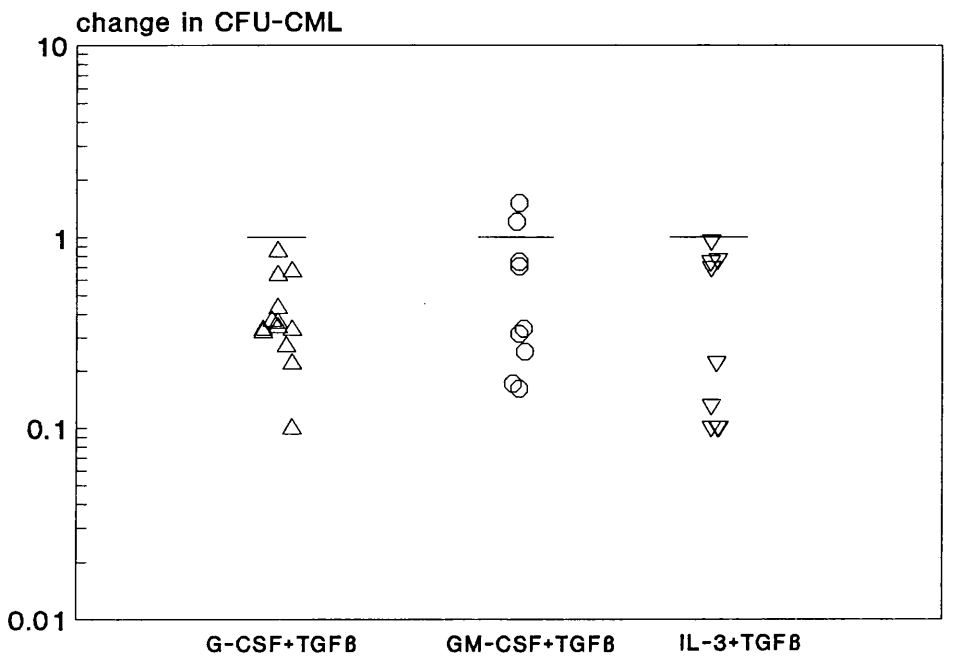


Figure 6: Effect of TGFß on CML BT MNC stimulated by G-CSF, GM-CSF and IL-3

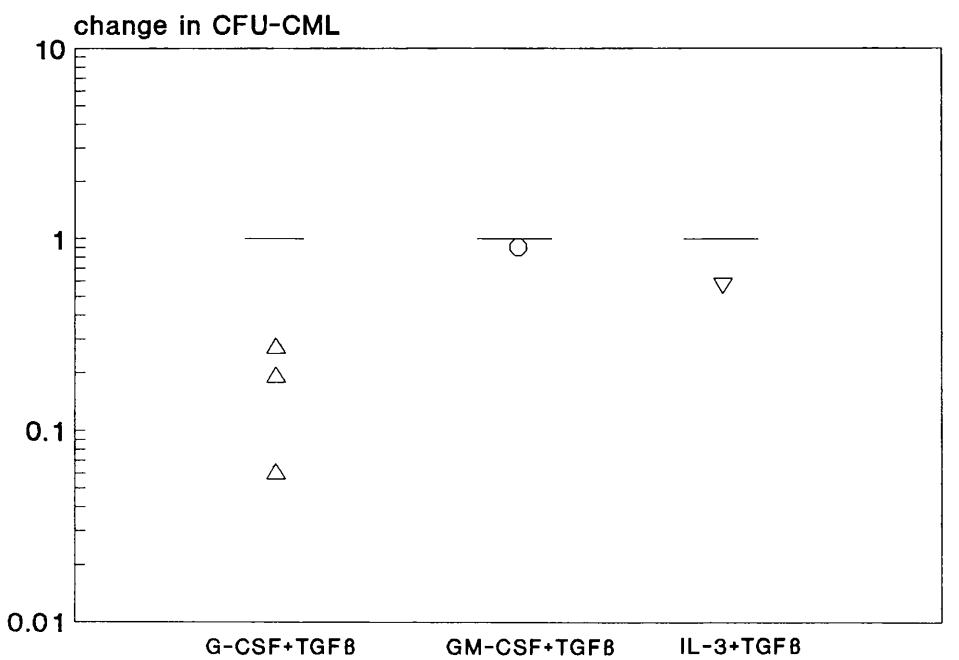


Table XII below, shows the effect of TGFβ on CML chronic phase peripheral blood mononuclear cells, stimulated by G-CSF, GM-CSF and IL-3. The cells were plated at $5X10^5$/plate. * - Ph negative CML; ND-Not done. In each instance the mean (of triplicate samples) CFU-CML are given. The concentrations of cytokines used per plate were G-CSF 15ng, GM-CSF 50ng, IL-3 50ng, TGFβ_1 5ng

UPN	G-CSF	G-CSF+ TGFβ	GM-CSF	GM-CSF+ TGFβ	IL-3	IL-3+ TGFβ
21	8.3	3.0	3.0	1.0	2.6	<1.0
22	52.7	22.3	55.3	8.7	35.7	<1.0
23	23.0	7.7	6.0	8.7	4.7	<1.0
24	30.0	8.0	13.0	0.0	9.0	0.0
25*	15.3	13.0	21.0	24.3	19.7	18.7
29	317.5	203.3	ND	ND	ND	ND
30*	45.0	30.0	42.0	7.0	32.0	4.0
31	377.5	127.5	65.0	ND	60.0	ND
36	104.0	23.3	ND	ND	ND	ND
37	90.7	33.7	67.3	47.3	43.0	32.7
38	67.0	21.7	79.0	24.7	63.0	46.7
39	0.0	0.0	0.0	0.0	0.0	0.0
54	0.0	0.0	0.0	0.0	0.0	0.0
55	73.0	24.0	25.3	18.7	19.3	13.3
56	0.0	0.0	0.0	0.0	0.0	0.0

Table XIIa below, shows the mean colony numbers obtained when CML blast transformation mononuclear cells were stimulated with G-CSF, GM-CSF and IL-3 alone and combination with TGFß. UPN 26$ was obtained from a patient in lymphoid blast transformation, the others were obtained from patients in myeloid blast transformation. UPN 26 and 27 were plated at $5x10^5$/plate, UPN 34 and 35 at $2x10^4$/plate and UPN54b at $2x10^5$/plate. The concentrations of cytokines used were:- G-CSF 15ng, GM-CSF 50ng, IL-3 50ng and TGFß 5ng. ND - not done. In each instance the mean of triplicate samples is shown.

UPN	G-CSF	G-CSF+ TGFβ	GM-CSF	GM-CSF+ TGFß	IL-3	IL-3+ TGFß
26$	11.0	0.0	4.0	0.0	<1.0	0.0
27	311.0	31.0	119.0	30.0	135	30.0
34	310.0	17.5	15.3	0.0	12.0	0.0
35	278.0	53.4	342.0	ND	55.0	ND
54b	334.3	89.7	493.0	445.7	837	489.0

Table XIII below, shows the effect of TGFβ on BM CFU-CML stimulated by G-CSF, GM-CSF or IL-3. Cells plated at 10^5/plate. ND - Not done
Concentration of cytokines used as above, in each instance the mean of triplicate samples is shown

UPN	G-CSF	G-CSF+ TGFβ	GM-CSF	GM-CSF+ TGFβ	IL-3	IL-3+ TGFβ
28	10.3	1.3	1.3	0.7	0.0	0.0
31	65.7	55.5	26.0	34.0	ND	ND
32	32.8	41.3	ND	ND	ND	ND
33	37.7	20.7	26.0	24.0	ND	N

Table XIV below, shows the effect of TGFβ ON CFU-AML stimulated by G-CSF, GM-CSF or IL-3. Cells were plated at $5x10^5$/plate except for UPN 47 & 49 which were plated at $5X10^4$/plate. Concentration of cytokines used were G-CSF 15ng, GM-CSF 50ng, IL-3 50ng, TGFß 5ng. ND - not done

UPN	FAB TYPE	G-CSF	G-CSF + TGFβ	GM-CSF	GM-CSF + TGFβ	IL-3	IL-3 + TGFβ
44	M1	26.3	14.7	0.0	0.0	0.0	0.0
46	M4	350.0	392.5	ND	ND	ND	ND
47	M2	21.0	26.7	104.0	30.7	115.0	0.0
48	2^{ry}	335.0	433.3	ND	ND	ND	ND
49	M4	25.3	10.0	230.0	ND	ND	ND

There was no consistent anti-proliferative effect of $TGF\beta_1$ on the proliferation

of G-CSF-induced proliferation of AML samples (see Table XIV).

A summary of the effects of $TGF\beta_1$ on G-CSF, GM-CSF and IL-3 induced proliferation of normal and CML cells are shown in **Table XV**, below. Mean colony numbers, taking into account all similar samples i.e all CML chronic phase samples, all normal bone marrow and all peripheral blood samples, are shown. AML samples are not shown as sample numbers were small.

COLONIES	G-CSF	G-CSF+ TGFβ	GM-CSF	GM-CSF+ TGFβ	IL-3	IL-3+ TGFβ
$CFU\text{-}GM_{BM}$	10.2	13.9	15.2	3.3	12.1	<1.0
$CFU\text{-}GM_{PB}$	4.0	3.5	4.2	3.2	7.7	3.7
$CFU\text{-}CML_{CPPB}$	80.3	34.5	26.0	11.7	17.6	9.0

Normal bone marrow samples n=8, normal PB samples n=4, CML CP samples with G-CSF n=15, GM-CSF n=13, IL-3 n=13

To summarise $TGF\beta_1$ reduced G-CSF, GM-CSF and IL-3-induced proliferation of CML cells (both chronic phase and blast transformation). $TGF\beta_1$ did not have a consistent anti-proliferative effect on normal or AML cells

Effect of IL-4 on G-CSF-induced proliferation in normal, CML and AML mononuclear cells

IL-4 is a small soluble glycoprotein that has diverse effects on B-lymphocytes and on other lymphoid and non-lymphoid cell types. IL-4 has an anti-proliferative effect on progenitor cells stimulated by GM-CSF or IL-3 *in vitro* (Peschel et al, 1987, Paul et al, 1991). In contrast IL-4 augments G-CSF-induced cell proliferation *in vitro* (Broxmeyer et al, 1988).

Objective

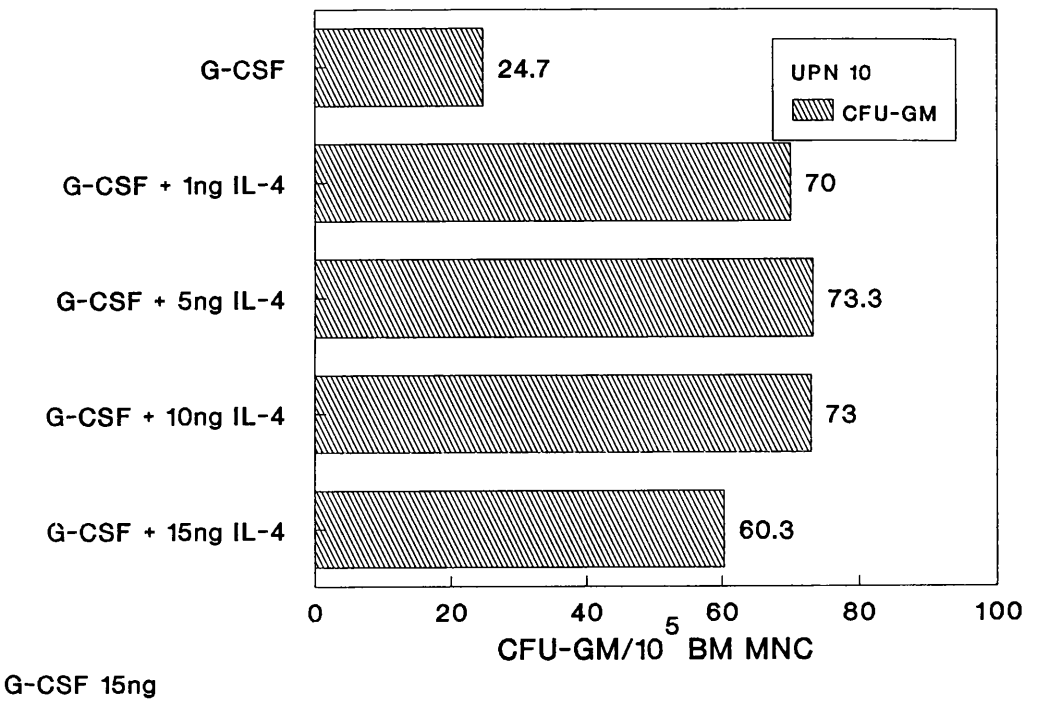
These experiments were done to determine if IL-4 augments G-CSF-induced proliferation in normal, CML and AML MNC.

Results

Preliminary experiments were carried out to determine the concentration of IL-4 which produced maximum colony numbers in combination with 15ng of G-CSF. Bone marrow mononuclear cells were plated at 10^5/plate and peripheral blood MNC were plated at $5x10^5$/plate, except UPN 34 and 35 which were plated at $2x10^4$/plate. Dose response curves were done on 13 normal samples, (UPN 10 is illustrated in Fig 7), 10 CML PB samples (UPN 32 and 34, chronic phase and blast transformation respectively are

illustrated in Fig 8a and 8b respectively), and 3 AML samples (UPN 48 is illustrated in Fig 9). From the dose response curves and in the dose ranges used, 5ng of IL-4 gave a maximum response in UPN 10; 1ng of IL-4 gave a maximum response upn 32; IL-4 had an inhibitory effect on G-CSF-induced CFU-CML, in UPN 34, 15ng of IL-4 gave maximal response in UPN 48. An intermediate dose of 5ng was therefore chosen for subsequent experiments.

Figure 7



G-CSF 15ng

Figure 7

The figure illustrates the effect of concentrations of IL-4 (1ng, 5ng, 10ng and 15ng) on G-CSF-induced CFU-GM in a normal donor BM MNC (UPN 10).

The addition of IL-4 augmented G-CSF-induced CFU-GM and a plateau in response was reached with 15ng of IL-4.

Figure 8a

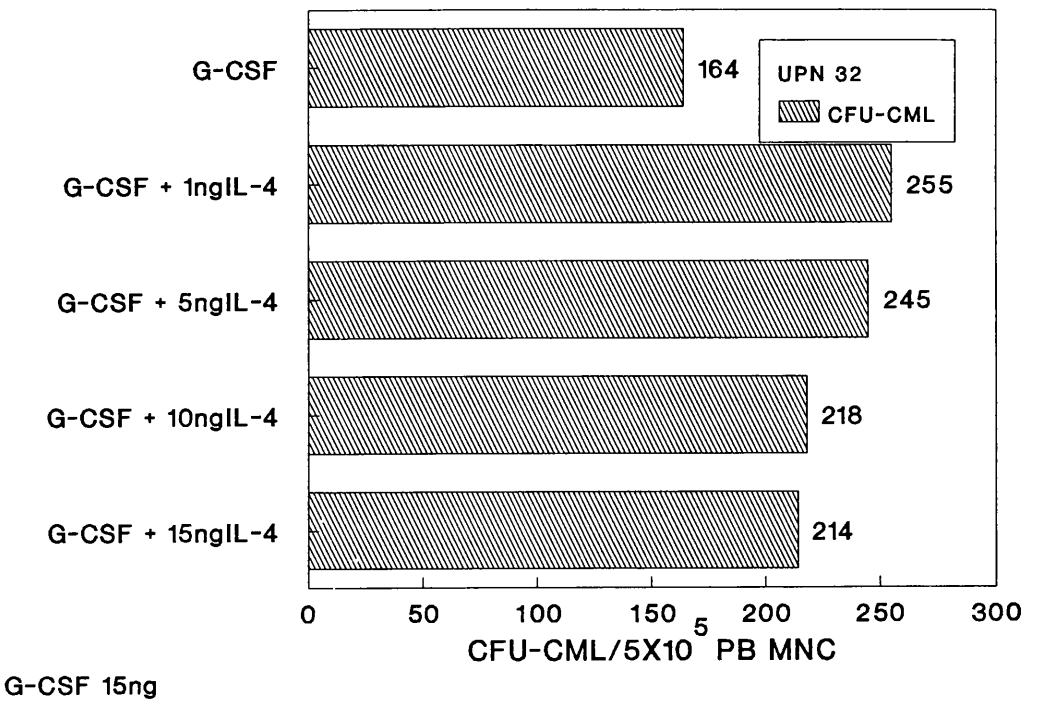


Figure 8a

The figure illustrate the effect of concentrations of IL-4 (1ng, 5ng, 10ng and 15ng) on G-CSF-induced CFU-CML in a patient with CML CP (UPN 32).

Similar to its effects on normal MNC, IL-4 augmented G-CSF-induced CFU-CML and a plateau in response was seen with 1ng IL-4.

Figure 8b

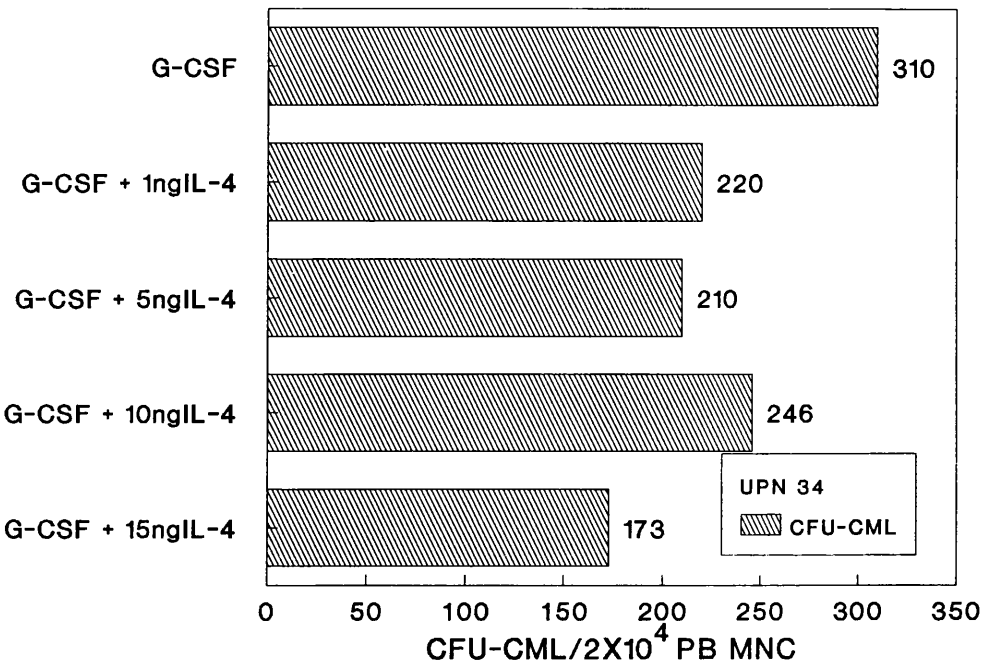


Figure 8b shows the effect of concentrations of IL-4 (1ng, 5ng, 10ng and 15ng) on G-CSF-induced stimulation of CML blast transformation MNC (UPN 34). The cells were plated at $2x10^4$/plate; concentration of G-CSF used was 15ng. In contrast to its effects on normal and CML CP MNC, IL-4 inhibited G-CSF-induced colony formation and a maximum inhibitory effect was seen with 15ng of IL-4.

Figure 9

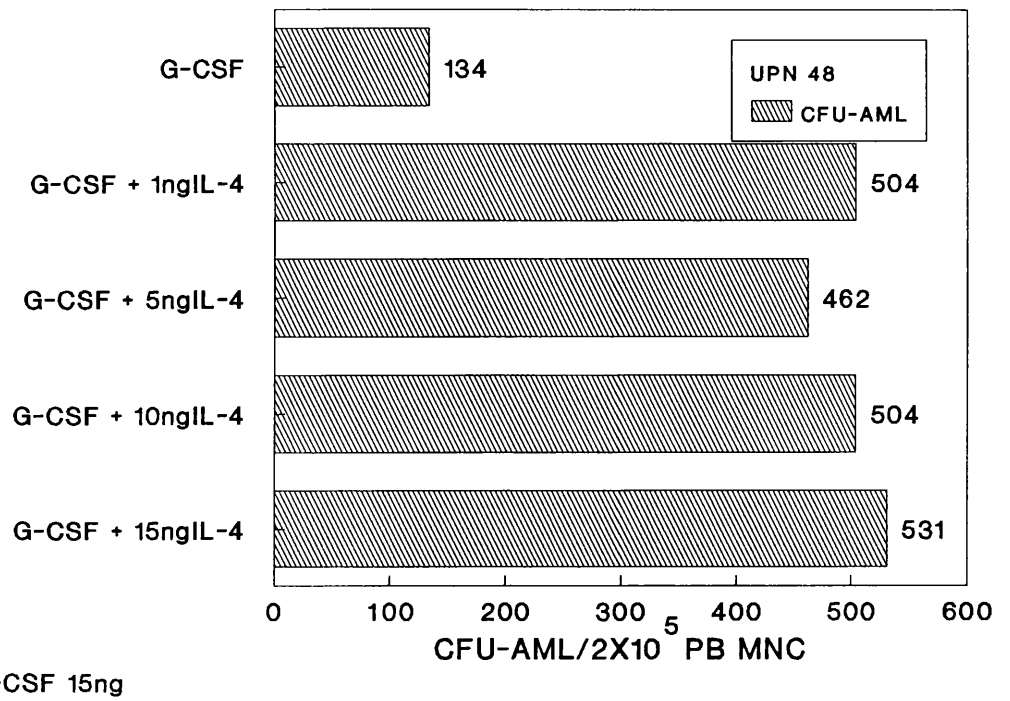


Figure 9 shows the effect of concentrations of IL-4 (1ng, 5ng, 10ng and 15ng) on G-CSF-induced stimulation of CFU-AML (UPN 48).

The *in vitro* response to G-CSF and IL-4 to MNC from patients with AML was similar to that of MNC from normal donors and patients with CML in chronic phase. IL-4 augmented G-CSF-induced CFU-AML and a maximum response was seen with 15ng of IL-4.

The effect of IL-4 on, all CML and all AML (Fig 10) cells stimulated with G-CSF are shown overleaf, in each instance the mean number of colonies for each group were used. Comparisons between samples containing G-CSF and IL-4 were normalised to colony numbers obtained with G-CSF alone. Actual colony numbers obtained and MNC plated are also shown below and overleaf in Tables XVI (normal bone marrow), XVII (normal peripheral blood), XVIII and XVIIIa (CML chronic phase and blast transformation peripheral blood MNC respectively) XIX (CML bone marrow MNC) and XX (AML mononuclear cells).

Table XVI below, shows the effect of IL-4 on G-CSF-induced proliferation on normal bone marrow MNC. Cells were plated at 10^5/plate. Concentrations of cytokines used are given in the table. Mean CFU-GM of triplicate samples are shown.

UPN	15ng G-CSF	15ng G-CSF+5ng IL-4
1	5.3	13.0
2	13.3	39.3
3	2.3	9.6
6	6.6	6.6
7	4.3	3.0
9	4.3	12.3
10	24.7	70.0
11	11.3	56.7
12	19.0	32.7
13	5.7	11.7
20	30.0	65.0

Fig 10: Effect of G-CSF and IL-4 on
CFU-CML and CFU-AML

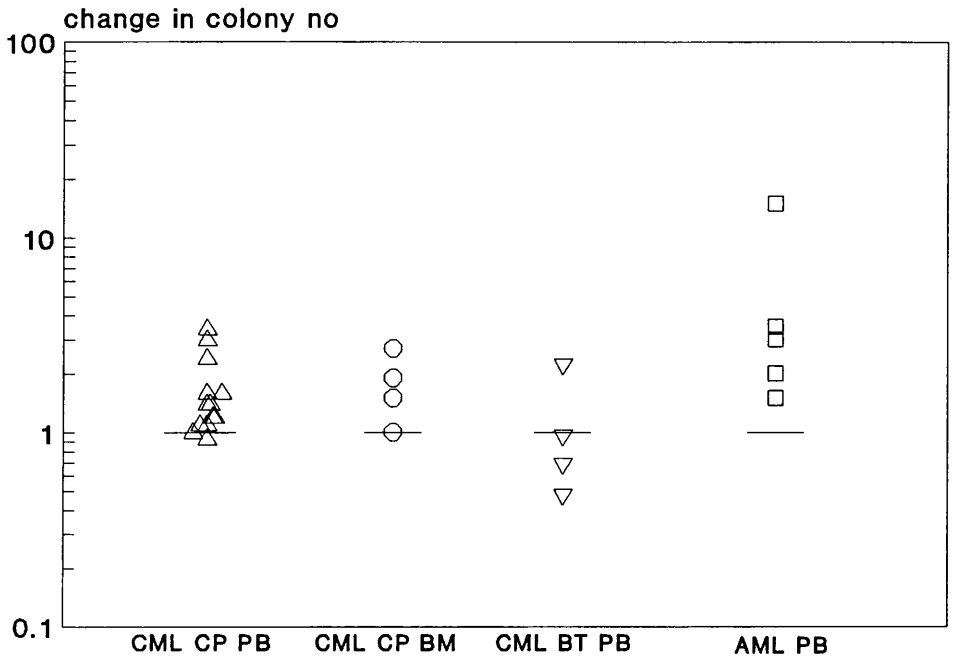


Table XVII below, shows the effect of G-CSF and IL-4 on normal PB MNC. Cells were plated at $5x10^5$/plate. Concentrations of cytokines used are given in the table. In each instance mean colony numbers of triplicate samples are shown.

UPN	15ng G-CSF	15ng G-CSF+5ng IL-4
4	6.0	10.0
5	4.6	5.0
8	1.3	1.6
14	7.6	25.0
15	17.0	20.6
16	<1.0	1.7
18	4.0	8.7

Table XVIII below, shows the effect of G-CSF and IL-4 on CML chronic phase peripheral blood MNC. Cells were plated at $5X10^5$/plate. Concentrations of cytokines used are given in the table. * Ph negative. Mean colony numbers (of triplicate samples) are shown.

UPN	G-CSF 15ng	15ng G-CSF+ 5ng IL-4
21	8.3	13.6
22	52.7	71.7
23	23.0	55.0
24	30.0	41.7
25*	15.3	16.7
29	317.5	332.5
30*	45.0	70.0
31	377.5	450.0
36	104.0	312.5
37	90.7	84.0
38	67.0	75.0
51	517.0	91.7
55	73.0	81.3

Table XVIIIa below, shows the effect of G-CSF and IL-4 on CML blast transformation peripheral blood mononuclear cells. UPN 26 was in lymphoid blast transformation, the remainder were in myeloid blast transformation. UPN 26 and 27 were plated at $5x10^5$/plate, UPN 34 and 35 at $2x10^4$/plate and UPN 54b at $2x10^5$/plate. The concentrations of cytokines used are given in the table. Mean colony numbers (of triplicate samples) are shown.

UPN	15ng G-CSF	15ng G-CSF+ 5ng IL-4
26$	11.0	7.1
27	311.0	680.0
34	310.0	210.0
35	278	130.6
54b	334.3	317.0

Table XIX below, shows the effect of G-CSF and IL-4 on CML bone marrow MNC. Cells were plated at 10^5/plate. Concentrations of cytokines used are given in the table. Mean colony numbers (of triplicate samples) are shown.

UPN	15ng G-CSF	15ng G-CSF+5ng IL-4
28	10.3	19.6
31b	65.7	180.0
32	32.8	49.1
33	37.7	39.3

Table XX below, shows the effect of G-CSF and IL-4 on AML MNC. The cell type (i.e peripheral blood - PB- or bone marrow-BM-) cell number plated and concentrations of cytokines are given in the table.

UPN	CELL TYPE	CELL NO	FAB TYPE	G-CSF 15ng	G-CSF+IL-4 15ng +5ng
44	PB	$5X10^5$	AML M1	26.3	52.6
46	PB	$5X10^5$	AML M4	4.5	15.0
46b	BM	10^5	AML M4	350.0	776.7
47	PB	$5X10^4$	AML M2	25.3	38.3
48	PB	$2X10^5$	2^{ry} AML	325.0	1153.0
49	PB	$5X10^4$	AML M4	21.7	329.3

IL-4 in combination with G-CSF had no appreciable effect on CFU-GM formation from normal bone marrow or peripheral blood MNC. The mean CFU-GM using PB MNC obtained with G-CSF was 5.9 (range <1.0-17) and this increased to 9.74 (range 1.7-25) on the addition of IL-4. In normal BM MNC, the mean CFU-GM obtained with G-CSF was 11.5(range 4.3-30) and this number increased to 26.3(range 3-70) when IL-4 was added to the cultures. Although IL-4 increased G-CSF-induced CFU-GM in normal peripheral blood and bone marrow samples, as the total colony numbers obtained was low, statistical analysis was not done.

IL-4 augmented G-CSF-induced colony formation in 11/13 CML CP peripheral blood MNC ($p<0.005$). The mean CFU-CML obtained when cultures were stimulated with G-CSF and G-CSF in combination with IL-4 were 132.5 (range 8.3-377.5) and 158 (range 13.6-450) respectively (see Table

XVIII). IL-4 augmented G-CSF-induced CFU-CML in 3/4 CML chronic phase BM MNC samples, the mean CFU-CML were increased from 36.6 (range 10.3-65.7) to 72 (range 19.6-180; see Table XIX). IL-4 also augmented G-CSF-induced CFU-AML in 5/5 AML samples.

In contrast to its effects on normal, CML chronic phase and AML cells IL-4 reduced G-CSF-induced colony formation in 2/4 samples from patients with CML myeloid blast transformation. IL-4 had no effect on G-CSF-induced stimulation of CFU-CML in the only patient with CML in lymphoid blast transformation.

In summary, IL-4 augmented G-CSF-induced colony formation in CML chronic phase and AML mononuclear cells. The effects of IL-4 on G-CSF-induced proliferation of normal and CML blast transformation cells was not consistent.

Effect of TGFβ_1 on combined stimulation with G-CSF and IL-4 on normal, CML and AML MNC

Objective

The aim of these experiments was to determine whether TGFβ_1 could inhibit potentiation of G-CSF and IL-4-induced colony formation in cultures of normal, CML and AML cells.

Results

Figures 12, 13a, 13b and 14 show the effect of TGFβ_1 on G-CSF- and G-CSF and IL-4-induced proliferation in normal (UPN 10), CML chronic phase (UPN32) CML blast transformation (UPN34) and AML MNC (UPN48).

Figure 11

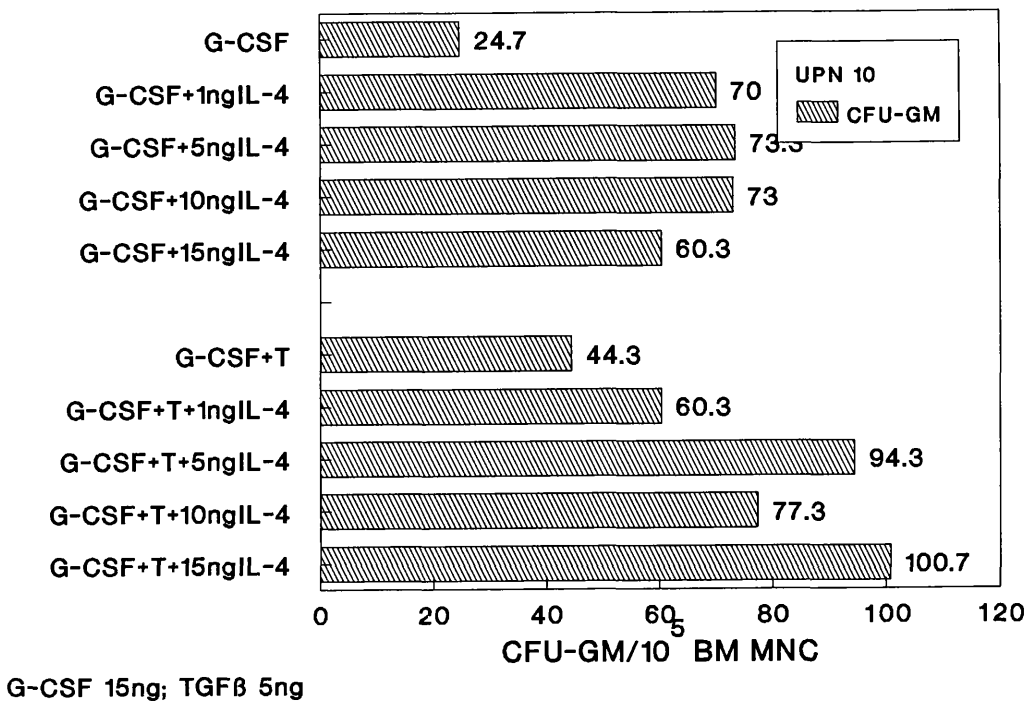
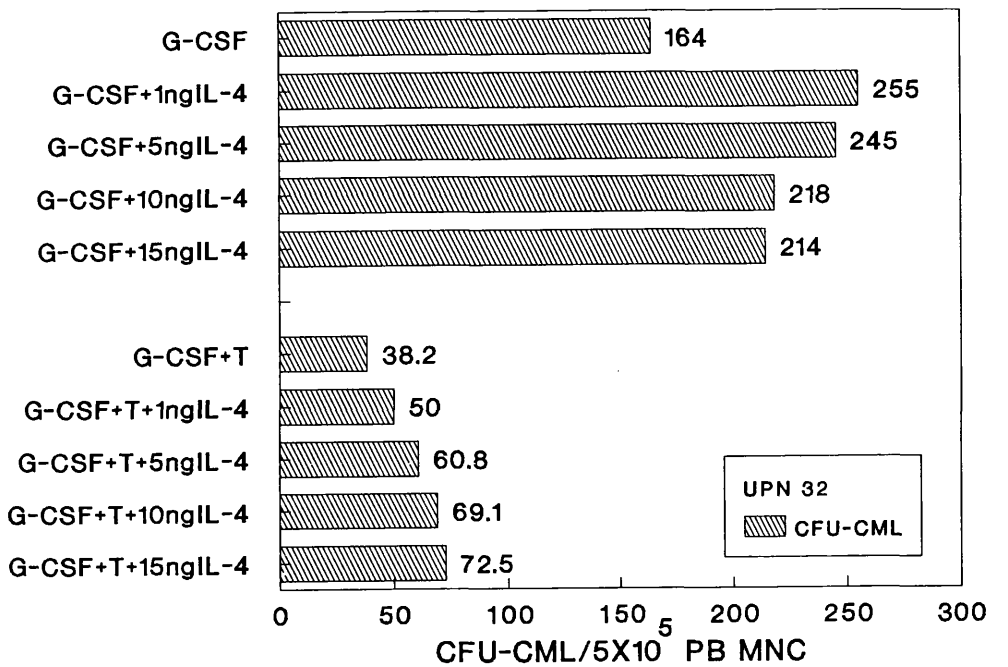


Figure 11

The effects of G-CSF, IL-4 and TGFβ_1 on CFU-GM in a normal BM sample (UPN 10) are shown.

TGFβ_1 and IL-4 when used separately augmented G-CSF-induced colony formation. TGFβ_1 in combination with G-CSF and IL-4 (1ng, 5ng and 10ng) had no effect on CFU-GM, compared to CFU-GM numbers obtained in cultures stimulated by G-CSF and IL-4 (1ng, 5ng and 10ng). However 15ng of IL-4 together with G-CSF and TGFβ_1 augmented CFU-GM-formation compared to G-CSF and IL-4 (100.7 and 60.3 CFU-GM respectively).

Figure 12a



G-CSF 15ng; TGFß 5ng

Figure 12a

The figure illustrates the effect of G-CSF, IL-4 and TGFβ_1 on CML chronic phase peripheral blood mononuclear cells.

TGFβ_1 inhibited CFU-CML formation in cultures stimulated with G-CSF (mean colony numbers were reduced from 164 to 38.2). IL-4 augmented G-CSF-induced colony formation and a maximum response was seen with 1ng of IL-4. The addition of TGFβ_1 to cultures containing G-CSF and IL-4 inhibited CFU-CML formation.

Figure 12b

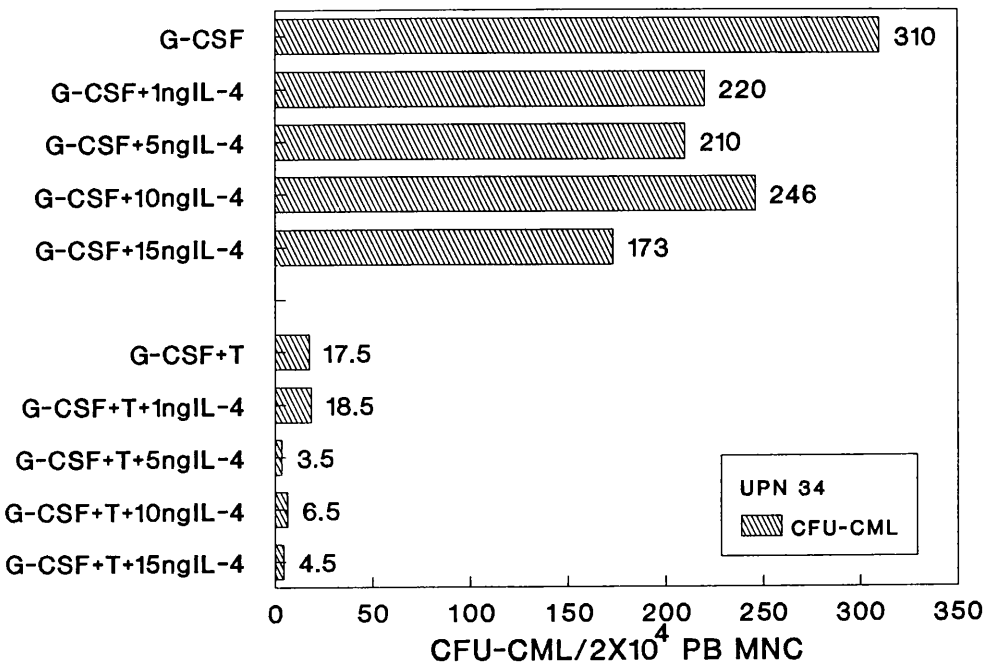


Figure 12b

The figure illustrates the effect of G-CSF, IL-4 and TGFβ_1 on CML blast transformation MNC (UPN 34).

IL-4 reduced G-CSF-induced CFU-CML and this inhibitory effect was further enhanced by the addition of TGFβ_1.

Figure 13

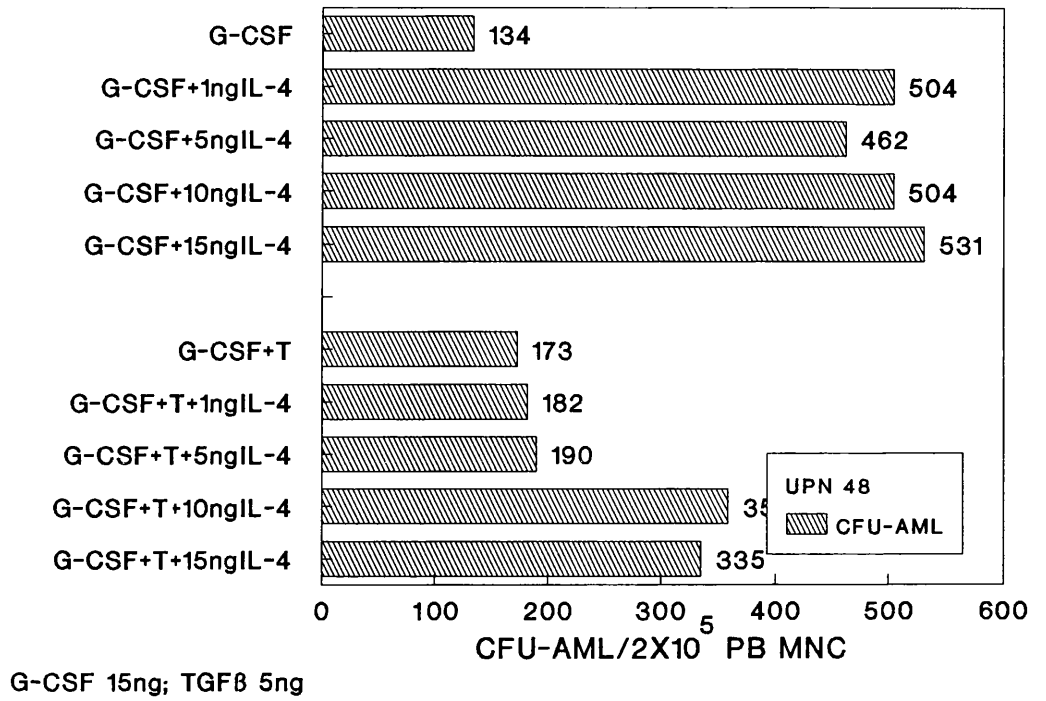


Figure 13

The figure illustrates the effect of G-CSF, IL-4 and TGFβ_1 on AML peripheral blood MNC.

IL-4 augmented G-CSF-induced CFU-AML and a maximum response was seen with 15ng of IL-4. TGFβ_1 augmented G-CSF-induced colony formation but inhibited CFU-AML stimulated by G-CSF and IL-4 in combination.

Table XXI below, shows the effect of G-CSF, IL-4 and TGFβ_1 on normal PB MNC. Cells were plated at $5X10^5$/plate. Concentration of cytokines used was G-CSF 15ng; IL-4 5ng; TGFβ_1 5ng. In each instance the mean of triplicate samples is shown

UPN	G-CSF	G-CSF+IL-4	G-CSF+TGFβ	G-CSF+IL-4+TGFβ
4	6.0	10.0	5.7	16.0
5	4.6	3.6	4.3	2.3
8	1.3	1.6	2.6	1.6
14	7.6	22.0	6.6	42.6
15	17.0	20.6	8.6	14.3
16	<1.0	1.7	1.3	2.0
18	4.0	8.7	2.0	3.0

Table XXII below, shows the effect of G-CSF, IL-4 and TGFβ_1 on normal BM MNC. Cells were plated at 10^5/plate. Concentration of cytokines used, as above. Mean (of triplicate samples are shown).

UPN	G-CSF	G-CSF+IL-4	G-CSF+TGFβ	G-CSF+IL-4+TGFβ
1	5.3	13.0	0.0	11.7
2	13.3	39.3	14.7	40.0
3	2.3	9.67	2.3	4.0
6	6.6	6.6	6.6	10.3
7	4.3	3.0	2.3	3.0
9	4.3	12.3	4.0	5.3
10	24.7	70.0	44.3	94.3
12	19.0	32.7	28.7	56.7
13	5.7	11.7	12.0	20.3
20	30.0	65.0	30.0	83.0

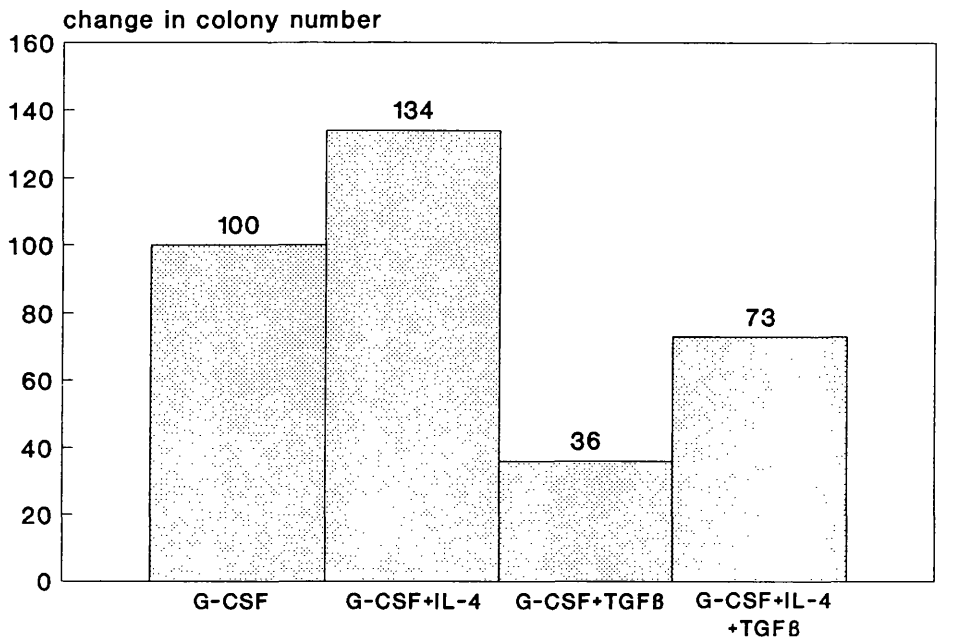
TGFβ_1 had no effect on normal peripheral blood MNC stimulated by a combination of G-CSF and IL-4. In the peripheral blood samples the mean

number of CFU-GM obtained with G-CSF was 5.8 (range 4-17; see Table XXI) and this increased to 11.7 (range 3-42.6; see Table XXI) when TGFβ_1 was added to cultures. In 2/7 normal PB samples (UPN 4 and 14) TGFβ_1 increased the G-CSF and IL-4-induced proliferation and in 5/7 samples CFU-GM were reduced or unaltered.

The mean number of normal BM CFU-GM obtained from cultures stimulated with G-CSF was 11.6 (range 2.3-30; see Table XXII) and this increased to 32.9 (range 3-83) when cultures were stimulated with G-CSF, IL-4 and TGFβ_1. In 4/10 normal BM mononuclear cells stimulated with G-CSF plus IL-4, the addition of TGFß$_1$ increased colony numbers further.

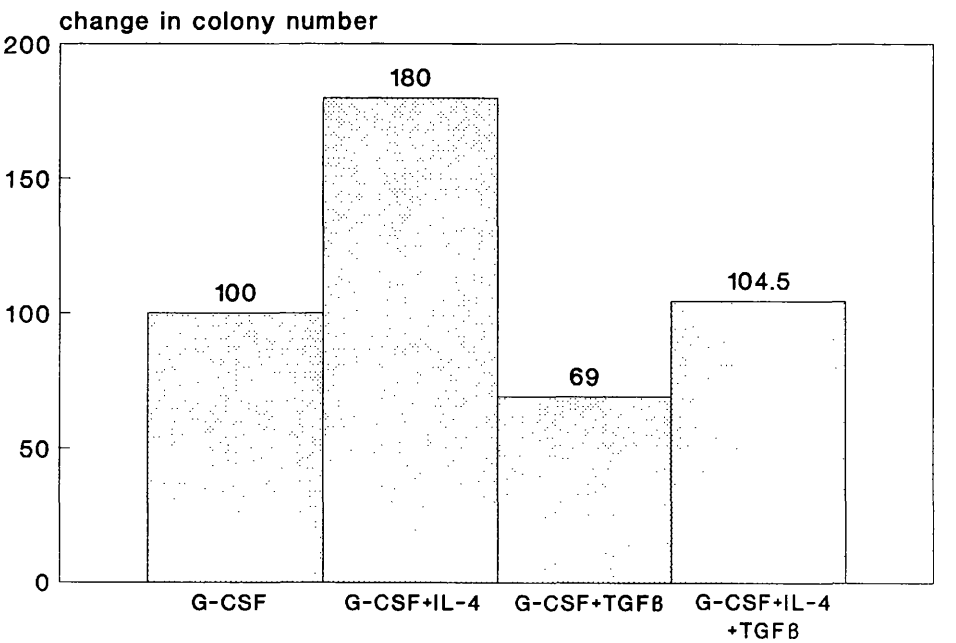
A summary of the effects of G-CSF, IL-4 and TGFβ_1 on all CML chronic phase peripheral blood and bone marrow, CML blast transformation and AML MNC are shown in Figures 14a, 14b (overleaf) 15 and 16 (page 151 and 152 respectively). The mean number of colonies obtained with the other samples was normalised to that obtained with G-CSF. Actual colony numbers obtained with the different cytokines are given in Table XXIII, XXIIIa, XXIV, XXIVa and XXV also overleaf.

Figure 14a: Effect of G-CSF, IL-4 and TGFß on CML CP PB.



G-CSF 15ng; IL-4 5ng; TGFß 5ng

Figure 14b: Effect of G-CSF, IL-4 and TGFß on CML CP BM.



G-CSF 15ng; IL-4 5ng; TGFß 5ng

Table XXIII below shows the effect of G-CSF, IL-4 and TGFβ_1 on CML chronic phase peripheral blood MNC. Cells were plated at $5X10^5$/plate. Concentrations of cytokines used were G-CSF 15ng, IL-4 5ng, TGFβ_1 5ng. * Ph negative. In each instance the mean (of triplicate samples) is shown.

UPN	G-CSF	G-CSF+IL-4	G-CSF+TGFβ	G-CSF+IL-4+TGFβ
21	8.3	13.6	3.0	8.0
22	52.7	71.7	22.3	93.7
23	23.0	55.0	8.0	16.0
24	30.0	41.7	8.0	26.7
25	15.3	16.7	13.0	14.0
29	317.5	332.5	203.3	133.3
30	45.0	70.0	30.0	25.0
31	377.5	450.0	127.5	282.5
36	104.0	169.0	23.3	76.7
37	90.7	84.0	33.7	50.0
38	67.0	75.0	21.7	35.0
51	518.0	593.0	91.7	72.7
55	73.0	81.3	24.0	43.7

Table XXIIIa below, shows the effect of G-CSF, IL-4 and TGFß on CML blast transformation cells. UPN 26 was in lymphoid BT, the others were in myeloid BT. UPN 26 and 27 were plated at $5x10^5$/ plate, UPN 34 and 35 at $2x10^4$/plate and UPN 54b at $2x10^5$/plate. Concentration of cytokines as previously used. In each instance mean (of triplicate samples) is shown.

UPN	G-CSF	G-CSF+IL-4	G-CSF+TGFß	G-CSF+IL-4+TGFß
26$	11	7.1	0.0	2.8
27	311	680	31	81.7
34	310	210	17.5	3.5
35	278	130.6	342	44.0
54b	334.3	317	89.7	182.3

Table XXIV below, shows the effect of G-CSF, IL-4 and TGFβ_1 on CML BM MNC. All patients were in chronic phase. Cells plated were 10^5/plate, the concentrations of cytokines as previously used.

UPN	G-CSF	G-CSF+IL-4	G-CSF+TGFβ	G-CSF+IL-4+TGFβ
28	10.3	19.6	1.3	7.3
31	65.7	180.0	55.5	113.0
32	32.8	49.1	41.3	41.3
33	37.7	39.3	20.7	18.6

TGFβ_1 reduced CFU-CML (chronic phase) in cultures exposed to G-CSF and IL-4 ($p<0.05$). The mean CFU-CML was reduced from 158.6 (range 13.6-593) to 67.4 (range 8-282.5; see Table XXIII). Furthermore in 8/13 CML chronic phase samples the number of CFU-CML in cultures containing G-CSF, IL-4 and TGFβ_1 was lower than in cultures exposed to G-CSF alone ($p<0.05$). The results suggest that in CML chronic phase mononuclear cells

TGFß$_1$ inhibits G-CSF and G-CSF plus IL-4-induced colony formation. The addition of IL-4 to these cultures partially reverses this effect.

Mean CFU-CML obtained with CML chronic phase PB and BM samples are shown in **Table XXIVa** below.

	G-CSF	G-CSF+ IL-4	G-CSF +TGFβ_1	G-CSF+ IL-4+ TGFβ_1
CFU-CML$_{CPPB}$ (n=13)	132.5 (8.3-518)	158.6 (13.6-593)	46.9 (3-203.3)	67.4 (2-282.5)
CFU-CML$_{CPBM}$ (n=4)	36.6 (10.3-65.7)	72.0 (19.6-180)	29.7 (1.3-55.5)	45.0 (7.3-113)

Figure 15: Effect of G-CSF, IL-4 and TGFß on CML myeloid BT MNC.

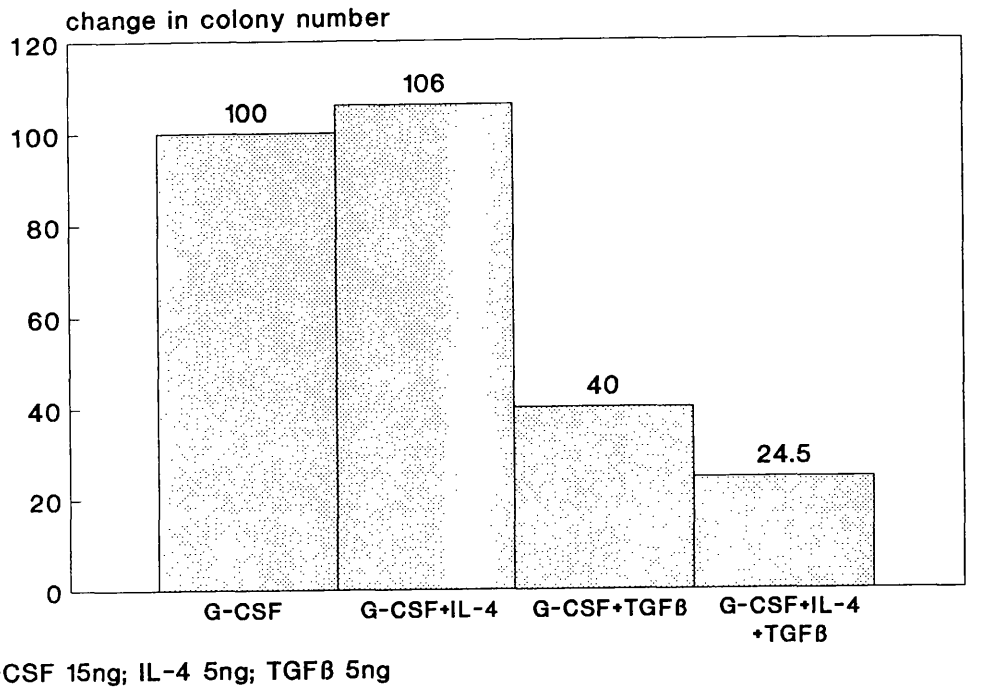
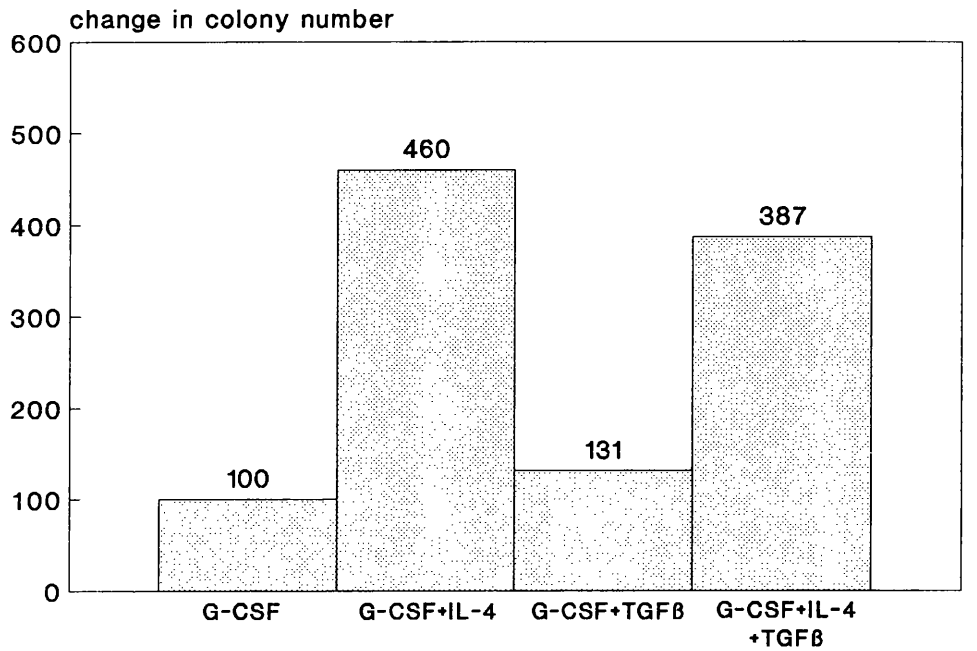


Figure 15

The effects of G-CSF, IL-4 and TGFβ_1 on CML myeloid blast transformation MNC are shown in Figure 15. The mean colony numbers in cultures exposed to mixtures of cytokines was normalised to the value for colony numbers in cultures exposed to G-CSF alone. In 3/4 patients in myeloid blast transformation, TGFβ_1 reduced G-CSF-induced CFU-CML. The actual colony numbers obtained with the different cytokines are given in Table XXIII.

Figure 16: Effect of G-CSF, IL-4 and TGFß on AML PB MNC.



G-CSF 15ng; IL-4 5ng; TGFß 5ng

Figure 16

The effects of G-CSF, IL-4 and TGFβ1 on AML PB MNC are shown above. The mean colony numbers in cultures exposed to mixtures of cytokines was normalised to the value for colony numbers obtained in cultures exposed to G-CSF alone. The effects of TGFß$_1$ on G-CSF and G-CSF plus IL-4-induced proliferation was variable. In 2/6 samples there was an increase (UPN 46b and 47), in 3/6 there was a decrease (UPN 44, 48 and 49), and in the remaining sample (UPN 46) there was no change. The actual CFU-AML numbers are given in the Table XXV, overleaf.

Table XXV below shows the effect of G-CSF, IL-4 and TGFβ_1 on AML MNC.

UPN	G-CSF	G-CSF+IL-4	G-CSF+TGFβ	G-CSF+IL-4+TGFβ
44	26.3	52.6	14.6	19.0
46	4.5	15.0	11.5	13.0
46b	350.0	776.7	392.5	1119
47	25.3	38.3	26.7	97.6
48	335.0	1155	433.0	475.0
49	21.7	329.3	ND	241.5

Cells were plated at $5X10^5$/plate except for UPN 47 and 49 which were plated at $5X10^4$/plate and UPN 46b which was plated at 10^5/plate. The samples were from peripheral blood except UPN 46b which was from bone marrow. Concentrations of cytokines used were G-CSF 15ng, IL-4 5ng, TGFß 5ng.

To summarise, TGFß$_1$ reduced CFU-CML formation by G-CSF and G-CSF plus IL-4 when CML chronic phase cells were used. There was a partial reversal of this inhibitory effect of TGFß$_1$ when IL-4 was added to cultures stimulated by G-CSF. The effects of TGFß$_1$ on G-CSF plus IL-4-induced proliferation of normal, AML and CML blast transformation MNC was more heterogeneous. However as sample and/or colony numbers were small this data may not be representative.

Morphological analysis

The morphology of colonies was assessed as described in Chapter 2. A typical CFU-CML from UPN 31 is shown in Plate1. Colonies from patients with CML in chronic phase showed myeloid progenitors in a distribution

similar to that seen in the original cytospin samples (see Plate 2), whereas colonies from patients in blast transformation revealed predominantly immature blasts.

Cytogenetic analysis

Satisfactory cytogenetic analyses were obtained from colonies from 5/8 CML samples (UPN 27, 29, 34, 36 and 47). In all 5 patients the chromosomal abnormality detected at diagnosis was described and they are detailed below:

UPN 27 - 46,XX,t(9;22),inv(16)(p13q12or13),iso(17q)

UPN 29 - 46,XY,t(9;22) with random loss

UPN 34 - 46,XY,t(3;21),t(9;22) and one cell with iso(17q)

UPN 36 - 46,XX,22q-

UPN 47 - 9 cells of normal female karyotype, 1 cell 45, XX,-5,?t(2;18)(p2;q2).

Plate 3 illustrates the chromosomal analysis performed on day 14 CFU-CML from UPN 27. The chromosomal karyotype is: 46, XY, t(9;22)(q34;q11), inv(16)(p13q12), i(17)(q10)

Discussion

The number of CFU-GM obtained with normal donor peripheral blood or bone marrow MNC was consistently lower than the number of CFU-CML or CFU-AML obtained when G-CSF, GM-CSF or IL-3 were used as the proliferative stimulus. In both CML and AML there are more committed progenitors at an arrested state of differentiation when compared with normal donor MNC and it is likely that these progenitors are more susceptible to the proliferative stimulus provided by G-CSF, GM-CSF or IL-3. Some AML blasts express high affinity receptors for the colony-stimulating factors and are therefore more likely to proliferate *in vitro* (Budel, et al, 1990). Similarly unlike in normal haemopoiesis autocrine haemopoietic growth may play an important role in myeloid leukaemogenesis (Lowenberg and Touw, 1992),

In the normal donor samples a greater number of colonies was obtained with the bone marrow MNC compared to the peripheral blood MNC (see Table V and VI). This was independent of whether G-CSF, GM-CSF or IL-3 was used to stimulate the cultures, reflecting the fact that in the normal state there are more progenitors in marrow than peripheral blood.

The low CFU-GM that were consistently obtained, from normal donors was disappointing. This may be due to technical reasons, or to the fact that as laboratory samples were frequently obtained at the end of the bone marrow harvest there may have been heavy contamination with peripheral blood. The number of CFU-GM obtained with normal samples were not increased by increasing the number of cells plated or by increasing the concentration of cytokines.

G-CSF unlike both GM-CSF and IL-3 acts on more mature myeloid progenitors (Demetri et al, 1991) and the number of G-CSFR increases with myeloid maturation. In CML there is an excess of myeloid activity resulting in an expanded myeloid compartment, with a left-shift in myeloid maturation. The data suggests that in CML there is an increase in committed progenitors that are more likely to respond to G-CSF than GM-CSF or IL-3. The mean number of CFU-CML obtained with G-CSF was more than that obtained with GM-CSF or IL-3. Alternatively the autocrine production of G-CSF in CML may be greater than either GM-CSF or IL-3. As may be expected the patient in lymphoid blast transformation (UPN 26) gave poor colony numbers with colony-stimulating factors which have an effect on mainly myeloid progenitors.

The increase in clonogenicity in response to G-CSF is also seen myeloid blast transformation. The data suggests that there is some expansion of selective progenitor cells that are more likely to respond to G-CSF. Table IX, overleaf summarises the mean and median number (taking into account all samples studied) of colonies/plate obtained with G-CSF, GM-CSF and IL-3 using normal and CML samples.

Table IX

COLONY TYPE	G-CSF 15ng		GM-CSF 50ng		IL-3 50ng	
	MEAN	MEDIAN	MEAN	MEDIAN	MEAN	MEDIAN
$CFU\text{-}GM_{PB}$ (n=4)	4.0	4.3	4.2	4.35	7.7	7.5
$CFU\text{-}GM_{BM}$ (n=8)	10.2	6.2	15.2	4.50	12.0	4.3
$CFU\text{-}CML_{CPPB}$	46.7 n=15	30.0	29.0 n=13	21.00	22.3 n=13	19.3
$CFU\text{-}CML_{BTPB}$ (n=4)	308.3	310.5	242.3	230.50	259.8	95.0

PB - peripheral blood CP - chronic phase
BM - bone marrow BT - myeloid blast transformation
PB - MNC were plated at $5x10^5$/plate, except for CML BT MNC which were plated at $2x10^4$/plate
BM - MNC were plated at 10^5/plate.
The colony data from AML samples are not included in the summary table as the total number of samples were small.

In AML lack of differentiation occurs early on in myeloid maturation resulting in an excess number of leukaemic blasts with very few mature myeloid progenitors present in the peripheral circulation. Of the AML samples that grew *in vitro*, G-CSF stimulated colony formation in all samples and GM-CSF and IL-3 in 2/4 and 1/3 samples respectively. This is different to the findings of Kelleher et al (Kelleher et al, 1987) who reported that GM-CSF and IL-3 had an equivalent activity in stimulating AML which was greater than G-CSF, this suggests that the proportion of cells capable of responding to G-CSF is less than that which can respond to GM-CSF or IL-3. The limited data obtained in this study suggests that the distribution of progenitor types at different stages of differentiation is different in CML compared to AML.

None of the AML or CML mononuclear cells grew autonomously *in vitro*, which is in contrast to the study by Reilly (Reilly et al, 1989) where all 70 AML samples tested grew autonomously. The different culture techniques used by Reilly et al is unlikely to account for this difference. In their study MNC were plated at 10^5/ml on Iscoves modified Dulbecco's medium containing 10% FCS and 0.8% methylcellulose. All AML samples in this study were obtained from cryopreserved samples. Cryopreservation of normal MNC is associated with reduced viability (Gorin, 1986) and a similar phenomenon may have occurred with the AML samples. Furthermore in this study the number of AML samples studied (18) was much smaller than the number studied by Reilly et al (70).

TGFβ_1 increased G-CSF directed CFU-AML in 2 samples (UPN 46, de novo AML; and UPN 48 2ry AML) whereas in the three remaining samples there was no discernible effect (see Table XIV). TGFß$_1$ reduced GM-CSF and IL-3-induced proliferation but had no effect on G-CSF-induced proliferation in UPN 47, the only sample where sufficient number of MNC were obtained to test all three cytokines. The sample numbers are too low for the results to be representative.

In this small study AML cells had a variable response to TGFß$_1$. Other workers have shown that the response of AML cells to TGFβ_1 is heterogeneous (Taetle et al, 1993) with both inhibitory and stimulatory effects documented. Morphology and agarose gel analysis of DNA showed that some AML cells underwent apoptosis when grown with GM-CSF and TGFβ_1 but not with M-CSF and TGFβ_1. Some of the effects of TGFβ_1 on AML may

therefore occur through programmed cell death.

TGFβ_1 failed to inhibit G-CSF-induced proliferation in MNC from one case of 2ry AML. This may have been due to a reduction in the numbers of high affinity binding sites for the cytokine (Masuya et al, 1993), compared with the numbers seen in de novo AML.

TGFβ_1 decreases IL-6 production by stromal cells (Lagneaux et al, 1993). Since IL-6 is a co-factor for myelopoiesis (Montes Borinaga et al, 1990) the inhibitory effects of TGFß$_1$ may be mediated by reducing IL-6 production.

Serum from patients with thrombotic thrombocytopenic purpura inhibits the proliferation of immature haemopoietic progenitors. When an antibody to TGFβ_1 was added this inhibitory effect was partially reversed (Zauli et al, 1993). It is possible that the sera of leukaemic patients may also contain inhibitory factors that suppress normal haemopoiesis.

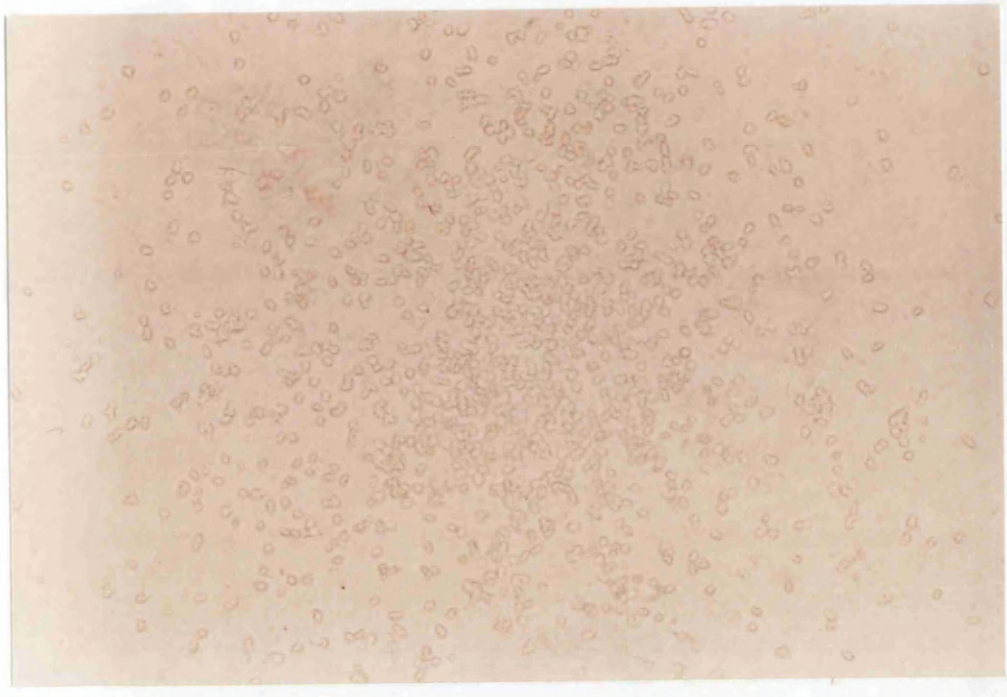
In normal MNC TGFβ_1 had no effect on CFU-GM stimulated by GM-CSF, IL-3 or G-CSF. In contrast TGFβ_1 significantly reduced G-CSF-induced colony formation by CML CP cell ($p<0.005$) and CML myeloid blast transformation cells. These results support the findings of other workers (Sing et al, 1988a; Aglietta et al, 1989). TGFβ_1 is thought to act on early myeloid progenitors (Sing et al, 1988a; Aglietta et al, 1989). Holyoake et al, 1993 using a clonogeneic assay which detects the human counterpart of the murine colony-forming unit, the CFU-A assay showed that the early progenitors from CML samples were inhibited by TGFβ_1 but showed no response with MIP-1α. In normal controls MIP-1α and TGFβ_1 inhibited the proliferation of CFU-A colonies.

In CML CP and AML samples G-CSF-induced CFU-C were augmented by the addition of IL-4. Other workers have shown that in normal MNC, IL-4 increased G-CSF-induced CFU-GM (Broxmeyer et al, 1988; Sonoda et al, 1990). Vellenga et al, 1990 showed that IL-4 augmented G-CSF-induced colony formation in normal and in 3/6 AML samples. By contrast IL-4 inhibited IL-3-induced colony formation in both normal and AML samples. Similar results were obtained with and without T-cell, B-cell and adherent cell depletion, suggesting that the effects of IL-4 were not mediated by accessory cells. By contrast IL-4 is thought to inhibit endogenous production of GM-CSF by blood mononuclear accessory cells (Sato et al, 1994). The possibility exists that IL-4 has different effects in association with different sources of accessory cells. The effect of IL-4 on G-CSF is on the early stages of proliferation (Sonoda et al, 1988). IL-4 inhibited G-CSF-induced proliferation of 2/4 CML myeloid cells. In mice with severe combined immunodeficiency (SCID) where *in vivo* growth of CML lymphoid blast transformation was established sub-cutaneous administration of IL-4 caused regression of the leukaemia (De Lord, personal communication). IL-4 also inhibits the growth of T-acute lymphoblastic leukaemia (Lowenberg and Touw, 1992). In the only patient in lymphoid blast transformation (UPN26) IL-4 had no effect on G-CSF-induced colony formation. It is possible that cells in blast transformation are saturated with endogenous IL-4 and the addition of further exogenous IL-4 does not augment cell proliferation. This is supported by the observation that certain leukaemic cell lines proliferate *in vitro* in the absence of growth factors (G.Christie, personal communication).

In CML chronic phase samples (see Table XXIII), TGFβ_1 reduced CFU-CML stimulated by G-CSF or by a combination of G-CSF and IL-4. Interleukin-2 is a helper cytokine for IL-4 production, and since TGFβ_1 impairs signal transduction through the IL-2 receptor (Holter et al, 1994), it may inhibit any synergistic effect that IL-4 and G-CSF have in combination.

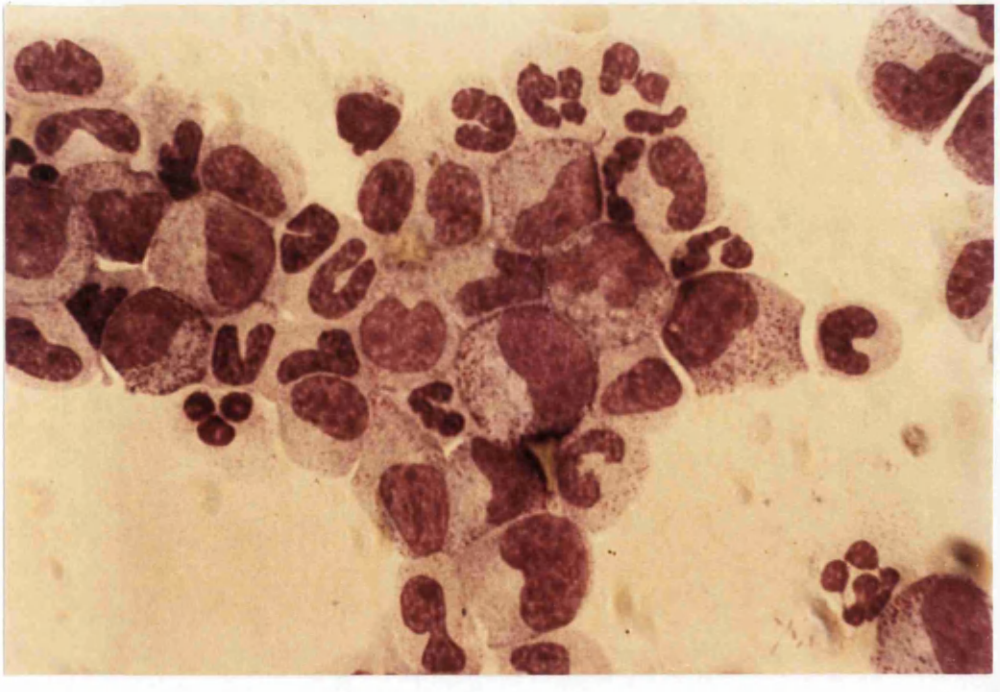
In CML chronic phase mononuclear cells IL-4 augmented G-CSF-induced proliferation whilst TGFß$_1$ had an inhibitory effect. The data suggests that in CML there may be a genetic change in the malignant clone which changes its response to TGFβ_1 and IL-4 as maturation occurs. Alternatively inhibition of G-CSF-induced proliferation by TGFβ_1 and augmentation of CFU-CML by IL-4 may reflect the distribution of cells along the myeloid lineage and the expansion of a specific progenitor cell compartment which has characteristics of both early and late progenitors. This compartment of cells may behave like early progenitors in their response to TGFβ_1 but like later progenitors in their response to G-CSF. If this is so, then in normal haemopoiesis there may be an equivalent sub-population of progenitor cells which are also inhibited by TGFβ_1 when G-CSF is the proliferative stimulus. Since TGFβ_1 had no significant inhibitory effect on the G-CSF-induced proliferation of samples from normal donors it seems likely that such cells would represent a small population in normal haemopoiesis and that any inhibitory effects of TGFβ_1 on G-CSF-induced proliferation are masked but in CML patients they form a larger part of the myeloid compartment.

Plate 1



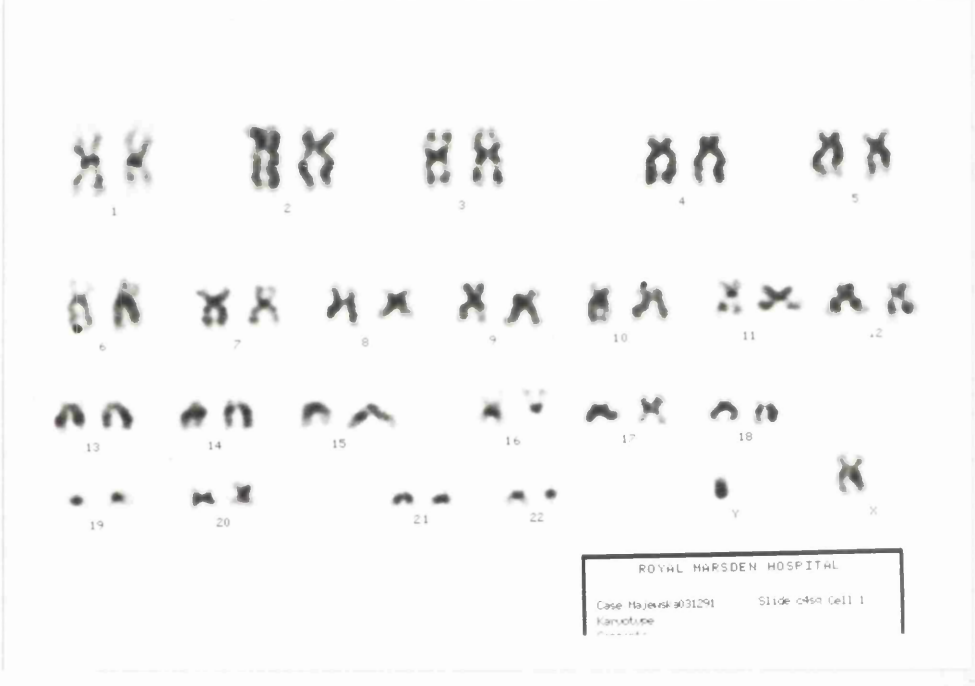
Peripheral blood CFU-CML from UPN 31 (CML CP), magnification x100

Plate 2



Cytospin of CFU-CML colonies from UPN 31, stained with May-Grunwald Giemsa, magnification x1000

Plate 3



Cytogenetic analysis on day 14 colonies from UPN 27 (CML BT).

The chromosomal abnormality was:-
46,XX,t(9;22)inv(16)p13q12or13,iso(17q)

CHAPTER 4: IMMUNOPHENOTYPIC STUDIES

CHAPTER 4

IMMUNOPHENOTYPIC STUDIES

Introduction

Immunophenotypic analysis in addition to morphological, cytogenetic and gene rearrangement studies have enabled more accurate characterisation of both normal and leukaemic cells.

In 1984, Civin et al, developed a mouse monoclonal antibody (anti-MY10) that bound to virtually all haemopoietic progenitors. Subsequently other groups discovered antibodies with similar specificities; 12.8, BI-3C5 and ICH-3 (Andrews et al, 1986; Tindle et al, 1985). These antibodies were subsequently assigned to the CD34+ cluster of differentiation (Hogg et al, 1987).

The gene encoding CD34+ has been localised to the long arm of chromosome 1 (Molgaard et al, 1989; Tenen et al, 1990). The human CD34+ gene spans 26-28 kilobases and the coding sequences have 8 exons (Satterthwaite et al, 1992; He et al, 1992). The human CD34+ gene shares significant coding sequence with the murine CD34+ gene which has also been cloned (Brown et al, 1991).

The CD34+ antigen is a glycoprotein with a molecular weight of 110kD (Civin et al, 1984). To date, the CD34+ antigen has not been attributed with any specific functions. Electron microscopy studies have shown that the CD34+ molecule is found predominantly at the endothelial junctions (Fina et al, 1990). These sites are crucial for leucocyte binding and migration to the

extravascular compartment. Thus CD34+ molecule may play a role in cell adhesion. The CD34+ molecule may also act as a binding site for lectins which are expressed on bone marrow stromal cells (Baumhueter at al, 1993). Hence the CD34+ molecule may play a role in early haemopoietic functions by mediating cell to cell or cell to stroma interactions.

The CD34+ antigen is expressed on 1-3% of normal bone marrow mononuclear cells (Andrews et al, 1986) and in about 0.1% of normal peripheral blood mononuclear cells (Bender et al, 1991). The CD34+ antigen is expressed early on in haemopoietic differentiation, all the colony-forming cells and both T and B-lymphocyte precursors express the CD34+ antigen (Civin et al, 1984; Andrews et al, 1986; Ryan et al, 1986; Srour et al, 1990). Subsets of CD34+ which do not coexpress any lineage associated antigens (lin-) have been identified, they do not form haemopoietic colonies *in vitro* but when cultured over irradiated stromal layers can give rise to multiple colonies of different cell types or alternatively to blast cell colonies (Sutherland et al, 1989; Andrews et al, 1990; Brandt et al, 1988). Hence CD34+ is expressed throughout the whole spectrum of haemopoietic cells, the expression however seems stage specific rather than lineage specific and decreases with cellular maturation. Primitive progenitors express high levels of the CD34+ antigen whilst the expression is weak or undetectable on cell that have matured beyond the committed progenitor stage (Sutherland et al, 1989: Strauss et al, 1986). CD34+ antigen is selectively expressed on haemopoietic stem cells and is not expressed on most non-haemopoietic cells, however it has been detected

on osteoclasts, marrow stromal progenitor cells, peripheral nerve sheath cells, fibroblastic cells and vascular endothelial cells (Holyoake and Alcorn, 1994). The HLA-DR antigen is expressed on both immature haemopoietic cells giving rise to CFC and on mature cells such as monocytes and B-lymphocytes. All CD34+ cells express the HLA-DR antigen but only a small proportion of HLA-DR+ cells are CD34+ (Civin et al, 1987), suggesting that the CD34+ antigen is expressed in early haemopoietic progenitors.

In AML, CD34 positivity predicts for a poorer outcome, with poorer response to induction treatment, lower response and survival rates (Vaughan et al,1988; Geller et al, 1990). Increased expression of the MDR-1 gene encoding for the P-170 glycoprotein, associated with drug resistance has been demonstrated in 50% of cases of AML. P-170 expression appears to be restricted to the CD34+ blast cell population and is significantly associated with lower remission rates (te Boekhorst et al, 1993). In B-ALL, CD34 positivity is associated with longer event free survival (Holyoake and Alcorn, 1994) but in T-ALL is associated with a increased likelihood of CNS disease (Pui et al, 1993).

The identification of the CD34+ antigen has been a major development to our understanding of haemopoiesis. Identification of this antigen in AML, may select out patients with a poorer prognosis and direct more aggressive treatments (such as bone marrow transplantation) early. However the real advance in haematology and oncology is likely to occur in the next few years when purified CD34+ cell populations will be used to enhance bone marrow

recovery after high dose chemotherapy and are likely to become targets for gene therapy. Transplantation using CD34+ cells has already been carried out (Shpall et al, 1994), but is likely to gain further popularity in the future.

Objective

The aims of immunophenotypic analyses in this study were 3 fold;

(1) to characterise surface antigen markers on CML and AML cell

(2) to isolate a primitive progenitor population (i.e CD34+) cells from patients with CML and AML and compare the effects of G-CSF, IL-4 and TGFβ_1 on these cells which have a higher clonogenecity with unmanipulated MNC (3) to quantitate the percentage of IL-4R on CML and AML cells and to correlate this with the data obtained from *in situ* hybridisation.

Results

The percentage of CD34+ cells from peripheral blood MNC, of patients with CML compared with disease stage, WBC count and CFU-CML obtained with 15ng of G-CSF is shown in **Table XXVI**, below.

UPN	STAGE OF DISEASE	% CD34+	WBC $x10^9/l$	CFU-CML
21	CML CP	1.0	87.2	8.0
22	CML CP	4.0	38.5	52.7
23	CML CP	20.0	69.0	23.0
24	CML CP	0.0	55.1	30.0
25	CML CP	4.0	48.0	15.3
30	CML CP	50.0	380.0	45.0
36	CML CP	2.0	123.0	104.0
38	CML CP	50.0	91.2	67.0
39	CML CP	2.0	250.0	0.0
51	CML CP	10.0	38.6	518.0
52	CML CP	90.0	98.9	0.0
53	CML CP	10.0	45.3	0.0
54	CML CP	20.0	350.0	0.0
55	CML CP	2.0	74.1	73.0
56	CML CP	10.0	38.4	0.0
26	CML lymphoid BT	10.0	26.5	11.0
34	CML myeloid BT	95.0	83.3	310.0
54b	CML myeloid BT	50.0	96.0	334.3
57	CML myeloid BT	80.0	81.0	0.0

Twelve of the 15 patients in chronic phase had CD34 positive cells of ≤20%. Three of the 15 patients in chronic phase had CD34 positivity ranging

from 50-95% (UPN30, 38 and 52). Plate 4 shows the appearance of CD34+ cells in UPN 38. The increase in CD34 positivity was correlated with satisfactory *in vitro* growth in UPN 30 and 38, but cells from UPN 52 which were 90% CD34+ did not grow *in vitro*. Among the 12 patients in CP with a low percentage of CD34 counts (≤ 20%) 8 produced colonies *in vitro* (UPN21, 22, 23, 24, 25, 36, 51, 55).

The 3 patients in myeloid blast transformation (UPN 34, 54b and 57) had a high percentage of CD34+ cells. Two of the 3 patients in myeloid blast transformation yielded satisfactory CFU-CML (UPN 34 and 54b). UPN 54b when in chronic phase (UPN 54) had a lower percentage of CD34 positivity, as might be expected.

A CD34+ count of >50% correlated with satisfactory CFU-CML numbers in all but two patient (UPN 52 and 57). The lack of *in vitro* growth is difficult to reconcile with the expected increased proliferative potential of these cells and may be a reflect a sub-population of cells, within the expanded population, which may for instance be CD34+/CD33- and are less likely to respond to G-CSF than CD34+/CD33+ cells (Ema et al, 1990).

The percentage of CD33, 34 and CD38 positive cells in CML PB MNC samples are shown in **Table XXVII**, below.

UPN	STAGE OF DISEASE	%CD34+	%CD33+	%CD38+	WBCx 10^9/l	CFU-CML
21	CP	1.0	10.0	70.0	87.2	8.0
22	CP	4.0	ND	90.0	38.5	52.7
23	CP	20.0	50.0	50.0	69.0	23.0
24	CP	0.0	50.0	1.0	55.1	30.0
25	CP	4.0	ND	ND	48.0	15.3
30	CP	50.0	ND	95.0	380.0	45.0
36	CP	2.0	ND	40.0	123.0	104.0
38	CP	50.0	10.0	40.0	91.2	67.0
39	CP	2.0	95.0	2.0	250.0	0.0
54	CP	20.0	20.0	50.0	350.0	0.0
55	CP	2.0	20.0	50.0	74.1	73.0
56	CP	10.0	30.0	50.0	38.4	0.0
26	LY BT	10.0	ND	ND	26.5	11.0
54b	MY BT	50.0	90.0	20.0	96.0	334.3
57	MY BT	80.0	0.0	50.0	81.0	0.0

The percentages have been correlated to the WBC count and the CFU-CML obtained with 15ng of G-CSF. ND - not done; CP - chronic phase; LY BT - lymphoid blast transformation; MY BT - myeloid blast transformation.

CD38 is an 45kD antigen found on 40% of BM MNC including myeloblasts and promyelocytes. Cells that coexpress CD34+ and CD38+ belong to a subset of lineage committed progenitors (Holyoake and Alcorn, 1994). Patients with CML may be expected to have a high percentage of these cells. Nine out of 11 patients in chronic phase had a CD38 positive count of

≥40%. UPN 24 had both a low CD38 (1.0%) and a low CD34 count (0.0%) but produced 30 CFU-CML/5x10^5 MNC with 15ng of G-CSF. UPN 39 too, had a low CD34 and CD38 count but unlike UPN 24 did not grow *in vitro.* Both these patients had not received any chemotherapy.

CD33 is a myeloid specific antigen, it binds strongly to monocytes and acute myeloid progenitor cells. It binds weakly to mature granulocytes. All 8 patients in chronic phase had a CD33+ count of ≥10%. Patients in myeloid blast transformation might be expected to have a high CD33+ count. This was so in UPN 54b, but not in UPN 57, who was also in myeloid blast transformation but was negative for the CD33 antigen.

***In-vitro* effects of various cytokines on CD34 purified cell populations**

CD34+ cells were purified from peripheral blood of CML patients as described in Chapter 2. The cells were plated at concentrations of between 5x10^3 and 8x10^4/plate. The concentration of cytokines used were as follows: G-CSF 15ng; IL-4 5ng; TGFβ_1 5ng; GM-CSF 50ng; IL-3 50ng. The results are shown in Table XXVIII, overleaf

Table XXVIII below, shows the effect of G-CSF alone and in combination with IL-4 and TGFβ_1 on CD34 enriched MNC from CML PB MNC.

UPN	STAGE	CELL NO	CD34%	G-CSF	G-CSF +TGFβ_1	G-CSF +IL-4	G-CSF +TGFβ_1 +IL-4
21	CP	$5x10^4$	1.0	1.0	0.0	5.3	2.3
22	CP	10^4	4.0	0.0	0.0	0.0	0.0
23	CP	$2x10^4$	20.0	71.0	ND	110.5	47.5
55	CP	$8x10^4$	2.0	160.3	24.0	136.0	48.67
25	CP	$5x10^3$	4.0	2.6	<1.0	1.6	1.6
24	CP	10^4	0.0	0.0	0.0	0.0	0.0
26	LY BT	$5x10^3$	10.0	0.0	0.0	0.0	0.0
36	CP	$2x10^4$	2.0	0.0	0.0	0.0	0.0
38	CP	$2x10^4$	50.0	0.0	0.0	0.0	0.0

The percentage of CD34 positivity prior to enrichment and the number of cells/plate are also shown. CP - chronic phase; LY BT - lymphoid blast transformation.

Only 2/9 CML samples produced colonies *in vitro* when CD34+ enriched cells were used (UPN 23 AND 55). IL-4 augmented G-CSF-induced colony formation in UPN 23 but not in UPN 55. TGFβ_1 had an inhibitory effect on G-CSF and G-CSF plus IL-4 induced colony formation in UPN 55. It also inhibited CFU-CML in cultures from patient UPN 23 stimulated with a combination of G-CSF and IL-4. Though sample numbers are small the data suggests that there are expanded sub-populations within the CD34+ compartment that are less likely to respond to G-CSF. During the enrichment process the CD34+/CD33+ cells which are more likely to be responsive to G-CSF may have preferentially been removed, compared to the CD34+/CD33- cells which are less likely to respond to G-CSF (Ema et al, 1990).

Enriched CD34+ cell population from UPN 55a gave rise to CFU-CML when stimulated by G-CSF, GM-CSF and IL-3 (mean colony numbers being 160, 118 and 75 respectively). In each instance TGFβ_1 reduced CFU-CML-stimulated by the 3 colony-stimulating factors (see Fig 17). The inhibitory effects of TGFβ_1 on G-CSF, GM-CSF or IL-3 stimulated, enriched CD34+ cells from CML patients was similar to its effects on non-enriched peripheral blood and bone marrow MNC from the same group of patients.

Figure 17, overleaf shows the effect of TGFβ_1 on G-CSF, GM-CSF and IL-3 induced cell proliferation using CD34+ cells from UPN 55.

Figure 17

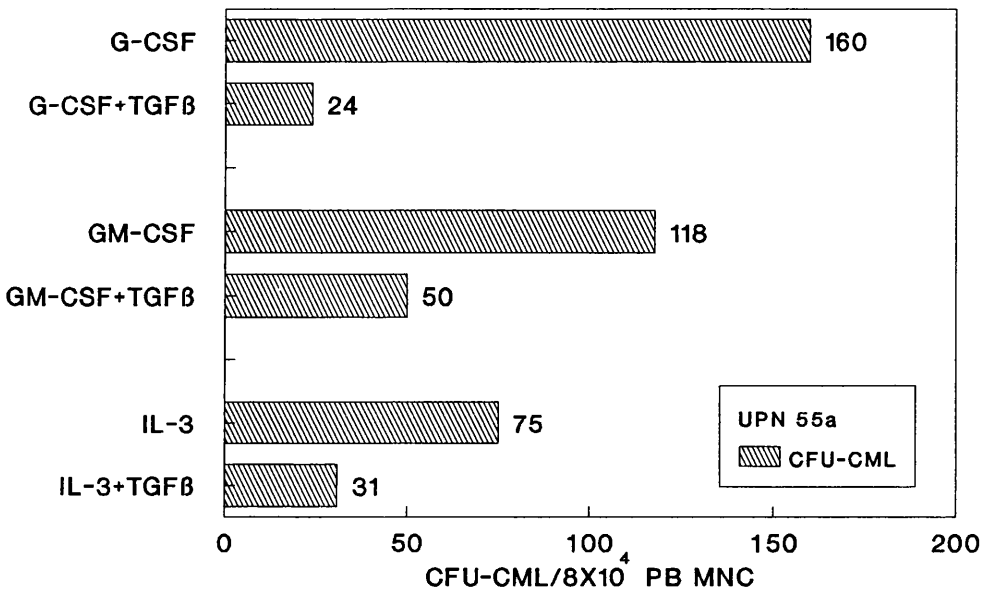


Figure 17

The effects of TGFβ_1 on G-CSF, GM-CSF and IL-3-induced cell proliferation of CD34 positive cells from UPN 55a (CML CP) are shown.

TGFβ_1 inhibited G-CSF, GM-CSF and IL-3-induced CFU-CML in each set of conditions.

Immunophenotyping in AML

The percentage of cells expressing the CD33, 34 and 38 antigen in AML peripheral blood MNC are shown in **Table XXIX**, below.

UPN	FAB-TYPE	%CD34	%CD33	%CD38	CFU-AML; 15ng G-CSF
40	2^{ry} AML	1.0	85.0	10.0	0.0
42	AML M3	90.0	50.0	90.0	0.0
44	AML M1	2.0	50.0	95.0	26.3
47	AML M2	70.0	ND	ND	58.7
48	2^{ry} AML	30.0	ND	ND	134.0

In the AML samples *in vitro* growth of CFU-AML could not be correlated to the percentage of CD34+ cells or the FAB classification. Patients with AML M2 and M3 have cells that are more differentiated than M1 and such cells may be expected to contain a low percentage of CD34+ cells, this was not reflected in the data. UPN44 a patient with AML M1 had 2% CD34+ cells whereas UPN42 and 47 who had AML M3 and M2 had 90% and 70% CD34+ respectively suggesting that the presence of the CD34 antigen is not confined to early progenitor cells in this disease.

Detection of the IL-4 receptor on the surface of CML and AML cells

Idzerda et al cloned the high affinity receptor for IL-4 in 1990 (Idzerda et al, 1990). Since then there has been much evidence to suggest that in

addition to the high affinity receptor there is a low affinity receptor for IL-4 (Foxwell et al, 1989). This receptor has thus far not been cloned. To indirectly detect the presence of the receptor on the surface of leukaemic cells a goat anti-human IL-4 neutralising antibody was used as described in Chapter 2. This antibody would bind to any endogenous IL-4 already bound to the receptor.

Objective

Since IL-4 augmented G-CSF-induced colony formation from normal, AML and CML chronic phase cells but reduced G-CSF-induced colony numbers in CML BT MNC experiments were done to determine whether there was quantitative differences between the expression of the IL-4R in CML, AML and normal MNC.

The percentage of cells expressing the receptor for IL-4 on CML MNC are shown in **Table XXX**, below. This number has been compared with CFU-CML numbers obtained with G-CSF, IL-4 and TGFβ. Normal or leukaemic MNC did not grow *in vitro* when stimulated with IL-4 alone. Samples were obtained from peripheral blood.

UPN	DISEASE	G-CSF	G-CSF+IL-4	G-CSF +TGFβ	G-CSF +IL-4 +TGFβ	% aIL-4R
21	CML CP	8.3	13.6	3.0	8.0	5.0
22	CML CP	52.7	71.7	22.3	93.7	0.0
23	CML CP	23.0	55.0	8.0	16.0	10.0
24	CML CP	30.0	41.7	8.0	26.7	5.0
36	CML CP	104.0	169.0	23.3	76.7	2.0
38	CML CP	67.0	75.0	21.7	35.0	50.0
39	CML CP	0.0	0.0	0.0	0.0	30.0
54	CML CP	0.0	0.0	0.0	0.0	1.0
55	CML CP	73.0	81.3	24.0	43.7	1.0
56	CML CP	0.0	0.0	0.0	0.0	50.0
34	CML BT	310.0	210.0	17.5	3.5	50.0
54b	CML BT	334.3	210.0	17.5	3.5	50.0
57	CML BT	0.0	0.0	0.0	0.0	10.0

Although IL-4 augmented G-CSF-induced colony formation CML cells in chronic phase there was no increase in CFU-CML in 3/4 patients in blast transformation (UPN 34, 35 and 54b, see Table XVIIIa). It could be speculated that in myeloid blast transformation clonogenic cells are saturated with endogenous IL-4 and that the addition of recombinant human IL-4 is unable to augment further CFU-CML. If this were the case the percentage

of cells from patients in blast transformation which express the IL-4R would be expected to be greater than in cells in chronic phase. However the percentage of CML MNC expressing the IL-4R did not correlate with the disease stage or CFU-CML. The 3 patients in blast transformation (UPN 34, 54b and 57) had 50%, 0% and 10% of MNC expressing the IL-4R. Three patients in chronic phase had a high percentage of cell expressing the IL-4R (UPN 38, 39 and 56). Plate 5 shows the expression of the IL-4R, in UPN 38. The data show that there was no correlation between the expression of IL-4R and enhanced colony numbers in cultures exposed to G-CSF and IL-4 compared to G-CSF alone, or that the expression of IL-4R on a significant number of MNC was indicative of proliferative potential *in vitro*.

Discussion

The results show that in CML, neither CD34 positivity nor the white cell count at diagnosis correlated with the growth of CFU-CML *in vitro* using G-CSF as the stimulus. In contrast Silvestri et al showed that the percentage of CD34+ cells in CML peripheral blood correlated with the growth of CFU-GM, although the growth factors used were not mentioned (Silvestri et al, 1991). Patients in blast transformation have more immature haemopoietic cells which would be expected to have a higher percentage of CD34+ cells. The 3 patients in myeloid blast transformation (UPN 34, 54b and 57) had a high percentage of CD34 positive cells (95%, 50% and 80% respectively).

The LTC-IC in CML CP peripheral blood are present at a much higher

concentration than in normal peripheral blood. Furthermore the LTC-IC in CML patients show features of proliferating or activated cells (CD34+ve, HLA-DR-ve, low Rhodamine-123 uptake and relative insensitively to 4-hydroperoxycyclophosphamide *in vitro)* compared with LTC-IC in normal peripheral blood which have features of a more quiescent cell population (Udomsakdi et al, 1992a; Udomsakdi et al, 1992b).

Nine of the 11 patients with CML ≥40% CD38+ cells and ≥10% CD33+ cells. Cells which express both the CD34 and CD33 antigens are committed to the myeloid lineage and can respond directly to G-CSF (Ema et al, 1990). Of the patients studied 5 had CD33 and 34 counts of ≥10% (UPN 23, 38, 54, 54b and 56). Three of these 5 patients produced CFU-CML *in vitro* (UPN 23, 38 and 56). However as the immunophenotyping was not done by dual staining it cannot be concluded that proliferation occurred from cells which were CD34+/CD33+. In normal bone marrow or peripheral blood only a minor subset of CD34+ cells coexpress CD33 antigen. The CD33 antigen is a lineage marker which is coexpressed with HLA-DR and CD13 on progenitors committed to the granulocyte-macrophage lineage (Pierelli et al, 1993). These authors also found a correlation between the percentage of CD34+/HLA-DR+ cells and the number of colony forming cells in unfractionated samples of BM and PB from patients with malignancies where there was no bone marrow involvement.

Geller et al in 1990, in a series of 96 patients with acute myeloid leukaemia found that CD33 and CD34 were expressed in 73% and 60% of

blasts respectively. Thus, a considerable proportion of AML blasts coexpress these antigens. The coexpression of CD33 and CD34 antigens on leukaemic blasts could reflect the neoplastic proliferation of a progenitor cell at the beginning of differentiation toward granulocyte-macrophage cells. This neoplastic clone retains a stem cell marker associated with a poor prognosis (Vaughan et al, 1988; Geller et al, 1990; Myint and Lucie 1992). Although spontaneous growth of AML cells *in vitro* has also been correlated to an adverse prognosis (Lowenberg et al, 1993) none of the AML samples in this study grew spontaneously *in vitro*.

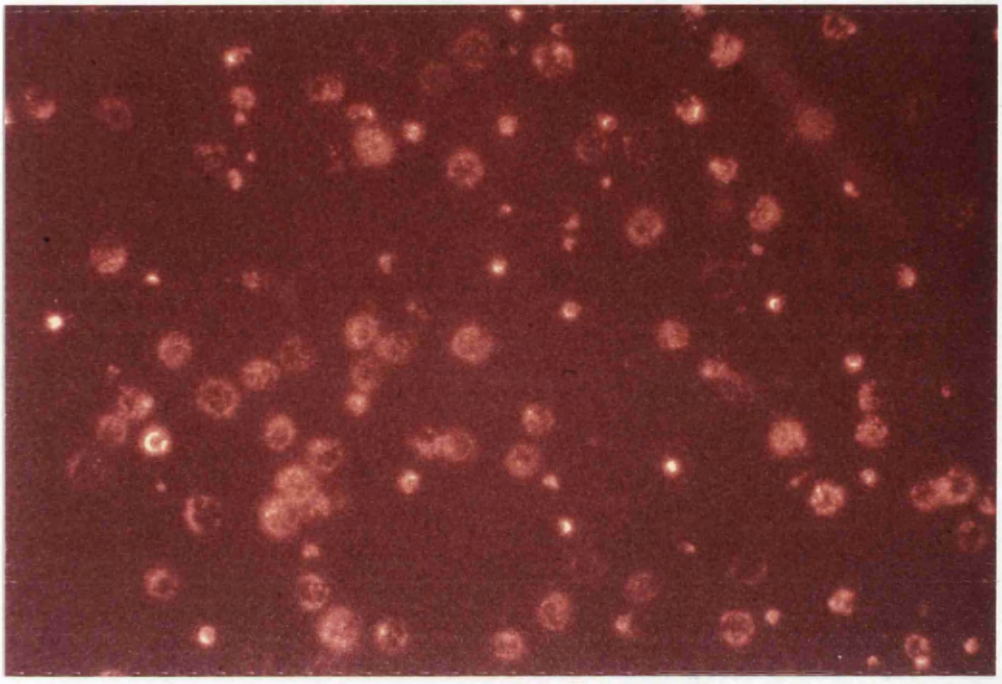
Akashi et al have shown that IL-4 alone stimulated *in vitro* growth of both CD34+ve and CD34-ve blasts from AML patients. The effects of IL-4 were enhanced by the addition of IL-3, GM-CSF and G-CSF. Expression of the IL-4R was not studied in these patients (Akashi et al, 1991).

Seven of the 13 CML patients studied had MNC expressing the IL-4R ($\geq$ 10.0%; of which 4 were in chronic phase and 3 in blast transformation). None of these samples grew *in vitro* when stimulated by IL-4 alone, although IL-4 increased the number of colonies in cultures exposed to G-CSF in one of these patients (UPN 23). There was no colony formation with either G-CSF or G-CSF combined with IL-4 in 3/7 samples. Furthermore there was a reduction in colony numbers when IL-4 was added to G-CSF from one patient in myeloid blast transformation (UPN 34).

Of the 8 CML samples in chronic phase only 2/8 grew *in vitro,* from purified CD34+ cells although unmanipulated MNC from all 8 patients grew

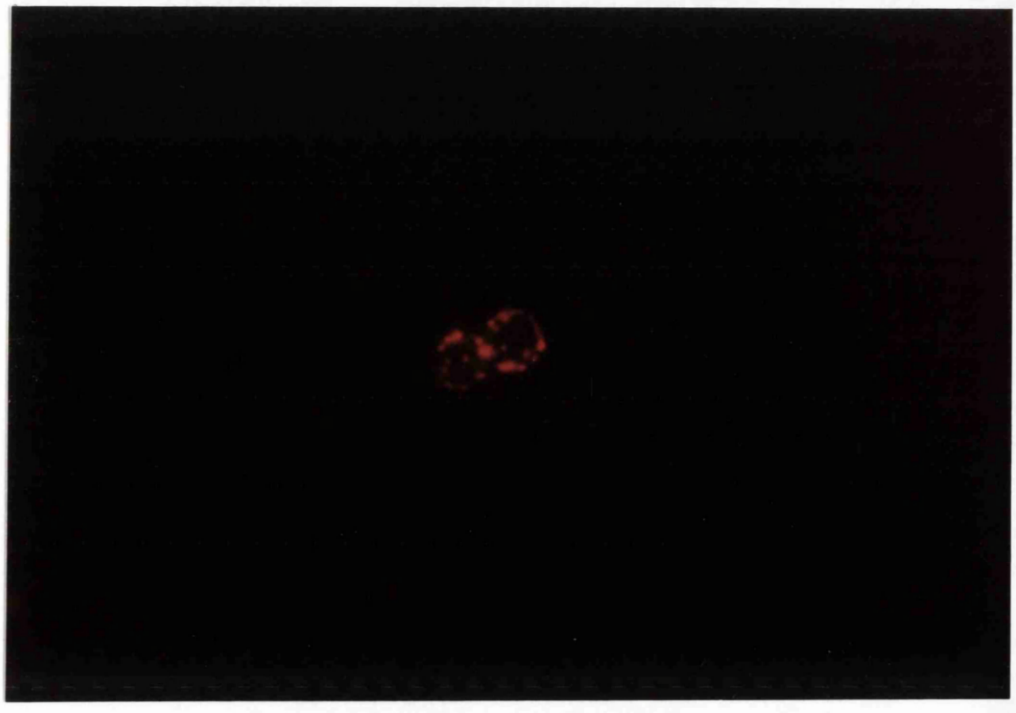
in vitro when stimulated by G-CSF or G-CSF plus IL-4. The data suggests that there may be significant inter-patient variation regarding the composition of the CD34+ compartment in CML. In normal haemopoiesis IL-4 synergizes with G-CSF to produce CFU-GM from CD34+ purified cells but inhibits GM-CSF and IL-3-induced colony formation (Snoeck et al, 1993). These effects were attributed predominantly to the differential response of CD34+/HLA-DR++ population compared with CD34+/HLA-DR+ cells to G-CSF (Snoeck et al, 1993). In CML there may be a similar population of CD34+/HLA-DR++ cells that are more likely to proliferate in response to G-CSF and IL-4.

Plate 4 - Immunophenotypic studies



Expression of CD34+ cells in PB MNC from UPN 38 (CML CP) magnification x1000

Plate 5



Expression of IL-4R on PB MNC from UPN 38, magnification x1000

CHAPTER 5: *IN-SITU* HYBRIDISATION

CHAPTER 5

IN-SITU HYBRIDISATION

Introduction

Over the past 30 years techniques employing immunological and molecular biological methods have had an increasing impact on the biological sciences and have resulted in an unparalleled increase in the number of growth factors, oncogenes, enzymes, peptide hormones, cell surface receptors and other proteins for which amino acid sequences and corresponding nucleotide sequences are known.

The past 17 years has seen the development of recombinant DNA techniques and sensitive methods for detection of specific DNA or RNA sequences by molecular hybridisation. Hybridisation can either be performed in solid supports, in solution (*in vitro*) and on tissue sections or cell preparations (*in situ*). The hybridisation techniques on solid supports involve the immobilisation of DNA fragments onto a filter (nitrocellulose or nylon membrane) followed by hybridisation onto a specific DNA probe (Southern blotting, Southern, 1975). Similarly different types of extracted RNA can be identified after electrophoretic separation and transfer onto a solid support prior to hybridisation (Northern blotting, Alwine et al, 1979). A dot blot analysis enables specific DNA or RNA sequences to be quantitated regardless of their length. Although molecular hybridisation *in vitro* or to membrane-bound nucleic acids allows the identification of DNA or RNA, it does not reveal the distribution of specific sequences in individual cells. *In situ*

hybridisation (ISH) was introduced in 1969 (Buongiorno-Nardelli and Amaldi 1969; Gall and Pardue 1969; John et al, 1969). It is also referred to as hybridisation histochemistry or cytological hybridisation. In contrast to the other methods available it enables the morphological demonstration of specific DNA or RNA sequences in individual cells in tissue sections, single cells or chromosome preparations. Thus ISH is the only method that allows the study of cellular location of DNA or RNA sequences in a heterogeneous population.

ISH is based on the principle that labelled single-stranded fragments of DNA or RNA containing complementary sequences (probes) are hybridised to cellular DNA or RNA under appropriate conditions forming stable hybrids. The sensitivity of ISH depends on the following variables: (a) the effect of tissue preparations on retention and accessibility of cellular DNA or RNA, (b)type of probe construct, efficiency of probe labelling and sensitivity of the method used for signal detection and (c) the effect of hybridisation conditions on the efficiency of hybridisation.

Detection of mRNA

The use of ISH for the detection of DNA is now well established. In the past decade the technique has been used to detect mRNA. The precursors of mRNA are transcribed from DNA in the nucleus by RNA polymerase II and are known as heterogeneous nuclear RNA. These molecules are processed into mature mRNA by the addition of the 5'-methyl cap and a 3'-tail of 200 adenyl residues (Perry, 1976). The transcripts are further processed by

removing the non-coding intron sequences by a process termed "splicing" in order to produce the complete mRNA which is transported from ribonuclear structures to the cytoplasm for translation. Therefore *in situ* hybridisation of mRNA can be sited within the nucleus or cytoplasm of the cell. mRNAs are generally very unstable being fairly readily degraded by RNAses. As cytoplasmic RNA represents only 3% of total cellular RNA their detection is dependent on the preservation of mRNAs by appropriate fixation of cells or tissue sections (Pringle et al, 1989)

Fixation

The process of fixation should retain the maximal level of cellular target DNA or RNA while maintaining morphological detail and allowing sufficient accessibility of probe. As mRNA is steadily synthesized and degraded enzymatically, for its localisation the type, time and concentration of the fixative are significant if loss of RNA is to be minimised. Paraformaldehyde a cross-linking fixative has been used in several studies using tissue sections (Brigati et al, 1983; Hafen et al, 1983; McAllister and Rock 1985, Hofler et al, 1986; Crabbe et al, 1992), though to date it has not been used successfully on bone marrow or peripheral blood MNC. Unlike glutaraldehyde, paraformaldehyde does not cross-link proteins so extensively as to prevent penetration of probes. Acetic acid-alcohol mixtures or Bouin's fixation are favoured by some workers (Gall and Pardue, 1971, Manuelidis et al, 1982; Berge-Lefranc et al, 1983). It must be emphasised that these fixatives were

used on tissue sections. Tissue sections and single-cell preparations must adhere to specially treated glass slides to avoid loss of cells during hybridisation. Gelatin chrome alum coated glass slides (Gall and Pardue, 1971), or treatment with poly-L-lysine (Huang et al, 1983) or 3-aminopropyltriethoxysilane (APES, Maddox and Jenkins, 1987) are the most commonly used.

Pretreatment of specimens with a detergent and/or proteinase digestion is standard procedure in most protocols, to increase probe penetration and accessibility. This process is of importance when dealing with paraffin embedded sections (Hofler, 1990). Triton X-100 and RNAse-free proteinase K are the most commonly used (Hofler, 1990). Lawrence and Singer (Lawrence and Singer, 1985) extensively studied the rate of RNA retention, influence of prolonged exposure to different fixatives and proteinase digestion on single-cell preparations. They concluded that paraformaldehyde fixation gave the highest specific signal and that detergent and proteinase pre-treatment is not always necessary.

Probes and labelling

Labelled DNA and RNA probes can be used to localise DNA and mRNA. Broadly there are 3 different DNA probes, double stranded DNA (ds DNA), single stranded DNA (ss DNA) and synthetic oligonucleotides. RNA probes used for the detection of mRNA are single stranded complementary RNA probes.

Double-stranded DNA probes: ds DNA probes are usually labelled by nick-translation or random priming. As the yield from random priming is low, it is not recommended for ISH where a large number of specimens are used. Synthetic oligodeoxyribonucleotides can be prepared from DNA synthesizers and have several advantages over cloned DNA probes, (a) a higher specificity (b) the possibility of synthesising probes from amino-acid sequences when the total DNA sequence is unknown and (c) the ability to generate discriminating sequences for similar genes (Hofler, 1990).

Single-stranded cDNA probes: With the introduction of the polymerase chain reaction (PCR) ssDNA probes with a high specificity can be generated (Gyllensten and Erlich, 1988). These probes have the theoretical advantage that re-annealing of the probe to the second strand cannot occur.

Single stranded anti-sense RNA probes: These probes can be synthesised using cDNA as a template in the presence of labelled or unlabelled ribonucleotides and RNA polymerase. These probes have a higher specificity and thermal stability than the DNA probes, in addition as they do not contain vector sequences they cause less non-specific binding (Hofler, 1990).

Antisense oligonucleotide probes: are made by sub-cloning of synthetic oligonucleotides (20-70 mers) into mRNA expression vectors. The probes are obtained by inserting specific cDNA sequences into an appropriate transcription vector containing an RNA polymerase promoter. Commonly used vectors include the plasmids pSP64 and pSP65, the Gemini vectors and Bluescript (Gibson and Polak, 1990). Sense and anti-sense probes can thus be

obtained by either using 2 separate plasmids or into those containing 2 different promoters on either side. This technique has several advantages, (a) it is easy to prepare large quantities of labelled probe (b) probes can be made for any gene whose protein sequence is known and (c) it has a high specificity (Chan and McGee 1990).

Labelling

There are 2 main types of labelling, direct labelling of a radioisotope onto the DNA or RNA and indirect where either a hapten (e.g digoxigenin or biotin) is attached to the probe and detected by a labelled binding protein (e.g. avidin) or the probe-hybrid is detected by a specific antibody. The most commonly used radioisotopes are ^{3}H, ^{32}P, ^{35}S, ^{14}C and ^{125}I. As a result of problems of safety, waste disposal and reduced stability non-isotopic probes have become more popular, the most favoured haptens being biotin and digoxigenin. The relative merits of the radioisotopes and the haptens are given below (from Hofler, 1990).

Label	Resolution	Sensitivity	Exposure (days)	Stability (weeks)
^{32}P	+	++	7	0.5
^{35}S	++	+++	10	6.0
^{3}H	+++	+++	14	>30
Biotin	+++	++	0.16	>52
Digoxigenin	+++	+++	0.16	>52

Hybridisation conditions

One of the main advantages of ISH is that the degree of specificity of the hybridisation reaction can be accurately controlled by varying the reaction conditions. The degree of specificity depends on the probe construction, temperature, pH and salt and formamide concentrations (Hofler, 1990). Stringency refers to the degree to which reaction conditions favour the dissociation of nucleic acid duplexes. Under conditions of high stringency only probes with a high degree of homology to the target sequence will form stable hybrids. At low stringency (i.e. reactions carried out at low temperature or high salt or low formamide concentrations) a probe may bind to sequences with only 70-90% homology thus resulting in non-specific binding. In general the maximum rate of hybridisation takes place at the melting temperature (T_m) minus 10-25^{0}C (Bonner et al, 1973). With oligonucleotide probes the rate of hybridisation rarely presents a problem, however it remains standard practice in most protocols to use overnight incubations and conditions approximating T_m-20^{0}C.

Objective

The purpose of the ISH studies was as follows: (1) to determine whether the mRNA for IL-4R and/or G-CSFR could be detected in normal, CML and AML peripheral blood and bone marrow samples (2) to determine whether TGFβ_1 down regulates the expression of the G-CSFR when CML MNC are incubated with both cytokines, (3) to determine whether the G-CSF

receptor is up-regulated by the addition of IL-4 to cultures containing G-CSF and (4) to determine if the inhibitory effects of TGFβ_1 were mediated via by the down-regulation of IL-4R mRNA in cultures of CML cells containing G-CSF, IL-4 and TGFβ_1.

To date there has been no published data on ISH being carried out on bone marrow or peripheral blood MNC. Several problems were encountered which have been detailed in Chapter 2. In total 3 different methods were tried however the results have been variable. The commonest problem was the fragility of bone marrow and peripheral blood MNC which was more marked in CML samples than normal or AML samples. Secondly in all 3 methods there was some non-specific staining which occurs independently of the addition of probe or antibody and which was localised predominantly to mature granulocytes.

Results

The development of a suitable method to examine individual mRNA *in situ* from MNC from peripheral blood and bone marrow formed a major part of the work and is ongoing. The results from the ISH studies using the 3 methods detailed in Chapter 2 are given below:

The results from ISH from Methods I, II and III are shown in **Table XXXI,** overleaf.

UPN	TYPE	RESULTS							
		I		11			III		
		CELLS	IL-4R	CELLS	IL-4R	G-CSFR	CELLS	G-CSFR	IL-4R
21	CP/PB	G	+	G	+	+	G	+	+
22	CP/BM	P	U/A						
23	CP/PB	F	N/S						
24	CP/PB	F	N/S				F	U/A	U/A
28	CP/BM	P	U/A						
29	CP/PB	P	U/A				G	N/S	N/S
39	CP/PB	P	U/A						
51	CP/PB						G	N/S	N/S
54	CP/PB	P	U/A						
55	CP/PB	F	N/A	G	N/S	N/S	G	N/S	N/S
56	CP/PB	G	N/A	P	U/A	U/A			
34	BT/PB	F	-	P	U/A	U/A			
54b	BT/PB	F	N/A	F	N/S	N/S			
57	BT/PB						F	N/S	N/S
42	M3/PB	P	U/A						
44	M1/PB	F	+						
47	M2/PB	P	U/A						
18	NPB						G	+	+
58	NBM	F	N/S						
59	NBM	P	N/S						
60	NPB	P	U/A						
61	NBM	P	U/A				G	+	+

Disease state CP/PB - CML chronic phase peripheral blood; CP/BM - CML chronic phase bone marrow; BT/PB - CML blast transformation peripheral

blood; M1/PB, M2/PB, M3/PB - are peripheral blood cells from patients with AML M1, M2 and M3; NBM - normal bone marrow; NPB - normal peripheral blood; Cells - describes the cellular integrity G - good, F - fair, P - poor; Expression of the mRNA for the IL-4R and G-CSFR was assessed as + - positive; - negative; U/A - unable to assess; N/S - non-specific binding.
Briefly the differences in methods were due to the method of fixation and/or pre hybridisation/hybridisation conditions; in Method I a 1:1 mixture of acetone and methanol, followed by 0.2% paraformaldehyde was used, whereas in Methods II and III the slides were fixed in 1:1 mixture of acetone-methanol only. In both methods I and II samples were exposed to formamide and salmon sperm before hybridisation with the probes, in Method III the prehybridisation step was omitted and a thermal cycler was used to provide the required stringency.
Results I,II and III - results from methods I, II and III respectively.

Several problems were encountered doing ISH on PB and BM MNC. Non-specific binding was seen in all 3 methods but at a reduced intensity with Method III. Paraformaldehyde even at a low concentration of 0.2% caused cellular destruction. Of the various fixatives tried the combination of acetone and methanol was best at retaining cellular integrity.

Plates 7 shows the expression of the G-CSF receptor using ISH in UPN 18 (normal PB), plate 6 shows the "no probe" negative control. Plate 9 and 10 shows the expression of the G-CSF receptor and IL-4 receptor in UPN 21 (CML CP PB) and plate 8 shows the "no probe" negative control.

To determine the effect of pre-incubation of MNC with growth factors, 5ml liquid cultures containing 10^6 MNC/ml from UPN 21, 24, 29, 51 and 55 were incubated with the following cytokines (1) 5ng G-CSF/ml, (2) 5ng G-CSF/ml and 5ng TGFβ_1/ml, (3) 5ng G-CSF/ml and 5ng IL-4/ml and (4) 5ng G-

CSF/ml, 5ng TGFβ_1/ml and 5ng IL-4/ml. After overnight incubation ISH was carried out as described in Chapter 2 (method III). In samples from UPN 21 a positive signal was to IL-4R and G-CSFR was detected in samples exposed to antisense probes to the respective mRNA irrespective of pre-incubation conditions. In the 4 different culture conditions the expression of the G-CSF and IL-4 receptor were similar. In each instance a total of 200 cells were counted and the percentage expressing the relevant receptor calculated. The range of expression of the G-CSF receptor in the 4 culture media was 65% to 71% and the range of expression of the IL-4 receptor was 38% to 46%. The cells expressing the signal were mainly myelocytes and promyelocytes.

Discussion

The results from the clonogenic studies showed that IL-4 augmented G-CSF-induced proliferation of CML CP and AML cells but not CML blast transformation MNC. TGFβ_1 inhibited G-CSF-induced colony formation of both CML CP and BT MNC. G-CSF-induced colony-formation of normal and AML cells were not inhibited by TGFβ_1. To try and determine the cause of this difference *in situ* hybridisation techniques were used to study the expression of the G-CSFR and IL-4R. As the cDNA sequence for the class 1 and III receptors for TGFβ_1 have not been cloned, the expression of this receptor was not studied. Unfortunately the technical difficulties encountered with ISH meant that no consistent results were obtained.

Other workers have shown that the mRNA of gelsolin (an actin-binding

protein that is also a constituent of normal plasma) in a megakaryocytic cell line could not be directly related by ISH but was detectable by reverse transcriptase polymerase chain reaction (PCR) *in situ* (Sninsky, personal communication). Thus the technique is limited by the number of copies of a particular mRNA and the ability to detect the mRNA using histochemical methods. Among the samples tested by each of the methods only cells from UPN21, who had CML in CP, were positive using each method and for both receptors. The morphology of the cells expressing both receptors indicated that they were mainly promyelocytes and myelocytes. The results from the clonogenic assays from UPN 21 are summarised below:

G-CSF	G-CSF+IL-4	G-CSF+TGFβ_1	G-CSF+IL-4+TGFβ_1
8.3	13.6	3.0	8.0

The inhibitory effects by TGFβ_1 on G-CSF-induced proliferation of CFU-CML were partially reversed by IL-4. By ISH however the mRNA expression of G-CSFR and IL-4R were unchanged in cultures containing G-CSF, TGFβ_1 and/or IL-4 suggesting that the difference maybe due either to changes in translation or signal transduction rather than inhibition of mRNA synthesis. UPN 21 consistently gave a positive result regardless of the method used suggesting that the aberrant malignant clone in this patient produced multiple copies of mRNA for both the G-CSFR and IL-4R.

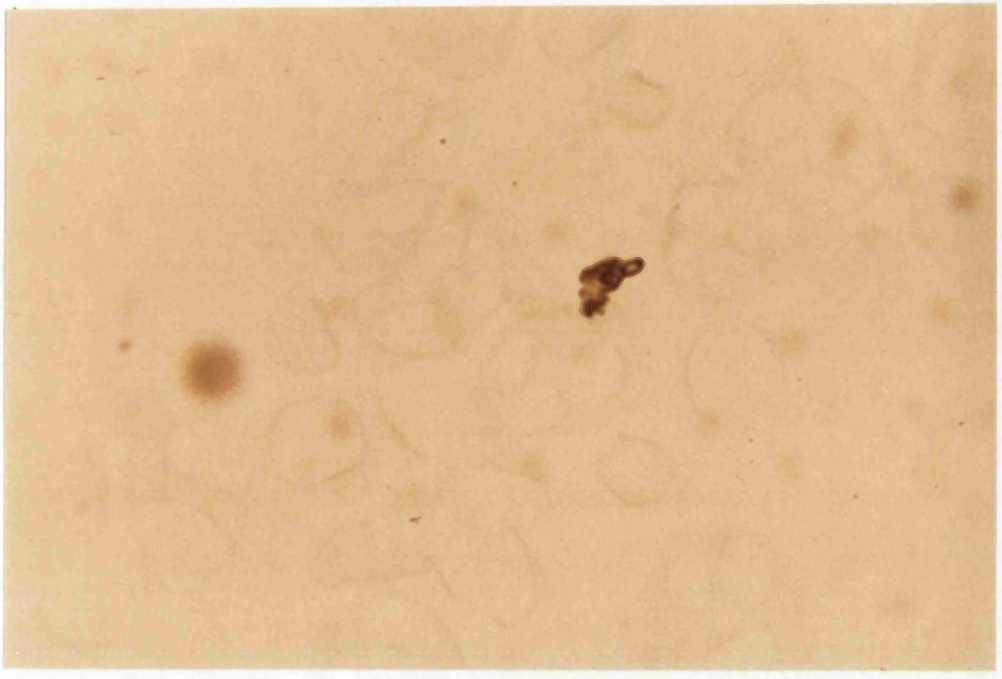
Peripheral blood from 2 normal donors, UPN 18 and 60, expressed the mRNA for the G-CSFR and IL-4R, the cells expressing the receptors were

mature neutrophils.

The cellular architecture was best maintained using Method III which avoided the use of paraformaldehyde. formamide and salmon sperm. In 6/8 samples studied using this method good cell morphology was retained, this contrasts with Method 1 where only 2/18 samples had good cellular integrity.

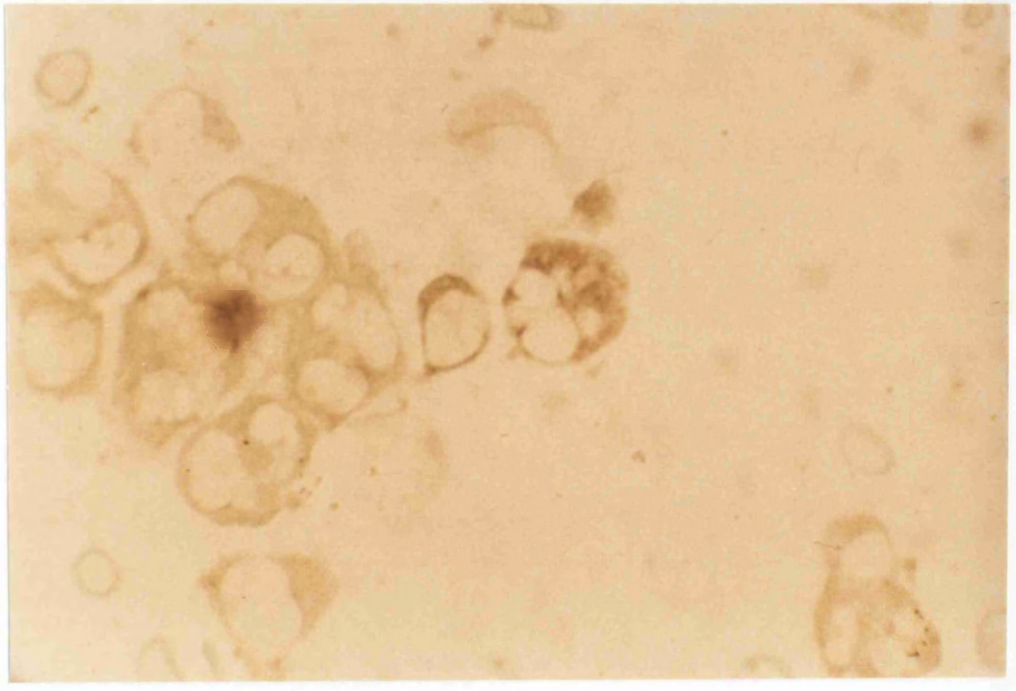
Although ISH studies using cytospin preparations of MNC is still at the developmental stage results suggest that in samples containing multiple copies of mRNA for specific receptors the use of DNA probes may enable their localisation within specific cells in heterogeneous populations. In summary, the effects of TGFβ_1 and IL-4 on G-CSF-induced stimulation of CFU-CML may be due to a difference in signal transduction or protein synthesis rather than down-regulation of the relevant receptor.

Plate 6 - *In situ* hybridisation studies
(using Method III)



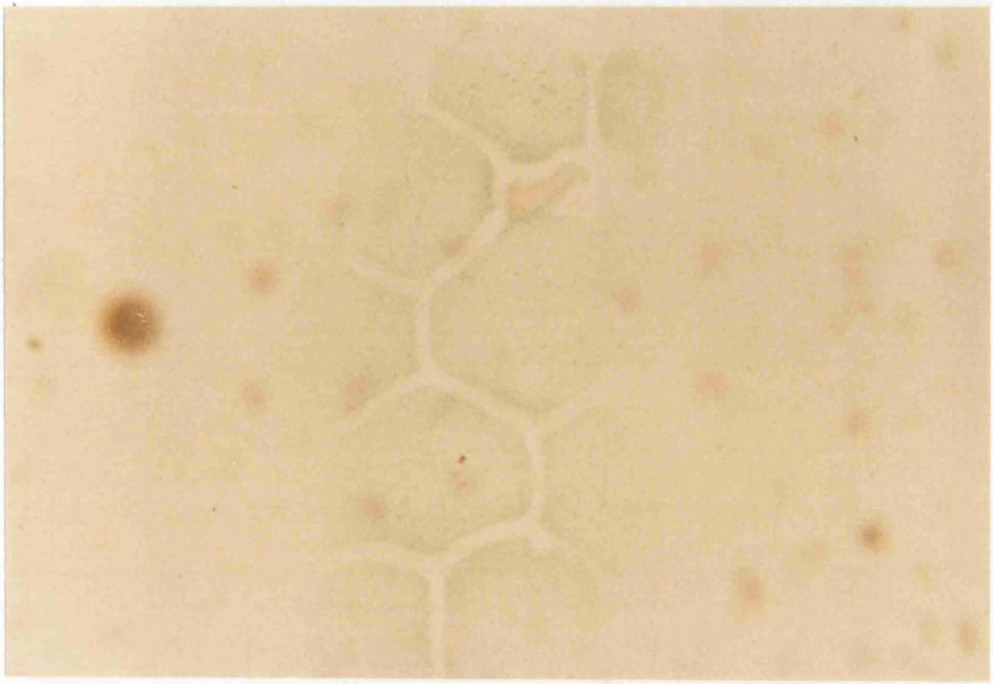
Negative control (No probe, no antibody) from normal PB MNC (UPN 18), magnification x1000

Plate 7



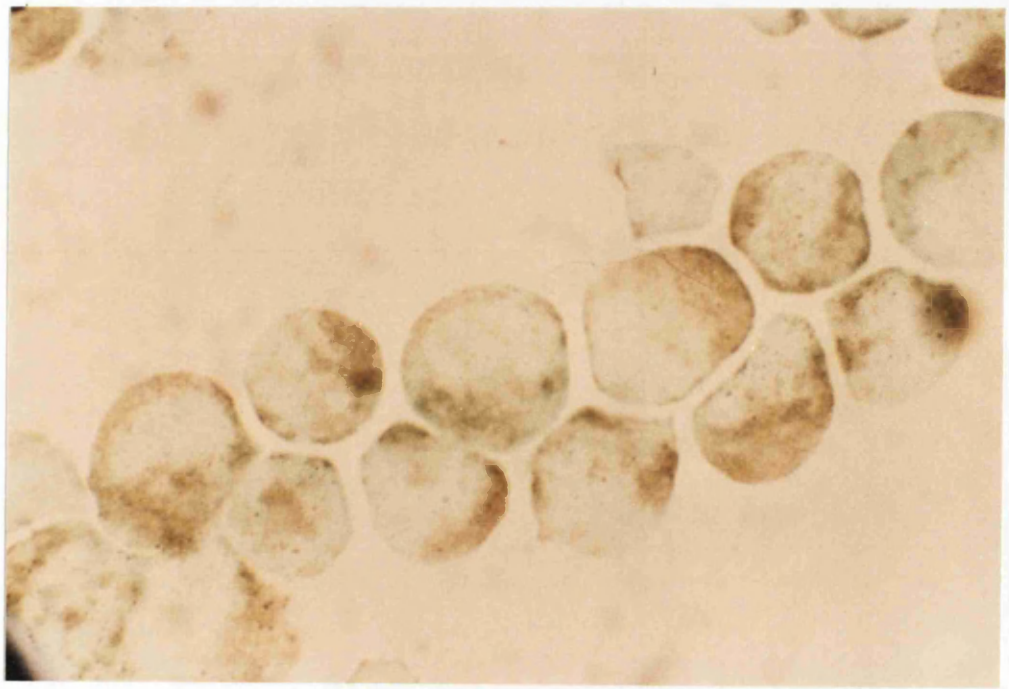
Expression of G-CSFR on normal PB MNC (UPN 18) magnification x1000

Plate 8 - *In situ* hybridisation studies
(using Method III)



Negative control (no probe, no antibody) from CML CP peripheral blood MNC (UPN 21), magnification x1000

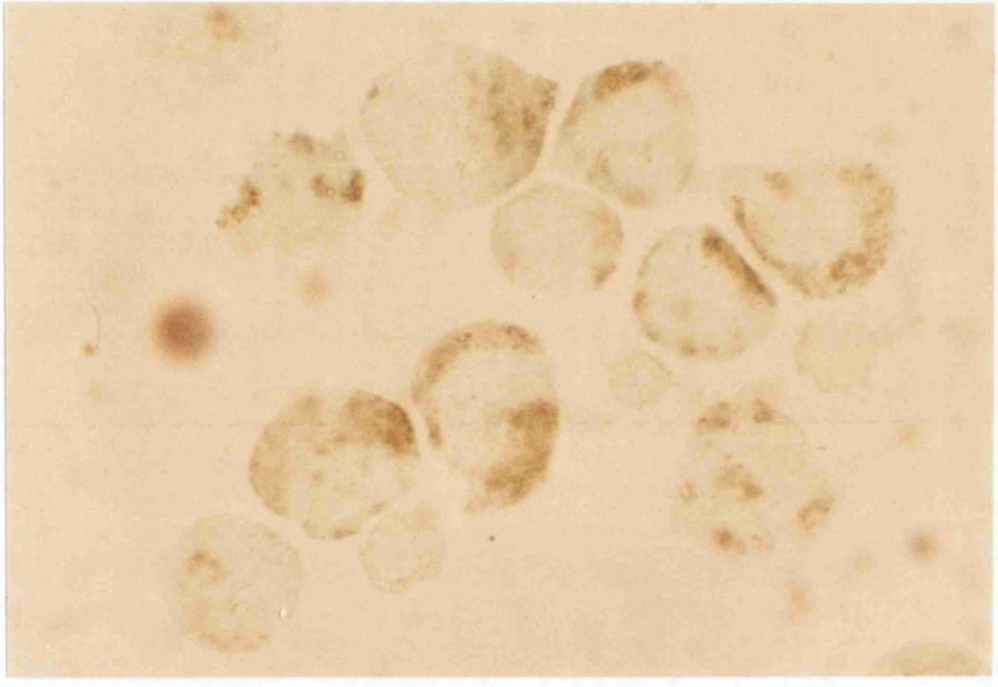
Plate 9



Expression of G-CSFR in CML CP peripheral blood MNC from UPN 21, magnification x1000

Plate 10 - *In situ* hybridisation studies

(using Method III)



Expression of IL-4R from CML CP peripheral blood MNC (UPN 21), magnification x1000

CHAPTER 6

SUMMARY OF CONCLUSIONS

Table XXXII below, summarises the results from the clonogenic assays

	Normal MNC	CML CP MNC	CML BT MNC	AML MNC
G-CSF	+	++	++	+
GM-CSF	+	+	++	+
IL-3	+	+	+	+
G-CSF+TGFβ_1	→	↓	↓	V
GM-CSF+TGFβ_1	→	↓	↓	-
IL-3+TGFβ_1	→	↓	↓	-
G-CSF+IL-4	→	↑	V	↑

↑ - colony numbers increased; ↓ - colony numbers decreased
→ - colony numbers unchanged; + and ++ - increased proliferative response to the 3CSF's. V - variable

Chronic myeloid leukaemia and acute myeloid leukaemia are pathologically and clinically distinct diseases. This study provides evidence that the proliferation of mononuclear cells from patients with CML are inhibited by TGFβ_1 *in vitro* irrespective of the proliferative stimulus whereas there is no consistent inhibition in either normal or AML mononuclear cells. Although colony numbers from normal bone marrow and peripheral blood were low G-CSF, GM-CSF or IL-3 were equally effective in stimulating CFU-GM whereas with CML mononuclear cells G-CSF induced the best proliferative response *in vitro*.

The inhibitory effects of TGFβ_1 on CML cells occurred independently of whether the cells were obtained in chronic phase or blast transformation or the nature of the colony-stimulating factor used. In contrast, TGFβ_1 had no consistent effect on normal MNC or those from patients with AML.

TGFβ_1 can be an activator or inhibitor of haemopoietic progenitors depending on the target cell population (Ottamann and Pelus, 1988; Sing et al, 1988; Keller et al, 1988a). In normal haemopoietic progenitor populations the earliest pluripotent cells are largely quiescent, while a larger proportion of later, lineage-restricted cells are in active phases of the cell cycle. Hatzfeld et al, 1991 have shown that anti-sense oligonucleotides to TGFβ_1 significantly enhanced the frequency of colony formation by multi-lineage, early erythroid and granulocyte-monocyte progenitors, but it did not affect colony formation by late progenitors. Using limiting dilutional analysis they showed that autocrine TGFβ_1 is produced by a sub-population of early progenitors. Anti-sense oligonucleotides to retinoblastoma susceptibility gene (Rb) produced a similar effect to anti-sense oligonucleotides to TGFβ_1, releasing multi-lineage progenitors from quiescence. Furthermore, Rb anti-sense partially reversed the effects of exogenous TGFβ_1. Their data suggest that the inhibitory effects of TGFβ_1 may be mediated by interaction with the Rb gene. Hatzfeld et al, 1991, also showed that with a combination of growth factors including IL-3, IL-6, G-CSF and Epo, CFU-GEMM and early BFU-E could be enhanced 2-3 fold by antisense TGFβ_1 oligonucleotides or by anti-TGFβ_1 serum. Antisense oligonucleotides to TGFβ_1 may have a role in the future of amplification of

normal haemopoietic stem cells by release of intracellular control mechanisms that prevent them from leaving G_0. Li et al, 1994, showed that Steel Factor in combination with antisense oligonucleotides to TGFβ_1, in cultures of CD34+ bone marrow, enhanced colony formation that was more than additive compared to cultures containing the single agents, thus suggesting that Steel Factor and TGFβ_1 act through distinct pathways.

The effects of TGFβ_1 on CD34+ purified cells depend on the proliferative stimulus. Whilst early progenitors stimulated by IL-3 in combination with IL-1 or SCF, on addition of TGFβ_1 are arrested specifically in the G_1 phase of the second cell cycle, more committed progenitors stimulated by IL-3 alone are either not arrested or undergo a slow retardation of growth (Lardon et al, 1994). The presence of a subset of CD34+ progenitor cells unaffected by the anti-proliferative effects of TGFβ_1 suggests the point at which cells become in sensitive to TGF-$ß_1$ is located within the CD34+ compartment.

During the past 5 years there has been an escalation in the use of peripheral stem cell transplants (PBSCT) in association with high dose chemotherapy to facilitate the recovery from neutropenia. The rationale that higher doses of a cytotoxic agent might have greater anti-tumour effect than lower doses is self-evident, but the dose administered is usually limited by the toxicity of the cytotoxic agent used. For the majority of cytotoxic drugs the limiting toxicity is myelosuppression. In the early 1950s, clinical studies were performed to try and ameliorate myelosuppression (McFarland et al, 1959).

By todays standard the dose of cytotoxics used was small and marrow inoculum inadequate. Enthusiasm was re-awaked in the 1970s, since when the number of autologous bone marrow transplants has risen exponentially each year (Armitage, 1994). Autologous bone marrow transplantation is not without its problems. In particular, there are protracted periods of pancytopenia with its attendant morbidity, mortality and financial costs. The administration of haemopoietic growth factors after autologous BMT has only made a minor impact on these parameters (Armitage, 1994). The use of stem cells collected from the peripheral blood instead of bone marrow represents a considerable further technical advance. Stem cells were first noted to be increased following chemotherapy in 1976 (Richman et al, 1976). In 1988, it was reported that administration of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor resulted in the mobilization of progenitor cells into the circulation (Dursten et al, 1988; Socinski et al, 1988). Chemotherapy induced mobilization may increase CFU-GM 100-fold over baseline values, the combination of chemotherapy plus either GM-CSF or IL-3 expands (5 to 10 fold) the number of progenitors (Holyoake and Alcorn, 1994). A number of issues concerning PBSC transplants still require resolution. The optimal mobilization schedules have yet to be determined, the quality and quantity of circulating haemopoietic progenitors required for rapid, complete and sustained engraftment is uncertain, and finally the degree of tumour mobilization and hence contamination is not known, tumour contamination however has been described (Bird et al, 1994; Moss et al, 1992).

The benefits of PBSC transplants are clear and include avoidance of a general anaesthetic, more rapid haematological recovery (Peters et al 1989; Khwaja et al, 1992) and lower costs (as a result of shorter in-patient stays, less antibiotic use, lower blood and platelet support). In addition no operating theatre costs are incurred. Against these savings must be set the costs associated with priming chemotherapy if used, mobilizing growth factors, the leukapheresis procedures and any quality control assays. The cost savings using PBSC as compared to bone marrow in autologous transplants is estimated at £3500/patient (Ager et al, 1994).

PBSC can also be used for allogeneic transplants (Russell et al, 1993; Dreger et al, 1993). G-CSF (with no cytotoxics) appears to be safe for normal donors and the avoidance of a general anaesthetic is clearly an advantage. There is concern that PBSC collections would contain more T cells and give rise to more graft-versus-host disease. A variant of using allogeneic peripheral stem cells for transplant is the use of umbilical cord blood. Several studies indicate that human umbilical cord blood contains a higher proportion of primitive haemopoietic progenitors than bone marrow (Broxmeyer et al, 1992; Hows et al, 1992). The place of human umbilical cord blood as a source of marrow repopulating cells remains to be established, but the emerging data from related and unrelated umbilical cord blood transplants is encouraging. To date, 35 such transplants have been performed. Comprehensive data is available on 26 patients. The median duration to neutrophil recovery

($0.5x10^9/l$) was 23 days (range 12-46), and the median time to platelet recovery ($50x10^9/l$) was 44.5 days (range 15-105). There were 4 cases of graft failure. (Nichol et al, 1994).

The quantity and quality of circulating haemopoietic progenitors required for rapid and sustained engraftment is unclear, though a threshold dose of approximately $15\text{-}50x10^4/kg$ CFU-GM has been recommended (Holyoake and Alcorn, 1994). Accurate measurement of CD34+ cell numbers may help to more accurately predict the most effective time to initiate apheresis procedures and also to avoid undesirable collections. The number of CD34+ progenitors obtained from mobilized peripheral blood may exceed that obtained from bone marrow 10 to 20-fold. Several groups have now shown that there also appears to be a correlation between CFU-GM numbers and CD34+ cell numbers obtained (Holyoake and Alcorn, 1994).

A further interest is the possibility of expanding PBSC *in vitro*. The potential advantages of *ex vivo* expansion are; reducing harvest time, expanding the most primitive cells required for long-term engraftment, which may be useful when performing autologous transplants from heavily pre-treated patients, the possibility of expanding normal haemopoiesis at the expense of the malignant clone and finally *ex vivo* expanded haemopoietic cells may be candidate cells for retroviral transduction and genetic manipulation. Brugger et al, 1993 studied the effects of several combinations of cytokines on a population of CD34+ enriched cells. Stem cell factor, epo, IL-1ß, IL-6, IL-3 and interferon-gamma were identified as the optimal combination of growth

factors for expanding both the total number of nucleated cells as well as clonogenic progenitor cells. The number of multilineage colonies increased 250-fold as did the absolute number of early progenitors (CD34+/HLA-DR-; CD34+/CD38-). Hence large scale expansion from small numbers of CD34+ cells is feasible and may be sufficient for repetitive use after multiple cycles of high-dose chemotherapy.

To demonstrate the potential for combined modality mobilization of PBSCs, Brugger et al, 1992, studied the effects of GM-CSF alone and in combination with IL-3. A greater number of progenitors were mobilized using GM-CSF and IL-3. Ganser et al, 1992 too showed that the number of progenitors giving rise to CFU-GM and BFU-E was enhanced when IL-3 was used with GM-CSF. Stem cell factor and G-CSF together or in combination with IL-3 may have a similar effect. Synergistic effects of combinations of growth factors is a rapidly expanding area and may show clinical benefit in the future.

In this study G-CSF and IL-4 had a synergistic colony-forming effect when CML cells in chronic phase were used. IL-4 had no effect on normal MNC stimulated by G-CSF (colony numbers however were low in all instances). In AML, the addition of IL-4 increased G-CSF-induced colony numbers. The number of AML samples studied were too small for this to be definitive. Further studies are required to determine the effects of IL-4 on G-CSF-induced CFU-GM of normal MNC, before G-CSF plus IL-4 can be used to mobilise PBSCs.

In CML, autologous transplants were carried out using unmanipulated peripheral blood cells collected at diagnosis, from patients in chronic phase (Hoyle et al, 1994). Recently autografts involving either *in vivo* or *in vitro* manipulations have evolved in the treatment of CML. Such approaches have been developed in an attempt to select benign haemopoietic progenitors and therefore restore normal, Ph negative haemopoiesis. The *in vivo* methods have involved giving chemotherapy and then collecting PBSCs mobilized into the circulation during the recovery phase. Various mobilizing regimens have been used including cyclophosphamide 5-7gm/m^2, ICE (idarubicin, cytarabine and etoposide), (Tringali et al, 1994, Carella et al,1991). The optimum mobilization regimen has yet to be defined and the follow up period is too short in this group of patients. What is interesting, is that normal progenitors appear to predominate over CML cells, when PBSCs are collected after chemotherapy, the reasons for this are not apparent. MIP-1α has an inhibitory effect on normal but not CML progenitors (Eaves et al,1993; Holyoake et al, 1993). Appropriate scheduling of MIP-1α together with intensive chemotherapy might may have future clinical application in the treatment of CML, that results in improved anti-leukaemic effect without the associated risks of severe marrow aplasia. In this study TGFβ_1 had no apparent effect on normal progenitors, however others have reported an inhibitory effect of TGFβ_1 on normal haemopoietic cells (Sing et al, 1988). If the effects of TGFβ_1 are not preferential it is unlikely to have a role in the treatment of CML. In CML part of the genetic lesion could be loss of sensitivity to inhibitory signals leading to

deregulated proliferation of primitive progenitor cells. If the effects of negative inhibitors such as TGFß$_1$ are preferential then drug regimens designed to ablate or reduce the leukaemic progenitors becomes a real possibility. Other potential uses of the negative regulatory proteins include MIP-1α to protect stem cells against myelotoxic treatments and antisense TGFß$_1$ oligonucleotides to amplify the normal haemopoietic compartment either following chemotherapy or to enhance mobilization of stem cells for transplantation.

The Vancouver group have recently published their results for autografting for CML using cultured marrow cells (Barnett et al, 1994). Thirteen of 16 patients autografted in first chronic phase are alive 1.0 to 5.7 years after the autograft. 4/13 are in complete cytogenetic remission. Thus autografting with cultured marrow cells may result in Ph negative haemopoiesis in some patients.

Okabe et al, 1992 have shown that IL-4 has an inhibitory effect *in vitro* on Ph positive acute lymphoblastic leukaemia cells (encoding a 190kD tyrosine kinase). This inhibitory effect was not mediated by the induction of TNF-α or interferon but was associated with a decreased activity of intracellular protein-tyrosine kinase. A phase 1 study of daily i.v bolus IL-4 in patients with refractory malignancies (colon, renal, sarcoma) produced an array of side effects the most prominent of which were nasal congestion, periorbital oedema, fatigue, diarrhoea and capillary leak syndrome (Atkin et al, 1992). The side effects were dose related and became severe at a dose of 15µg/kg.

In this study, IL-4 had a variable effect on G-CSF-induced colony

formation from CML myeloid blast transformation mononuclear cells. In 2/4 patients colony numbers were reduced, in 1/4 it was increased and in the remaining patient CFU-CML induced by G-CSF were unchanged by the addition of IL-4. IL-4 inhibits the production of granulocyte-macrophage colony-stimulating factor by blood mononuclear accessory cells in normal human peripheral blood (Sato et al, 1994). As a negative regulator, this mechanism of action is different from TGFβ_1 which directly inhibits haemopoietic progenitor cells (Sing et al, 1988; Keller et al, 1988a). IL-4 suppresses the spontaneous proliferation of chronic myelomonocytic leukaemia by inhibiting their production of IL-6 and/or GM-CSF, both of which could act as an autocrine growth factor for chronic myelomonocytic cells (Akashi et al, 1991a). IL-4 could similarly have an inhibitory effect on CML blast transformation cells by inhibiting accessory cells or autocrine growth factors. As the prognosis for patients with CML in blast crisis is so poor (Goldman, 1989b) and as IL-4 may potentially be of benefit further *in vitro* studies of the effects of this cytokine on CML blast transformation MNC are justified.

In patients with CML, the degree of CD34 positivity did not correlate with the WBC at diagnosis or the growth of CFU-CML *in vitro*. Patients in chronic phase have the whole spectrum of myeloid progenitors and these more mature cells are less like to express the CD34+ antigen. By contrast, 3/3 patients in myeloid blast transformation had a high percentage of CD34+ cells. In AML cells the degree of CD34+ positivity at diagnosis correlates with a poorer prognosis (Geller et al, 1990).

In normal haemopoiesis cells expressing CD34+ antigen, constitute a heterogeneous population, many of the cells of which are already committed to a particular lineage (Sutherland et al, 1989). The antigens that define lineage commitment include CD10 and CD19 on pre-B lymphoid cells, CD5 and CD7 on pre-T lymphoid cells, CD13, CD33 and CD45RA on myeloid progenitors, CD41 on megakaryocytic progenitors and CD71 and CD45RO on erythroid precursors (Holyoake and Alcorn, 1994). A more primitive progenitor population can be defined that does not coexpress any lineage antigens and is CD34+, lin-, HLA-DR-, Rho^{dull}, CD45RO. The primitive nature of these cells is confirmed by their ability to establish haemopoiesis on fibroblast feeder layers (LTC-IC), (Sutherland et al, 1989). Recently Huang and Terstappen, 1992 reported the presence of 2 distinct subsets of stem cells from foetal human bone marrow. One subset was CD34+, CD38-, HLA-DR+ and could differentiate into all haemopoietic lineages. The other subset was more primitive and had the immunophenotype CD34+, CD38-, HLA-DR-. These cells could differentiate into haemopoietic precursors and could form stromal structures capable of supporting the differentiation of these precursors. These cells were termed "common (haemopoietic/stromal) stem cells". They could represent the first identification of a single cell capable of reconstituting haemopoietic cells and their microenvironment.

CD34+ cell populations can be isolated by fluorescence activated cell sorting (FACS), giving yields of high purity. Because of the large cell numbers required this technology is not suitable for collecting cells for transplantation

(Holyoake and Alcorn, 1994). Adequate numbers of cells for this purpose can be purified using several commercially available devices that are available. The most widely used system (CellPro) exploits the high affinity interaction between the protein avidin and the vitamin biotin. For positive selection cells are labelled with an biotinylated anti-CD34+ and then passed through a column of avidin-coated polyacrylamide beads. CD34+ cells are retained and can be recovered by gentle agitation (Holyoake and Alcorn, 1994).

Brenner et al, 1993 first showed using an neomycin resistance gene marker, that in autologous transplantation for AML or neuroblastoma an apparent remission marrow can contribute to relapse. Since then other workers have shown that there is tumour contamination of PBSC collections and this depends on the type of primary tumour, its stage and prior chemotherapy (Bird et al, 1994; Moss et al, 1992). A major advantage in using CD34+ cells for transplantation would be the possible reduction in contaminating tumour cells. In the majority of tumours, malignant cells contaminating the blood or bone marrow do not express the CD34+ antigen. Lebkowski et al, 1992 showed that CD34+ cells enriched from bone marrow to which defined numbers of radiolabelled tumour cells were added, were 99.9% depleted of tumour cells. The tumour cells studied included AML, neuroblastoma, breast cancer and small cell lung cancer. The potential benefits of selected CD34+ for transplantation include reduced tumour contamination, faster neutrophil and platelet engraftment times, reduced storage requirements, smaller volumes being re-infused to the patient thus reducing

the effects of DMSO toxicity and fluid overload. Positively selected PBSC transplants have now been carried out (Berenson et al, 1991, Shpall et al, 1994). It is likely that in the next decade the use of CD34+ will significantly reduce the period of pancytopenia following high-dose chemotherapy so that the procedure may be carried out in the out-patient clinic.

Dubois et al, 1990 showed that specific binding of IL-1 to growth factor dependent myeloid progenitor cell lines was inhibited by incubation with TGFβ_1. TGFβ_1 inhibits the ability of IL-3 to up-regulate IL-1 receptors and the expression of IL-1 receptors on lymphoid cells (Ruscetti et al, 1992). Thus the mechanism of growth inhibition by TGFβ_1 could be relevant to other surface receptors as well. Attempts to elucidate the mechanisms by which TGFβ_1 inhibited G-CSF and G-CSF plus IL-4-induced CFU-CML using *in situ* hybridisation was largely unsuccessful. The expression of the G-CSFR and IL-4R were established in one CML sample and 2 normal donor peripheral blood samples. In the CML sample the expression of the G-CSFR and IL-4R were unchanged in cultures stimulated with G-CSF, G-CSF and IL-4, and G-CSF in combination with both IL-4 and TGFβ_1. Clearly definitive results cannot be drawn from this data. It does however suggest that the inhibitory effects of TGFβ_1 may be mediated by a difference in signal transduction or protein synthesis rather than at the level of transcription.

Over the past decade the haemopoietic growth factors have provided new insights into the mechanisms of haemopoiesis and the differentiation of blood cells. Cytokines such as erythropoietin, G-CSF, GM-

CSF, IL-2 and IL-3 have been used in clinical trials and G-CSF, GM-CSF and erythropoietin have found niches in different clinical situations. Other growth factors such as IL-4 and TGFβ_1 have not been studied extensively in humans but hold promise for the future.

APPENDIX

TABLE 1: CLINICAL DETAILS OF PATIENTS WITH CML

UPN	AGE	SEX	DISEASE	Hb g/dl	WBC 10^9/l	Plat 10^9/l	Treatment
21	39	M	Ph CML CP	14.4	87.2	210	Nil
22	49	F	Ph CML CP	12.9	38.5	702	Nil
23	38	F	Ph CML CP	9.8	69	519	Nil
24	43	F	Ph CML CP	13.1	55.1	310	Nil
25	53	F	Ph^{-ve} CML	12.0	48.0	170	Nil
26	11	M	Ph CML BT	11.1	26.5	54	Yes chemo
27	31	F	Ph CML BT	9.0	2.9	115	Yes chemo
28	36	F	Ph CML CP	13.6	9.0	424	Nil
29	29	M	Ph CML CP	8.9	35.0	102	Yes Bus
30	39	M	Ph^{-ve} CML	7.7	380	207	Nil
31	31	M	Ph CML CP	10.2	22.6	496	Nil
32	47	M	Ph CML CP	11.5	65.1	371	Nil
33	32	M	Ph CML CP	12.3	53.0	222	Nil
34	24	M	Ph CML BT	15.1	83.3	688	Yes chemo
35	46	M	Ph CML BT	11.3	51.8	43	Yes A/G
36	34	F	Ph CML CP	11.4	123	348	Nil
37	36	M	Ph CML CP	14.0	52.0	253	Nil
38	49	M	Ph CML CP	12.9	91.2	127	Nil
39	33	M	Ph CML CP	13.8	250	589	Nil
51	37	F	Ph CML CP	10.1	38.6	946	Yes OH urea
52	48	M	Ph CML CP	15.1	98.9	490	Yes OH urea
53	41	F	Ph CML CP	13.2	45.3	380	Yes OH urea
54	49	F	Ph CML CP	7.2	350	98	No
54b	49	F	Ph CML CP	11.2	96.1	54	Yes chemo
55	31	F	Ph CML CP	13.0	74.1	298	No
56	46	M	Ph CML CP	10.0	38.4	378	Yes OH urea
57	36	M	Ph CML BT	9.9	81.0	32	Yes, chemo

UPN 26 - lymphoid blast transformation; UPN 34, 35, 54b and 57 - myeloid blast transformation

UPN 25 & 30 - Ph^{-ve} CML; A/G - autograft; Bus - busulphan; OH urea - hydroxyurea

TABLE II: CLINICAL DETAILS OF PATIENTS WITH AML

UPN	AGE	SEX	FAB TYPE	Hb g/dl	WBC 10^9/l	Plat 10^9/l	Treatment
40	35	F	2^{ry} AML	14.1	72.5	73.0	Yes
42	23	M	AML M3	4.5	137.0	71.0	Nil
44	69	4	AML M1	14.5	71.6	23.0	Nil
46	33	M	AML M4	9.5	43.8	10.0	Nil
47	21	F	AML M2	8.8	76.0	78.0	Nil
48	59	M	2^{ry} AML	10.5	4.1	15.0	Yes
49	41	M	AML M4	8.1	3.2	61.0	Nil

TABLE III: Composition of Eagles minimal essential medium (alpha-medium)

INGREDIENT	MEM mg/litre	INGREDIENT	MEM mg/litre
L-Alanine	25.00	L-Tyrosine	45.00
L-Arginine	126.4	L-Valine	46.90
L-Asparagine	50.00	Ascorbic acid	50.00
L-Aspartic acid	30.00	Biotin	0.10
L-Cysteine	89.74	Folic acid	1.00
L-Cystine	30.22	i-Inositol	2.00
L-Glutamic acid	75.00	Nicotinamide	1.00
L-Glutamine	292.00	Pyridoxine	1.00
Glycine	50.00	Riboflavin	0.10
L-Histidine	41.90	Thiamin HCl	1.00
L-Isoleucine	52.50	Vitamin B12	1.36
L-Leucine	52.50	$CaCl_2$	264.90
L-Lysine HCl	73.06	KCl	400.00
L-Methionine	14.90	$MgS0_4.7H_2O$	200.00
L-Phenylalanine	33.02	NaCl	6000.800
L-Proline	40.00	$NaHCO_3$	2000.00
L-Serine	25.00	$NaH_2P0_4.2H_2O$	158.3
L-Threonine	47.64	D-Glucose	1000.00
L-Tryptophan	10.20	Phenol red sodium salt	10.00

TABLE IV: COMPOSITION OF RPMI 1640 CULTURE MEDIUM

INGREDIENT	RPMI 1640 mg/litre	INGREDIENT	RPMI 1640 mg/litre
L-Arginine	200.00	L-Tyrosine	24.83
L-Asparagine	56.82	L-Valine	20.00
L-Aspartic acid	20.00	Biotin	0.20
L-Cystine	62.92	D-Calcium pantothenate	0.25
L-Glutamic acid	20.00	Choline Chloride	3.00
Glutathione	1.00	Folic acid	1.00
L-Glutamine	300.00	i-Inositol	35.00
Glycine	10.00	Nicotinamide	1.00
L-Histidine	15.00	p-Aminobenzoic acid	1.00
L-Hydroxyproline	20.00	Pyridoxine HCl	1.00
L-Isoleucine	50.00	Riboflavin	0.20
L-Leucine	50.00	Thiamin HCl	1.00
L-Lysine HCl	40.00	Vitamin B12	0.005
L-Methionine	15.00	$Ca(NO_3)_2$	69.48
L-Phenylalanine	15.00	KCl	400.00
L-Proline	20.00	$MgS0_4.7H_2O$	100.00
L-Serine	30.00	NaCl	6000.00
L-Threonine	20.00	D-Glucose	800.7
L-Tryptophan	5.00	Phenol red sodium salt	5.00

Figure 2

ctggactgca gctggtttca ggaacttctc ttgacgagaa gagagaccaa ggaggccaag
caggggctgg gccagaggtg ccaacatggg gaaactgagg ctcggctcgg aaaggtgaag
taacttgtcc aagatcacaa agctggtgaa catcaagttg gtgctatggc aaggctggga
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gcctgccttt taaaggcctg agctagctgg agaagagggg agggtccata agcccatgac
taaaaactac cccagcccag gctctcacca tctccagtca ccagcatctc cctctcctcc
caatctccat aggctgggcc tcccaggcga tctgcatact ttaaggacca gatcatgctc
catccagccc cacccaatgg ccttttgtgc ttgtttccta taacttcagt att

Sequence of human mRNA coding for granulocyte colony-stimulating factor receptor.
Source tissue: placenta
Source clone: HuGCSFR-25-1
From: Larsen, A., Davis, T., Curtis, B.M., Gimpel, S., Sims, J., Cosman, D., Park, L., Sorensen, E., March, C.J., Smith, C. (1990) Expression cloning of a human granulocyte colony-stimulating factor receptor: a structural mosaic of hematopoietin receptor, immunoglobulin and fibronectin domains. Journal of Experimental Medicine, 172, 1559-1570.
Submitted on 02-Oct-1990 on tape to the EMBL Data Library.

Figure 3

```
   1  GGCGAATGGA GCAGGGGCGC GCAGATAATT AAAGATTTAC ACACAGCTGG AAGAAATCAT
  61  AGAGAAGCCG GGCGTGGTGG CTCATGCCTA TAATCCCAGC ACTTTTGGAG GCTGAGGCGG
 121  GCAGATCACT TGAGATCAGG AGTTCGAGAC CAGCCTGGTG CCTTGGCATC TCCCAATGGG
 181  GTGGCTTTGC TCTGGGCTCC TGTTCCCTGT GAGCTGCCTG GTCCTGCTGC AGGTGGCAAG
 241  CTCTGGGAAC ATGAAGGTCT TGCAGGAGCC CACCTGCGTC TCCGACTACA TGAGCATCTC
 301  TACTTGCGAG TGGAAGATGA ATGGTCCCAC CAATTGCAGC ACCGAGCTCC GCCTGTTGTA
 361  CCAGCTGGTT TTTCTGCTCT CCGAAGCCCA CACGTGTATC CCTGAGAACA ACGGAGGCGC
 421  GGGGTGCGTG TGCCACCTGC TCATGGATGA CGTGGTCAGT GCGGATAACT ATACACTGGA
 481  CCTGTGGGCT GGGCAGCAGC TGCTGTGGAA GGGCTCCTTC AAGCCCAGCG AGCATGTGAA
 541  ACCCAGGGCC CCAGGAAACC TGACAGTTCA CACCAATGTC TCCGACACTC TGCTGCTGAC
 601  CTGGAGCAAC CCGTATCCCC CTGACAATTA CCTGTATAAT CATCTCACCT ATGCAGTCAA
 661  CATTTGGAGT GAAAACGACC CGGCAGATTT CAGAATCTAT AACGTGACCT ACCTAGAACC
 721  CTCCCTCCGC ATCGCAGCCA GCACCCTGAA GTCTGGGATT TCCTACAGGG CACGGGTGAG
 781  GGCCTGGGCT CAGTGCTATA ACACCACCTG GAGTGAGTGG AGCCCCAGCA CCAAGTGGCA
 841  CAACTCCTAC AGGGAGCCCT TCGAGCAGCA CCTCCTGCTG GGCGTCAGCG TTTCCTGCAT
 901  TGTCATCCTG GCCGTCTGCC TGTTGTGCTA TGTCAGCATC ACCAAGATTA AGAAAGAATG
 961  GTGGGATCAG ATTCCCAACC CAGCCCGCAG CCGCCTCGTG GCTATAATAA TCCAGGATGC
1021  TCAGGGGTCA CAGTGGGAGA AGCGGTCCCG AGGCCAGGAA CCAGCCAAGT GCCCACACTG
1081  GAAGAATTGT CTTACCAAGC TCTTGCCCTG TTTTCTGGAG CACAACATGA AAAGGGATGA
1141  AGATCCTCAC AAGGCTGCCA AAGAGATGCC TTTCCAGGGC TCTGGAAAAT CAGCATGGTG
1201  CCCAGTGGAG ATCAGCAAGA CAGTCCTCTG GCCAGAGAGC ATCAGCGTGG TGCGATGTGT
1261  GGAGTTGTTT GAGGCCCCGG TGGAGTGTGA GGAGGAGGAG GAGGTAGAGG AAGAAAAAGG
1321  GAGCTTCTGT GCATCGCCTG AGAGCAGCAG GGATGACTTC CAGGAGGGAA GGGAGGGCAT
1381  TGTGGCCCGG CTAACAGAGA GCCTGTTCCT GGACCTGCTC GGAGAGGAGA ATGGGGGCTT
1441  TTGCCAGCAG GACATGGGGG AGTCATGCCT TCTTCCACCT TCGGGAAGTA CGAGTGCTCA
1501  CATGCCCTGG GATGAGTTCC CAAGTGCAGG GCCCAAGGAG GCACCTCCCT GGGGCAAGGA
1561  GCAGCCTCTC CACCTGGAGC CAAGTCCTCC TGCCAGCCCG ACCCAGAGTC CAGACAACCT
1621  GACTTGCACA GAGACGCCCC TCGTCATCGC AGGCAACCCT GCTTACCGCA GCTTCAGCAA
1681  CTCCCTGAGC CAGTCACCGT GTCCCAGAGA GCTGGGTCCA GACCCACTGC TGGCCAGACA
1741  CCTGGAGGAA GTAGAACCCG AGATGCCCTG TGTCCCCCAG CTCTCTGAGC CAACCACTGT
1801  GCCCCAACCT GAGCCAGAAA CCTGGGAGCA GATCCTCCGC CGAAATGTCC TCCAGCATGG
1861  GGCAGCTGCA GCCCCCGTCT CGGCCCCCAC CAGTGGCTAT CAGGAGTTTG TACATGCGGT
1921  GGAGCAGGGT GGCACCCAGG CCAGTGCGGT GGTGGGCTTG GGTCCCCCAG GAGAGGCTGG
1981  TTACAAGGCC TTCTCAAGCC TGCTTGCCAG CAGTGCTGTG TCCCCAGAGA AATGTGGGTT
2041  TGGGGCTAGC AGTGGGGAAG AGGGGTATAA GCCTTTCCAA GACCTCATTC CTGGCTGCCC
2101  TGGGGACCCT GCCCCAGTCC CTGTCCCCTT GTTCACCTTT GGACTGGACA GGGAGCCACC
2161  TCGCAGTCCG CAGAGCTCAC ATCTCCCAAG CAGCTCCCCA GAGCACCTGG GTCTGGAGCC
2221  GGGGGAAAAG GTAGAGGACA TGCCAAAGCC CCCACTTCCC CAGGAGCAGG CCACAGACCC
2281  CCTTGTGGAC AGCCTGGGCA GTGGCATTGT CTACTCAGCC CTTACCTGCC ACCTGTGCGG
2341  CCACCTGAAA CAGTGTCATG GCCAGGAGGA TGGTGGCCAG ACCCCTGTCA TGGCCAGTCC
2401  TTGCTGTGGC TGCTGCTGTG GAGACAGGTC CTCGCCCCCT ACAACCCCCC TGAGGGCCCC
2461  AGACCCCTCT CCAGGTGGGG TTCCACTGGA GGCCAGTCTG TGTCCGGCCT CCCTGGCACC
2521  CTCGGGCATC TCAGAGAAGA GTAAATCCTC ATCATCCTTC CATCCTGCCC CTGGCAATGC
2581  TCAGAGCTCA AGCCAGACCC CCAAAATCGT GAACTTTGTC TCCGTGGGAC CCACATACAT
2641  GAGGGTCTCT TAGGTGCATG TCCTCTTGTT GCTGAGTCTG CAGATGAGGA CTAGGGCTTA
2701  TCCATGCCTG GGAAATGCCA CCTCCTGGAA GGCAGCCAGG CTGGCAGATT TCCAAAAGAC
2761  TTGAAGAACC ATGGTATGAA GGTGATTGGC CCCACTGACG TTGGCCTAAC ACTGGGCTGC
2821  AGAGACTGGA CCCCGCCCAG CATTGGGCTG GGCTCGCCAC ATCCCATGAG AGTAGAGGGC
2881  ACTGGGTCGC CGTGCCCCAC GGCAGGCCCC TGCAGGAAAA CTGAGGCCCT TGGGCACCTC
2941  GACTTGTGAA CGAGTTGTTG GCTGCTCCCT CCACAGCTTC TGCAGCAGAC TGTCCCTGTT
3001  GTAACTGCCC AAGGCATGTT TTGCCCACCA GATCATGGCC CACGTGGAGG CCCACCTGCC
3061  TCTGTCTCAC TGAACTAGAA GCCGAGCCTA GAAACTAACA CAGCCATCAA GGGAATGACT
3121  TGGGCGGCCT TGGGAAATCG ATGAGAAATT GAACTTCAGG GAGGGTGGTC ATTGCCTAGA
3181  GGTGCTCATT CATTTAACAG AGCTTCCTTA GGTTGATGCT GGAGGCAGAA TCCCGGCTGT
3241  CAAGGGGTGT TCAGTTAAGG GGAGCAACAG AGGACATGAA AAATTGCTAT GACTAAAGCA
3301  GGGACAATTT GCTGCCAAAC ACCCATGCCC AGCTGTATGG CTGGGGGCTC CTCGTATGCA
3361  TGGAACCCCC AGAATAAATA TGCTCAGCCA CCCTGTGGGC CGGGCAATCC AGACAGCAGG
3421  CATAAGGCAC CAGTTACCCT GCATGTTGGC CCAGACCTCA GGTGCTAGGG AAGGCGGGAA
```

Sequence of mRNA coding for human interleukin-4 receptor.
Source: peripheral blood T-cells
Source clone: T22-8
From: Idzerda, R.L., March, C.J., Mosley, B., Lyman, S.D., Gimpel, S.D., Din, W.S., Grabstein, K.H., Widmer, M.B., Park, L.S., Cosman, D., Beckmann, M.P. (1990) Human interleukin-4 receptor confers biological responsiveness and defines a novel receptor superfamily. Journal of Experimental Medicine, 171, 861-873.
Submitted to EMBL Data Library on 02-Nov-1990.

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