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ASPECTS OF BLAST CELL PROLIFERATION IN ACUTE MYELOBLASTIC LEUKAEMIA

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Leukaemia was first documented by Hippocrates in 430 BC, when he related the symptoms of pallor and weakness to corruption of blood "Phthore Haimatos". Despite this early clinical diagnosis it was not until some two thousand years later, with the advent of microscopy, that the morphological basis of the disease could be studied. In stark contrast the last three hundred years has seen an exponential increase in the amount of information acquired concerning the disease.

Strict regulation of the differentiation and proliferation of the progenitor cells seen in normal haemopoiesis is absent in leukaemia. It is the accumulation of immature blasts in this malignant condition which will stifle normal haemopoiesis and eventually cause death. In an effort to improve treatment of leukaemia the biological differences between leukaemic blasts and normal haemopoietic cells is where current knowledge needs to be improved. This thesis will attempt to enhance this knowledge by studying the proliferation characteristics of leukaemic blast cells in the hope that as a small piece of a rather large jigsaw puzzle it will find its rightful place and one day the whole picture will be complete.

ABSTRACT

ASPECTS OF BLAST CELL PROLIFERATION IN ACUTE MYELOBLASTIC LEUKAEMIA.

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The transferrin receptor expression (TfR) of acute myeloblastic leukaemia (AML) blast cells was investigated. Whilst a high percentage ($\geq$58%) of AML blast cells were found to express TfRs the degree of TfR expression within each patient's blast cell population was heterogeneous. Suspension culture of the AML blast cells also revealed that their proliferation, as assessed by DNA synthesis, was proportional to their TfR expression.

A fluorescent activated cell sorter allowed AML blast cell populations to be separated into two fractions on the basis of their TfR expression. The fractions with the highest TfR expression were found to contain the majority of blast colony forming cells (AML-CFU). These results indicate AML-CFU are highly proliferative and express high levels of TfR which supports the concept that AML-CFU function as leukaemic stem cells.

AML blast cell proliferation was significantly increased ($p \leq 0.05$) at low cell concentrations ($\leq 62.5 \times 10^3$/ml) in round bottomed wells when compared to flat bottomed wells. The enhanced proliferation in round bottomed wells was abrogated when cell to cell contact was inhibited by the addition of latex beads. Following short term suspension cultures of AML blast cells at a low cell concentration(31.25×10^3/ml) a significantly ($p \leq 0.05$) greater number of AML-CFU were recovered from round bottomed wells than from flat bottomed wells. These results demonstrate AML blast cell proliferation is enhanced in crowded cultures and that this phenomenon requires direct cell to cell contact.

As some AML blast cells displayed autonomous proliferation an Autostimulatory Index (ASI) was calculated (no. of colonies without stimulation ÷ no. of colonies with stimulation) and then used to classify patients into four groups. Group 1 non-growers; Group 2 require stimulation (ASI<0.1); Group 3 partially autonomous (ASI 0.1-0.8); Group 4 totally autonomous (ASI>0.8).

Group 3 and Group 4 patients blast cells produce GM-CSF which may regulate their proliferation via autocrine loops. In Group 3 patients blast cells this autocrine loop is inhibited by a specific anti-GM-CSF antibody. In contrast this anti-GM-CSF has no effect on Group 4 patients blast cell proliferation which suggests GM-CSF may act via an intracellular mechanism. These results indicate that GM-CSF production by some AML blast cells can act via autocrine loops. These loops are responsible for the autonomous proliferation of AML blast cells but through heterogeneous mechanisms.

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ABBREVIATIONS

AIF	Acidic isoferritins
AML	Acute myeloblastic leukaemia
AML-CFU	Acute myeloblastic leukaemia colony forming units
ASI	Autostimulatory Index
BCCM	Blast cell conditioned medium
BFU-e	Burst forming unit - erythroid
BMM	Bone marrow microenvironment
CD	Cluster of differentiation
CFU-BLAST	Colony forming unit - blast cells
CFU-e	Colony forming unit - erythroid
CFU-Eo	Colony forming unit - eosinophillic
CFU-G	Colony forming unit - granulocytes
CFU-GE	Colony forming unit - granulocytes/erythrocytes
CFU-GEMM	Colony forming unit - granulocytes/erythrocytes/ megakaryocytes/macrophages
CFU-GEMMT	Colony forming unit - granulocytes/erythrocytes/ megakaryocytes/macrophages/ T lymphocytes
CFU-GM	Colony forming unit - granulocytes/macrophages
CFU-M	Colony forming unit - macrophages
CFU-S	Colony forming unit - spleen
CFU-Mega	Colony forming unit - megakaryocytes
CGL	Chronic granulocytic leukaemia
CSF	Colony stimulating factor
ECM	Extracellular matrix
Ep	Erythropoietin
FAB	French American British system
FACS	Fluorescent Activated Cell Sorter
FCS	Foetal calf serum

FITC	Fluorescein isothiocyanate
FITC-Tf	Fluorescently conjugated transferrin
FN	Fibronectin
G-6-PD	Glucose-6-phosphate dehydrogenase
GAM-FITC	Goat anti-mouse fluorescein isothiocyanate
GCT-CM	Giant Cell Tumour conditioned medium
GM-CSF	Granulocyte-macrophage colony stimulating factor
G-CSF	Granulocyte colony stimulating factor
H-CAM	Haemopoietic cellular adhesion molecule
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
IM	Incubation media
IMDM	Iscoves Modified Dulbeccos Medium
LAI	Leukaemia associated inhibitor
LB	Leukaemic blood
Lf	Lactoferrin
LFA	Lymphocyte function antigen
LPS	Lipopolysaccharide
LSC	Leukaemic stem cell
LSM	Lymphocyte separation medium
M-CSF	Macrophage colony stimulating factor
MCI	Mean cellular immunofluorescence
ml	Millilitre
MNF	Mononuclear cell fractions
MPC	Magnetic particle concentrator
NBM	Normal bone marrow
NCM	No conditioned medium
NPBM	Normal peripheral blood mononuclear cells

PB	Peripheral blood
PBS	Phosphate buffered saline
PGE	Prostaglandin E
PHA-LCM	Phytohaemagglutinin - leukocyte conditioned medium
PHA-TCM	Phytohaemagglutinin - T lymphocyte conditioned medium
Tf	Transferrin
TfR	Transferrin receptor
TGF	Transforming growth factor
TNF	Tumour necrosis factor
µg	micrograms
5637-CM	Bladder cell carcinoma conditioned medium

INTRODUCTION

1. INTRODUCTION

In normal haemopoiesis strict control of peripheral blood cell production is maintained. A small population of pluripotent stem cells residing in the bone marrow provides all the myeloid, erythroid, lymphoid and megakaryocytic lineages present in healthy individuals. The stem cell's ability for self renewal and differentiation helps sustain the inexhaustable supply and diverse functions of the mature circulating haemopoietic cells. Whilst the stem cells remain within the bone marrow microenvironment they are influenced by a number of factors. It is cell-cell interactions within this microenvironment together with the action of various cytokines on stem cells which leads to the production and subsequent release of committed progeny into the peripheral blood.

Unfortunately as with all intricate regulatory mechanisms haemopoiesis has it's aberrations. Acute myeloblastic leukaemia is a pathological condition where the precise control seen in normal haemopoiesis is no longer operational. Patients with the disease are found to have leukaemic blast cells in their blood and bone marrow. Accumulation of these malignant blasts eventually suppresses normal haemopoiesis. The objective of clinical treatment is the reduction or ideally complete elimination of leukaemic blasts. Present treatment regimes are not particularly successful at complete eradication of the leukaemic cell

population as relapses due to residual disease are still common. A better understanding of normal and leukaemic haemopoiesis may offer avenues for control or removal of malignant cells whilst leaving the normal stem cell population unaffected. Current knowledge of normal and leukaemic haemopoiesis is described in the following chapter.

1.1.1 EARLY STEM CELL STUDIES

Haemopoiesis is a complex mechanism requiring a great deal of careful dissection in order to comprehend it fully. Current understanding of the mechanism evolved from the notion first suggested by Maximow in 1924 that all haemopoietic cells develop from one particular type of early cell [1]. In the following years this notion of a stem cell was the source of much speculation and debate. Experimental techniques which would support the stem cell thoery were to become available in the early 1960s. Till and McCulloch, in 1961, showed that an injection of syngenic bone marrow produced discrete spleen colonies of mixed cell lineage in mice following ablation of the haemopoietic tissue by irradiation [2]. These spleen colonies which developed after some 7 - 10 days were referred to as colony forming units - spleen (CFU-S). Using donor marrow which had multiple radiation induced chromosome markers CFU-S were shown to be truly clonal in origin [3, 4]. The CFU-S capability for self renewal was elucidated when it was shown that a cell suspension prepared from a single colony, when infused into irradiated mice, produced secondary CFU-S [5].

From these preliminary experiments, and many others which followed, it became clear that a murine pluripotent cell with the capacity for self renewal did exist [6]. In these early murine studies severe in vivo experiments were performed but clearly, for ethical

reasons, alternative methods were necessary to confirm the existence of the human pluripotent stem cell.

Evidence supporting a pluripotent stem cell in man was first obtained from genetic studies. There are two particular markers that help confirm the clonal nature of haemopoiesis. The first of these is the Philadelphia (PH') chromosome first reported in the disease Chronic Granulocytic Leukaemia (CGL) by Nowell and Hungerford in 1960 [7]. Translocation of the long arm of chromosome 22 produces the PH' chromosome which is known to exist in the majority of CGL cases. The discovery of the PH'chromosome in the erythroid, granulocytic and megakaryocytic cells of each individual CGL patient [8, 9] suggested that the chromsomal aberration occured in a progenitor cell which could give rise to these seperate lines of cellular differentiation.

Secondly the X-linked enzyme glucose-6-phosphate dehydrogenase (G-6-PD) is found in two forms, A and B, the expression of which is dependant of the genes Gd^A and Gd^B.

The random event of X chromosome inactivation [10] determines which form of the enzyme is produced and once X-inactivation has occurred within a particular cell the same inactive chromosome persists in all of the cell's progeny [11].

In normal heterozygous (Gd^AGd^B) females haemopoietic cells with both A and B forms of the G-6-PD enzyme are present. Whilst in heterozygous CGL females

only one form of the G-6-PD enzyme has been found in their erythrocytes, granulocytes, platelets and macrophages even though both forms were present in their fibroblasts [12-14]. Therefore the presence of only one G-6-PD enzyme type in different haemopoietic cells of individual heterozygous CGL females suggests a clonal basis for the disease.

These two genetic investigations of CGL both support the idea that a stem cell exists with the capacity to differentiate into a variety of haemopoietic end stage cells. In the wake of these genetic discoveries came the development of in-vitro culture techniques for human haemopoietic cells which would enable further evidence for the stem cell theory to be accumulated.

1.1.2 NORMAL HAEMOPOIETIC PROGENITOR CELLS AND THE BONE MARROW MICROENVIRONMENT

Following the development of in vitro techniques for the growth of murine bone marrow [15, 16] these methods were successfully adapted for the culture of normal human bone marrow [17]. The use of semi-solid media such as agar allowed cell division to occur whilst preventing the cells from dispersing. Colonies which developed could then be removed and stained for identification. In these initial studies it was clear a growth factor was required if colonies were to develop.

At first mouse kidney tubules [17] and human peripheral leukocytes [18] were both used as feeder cells to provide the required growth factors. Later studies found that medium conditioned by human peripheral leukocytes could replace the feeder cell requirement [19].

Initially these methods produced colonies of only one cell type but their pioneering work in the field of growth factors was to prove essential in the quest for the pluripotent cell. It was the medium conditioned by peripheral leukocytes which produced the first colonies of mixed lineage [20]. These mixed colonies contained granulocytes and macrophages and were to become known as CFU-GM. The introduction of conditioned medium prepared by stimulating peripheral blood leukocytes with phytohaemagglutinin (PHA-LCM) [21] also produced mixed lineage colonies. When PHA-LCM and another growth factor, erythropoietin [22], were combined the colonies which developed contained a mixture of granulocytes and erythrocytes (CFU-G/E) [23].

Some of these CFU-G/E were shown in later studies to also contain eosinophils, megakaryocytes and macrophages, these colonies being termed CFU-GEMM [24]. It is clear a pluripotent cell is responsible for myelopoiesis but does this cell have a link with lymphopoiesis?

When phytohaemagglutinin stimulated T lymphocyte conditioned medium (PHA-TCM) [25] was incorporated into

the CFU-GEMM culture system some of the mixed colonies were found to contain T lymphocytes (CFU-GEMMT) [26]. B lymphocytes were also found to be generated from a myelolymphopoietic stem cell when studied via in vitro techniques [27].

Existence of a pluripotent cell in man responsible for both the myeloid and lymphoid lineages has clearly been confirmed through the utilization of in vitro assays. With these established assays it was then possible to verify the self renewal capabilities of the CFU-GEMM. CFU-GEMM colonies could be removed from culture, dispersed into single cell suspensions and replated. It was found that approximately 20% of the initial CFU-GEMM formed secondary colonies [28, 29]. More recent studies have reported a cell termed the CFU-BLAST which produces blast cell colonies with secondary plating efficiencies ranging from forty to seventy-five percent [30,31]. With their self renewal capacity significantly greater than that of the CFU-GEMM, CFU-BLAST are thought to be the earliest human progenitor cell capable of being assayed in vitro at present.

Even though they have a high self renewal capacity CFU-BLAST cells are still not considered to be the primary haemopoietic stem cell. The cell from which the CFU-BLAST is thought to arise has not yet been positively identified. General opinion suggests these primary stem cells account for less than 0.1% of bone marrow mononuclear cells and are small with a lymphoid like appearance [32].

The CFU-BLAST is the result of the first step in a hierarchial system which produces all the fully differentiated cells required for normal haemopoietic function. This hierarchial system is an ordered sequence of events whereby undifferentiated progenitor cells seen in the early stages of haemopoiesis gradually differentiate towards mature fully functional end cells [33, 34]. (See Figure 1.1) As progenitor cells differentiate their capacity for self renewal decreases.

The CFU-BLAST [30, 31], CFU-GEMM [24] and CFU-GM [20] shown in Figure 1.1 have been alluded to previously. There are, however, a number of elements in Figure 1.1 which require elucidation. Firstly the colony-forming units megakaryocytes (CFU-Mega) have been described in vitro as colonies consisting purely of megakaryocytes [35]. Intermediate stages of erythropoiesis are termed the burst forming unit erythroid (BFU-e) and the colony forming unit erythroid (CFU-e) [36]. The BFU-e is the most primitive precursor and obtains its description from its appearance of small dispersed erythroid clusters in culture. CFU-e develop from BFU-e and appear in culture as tight colonies consisting entirely of erythroblasts. In vitro cultures also provide clear evidence of a distinct eosinophillic colony forming unit (CFU-Eo) [37, 38].

In normal individuals the majority of stem cells and committed progenitors are confined to the bone marrow [32, 39] with only a limited number being found

HAEMOPOIESIS

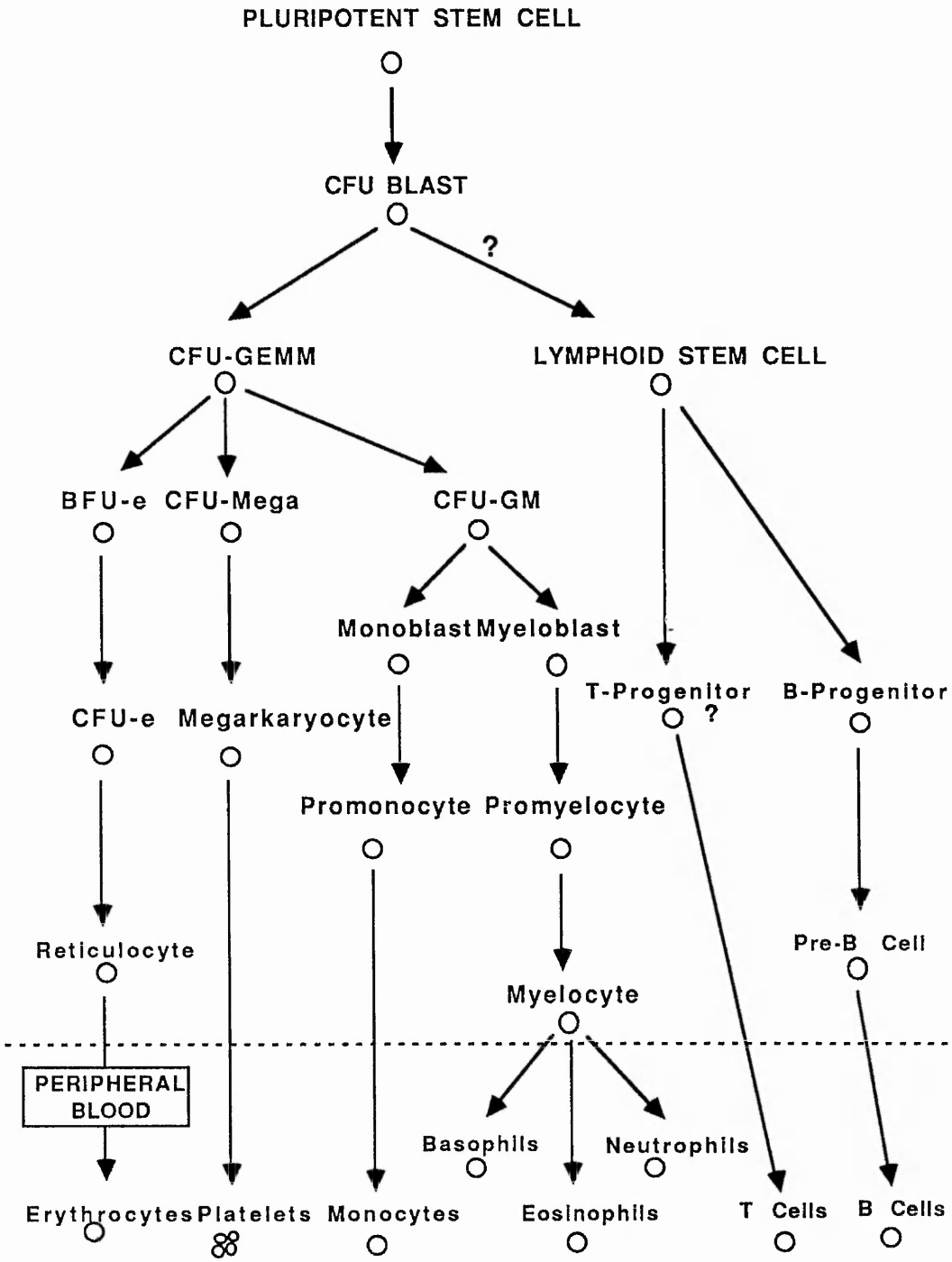


Figure 1.1

in the peripheral blood [40]. Along with the haemopoietic progenitors the bone marrow also consists of fibroblasts, fat cells, macrophages and endothelial cells which together are known as the stroma [41]. This mixture of cells are all bound within a layer of collagen, fibronectin, proteoglycans and laminin which are collectively termed the extracellular matrix (ECM) [42].

Haemopoietic progenitors are retained in this rather complex bone marrow microenvironment (BMM) by a number of specific binding mechanisms [39] . Some haemopoietic progenitors possess surface membrane proteins, including chondroitin sulphate and a 110Kd lectin known as homing protein, which bind to stromal cell glycoproteins such as fibronectin (FN) [43, 44]. Erythroid progenitors BFU-e and CFU-e are also known to possess membrane receptors for FN [45, 46], whilst granulocytic cells appear to have specificity for a protein component of the ECM known as haemonectin [47].

Other haemopoietic progenitor cell surface antigens such as lymphocyte function association antigen (LFA-1) [48] and the recently discovered haemopoietic cellular adhesion molecule (H-CAM) [49] bind to endothelial cells. LFA-1 has specificity for the intracellular adhesion molecule (ICAM-1) [50, 51] whilst the endothelial component to which H-CAM binds is still unknown.

Whilst bound within the BMM the haemopoietic

progenitor cells proliferate to produce the carefully regulated numbers of fully differentiated cells required to maintain basal haemopoiesis but must also be able to supply the vastly increased numbers of cells needed during episodes of haemopoietic stress such as haemorrhage or acute infection. The proliferation characteristics of haemopoietic progenitor cells are therefore crucial to the maintenance of normal haemopoietic function.

1.1.3 PROLIFERATION OF NORMAL HAEMOPOIETIC PROGENITOR CELLS

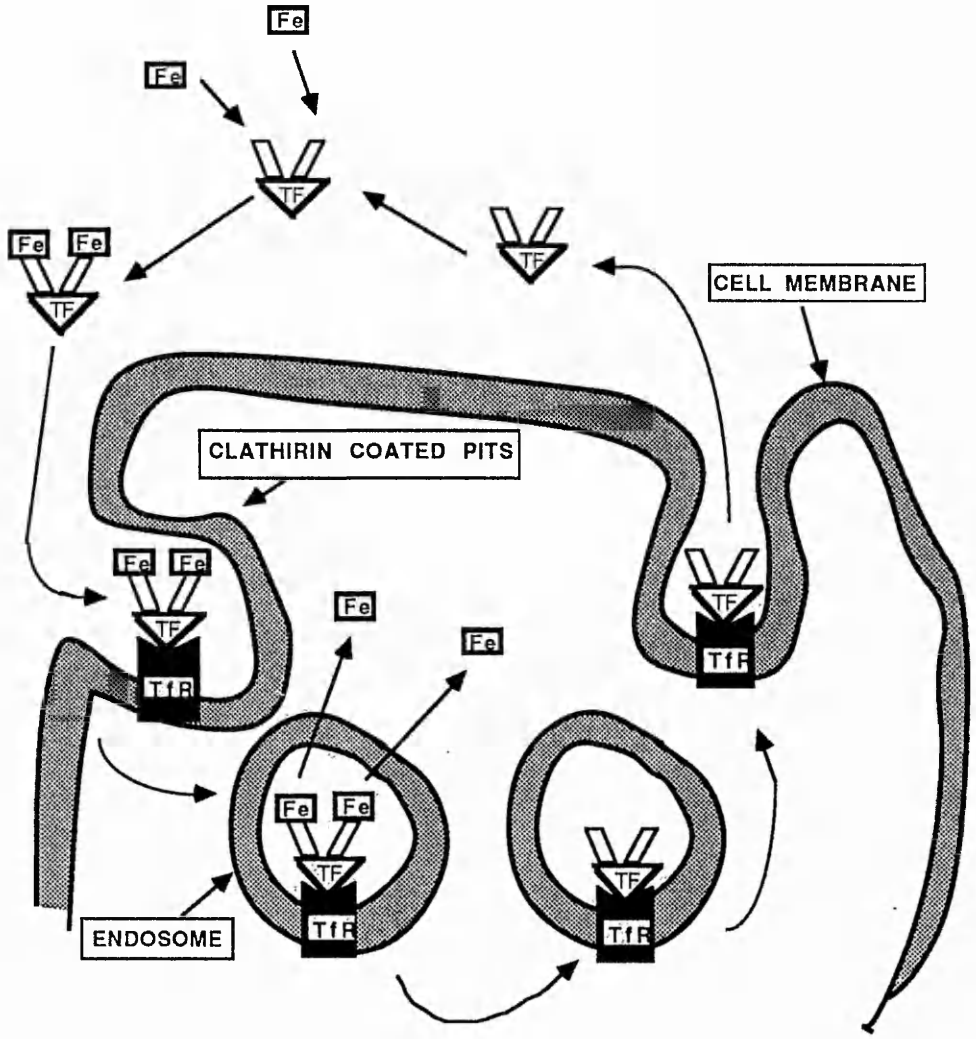
In basal haemopoiesis only the production of an adequate number of cells necessary to replace the degenerate cells, which have reached the end of their life span, are required. When the haemopoietic system is stressed it responds by significantly increasing the number of cells produced [32]. Increased proliferation of haemopoietic progenitor cells is responsible for the rise in production of end cells [52]. A number of different methods have been employed to study the proliferation characteristics of haemopoietic progenitor cells.

Proliferating cells require iron for essential intracellular enzymes. Iron is transported across the cell membrane by the serum glycoprotein transferrin [53]. This glycoprotein has two binding sites which both have a high affinity for iron at normal physiological pH [54]. In vivo apo, monoferric and diferric forms of transferrin are present. The diferric

form is the favoured iron donor as its avidity for the specific cellular membrane receptor (TfR) is approximately three fold higher than that of the other two forms [53]. Once diferric transferrin is bound to the TfR the complex is internalized via an endosome [55]. Within this endosome a proton pump serves to lower the pH to~5 at which the iron dissociates from both binding sites [56]. Following this release into the endosome the iron is subsequently liberated into the cytoplasm [57]. The endosome the returns to the cellular membrane where the apotransferrin is released into the extracellular environment. The apotransferrin then acquires more iron and the cycle resumes (see Figure 1.2). Transferrin is then the major route through which proliferating cells acquire iron [58]. Under normal conditions significant levels of TfR expression have been recorded on the committed progenitors CFU-GM, BFU-e and CFU-e, whilst on earlier progenitors such as CFU-GEMM no TfR expression is detectable [59-61].

A number of different in vitro culture techniques have been utilized to study the proliferation status of progenitor cells. Tritiated thymidine and hydroxyurea are cell cycle specific agents which will kill cells that are in the actively proliferating S phase. Using these two compounds confirmed that under normal conditions the committed progenitors are in a

TRANSFERRIN RECEPTOR CYCLE



TF = Transferrin

TfR = Transferrin receptor

Figure 1.2

proliferative state [32, 52] whilst the majority of early progenitors such as CFU-GEMM and CFU-BLAST remain non-proliferative [52, 62-65]. In contrast there is a significant rise in the number of proliferating CFU-GEMMs when the bone marrow regenerates following insults such as haemorrhage or acute infection [52].

These observation support the theory that basal haemopoiesis is maintained by a small actively proliferating fraction of a largely quiescent stem cell population. During haemopoietic stress a significant number of the quiescent stem cells are triggered into active proliferation. Once the stress is negated the system returns to basal conditions.

Regulating the proliferation of the stem cell and its progeny is therefore crucial for normal haemopoietic function. Interest in the mechanisms by which haemopoietic regulation is maintained has led to increase in research in this area over the last few years.

1.1.4 THE STIMULATION OF HAEMOPOIESIS BY CYTOKINES

Cytokines are a group of proteins which are produced and secreted by a variety of cells [66]. Normally cytokines are produced as a result of stimulation but constitutive expression by particular cells is not uncommon. These cytokines can directly and indirectly affect a number of cellular responses in both an autocrine and paracrine manner. Cell surface receptor

expression, cytokine production and release and cell proliferation and differentiation are all capable of being influenced by cytokines. These effects are triggered by binding of a cytokine to it's specific membrane receptor [67-69].

Early in vitro assays illustrated that for proliferation of normal haemopoietic progenitor cells an exogenous source of growth factors was an absolute necessity [70]. It is now known that a number of cytokines play a significant role in the control of normal haemopoiesis. Three of these cytokines are known as colony stimulating factors (CSFs) and stimulate the proliferation and differentiation of a number of different haemopoietic progenitor cells.

Granulocyte-macrophage CSF (GM-CSF) stimulates CFU-BLAST, CFU-GM, CFU-M, CFU-G, CFU-Meg and CFU-Eo [71-74]. GM-CSF will also stimulate CFU-GEMM and BFU-e but only in concert with erythropoietin (Ep) [75], a cellular protein which is produced by the kidney in response to hypoxia [76, 77] . GM-CSF is produced by T lymphocytes, monocytes and endothelial cells following exposure to antigen or stimulation with cytokines such as interleukin-1 (IL-1) and tumour necrosis factor (TNF) [78-86]. Only in fibroblasts is a low level constitutive production of GM-CSF seen but this level is also significantly increased after stimulation with IL-1 or TNF [85,87].

Granulocyte CSF (G-CSF) is extremely specific in that it only stimulates the committed progenitor CFU-G

[88]. Low level constitutive production of G-CSF occurs in fibroblasts but this is increased in the presence of IL-1 and TNF [87, 89]. Endothelial cells, monocytes and neutrophils also produce G-CSF but only in response to a variety of stimuli which include endotoxin, IL-1, TNF, GM-CSF and interleukin-3 (IL-3) [81, 83, 84, 86, 90-93].

Macrophage CSF (M-CSF) is also specific in that it only affects CFU-M [94] . Endothelial cells and fibroblasts both produce M-CSF constitutively but endothelial cells will increase production on stimulation with IL-1, TNF or bacterial lipopolysaccharide (LPS) [81, 86, 95]. TNF and IL-1 as well as GM-CSF, IL-3 and another cytokine, gamma interferon (IFN-γ), will also stimulate M-CSF production in monocytes [90, 92, 96-99]. Neutrophils are also reported to secrete M-CSF following stimulation with GM-CSFs [93].

Apart from the CSFs there are other cytokines which control haemopoiesis. Interleukins are a large group of cytokines many of which exert an effect on haemopoiesis. There are three interleukins (IL) which directly stimulate a variety of haemopoietic progenitors. Although previously mentioned as a stimulator of CSF production IL-3 is itself capable of stimulating CFU-BLAST, CFU-GM, CFU-G, CFU-M, CFU-Eo and CFU-Meg [31, 100-104]. In conjunction with Ep, IL-3 will also stimulate CFU-GEMM and BFU-e [105, 106]. IL-5

meanwhile has a specific role in stimulating just CFU-Eo [107] . Similarly IL-7 only serves to expand the lymphocyte population through stimulation of early B and T cell progenitors [108]. IL-3 and IL-5 are both produced by activated T lymphocytes whilst the cells responsible for the production of IL-7 still remain to be elucidated [109, 110].

There are then a number of cytokines capable of directly stimulating haemopoiesis. The need for expansion of haemopoietic cell populations is obvious at times of insult, but on removal of the insult a rapid return to basal haemopoietic levels is required. Suppression of haemopoiesis is achieved by release of cytokines which can inhibit the proliferation of haemopoietic progenitors.

1.1.5 INHIBITION OF HAEMOPOIESIS BY CYTOKINES

There are certain cytokines capable of inhibiting the proliferation of haemopoietic progenitors. Although TNF is known to stimulate CSF release in complete contrast it is also a potent inhibitor of CFU-GM and BFU-e [111-113]. Activated lymphocytes, as well as endotoxin treated or virus infected monocytes, are known to secrete TNF [114-121].

Interferons (IFNs) are secreted in three main forms α, β and γ all of which significantly inhibit CFU-GEMM, CFU-GM and BFU-e [122-124]. Lymphocytes, macrophages and fibroblasts secrete IFNs in response to activation

with a variety of stimuli including viruses, bacteria, mitogen or the cytokines IL-1 and IL-2 [125-133]. Another cytokine known to inhibit CFU-GEMM, CFU-e, BFU-e and the less committed 14 day CFU-GM is transforming growth factor β (TGF-β) [134-138]. The action of TGF-β on the more committed 7 day CFU-GM still remains to be clarified as a number of studies have shown conflicting results [134, 136, 138]. Whilst lymphocytes, monocytes and fibroblasts all produce TGF-β the α granules of platelets are the main storage site. Following platelet activation large amounts of TGF-β are released from these granules [139-144].

Apart from TNF, IFNs and TGF-β other cytokines perform a minor role in the inhibition of haemopoiesis. Leukaemia associated inhibitor (LAI) is produced by a subpopulation of mononuclear cells found in both normal peripheral blood and bone marrow and is known to inhibit CFU-GM [145]. Acidic isoferritins (AIF) and prostaglandin E (PGE) are both produced by monocytes, the latter is also secreted by fibroblasts and endothelial cells [146-151]. CFU-GEMM, CFU-GM and BFU-e are all inhibited by AIF whilst PGE only acts on CFU-GM [152-155].

1.1.6 INDIRECT EFFECTS OF CYTOKINES ON HAEMOPOIESIS

Although haemopoiesis is directly controlled by various cytokines there are also indirect routes through

which cytokines exert effects on haemopoietic progenitor cells. For instance directly acting cytokines often depend on the stimulation of cells by dissimilar cytokines for their production and release. It has also been shown that some cytokines, such as IL-1 and TNF, perform a dual role as both stimulators and inhibitors of haemopoiesis.

IL-1 is produced by endothelial cells, epithelial cells, neutrophils, monocytes, lymphocytes and fibroblasts following stimulation by a number of factors [156-168]. Antigen, M-CSF, GM-CSF, TNF, IL-2, TGF-β and in an autocrine fashion IL-1 itself are all able to stimulate IL-1 production [160,162-165,166,167, 169]. Apart from the functions already described for IL-1 it also stimulates the release of PGE, IL-1, IL-2 and another cytokine with a haemopoietic function, IL-6, from a variety of cells including fibroblasts, endothelial cells, monocytes and T lymphocytes [151, 161, 164, 166, 170-174]. IL-1 has also been shown to increase IL-2 receptor expression on T lymphocytes [166]. With such wide ranging effects IL-1 clearly plays a significant role in the control of haemopoiesis. The importance of IL-1 in haemopoiesis has recently been described in two excellent reviews by Moore [175] and Bagby [176].

Apart from it's previously mentioned roles in haemopoietic inhibition and IL-1 secretion TNF also stimulates release of PGE and IL-6 from endothelial cells and fibroblasts [150, 173, 174, 177]. TNF also

synergizes with IL-1 to enhance fibroblast secretion of PGE [150].

IL-6 has already been shown to be released by a number of cells in response to IL-1 and TNF. IL-6 is in fact constitutively secreted by endothelial cells and can also be released by lymphocytes following mitogen stimulation [178, 179]. IL-6 can affect haemopoiesis in a number of ways. IL-6, by activating T lymphocytes, serves to expand the T lymphocyte population whilst B lymphocytes require IL-6 for differentiation [180, 181]. IL-6 has also been shown to suppress the release of IL-1 and TNF by activated mononuclear cells [182]. Possibly the most important haemopoietic effect of IL-6 is on early progenitors such as CFU-BLAST and CFU-GEMM. In the presence of IL-6 these early progenitors display a shorter resting phase (Go) within their cell cycle, which in turn renders them more responsive to other cytokines such as IL-3 [183]. An increase in CFU-BLAST and CFU-GEMM proliferation is therefore apparent when IL-6 is present in conjunction with IL-3 [183].

Some of the effects of IL-3 on haemopoiesis have already been revealed but there are others which require explanation. T lymphocyte proliferation and B lymphocyte differentiation are both enhanced when IL-3 is used in concert with IL-2 [184, 185]. In monocytes IL-3 can enhance the transcription of TNF, which leads to augmented secretion of this cytokine following exposure to a second activation signal [186]. In the

same cells IL-3 also increases transcription and translation of IL-1, when used in conjunction with bacterial lipopolysaccharide [187].

IL-2 is secreted by activated T lymphocytes and has been shown to work in concert with other cytokines [188]. IL-2, by stimulating proliferation of T lymphocytes, acts in an autocrine fashion but can also cause production and release of IFN-γ from inactivated T cells [188-190]. Another route through which IL-2 causes the release of IFN-γ is by regulating the proliferation of large granular lymphocytes and natural killer cells both of which produce IFN-γ [191, 192]. In conjunction with mitogen IL-2 also stimulates secretion of TNF by mononuclear cells [193].

Another product of activated lymphocytes is IL-4 the primary function of which is to prime haemopoietic progenitor cells such as CFU-GEMM, CFU-G, BFU-e, CFU-e and CFU-Mega in preparation for stimulation with other cytokines [194-197]. Whereas priming by IL-4 leads to the enhanced proliferation of most haemopoietic progenitor cells after stimulation with various cytokines, in the case of CFU-GM it has actually been shown to antagonise the response to IL-3 [198]. IL-4 also stimulates proliferation of activated lymphocytes and increases secretion of GM-CSF, G-CSF and M-CSF by activated T lymphocytes and monocytes [199-202]. TNF and IL-1 gene expression in monocytes is also down regulated by the presence of IL-4 [203].

Apart from the ILs there are a number of indirect

ways in which the CSFs can affect haemopoiesis. G-CSF and GM-CSF can stimulate endothelial cell proliferation whilst the latter will also enhance IL-2 driven T lymphocyte proliferation [184, 204]. Monocytes, if pretreated with M-CSF, will release significantly higher amounts of IFN, TNF and CSFs [205].

The IFNs are another group of cytokines with a dual role in haemopoiesis. Although IFNs are known inhibitors they also enhance the release of a variety of cytokines including IL-1, TNF and CSFs from activated monocytes and T lymphocytes [99, 164, 206-208]. IFN synergizes with PGE in the inhibition of CFU-GM proliferation but has contrasting effects with regard to PGE production [209]. The release of PGE by activated monocytes is inhibited by IFN whilst secretion by fibroblasts is stimulated [210, 211]. IFN, depending on which type is present, can also lead to the stimulation or inhibition of fibroblast proliferation [212, 213]. B lymphocyte proliferation and differentiation are both enhanced by IFN [214, 215].

Apart from its role as an inhibitor PGE also serves to indirectly affect haemopoiesis. PGE has been shown to suppress: the production of TNF and IL-1 by activated monocytes, secretion of IL-2 by lymphocytes, secretion of the inhibitor LAI and B lymphocyte activation [145, 149, 216-218].

Normal haemopoiesis is obviously a finely balanced system in which a number of cytokines have a vital role.

The complexity of the system is already apparent and is made even more so by the fact that other circulating proteins, in addition to the cytokines, can affect haemopoiesis.

Two iron carrying proteins, lactoferrin and the previously mentioned transferrin, are both known to indirectly affect secretion of various cytokines. Transferrin suppresses GM-CSF secretion by T lymphocytes [219]. Lactoferrin (Lf) is produced by immature granulocytes and is stored in secondary granules of mature neutrophils [220]. When neutrophils degranulate Lf is released and has been shown to inhibit the secretion of AIF, IL-1, GM-CSF and PGE by monocytes [220, 221-223].

1.1.7 SUMMARY

The preceding sections illustrate the enormous volume of information currently available on normal haemopoiesis but due to the amount of research presently being undertaken on this subject it has more than likely been added to during the writing of this thesis. Even though a complete understanding of normal haemopoiesis has not yet been achieved there is still a great deal known about this rather complex system.

To summarize, normal haemopoiesis is dependent on a small number of mainly quiescent stem cells which have a high capacity for self-renewal as well as the ability to differentiate. Self-renewal is required for generation of new stem cells whilst differentiation gives rise to

more committed progenitor cells. These committed progenitor cells also proliferate and produce large numbers of haemopoietic cells which are then released into the peripheral circulation. Within this hierarchy a cell's ability for self-renewal is gradually lost as its degree of differentiation is increased.

The stem cells and committed progenitor cells are retained within the bone marrow microenvironment (BMM) by a number of factors, including elements of the ECM as well as certain cell surface proteins. Proliferation and differentiation of these cells, whilst bound within the BMM, are directly and indirectly affected by a variety of cytokines.

The strict control of haemopoiesis, exerted by cytokines, allows for the sudden increase in mature end cell numbers required at times of insult, but is also responsible for a swift return to basal levels following abrogation of the insult. Cytokines are then an obvious necessity for stem cell proliferation but what actually determines the switch between self-renewal or differentiation of these stem cells still remains unanswered. In a recent review Metcalf [224] proposed that this switch may be due to a single genetic element which is controlled by precise cytokine concentrations.

In normal individuals, through strict control, haemopoiesis remains a finely balanced system. Unfortunately there is an extremely complex network required to maintain this balance which consequently

lends itself to a high probability of dysfunction.

Acute myeloblastic leukaemia (AML) is just one of a number of haematological disorders and is characterized by the appearance of malignant blast cells in the bone marrow and blood of affected individuals. Even though haematological in origin these AML blast cells evade the rigid control typically seen with normal haematological cells. It is this loss of normal control that leads to the uncontrolled proliferation of AML blasts. Current ideas on mechanisms which may be responsible for this uncontrolled proliferation are outlined in the following sections.

1.2.1 HISTORICAL ASPECTS OF AML

In the late 1800s it was the reports of Donne, Bennett and Virchow of excessive white cells in the blood of affected individuals which indicated the pathological basis of the condition described by Virchow in 1847 as "white blood" or in Greek "leukaemia" [225]. In 1868 Neumann theorized that the bone marrow was the site of white blood cell production and as such is the site of dysfunction in leukaemia [226]. Further evidence for involvement of the bone marrow in leukaemia was obtained following development of cellular staining techniques by Ehrlich in 1877 [226]. Using these techniques Naegeli, in 1900, identified cells within the bone marrow he considered as ancestors of all granular cells and which he termed myeloblasts and also reported that these myeloblasts appeared in grossly increased numbers in many acute leukaemias [226]. In later years these morphologically based tenets suggesting a dysfunctional bone marrow as the physiological basis of leukaemia proved to be correct.

Acute myeloblastic leukaemia (A.M.L.) is now seen as a haemopoietic neoplasia stemming from overproduction of immature blasts within the bone marrow which eventually leads to their release into the bloodstream [34]. Accumulation of these A.M.L. blasts in the bone marrow gradually suppresses normal haemopoietic function leading to anaemia, thrombocytopenia and neutropenia [227]. It is this suppression of normal haemopoiesis along with rapidly increasing numbers of

undifferentiated leukaemic blasts in peripheral blood and bone marrow which will eventually cause death.

Complete elimination, or at the very least cessation of uncontrolled proliferation of these leukaemic blasts, together with a minimum of disruption to normal haemopoiesis should be the aim of treatment regimes. It is fundamental differences in the proliferation characteristics of normal haemopoietic stem cells and leukaemic blasts that need to be addressed, then exploited, if this goal is to be achieved.

1.2.2 IN VITRO ASSAYS FOR AML BLAST CELLS

The in vitro proliferation characteristics of leukaemic blast cells were first studied using a modification of the technique used to culture normal haemopoietic progenitor cells [17, 18]. These initial leukaemic blast cell assays used an agar bilayer system with normal leukocytes in the bottom layer as a source of stimulating factors [228]. Cytogenic analysis confirmed that the colonies which developed in this bilayer technique consisted of leukaemic blast cells [229]. These early assays yielded very low colony numbers and more efficient techniques for leukaemic blast cell culture were soon described.

By using PHA-LCM, which had previously been described as a stimulating factor for normal progenitor

cells [21], in a single layer of methyl cellulose McCulloch's group, in 1977, developed what was to become the standard technique for assaying leukaemic blast cell colonies [230]. Colonies which developed using McCulloch's technique contained morphologically homogeneous blast cells which carried cytogenic AML markers and showed a limited degree of aberrant differentiation when analysed by surface marker analysis [231-233]. With the discovery that a small population of leukaemic blast cells, recently termed AML-colony forming units (AML-CFU), were responsible for colony formation in vitro their in vivo implications were soon to become evident.

1.2.3 LEUKAEMIC STEM CELL THEORIES

Genetic studies have shown that only one form of the G-6-PD enzyme occurs in the leukaemic blast cells of an individual patient [234]. The finding that AML is a clonal disease suggests the existence of a leukaemic stem cell (LSC). The idea of LSCs was also supported by in vivo studies which showed only a small percentage of the AML blast population was actively proliferating [235].

The fact that AML-CFU were responsible for colony formation yet showed morphological homogeneity with the non-proliferating blast cells, led to a suggestion that they may function as these leukaemic stem cells (LSC) by

maintaining the entire leukamic cell population [34, 227]. This theory of a LSC was investigated by studying the proliferation characteristics of the AML-CFU.

In vitro studies using the previously referred to tritiated thymidine and hydroxyurea techniques were utilized to determine the cell cycle characteristics of AML-CFU. These studies showed that a high proportion of the AML-CFU were in the active stage (S-phase) of the cell cycle, which is one of the features indicative of stem cells [236]. Although TfR expression occurs in a high percentage of the leukaemic blast cell population the relationship between AML-CFU and the level of expression has not yet been resolved.

If AML-CFU are the cells in the leukaemic blast population with the highest level of TfR expression it would correlate with the cell cycle studies and confirm that these cells constitute the actively proliferating population of in vivo AML blast cells. To be considered as LSCs AML-CFU must be capable of self renewal. Primary AML-CFU colonies, following recovery, were dispersed into single cell suspensions and replated in a blast cell colony assay. This technique produced secondary leukaemic blast cell colonies which, when counted, gave an estimate of the so called secondary replating efficiency (PE2) [237, 238]. Although PE2 values show inter patient variation they remain constant for individual patients and are usually found to be <10%

[227]. The fact that AML-CFU can form secondary leukaemic blast cell colonies indicates their capacity for self renewal.

Finally AML-CFU must also show an ability to differentiate which is another characteristic indicative of stem cells. Early cytological examination of the cells present in AML-CFU colonies suggested some degree of differentiation had occurred [239-241]. The levels of differentiation have been more accurately determined by more recently available surface marker systems. These systems use monoclonal antibodies to detect cell surface antigens which occur at specific stages of normal haemopoietic progenitor cell differentiation. These monoclonal antibodies confirmed the earlier cytological findings regarding the ability of AML-CFU to differentiate [233]. Many studies have now shown that the blast cells which arise during colony formation often show aberrant expression of differentiation markers but that the cell surface antigens present on these leukaemic blast cells are no different from those found on normal haemopoietic progenitors [232].

By displaying the properties of active proliferation, self renewal and differentiation the AML-CFU appear to fulfill the criteria of a LSC. If AML-CFU are responsible for maintaining the entire leukaemic blast cell population then understanding the proliferation mechanisms of these cells should give an insight into the biology of AML.

1.2.4 THE ROLE OF CYTOKINES IN AML BLAST CELL PROLIFERATION

When AML-CFU were first cultured in vitro stimulation was required for these cells to form colonies. Initially leukocyte feeder cells, as well as PHA-LCM, served as the sources of rather crude stimulating factors [228, 230]. With the advent of recombinant cytokines it was possible to isolate the particular growth factors responsible for the proliferation of AML blast cells. A number of the cytokines which stimulate the proliferation and differentiation of normal haemopoietic progenitors are now known to affect AML blast cell proliferation and differentiation.

GM-CSF, G-CSF, IL-2, IL-3 and IL-4 have all been shown to stimulate the proliferation of AML blast cells, although it is recognised that there is heterogeneity between patients [242-247]. A limited degree of AML blast cell differentiation can also occur following treatment with GM-CSF, G-CSF, M-CSF, IL-3 and IL-4 [242, 243, 245-248].

Cytokines, however, do not always act in isolation. Synergistic, additive and antagonistic responses have been reported for a number of these growth factors. GM-CSF and IL-3 have both shown synergistic or additive effects with G-CSF, M-CSF, IL-1 and IL-6 [245, 249-251]. G-CSF synergizes with IL-1, is additive with M-CSF but its stimulation of AML-CFU is antagonized by TNFα

[245, 250, 252, 253]. It should also be noted that cytokine interactions often display contrasting effects, for instance TNFα displays synergy for GM-CSF and IL-3 stimulated proliferation in some patients but has been reported to antagonize IL-3 stimulated AML-CFU growth in others [251-253] . Similarly IL-4 has the capacity to either enhance or suppress GM-CSF, G-CSF or IL-3 stimulated AML blast proliferation [247, 248]. Inhibitors of normal haemopoietic progenitor cells, such as TGFβ and IFNs, are also found to suppress leukaemic blast cell proliferation [254, 255].

Although AML blast cells proliferate and differentiate in response to the various cytokines produced by normal fully differentiated haemopoietic cells recent studies have reported a small number of AML patients whose leukaemic blast cells proliferate without the addition of exogenous cytokines [256-260]. As many malignant cell lines show autonomy due to the secretion of cytokines it was suggested that this may also be responsible for the autonomous proliferation characteristics of some AML blast cells.

Leukaemic blast cells from a number of patients show constitutive production and secretion of IL-1, IL-6, G-CSF, LAI and AIF [145, 256-258, 261, 262]. The IL-1 secreted also stimulates the production of TNFα and GM-CSF by AML blast cells in an autocrine fashion [256, 258, 260]. But whether IL-1 is an absolute necessity for TNFα and GM-CSF release is still a matter for conjecture as some studies suggest they are released

constitutively [256, 259, 260]. The question of GM-CSF and TNFα production by AML blasts is further complicated by the fact that TNFα, having stimulated the release of GM-CSF, then combines with this cytokine to cause the release of IL-1β [252]. Constitutive expression of M-CSF.mRNA has also been reported but M-CSF will only be secreted following stimulation of the AML blasts with a variety of cytokines [256, 263, 264]. Higher levels of LAI and AIF are reported to be produced by leukaemic blast cells when compared to those observed from normal haemopoietic cells [145, 262].

A number of cytokines are clearly produced by AML blast cells although constitutive secretion of some of these cytokines still remains to be verified. Many of these cytokines stimulate proliferation of AML blasts as well as the release of cytokines from a number of fully differentiated haemopoietic cells [265]. By operating in these autocrine and paracrine fashions secretion of cytokines by AML blasts may have important implications for the biology of the disease.

The role of cytokines in the autonomous proliferation of leukaemic blast cells is still unclear but autocrine loops may be an area for consideration. In vivo the paracrine effects of the cytokines secreted by AML blast cells could very well lead to acceleration of the disease. In contrast TNF, LAI and AIF all inhibit normal haemopoietic progenitors yet in most cases have no effect on leukaemic blast cell

proliferation [251-253, 262, 266]. With their capacity to release a number of cytokines AML blasts appear to have the ability to accelerate disease whilst suppressing normal haemopoiesis. These two factors may enable AML blasts to gain a proliferative advantage over normal haemopoietic progenitors. The autonomous proliferation characteristics of AML blasts and the role of cytokines in this phenomenon are therefore factors requiring further investigation.

1.3 EXPERIMENTAL AIMS

The previous sections are a review of the proliferation characteristics found in normal haemopoietic progenitor cells and AML blast cells. This review has revealed that a number of the factors regulating the proliferation of AML blast cells may differ from those seen in normal haemopoietic progenitor cells but that these differences remain to be verified as they have not yet been thoroughly investigated.

It is well documented that during basal haemopoiesis the majority of normal early haemopoietic progenitors, such as CFU-BLAST and CFU-GEMM, are quiescent and consequently express no TfRs. In contrast AML blast cells have been shown to express significant levels of TfRs but whether this expression is higher on the proposed leukaemic stem cells (AML-CFU) is still unknown.

A difference in TfR expression between normal haemopoietic progenitor cells and AML blast cells has important implications for targetting of cytotoxic therapy. At present there are no cell surface antigens specific for AML blast cells so high TfR expression on AML-CFU would provide a potential site at which to direct cytotoxic agents. Therefore flow cytometry and fluorescent microscopy techniques were employed in studies designed to assess the TfR expression of AML-CFU.

The recent discovery that AML blast cells from

some patients secrete cytokines has led to speculation regarding the mechanisms by which the uncontrolled proliferation of AML blast cells is sustained. This cytokine release by AML blasts is in complete contrast to normal haemopoietic progenitor cells which do not secrete cytokines and are thus totally dependent on exogeneous stimulation for proliferation which suggests a fundamental difference between the biology of normal haemopoiesis and AML.

Stimulation of cytokine secretion via paracrine loops occurs in a number of fully differentiated cell types and it is possible that cytokines released by AML blast cells may act on these loops in a similar manner to that seen with cytokines which are released by normal cells. If AML cytokines stimulate these paracrine loops this would lead to an increase in blast cell proliferation due to an augmentation of cytokine secretion. It has also been suggested that AML blast cells are able to autonomously proliferate due to autocrine loops involving the endogenously secreted cytokines. Such autocrine loops, if operating, would also serve to enhance the proliferation of AML blast cells.

As information regarding these paracrine and autocrine loops in AML is still rather scant in vitro studies were performed in an effort to investigate the blast cell proliferation characteristics of a number of AML patients . By culturing the blast cells under both stimulated and unstimulated conditions any which

displayed autonomous characteristics could be identified. These autonomous blast cells were then subjected to further examination in an effort to determine the mechanisms through which this autonomous proliferation was maintained. Understanding the mechanisms through which AML blast cells proliferate is important to the clinical management of patients as it may lead to improvements in the treatment regimes employed to inhibit the proliferation of these malignant cells.

METHODS

2.1 STERILITY

Throughout this study aseptic techniques were applied in order to eliminate bacterial and fungal contamination. Sterility was maintained through the use of tissue culture disposable plastic ware or equipment that had either been autoclaved or dry heat sterilised. Unsterile heat labile solutions required for experiments were sterilised by means of 0.2μm filtration. All manipulations were carried out in filtered air environments provided by laminar flow cabinets.

2.2 COLLECTION OF A.M.L. SAMPLES

At presentation, following patient consent, peripheral blood was obtained from A.M.L. patients by venepuncture and placed into preservative free heparin (final concentration 10 units/ml).

Diagnosis of AML was based on examination of peripheral blood and bone marrow cells by Romanowsky and cytochemical staining, supplemented with analysis of cell surface markers by immunophenotyping techniques. Following diagnosis patients were classified according to the French-American-British system (FAB), which is based on the direction of differentiation and degree of maturation displayed by the leukaemic blasts (Bennett *et al* 1976) (Figure 2.1) [267]. Typically the patients used for study had >90% leukaemic blasts in the mononuclear cell fraction of their peripheral blood.

CLASSIFICATION OF ACUTE MYELOBLASTIC LEUKAEMIAS

FAB TYPE	LEUKAEMIC BLAST CELL CHARACTERISTICS
M1	Myeloblastic with some degree of granulocytic differentiation but no maturation.
M2	Myeloblastic with further degree of granulocytic differentiation and maturation.
M3	Promyelocytic with granulocytic differentiation and maturation even more advanced than observed in M2. Identification of promyelocytes.
M4	Myelomonocytic with both granulocytic and monocytic differentiation discernable.
M5(a)	Monocytic with detection of monoblasts.
(b)	Monocytic with detection of monoblasts, promonocytes and monocytes.
M6	Erythroblastic.
M7	Megakaryoblastic

Figure 2.1

2.3 BONE MARROW SAMPLES

Human ribs removed during thoracotomy operations, performed on haematologically normal individuals, were used as a supply of normal bone marrow cells. Within thirty minutes of removal from the patient the rib was sectioned and placed into an incubation medium (IM) consisting of RPMI 1640 tissue culture medium (RPMI) supplemented with preservative free heparin (final concentration 10 units/ml).

Following thirty minutes incubation at room temperature bone marrow was obtained by flushing the sections with fresh IM. This was performed by needle and syringe with samples being collected into petri dishes.

The contents of the petri dishes served as the source of normal bone marrow (NBM).

2.4 MONONUCLEAR CELL FRACTIONATION

Following collection NBM samples were diluted 1:3 with IM, whilst leukaemic blood samples (LB) were diluted 1:3 with just RPMI. Density gradient centrifugation techniques were then employed to isolate the mononuclear cell fractions (MNF) from both NBM and LB [268]. Briefly, 15mls of diluted sample was carefully layered onto 5mls of lymphocyte separation medium (LSM), a commercially available preparation (ICN-Flow

Laboratories) of ficoll hypaque, specific gravity 1.077 g/cm^3 in a plastic universal container.

After centrifugation at 400g for 20 minutes at room temperature the MNF (density <1.077 g/cm^3) accumulates at the interface between the diluting medium and the LSM. The MNF were carefully removed from the interface with glass pasteur pipettes and washed twice in RPMI at 400g for 10 minutes.

2.5 CELL COUNT AND VIABILITY

Following resuspension in RPMI small volumes of the MNF were removed, diluted in 0.1% trypan blue and counted in a haemocytometer. Trypan blue exclusion by cells with intact membranes was used to calculate the percentage viability of each particular sample.

With fresh samples of NBM and LB it was usual to find 100% viability but in the rare cases of reduced viability samples were excluded from any further investigation.

Experiments which required NBM cells were always commenced within 24 hours of their collection.

Some LB cells were also used within 24 hours of collection but the majority were frozen for future examination.

2.6 FREEZING AND THAWING OF A.M.L. CELLS

AML cells were centrifuged at 400g for 10 minutes and the supernantant (RPMI) removed. Iscoves Modified Dulbeccos Medium (IMDM) supplemented with 10% dimethylsulphoxide and 20% foetal calf serum (FCS) was slowly added to the cell pellet, with gentle agitation, to give a series of final cell concentrations ranging from $2x10^7$ to $4x10^8$/ml cells. After a thorough final mix 1ml aliquots of the cell suspension were transferred into 1.2ml cryotubes and frozen in a Kryo 10 programmable cell freezer. Freezing was performed in three stages firstly from room temperature down to -30°C at -1°C/minute, then at -5°C/minute to -70°C, the cells finally being placed in liquid nitrogen for storage at -196°C.

When required for use cells were removed from liquid nitrogen and placed immediately into a 37°C water bath. Once thawed the cells were placed into centrifuge tubes to which 9mls of thaw solution (RPMI, 20% FCS and preservative free heparin 10units/ml) was added dropwise with continuous mixing.

The cells were then centrifuged at 400g for 10 minutes, washed twice more with thaw solution, again at 400g for 10 minutes, resuspended in RPMI and their concentration and viability assessed as described in section 2.5. Consistent viabilities of >90% were achieved for thawed

AML cells so only samples with high viabilities were considered for subsequent studies.

2.7. T CELL DEPLETION OF SAMPLES

In order to prevent cytokine production and colony formation by T lymphocytes samples were routinely T depleted. Initially contaminating T cells were labelled with anti-CD3 mouse monoclonal antibody, then incubated with anti mouse immunoglobulin coated magnetic beads (Dynal) at the recommended bead:target cell ratio of 20:1, following which the bead-cell complex was removed using a magnetic particle concentrator (MPC) [269].
Development of anti-CD2 coated magnetic beads (Dynal) meant T depletion of later samples required only one incubation step at the appropriate bead:target cell ratio of 20:1 followed by removal once again using a MPC.
Immunofluorescent staining techniques indicated that following T depletion <1% of the cell population consisted of T lymphocytes.

2.8. DNA Analysis

In experiments where cell proliferation assays were required a standard technique using tritiated thymidine ([^{3}H]TdR) incorporation was employed as a measure of DNA synthesis [270]. Each data point was performed in

quadruplicate by adding 0.5μCi of (methyl-^{3}H)-thymidine ([^{3}H]TdR) {5.0Ci/mmol, Amersham, UK} to the individual microtitre plate wells. Cells were pulsed with [^{3}H]TdR for 6 hours at 37°C in an atmosphere of 5% CO_2 in air. Cells were lysed with distilled water and their DNA collected onto glass fibre filter paper using a Dynateck Automash 2000. Following solubilisation of the filter paper with 0.01M KOH and addition of Beckman Ready Protein Scintillation Cocktail, incorporation of [^{3}H]TdR into DNA was assessed using a Packard Tricarb Scintillation Counter.

2.9. PREPARATION OF IRON LOADED AND FLUORESCEIN ISOTHIOCYANATE CONJUGATED TRANSFERRIN

Human transferrin (Tf) was 95% saturated with iron using a modification of the Gutteridge method [271]. Briefly 13.57 mg of ferrous ammonium sulphate and 20 mg of Tf were each dissolved in 1ml of 0.1M tris HCl buffer containing 20mM $NaHCO_3$, pH 7.4. When fully dissolved the two 1ml volumes were mixed together and incubated at room temperature (25°C) for 30 minutes. The mixture was then dialyzed against three changes of a 500 fold excess of 0.1M tris HCl buffer containing 20mM $NaHCO_3$, pH 7.4, over a twenty-four hour period at 4°C. Iron loading of the Tf was determined by measuring the A_{465nm}/A_{280nm} ratio which was routinely found to be $\geq$0.040, consistent with 95% saturation.

Commercially available diferric Tf was labelled with fluorescein isothiocyanite (FITC) using the Van Renswoude technique [272]. Diferric Tf (8mg) and FITC (isomer I on Celite:approximately 10% FITC) (1.3mg) were separately dissolved in 0.5ml aliquots of 0.1M sodium borate buffer, pH 9.3. On ice the FITC was added dropwise with gentle agitation to the Tf, the mixture remaining on ice for a further 30 minutes. The reaction was stopped by removing the FITC-celite particles by centrifuging the mixture for 5 minutes at 13,000G in a microcentrifuge. The unbound FITC was then removed by overnight dialysis at 4°C against three changes of a 500 fold excess of 0.15M NaCl/0.02M tris HCl buffer, pH 7.3. The molar ratio of FITC to Tf was consistently found to be approximately 3:1 when calculated by the Jobagy and Kiraly formula [273].

2.10. IMMUNOFLUOESCENT LABELLING OF THE TfR

The cluster of differentiation (CD) system is now the standard method for the classification of cell surface antigens [274]. Using this system TfR is designated CD71. Therefore to determine TfR expression of leukaemic blasts by immunofluorescence an anti-CD71 antibody (OKT9) was used [60]. All receptor studies were performed at 4°C or on ice and cell washes were routinely carried out with phosphate buffered saline (PBS) at 400G for 10 minutes.

Aliquots of cells (10^6/ml) in PBS were incubated for 30 minutes with 5µl of neat anti-CD71 (40µg/ml) then washed twice. Following resuspension in PBS at 10^6/ml the cells were incubated for a further 30 minutes with 10 µl of neat goat anti-mouse fluorescein isothiocyanate antibody GAM-FITC antibody (0.8mg/ml). After labelling with GAM-FITC the cells were washed twice and resuspended in 1ml of PBS. Non-specific binding of the GAM-FITC was assessed by performing a second label only step on an identical number of cells. To test for non-specific positivity due to irrelevant Fc receptor binding an antibody with the same immunoglobulin subclass as anti-CD71 was used, in this case anti-CALLA (anti-CD10). Binding of Tf to the TfR was detected by incubating 1ml of cells (10^6/ml) with 10µl (1mg/ml) diferric Tf dissolved in PBS for 30 minutes. Following two washes the cells were resuspended in 1ml of PBS and incubated with 10µl of rabbit anti-human transferrin antibody for 30 minutes, after which came a further two washes. Resuspended in 1ml PBS the cells were then treated with 20µl of FITC-conjugated goat anti-rabbit IgG for 30 minutes. After two final washes the cells were resuspended in 1ml PBS.

The FITC-Tf prepared as detailed in section 2.9 was also used for binding studies. Briefly, 1ml of cells (10^6/ml) in PBS were incubated with either $5x10^{-7}$M or 10^{-7}M FITC-Tf for 30 minutes followed by two washes and resuspension in 1ml of PBS. Specificity of FITC-Tf

binding was tested by competitive inhibition in the presence of 100 fold excess diferric Tf.
In all binding studies the cells, once resuspended in PBS, remained on ice until examination.

2.11. ANALYSIS OF TfR EXPRESSION

Once TfR were fluorescently labelled, surface expression on individual cells was assessed by fluorescent microscopy or on a Fluorescent Activated Cell Sorter (FACS). For microscopy small samples of the labelled cells were placed on glass slides which were then mounted with cover slips. Visual determination of the percentage of positive cells in a given sample was performed on a Leitz Ortholux microscope fitted with a ploemopak 2.2, mercury arc vapour lamp and the appropriate FITC filter.
For FACS analysis the samples were processed through a FACS IV flow cytometer (Becton Dickinson). Fluorescence was excited using 200mW of 488nm light from an argon laser and analyzed via a 30nm bandwidth pass filter centred at 530nm. Under these conditions fluorescent intesity was measured between a minimum channel of 0 and a maximum of 255. These measurement conditions were standardized so that one channel increase represents the equivalent of 280 molecules of fluorescein per cell.

FACS analysis allowed the degree of TfR expression of a given cell population to be calculated in terms of the mean cellular immunofluorescence (MCI) [275].

Control cells with FITC second label only were always run concurrently with each fully labelled sample to measure non-specific fluorescence. The true MCI was then calculated by subtracting the mean non-specific fluorescence from the mean total fluorescence of a fully labelled sample.

Cells labelled with anti-CD71 were sorted on the FACS into positive and negative TfR expressing fractions. In some instances the numbers of the TfR negative cells were extremely low. In such cases cells were separated into TfR positive (fraction 1) and TfR weakly positive/negative fractions (fraction 2). The separation between fractions was usually performed at fluorescence gate 60, but due to variations in the fluorescence intensity of each patient's blast cell population slight alterations in the gate settings were sometimes necessary.

Sorted fractions were then analysed by semi-solid culture techniques for the prescence of colony forming cells.

2.12. COLONY ASSAYS

(a) Normal Marrow Progenitors

A modification of Iscove's technique [19] was used to assay normal CFU-GM. NBM cells were suspended at $2x10^5$/ml in 1.3% methylcellulose (4000cps), 10% FCS, penicillin (50 units/ml), streptomycin (50μg/ml), 2mM L-Glutamine with stimulation provided by either Giant Cell Tumour conditioned medium (GCT-CM) from Gibco or bladder carcinoma cell line conditioned medium (5637-CM) (kindly donated by Dr J. Fogh, Sloan Kettering Institute, New York) . Both GCT-CM and 5637-CM are known to contain a variety of cytokines including GM-CSF, G-CSF and IL-1. [276-280]

After thorough mixing 1ml samples of the cell suspension were plated in 35mm petri dishes in triplicate. The petri dishes were then incubated at 37°C in an atmosphere of 5%CO_2 in air for 10 days after which time colonies of $\geq$ 40 cells could normally be visualized and were counted using a Leitz Diavert microscope.

(b) Leukaemic Blast Cells

A modification of Buick's blast cell colony assay was employed to measure numbers of leukaemic progenitor cells [230]. Cells at 10^5/ml or $2x10^5$/ml were cultured

with or without stimulation in 0.8% methylcellulose containing 10% FCS, Penicillin (50 units/ml), streptomycin (50μg/ml), 2mM L-Glutamine and IMDM. Maximal stimulation of blast cells was achieved using the previously referred to GCT-CM and 5637-CM when used at a concentration of 10% (vol:vol). Experiments were also conducted where no conditioned medium (NCM) was supplied in an effort to confirm autonomous proliferation of AML blasts.

Following thorough mixing 0.1ml aliquots of the cell suspension were plated into 96 well, flat bottomed microtitre plates in quadruplicate. The plates were then incubated at 37°C in an atmosphere of 5% CO_2 in air for 5 days after which time colonies $\geq$ 20 cells could normally be visualized and were counted using a Leitz Diavert microscope (Figure 2.2).

In FACS studies which utilised blast colony assays control cultures in all experiments were performed with unsorted cells that had been treated with anti-CD71, this treatment would indicate any detrimental effects of the antibody on the blast

2.13. Autostimulatory Index (ASI)

An Autostimulatory Index for each patient was calculated according to the following equation :

$$ASI = \frac{\text{colony numbers in an unstimulated blast colony assay}}{\text{colony numbers in a stimulated blast colony assay}}$$

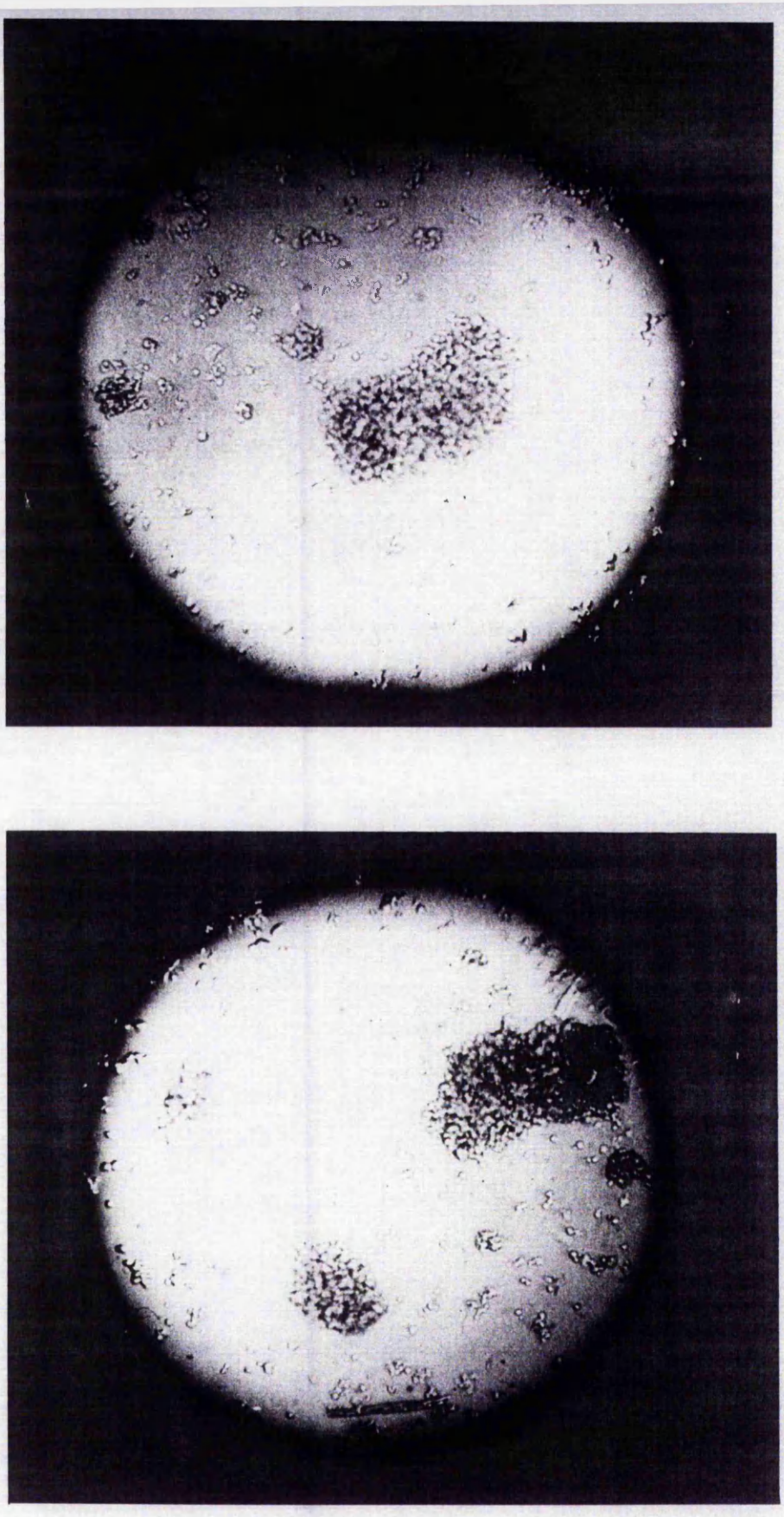


Figure 2.2

Microscopic appearance of typical AML blast cell colonies.

The blast colony assay was performed as described in section 2.12 (b) with the cells at a concentration of $2x10^5$/ml.

The ASI was then used to group patients with respect to their blast cells' capacity for autonomous proliferation. Group 1 patients' cells showed no proliferation in either stimulated or unstimulated cultures. Patients assigned to Group 2 had an ASI<0.1, Group 3 had an ASI between 0.1 and 0.8 and Group 4 had an ASI>0.8 [281].

2.14 Suspension cultures of AML blasts

(a) For TfR Analysis

Anti-CD71 and FITC-Tf labelling techniques previously described in section 2.11, were employed to analyze TfR expression on AML blast cells prior to and following a 48 hour period of suspension culture. Blasts were cultured at 10^6/ml in IMDM containing 10% FCS, Penicillin (50 units/ml), Streptomycin (150μg/ml), 2mM L-Glutamine and 10%(vol:vol) GCT-CM at 37°C in an atmosphere of 5% CO_2 in air.

In order to compare TfR expression of pre and post cultured cells with their level of proliferation 0.1ml volumes of cells from both conditions were aliquoted into microtitre plates with flat bottomed wells and DNA synthesis was measured (see section 2.8).

(b) Cell Crowding Experiments

AML blast cell proliferation was investigated under crowded and uncrowded conditions. Unstimulated and stimulated (GCT-CM or 5687-CM 10% vol:vol) cells at a range of concentrations from $15.6x10^3$ to $500x10^3$/ml were suspended in RPMI containing 10% FCS, Penicillin (50 units/ml), Streptomycin (50µg/ml) and 2mM L-Glutamine. 0.1ml volumes of the cell suspensions were aliquoted into microtitre plates with either round or flat bottomed wells, thereby simulating crowded and uncrowded conditions (Figure 2.3i). The microtitre plates were then cultured for 48 hours at 37°C in an atmosphere of 5% CO_2 in air, following which DNA synthesis was measured (see section 2.8).

In some of these experiments direct cell to cell contact was reduced by adding latex beads which were approximately the same size as the cells (15.8µm diameter). Beads were added to crowded and uncrowded cultures of stimulated AML blast cells at $15.6x10^3$/ml and $31.25x10^3$/ml, with cell to bead ratios being 1:40 and 1:100 (Figure 2.3ii). Following incubation at 37°C in an atmosphere of 5% CO_2 in air for 48 hours DNA synthesis was measured (see section 2.8).

The effect of crowding on the production of leukaemic progenitor cells was also studied. Stimulated AML blast cells were cultured in both round and flat bottomed wells at $31.25x10^3$/ml and $250x10^3$/ml in RPMI, 10% FCS, Penicillin (50units/ml), Streptomycin (50µg/ml) and 2mM

L-Glutamine at 37°C in an atmosphere of 5% CO_2 in air. After 48 hours the cells were washed at 400g for 10 minutes and then cultured with stimulant in a blast cell colony assay (see section 2.12[b]).

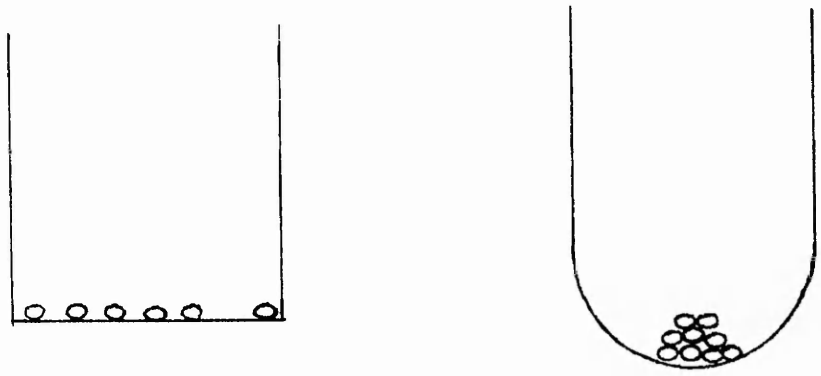
(c) Preparation of Blast Cell Conditioned Medium (BCCM)

The autonomous secretion of growth factors by AML blasts was also investigated. Blast cell conditioned medium (BCCM) was prepared from unstimulated AML blast cells cultured at 10^6/ml in RPMI, 10% FCS, Penicillin (50 units/ml), Streptomycin (50μg/ml) and 2mM L-Glutamine. Following 48 hours at 37°C in an atmosphere of 5% CO_2 in air the BCCM was harvested and centrifuged at 600g for 10 minutes to pellet any contaminating cells. The BCCM was then carefully aspirated and spun at 30,000 rpm (50,000g) for 60 minutes at 4°C in a Beckman L8-70M ultracentrifuge. This second high speed centrifugation step was required to remove any cell membrane components which may have been shed during culture. After this second centrifugation step the BCCM was again carefully aspirated, filter sterilized and stored at -70°C.

(d) Effects of BCCM on Leukaemic Blast Cell Proliferation

BCCMs were tested for their ability to stimulate both homologous and autologous AML blast cells. In the case of homologous stimulation both the blast colony assay

(i)



(ii)

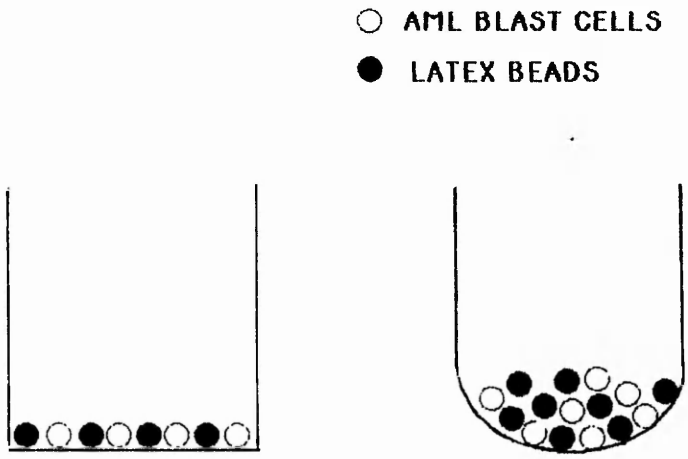


Figure 2.3

(i) Representation of crowded and uncrowded suspension cultures.

(ii) Reduction of cell to cell contact by addition of latex beads.

described in section 2.12(b) as well as suspension cultures were carried out. Only suspension cultures were conducted for proliferation studies of an autologous nature. Suspension cultures were performed in flat bottomed wells in RPMI, 10% FCS, Penicillin (50units/ml), Streptomycin (50μg/ml) and 2mM-L-Glutamine at cell concentrations of either $3.25x10^3$/ml or $250x10^3$/ml (as indicated in the text) whilst in the blast colony assay the cell concentration was $2x10^5$/ml. For both blast colony assays and suspension cultures the BCCMs were tested at a concentration of 20% vol:vol.

In some of these experiments the possibility that GM-CSF was a constituent of the BCCMs was also investigated. For these studies the BCCMs were pre-incubated for thirty minutes with a polyclonal anti-GM-CSF antibody prior to incorporation into the suspension cultures. This antibody binds to GM-CSF and in doing so prevents this cytokine binding to it's specific membrane receptor. The antibody concentrations used are indicated in the text. The antibody was a kind gift of Dr. A. Gearing, National Institute for Biological Standards and Control, U.K.

After incubation for 48 hours at 37°C in an atmosphere of 5% CO_2 in air all autologous and homologous suspension cultures were measured for DNA synthesis (see section 2.8).

(e) Effects of BCCM on Normal CFU-GM

The proliferation of normal CFU-GM in response to BCCMs from AML patients was also tested. Colony assays as described in section 2.12(a) were used. For all experiments the cell concentration was $2x10^5$/ml and the BCCMs added at 20% vol:vol.

Polyclonal anti-GM-CSF antibody (5µg/ml) was again incorporated into the assays in an effort to confirm the presence of GM-CSF.

2.15 Proliferation Characteristics of Group 3 and Group4 Patients blast cells

(a) Stimulated and Unstimulated Cultures

Methods used to investigate the effect of crowding on AML blast cell proliferation [refer to section 2.14(b)] were adopted to study proliferation characteristics of Group 3 and 4 patients. Unstimulated and stimulated cells (GCT-CM or 5637-CM) at a range of concentrations from $15.6x10^3$/ml to $250x10^3$/ml were cultured in an atmosphere of 5% CO_2 in air. After 48 hours DNA synthesis by the cells was measured (see section 2.8).

(b) Effect of Polyclonal Anti-GM-CSF- on Proliferation

Autonomous proliferation of Group 3 and Group 4 patients' cells due to autocrine production of GM-CSF was investigated with polyclonal anti-GM-CSF antibody.

These experiments were carried out in a similar manner to those described in section 2.14(b). Unstimulated cells at $250x10^3$/ml were cultured in flat bottomed wells with or without the antibody (5μg/ml) at 37°C in an atmosphere of 5% CO_2 in air. After 48 hours DNA synthesis by the cells was measured (see section 2.8).

(c) Production of Subcellular Fractions

Studies were performed to test for the presence of GM-CSF in subcellular fractions of Group 3 and Group 4 patients. The unstimulated blast cells that had been cultured for the production of BCCM (refer to 2.14[c]) were harvested and processed for subcellular fractions. The method used for the production of subcellular fractions has been previously described by Matsushima [282].

After culture the cells were washed three times with PBS and resuspended in 1ml of ice cold buffer A consisting of 25mM Hepes, 2mM EDTA, 5mM EGTA, 4mM 2-Mercaptoethanol and 1mM Phenylmethylsulfonylfluoride at pH 7.5. After a 10 minute incubation on ice the cells were checked microscopically for evidence of turgidity. Physical disruption of the cells was then performed on ice by sonicating with a Lucas Dawe Soniprobe.

Cells received two 30 second sonication pulses with a 30 second pause between pulses. Each pulse had an amplitude of level 3 and an output of 20%, cell disruption was confirmed by microscopy. One ml of 0.66M

sucrose dissolved in buffer A was added to the disrupted cell suspensions. After centrifugation at 200g for 10 minutes at 4°C, to remove nuclei and undisrupted cells, the supernatant was stored overnight at 4°C.

The following day the supernatant was carefully layered onto 1.5 ml of buffer A containing 40% sucrose. Tubes were then ultracentrifuged at 40,000 rpm (100,000g) for 60 minutes at 4°C.

After centrifugation the subcellular fractions were harvested as indicated (Figure 2.4). The supernatant containing the cytosol was carefully removed. As the membrane fraction accumulates at the interface it was necessary to collect a small proportion of the cytosolic fraction as well as all the remaining buffer, along with the interface, to ensure that 100% of the membrane fraction was collected. The particulate fraction was discarded.

The membrane fraction was diluted 10 fold in buffer A and ultracentrifuged at 40,000 rpm (100,000g) for 60 minutes at 4°C to isolate the membrane components from any contaminating cytosol. The membrane pellet obtained was resuspended in 1ml 9mM CHAPS dissolved in PBS and kept on ice for 30 minutes. Cytosolic and membrane fractions were transferred into dialysis tubing (molecular weight cut off 10,000) and dialyzed against 100 fold excess PBS over a 48 hour period at 4°C. Fractions were then filter sterilized and stored at -70°C prior to use.

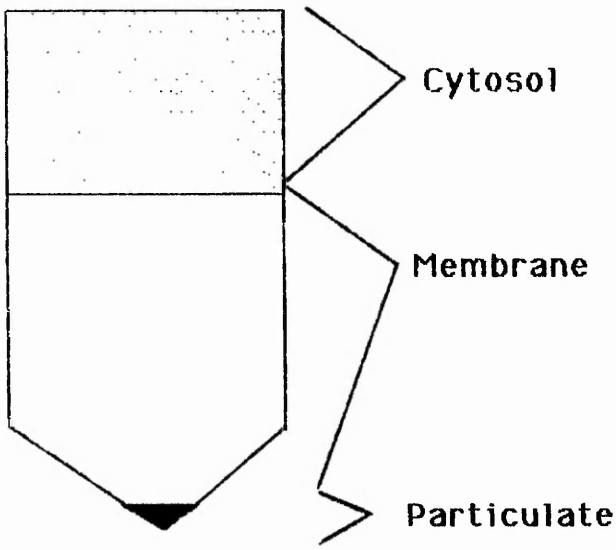


Figure 2.4

Collection of subcellular fractions following ultracentrifugation

(d) Characterization of Subcellular Fractions

The stimulatory activity of subcellular fractions was assessed against homologous AML blast cells. A similar methodology as that described for BCCM characterization in 2.14(d) was used in these experiments. A Group 2 patient (No 6) cells were stimulated with membrane and cytosol fractions at 10% vol:vol in flat bottomed wells at a concentration of $250x10^3$/ml. Polyclonal anti-GM-

CSF antibody (5μg/ml) was incorporated into some of the cytosol stimulated cultures. Controls were performed to measure unstimulated (no CSF/subcellular fractions) and maximally stimulated (5637-CM) levels.

In an effort to confirm the specificity of the polyclonal anti-GM-CSF antibody an irrelevant antibody, anti-CD3 (OKT3), as well as the anti-GM-CSF antibody (5μg/ml) was incorporated into 5637-CM stimulated cultures.

All cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air. After 48 hours cell proliferation was measured by DNA synthesis measurements (see section 2.8).

2.16. Statistical Methods

Data were analyzed by nonparametric methods [283], thereby avoiding assumptions about the distributions of the variables under study. Pairwise comparisons were analysed using the Wilcoxon rank-sum test.

RESULTS

3.1 TfR Expression on AML Blasts

Immunofluorescent techniques were used to study the TfR expression on AML blasts. This expression was initially examined in three patients by FACS analysis following cell surface labelling with anti-CD71. Results (figure 3.1) indicate that in all three patients a high percentage ($\geq$ 58%) of the cells were anti-CD71 positive. In each patient the fact that three separate FACS analyses for TfR expression displayed strong correlation illustrated the reproducibility of this technique. With FACS analysis established as a suitable method for studying TfR expression, anti-CD71 labelling of a further eight patients' cells were performed.

Table I indicates the FAB types of all AML patients studied and shows that TfR expression, when detected by anti-CD71 , was found on the majority of blast cells. To substantiate the anti-CD71 results, TfR expression on nine of these patients was also studied by analysing surface bound Tf. Following staining of surface bound Tf with a polyclonal anti-transferrin antibody the percentage of positive cells was assessed by fluorescent microscopy. As a substantial proportion of blast cells in nine out of nine cases studied were found to have detectable levels of surface bound Tf (see table I) these results were consistent with the anti-CD71 findings. Controls were conducted during analyses for two reasons. Firstly, as a measure of non-specific

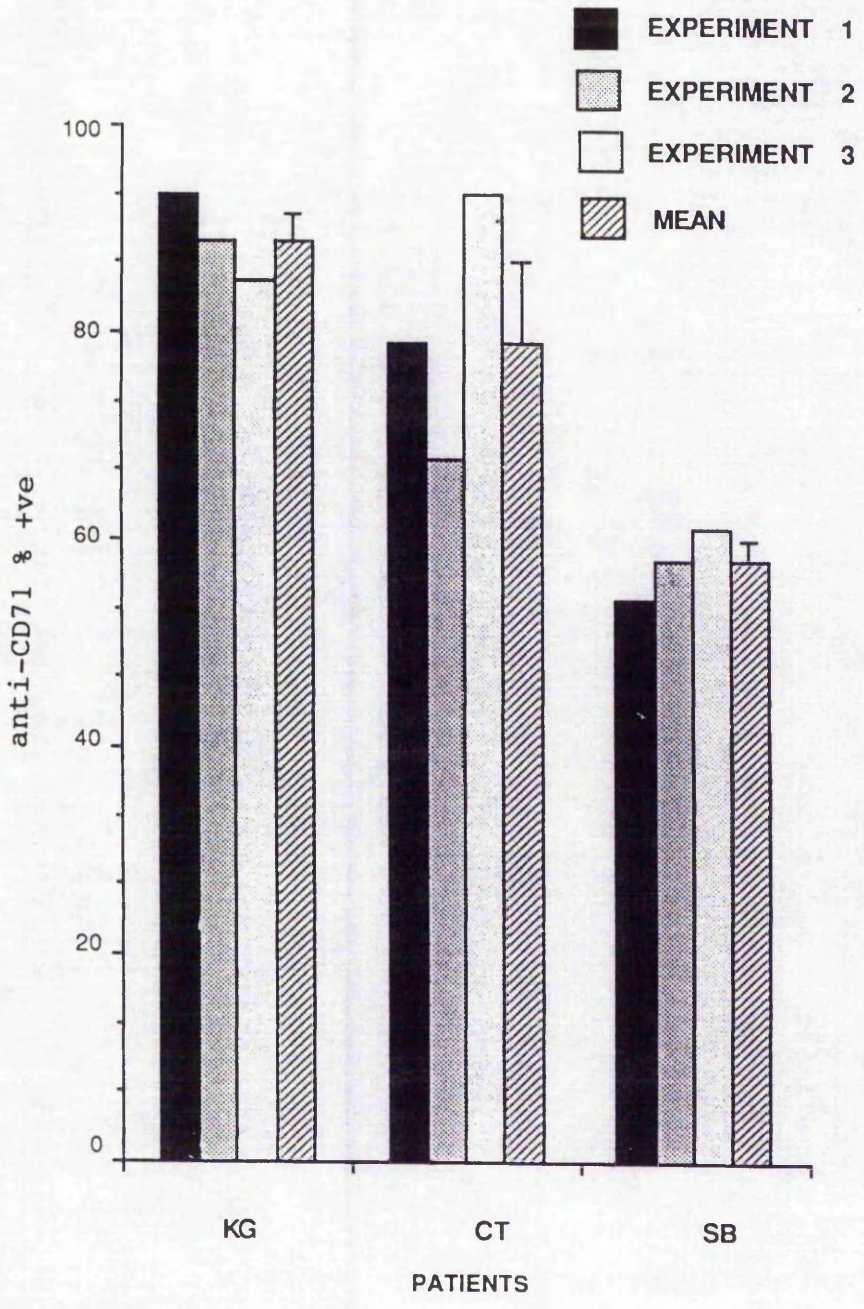


Figure 3.1

The percentage of AML blast cells staining with anti-CD71 as measured by flow cytometry.

The results are shown as individual experiments along with the mean±SEM for each particular patient.

Patient	FAB type	anti-CD71 positive (%)	Tf+anti Tf positive(%)	Blast cell colony growth
DA	M4	88	69	+
KG	M5	85	60	+
LP	M4	87	95	+
SB	M4	58	95	+
CT	M2	93	73	+
DW	M4	45	NT	+
MS	M4	84	NT	-
DM	M4	70	95	+
JT	M2	51	69	-
EW	M5	47	NT	+
SH	M1	67	NT	+
PB controls		<5%	<10%	

Table I.

TfR expression on AML blasts.

The percentage of peripheral blood (PB) blasts staining with anti-CD71 was determined by flow cytometry whereas the percentage of blasts staining with anti-transferrin was determined by fluorescent microscopy. In all cases the percentage of blast cells in the mononuclear cell fraction was >90%.

NT = not tested

binding, a second label only with GAM-FITC was performed in each case. Secondly, irrelevant Fc receptor binding was tested by incubating with an inappropriate antibody anti-CALLA (anti-CD10) which has the same immunoglobulin subtype as anti-CD71. These controls were consistently negative, thereby supporting the specificity of the immunofluorescent techniques employed. In contrast to the AML blast cells, TfR expression on normal peripheral blood mononuclear cells (NPBM) was very low (<10%) when labelled with anti-CD71 or anti-transferrin (see table I).

3.2 Relationship of TfR Expression to Blast Cell Proliferation

Whether the AML blast cells with significant TfR expression were in fact highly proliferative was then examined. TfR expression of blast cells was analysed by anti-CD71 and FITC-Tf binding prior to and following 48 hours in culture. Cell proliferation at both time points was also assessed by measuring DNA synthesis.

For these studies FITC-Tf was not commercially available so it was produced in-house and its specificity was then checked (see figure 3.2). At both concentrations of FITC-Tf the binding was reduced to control levels by the addition of 100 fold excess unconjugated diferric transferrin. As the control levels are simply the autofluorescence values for unlabelled cells these results demonstrate the

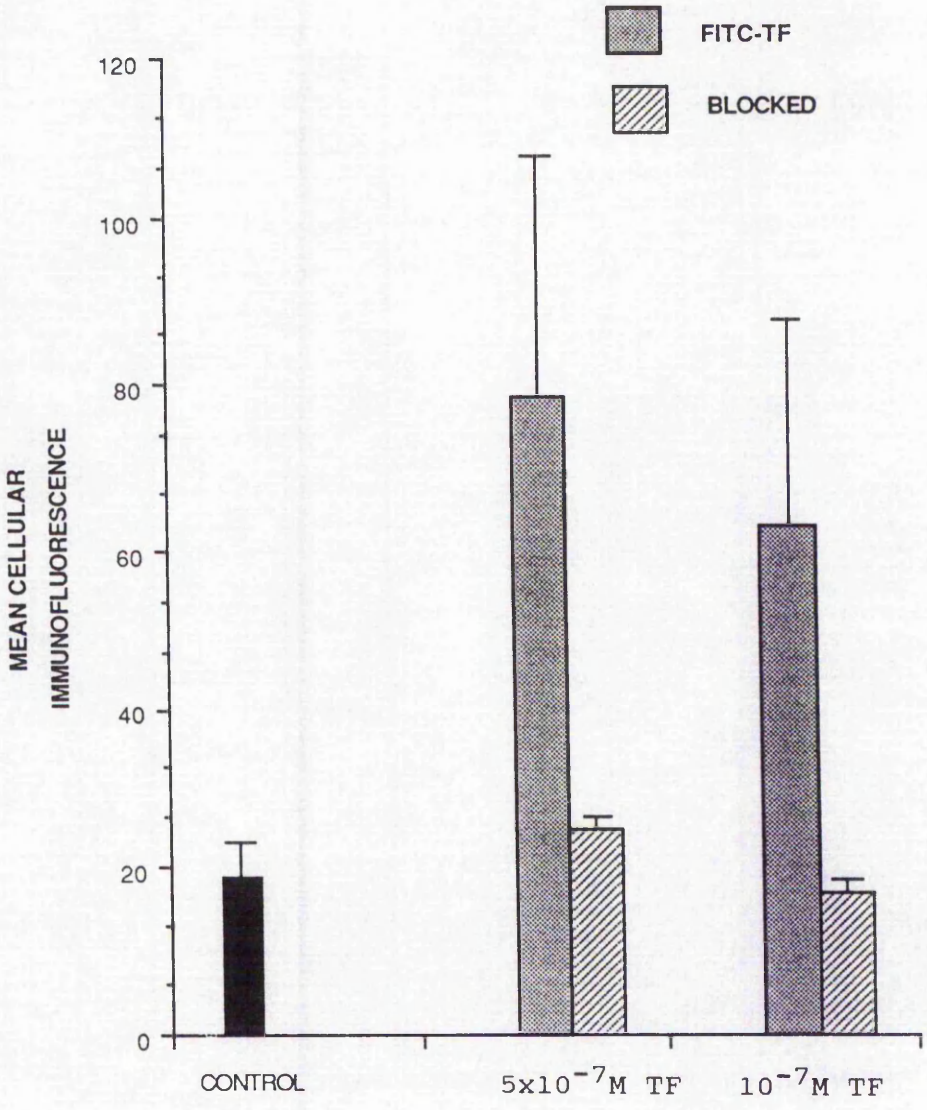


Figure 3.2

Binding of $5x10^{-7}M$ and $10^{-7}M$ FITC-Tf to AML blast cells as assessed by flow cytometry. FITC-Tf specificity was demonstrated by blocking with 100 fold excess diferric transferrin. In each experiment a control of unlabelled cells was performed to measure the level of non-specific autofluorescence.

Results are the mean±SEM of 4 patients.

specificity of FITC-Tf for the TfR.

As figure 3.2 also showed that both FITC-Tf concentrations gave similar MCI readings it was decided that FITC-Tf at 10^{-7}M be employed in further analyses.

At both time points anti-CD71 and FITC-Tf binding were assessed by FACS analysis. Fluorescence histograms (figure 3.3) were produced which illustrated the heterogeneous, but rather low level, of TfR expression detected by both anti-CD71 and FITC-Tf binding prior to culture. After 48 hours of culture the TfR expression still remained heterogeneous but these levels appeared increased when compared to those seen prior to culture.

Controls conducted in Figure 3.3 were second label only (GAM-FITC) in the case of anti-CD71 and 100 fold excess diferric transferrin in the case of FITC-Tf. The results of these controls again clearly indicate the specificity of anti-CD71 and FITC-Tf for the TfR.

Although the fluorescence histogram in Figure 3.3 gave an indication of a possible increase in TfR expression following 48 hours in culture, it was the MCI figures along with the corresponding DNA synthesis measurements which required comparison in an effort to show a link between TfR expression and cell proliferation.

In table II the MCIs for six patients obtained by FACS analysis following labelling with anti-CD71 and FITC-Tf are shown along with the corresponding DNA synthesis levels as measured by [^{3}H]-TdR incorporation.

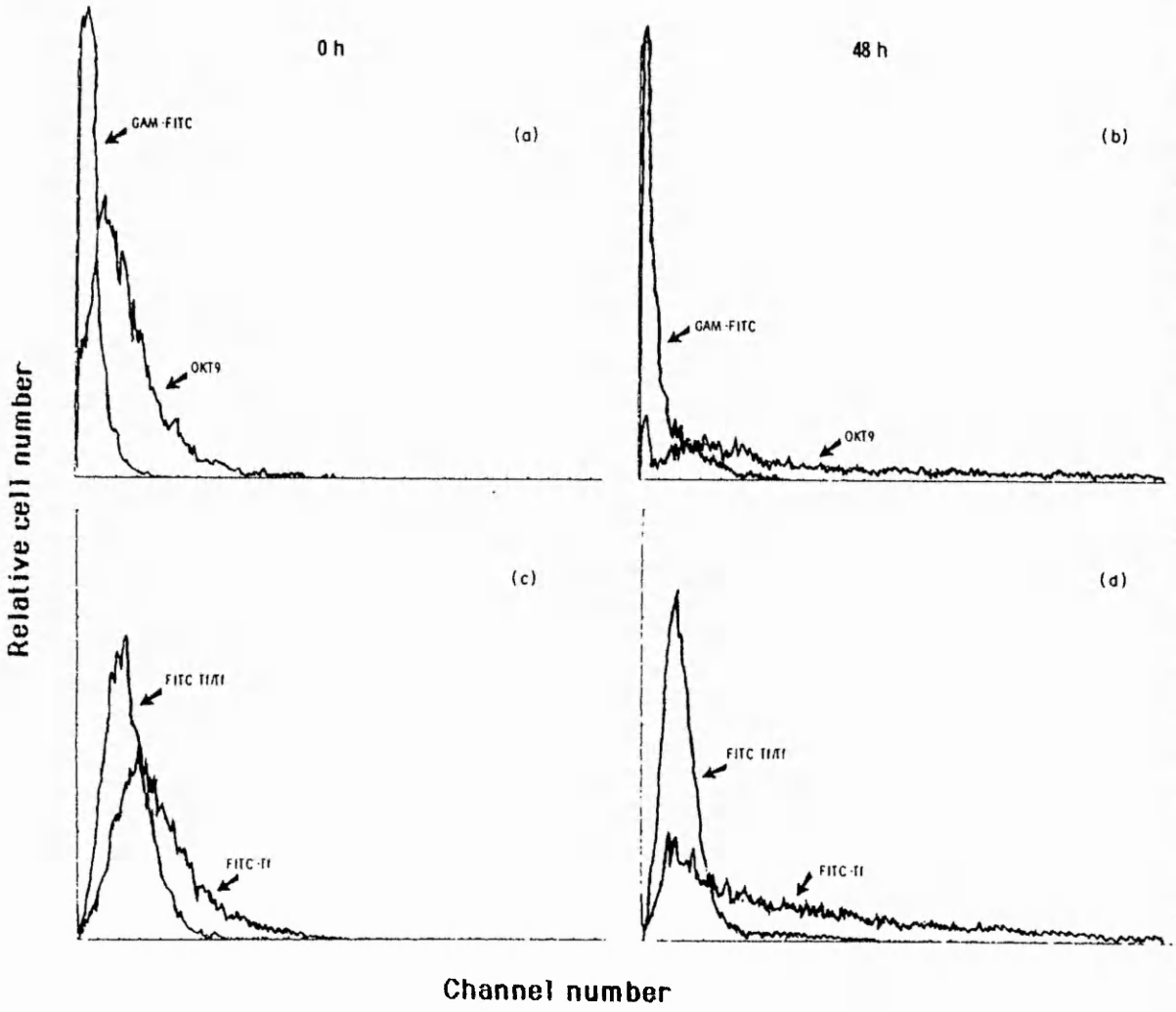


Figure 3.3

Fluorescence histograms of AML cells (patient DM) labelled with anti-CD71 and goat anti-mouse fluorescein isothiocyanate (GAM-FITC). As a control, cells incubated with GAM-FITC only were used. Panel (a) shows the fluorescence profile of fresh cells and panel (b) the profile following 48 hours suspension culture in complete medium containing 10%GCT-CM. Panels (c) and (d) show fluorescence histograms of the same cells (0 hour and 48 hours) stained with FITC-Tf (10^{-7}M) in the presence and absence of a 100-fold excess diferric transferrin (FITC-TF/Tf).

Patient		cpm/10^4 cells	MCI* anti CD71	FITC-Tf
DM	Pre-culture	420	74	15
	Post-culture(48 hrs)	5900	480	140
CT	Pre-culture	110	26	5
	Post-culture(48 hrs)	3180	142	29
EW	Pre-culture	170	42	6
	Post-culture(48 hrs)	3240	147	36
DW	Pre-culture	150	16	2
	Post-culture(48 hrs)	2240	55	10
SB	Pre-culture	30	57	NT
	Post-culture(48 hrs)	600	384	NT
JT	Pre-culture	40	42	NT
	Post-culture(48 hrs)	40	48	NT

Table II.

Surface membrane TfR expression and [^{3}H]TdR incorporation following suspension culture of AML blasts.

* Mean cellular immunofluorescence

NT Not tested

In five patients there is a strong correlation between the relative increases in TfR expression and [^{3}H]-TdR incorporation seen after 48 hours of culture. One patient (JT) served as a control, in that when no increase was seen in TfR expression this was also the case for [^{3}H]-TdR incorporation.

3.3 Blast Cell Colony Growth Following FACS Sorts

As TfR expression is then a measure of cell proliferation it was used to confirm that highly proliferative cells within leukaemic blast populations are in fact leukaemic progenitor (clonogenic) cells. Following anti-CD71 labelling it was possible, using the FACS, to divide leukaemic blast cell populations into separate fractions according to their TfR expression. These fractions were then cultured in blast cell colony assays.

In four cases separate TfR positive and TfR negative cell fractions were collected (table III). In all four patients studied a high proportion of the blast cell colonies present in the unsorted controls were recovered in the TfR positive fraction. Unfortunately in two cases insufficient cells were obtained in the TfR negative fraction to set up a blast colony assay, but in the two cases where sufficient cell numbers were obtained from the TfR negative fraction there were either no colonies seen or the number was only two thirds of that seen in the corresponding TfR positive

		Colonies/10^4 cells*#		
Patient	%anti-CD71 positive(FACS)	Control@	anti-CD71 positive	anti-CD71 negative
LP	87	155	94	0
DA	88	83	96	NS
CT	93	30	16	NS
DW	45	24	36	24

Table III.

Blast cell colony growth in anti-CD71 positive and anti-CD71 negative fractions.

NS = Insufficient cells obtained for culture

* In each case the number of colonies (defined as >20 cells) is the mean of quadruplicate cultures.

\# The total number of cells recovered after FACS sorting varied between 50% and 85%.

@ Control (refer to section 2.12[b])

fraction. As the unsorted control cells were treated with anti-CD71 prior to culture the results indicate the presence of this antibody was not detrimental to blast cell colony formation.

In a further three cases it was not possible to discern definite TfR positive and TfR negative fractions. In these cases it was decided to divide the blast cell populations into TfR strongly and weakly positive fractions (table IV). In all three cases the majority of blast cell colonies present in the unsorted control were recovered in the TfR strongly positive fractions. In the two cases where some colony growth was observed in the TfR weakly positive fractions a more detailed analysis of colony size was undertaken (table V). This analysis showed that in both cases the vast majority of colonies of greater than 40 cells in size were present in the strongly TfR positive fraction. In one case it was also clear that more colonies of 20-40 cells in size were seen in the TfR positive fraction.

When leukaemic blast populations were divided on the basis of TfR expression results obtained from subsequent blast colony assays support the notion that the cells which constitute the leukaemic progenitors (clonogenic cells) do in fact express high levels of TfR.

3.4 The Effect of Cell Crowding on Proliferation

The observation noted in a number of early leukaemic blast colony assays that cell concentration may be a

		Colonies/10^4 cells*		
Patient	% cells strongly anti-CD71 positive	Control@	Strongly anti-CD71 positive [Fract. 1]	Weakly anti-CD71 positive [Fract. 2]
KG	85	34	43	23
DM	70	37	26	1
SB	58	31	30	14

Table IV.

Blast cell colonies in strongly anti-CD71 positive and weakly anti-CD71 positive fractions.

The cut off on the FACS for all samples of the positive fraction was greater than gate 60.

* In each case the number of colonies (defined as >20 cells) is the mean of quadruplicate samples.

@ Control (refer to section 2.12[b])

Patient		Colonies/10^4 cells		
		>40 cells	20-40 cells	10-20 cells
KG	Control*	3	31	41
	Fraction 1 (strongly pos.)	4	39	49
	Fraction 2 (weakly pos.)	1	22	42
SB	Control*	17	14	14
	Fraction 1 (strongly pos.)	18	13	13
	Fraction 2 (weakly pos.)	2	12	12

Table V.

Analysis of colony size in strongly anti-CD71 positive and weakly anti-CD71 positive fractions.

In this study colonies were divided into >40 cells, 20-40 cells and clusters of 10-20 cells (mean of quadruplicate cultures).

* Control (refer to section 2.12[b])

critical factor controlling leukaemic cell growth was investigated by a series of experiments in which the relationship between cell crowding and proliferation was examined.

Suspension cultures of 5637-CM stimulated (figure 3.4) and unstimulated (figure 3.5) leukaemic blast cells from seven patients were placed in microtitre plates with round bottomed and flat bottomed wells to simulate crowded and non-crowded conditions (see fig 2.3(i)). When stimulated and unstimulated blast cells were cultured in flat bottomed wells cell proliferation, as measured by DNA synthesis, was directly proportional to cell numbers up to a concentration of $250x10^3$ cells/ml. In contrast in round bottomed wells the dependence of DNA synthesis on cell density was abolished in the stimulated cultures and reduced in unstimulated cases.

In fact in both unstimulated and stimulated cultures there was a significant increase ($p \leq 0.05$) in the rate of DNA synthesis in the round bottomed wells at each of the three lower concentrations ($\leq 62.5x10^3$/ml) compared to the flat bottomed wells. Therefore cell proliferation at low cell concentration ($\leq 62.5x10^3$/ml) was increased under crowded conditions even when stimulated cells were cultured.

To investigate whether actual cell to cell contact was responsible for this crowding effect contact was reduced by addition of latex beads of similar diameter (15.8μm) to the cells (see fig 2.3 (ii)). Beads were

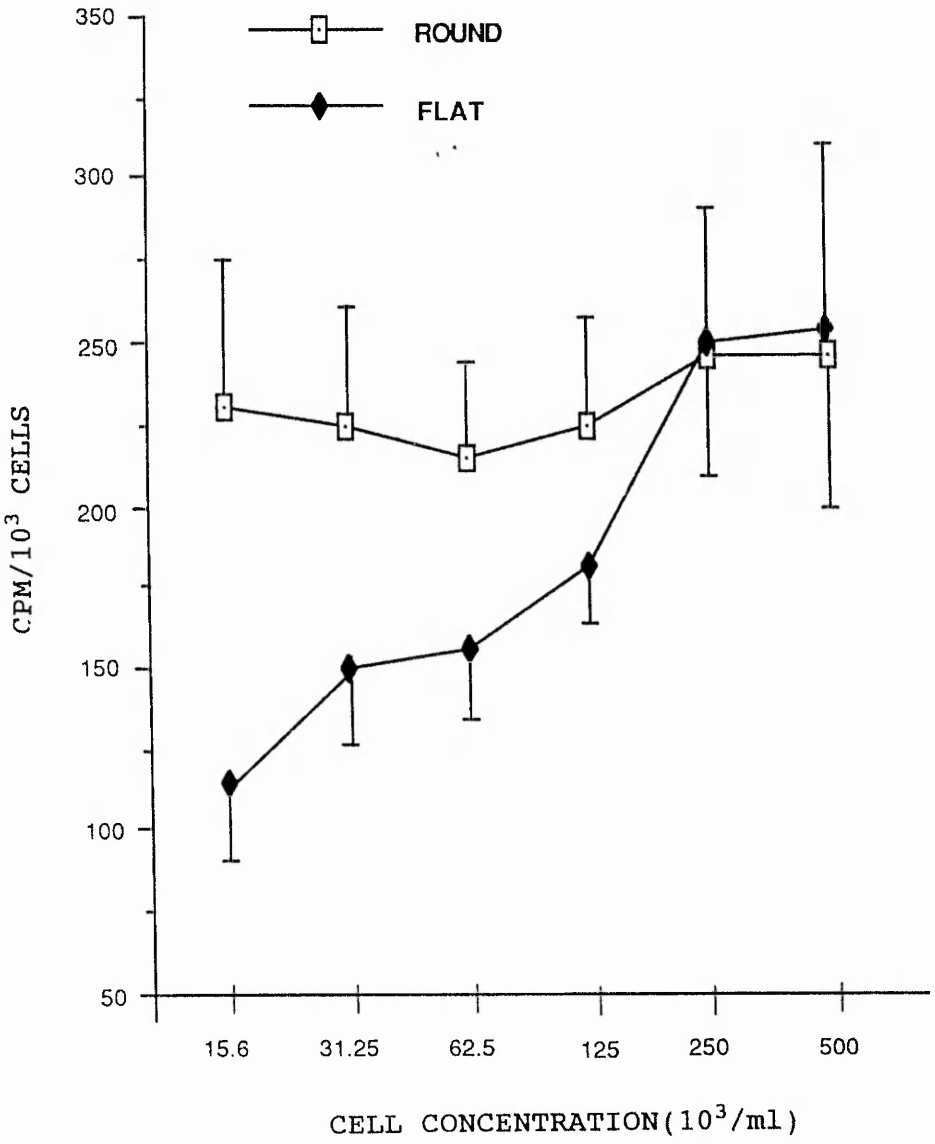


Figure 3.4

DNA synthesis ([^{3}H]TdR incorporation) of stimulated AML blast cells cultured at specified concentrations in round bottomed and flat bottomed wells. At concentration $\leq$ 62.5x10^3/ml DNA synthesis was significantly greater ($p \leq 0.05$) in round bottomed as compared to flat bottomed wells.

Results are the mean$\pm$SEM of 7 patients.

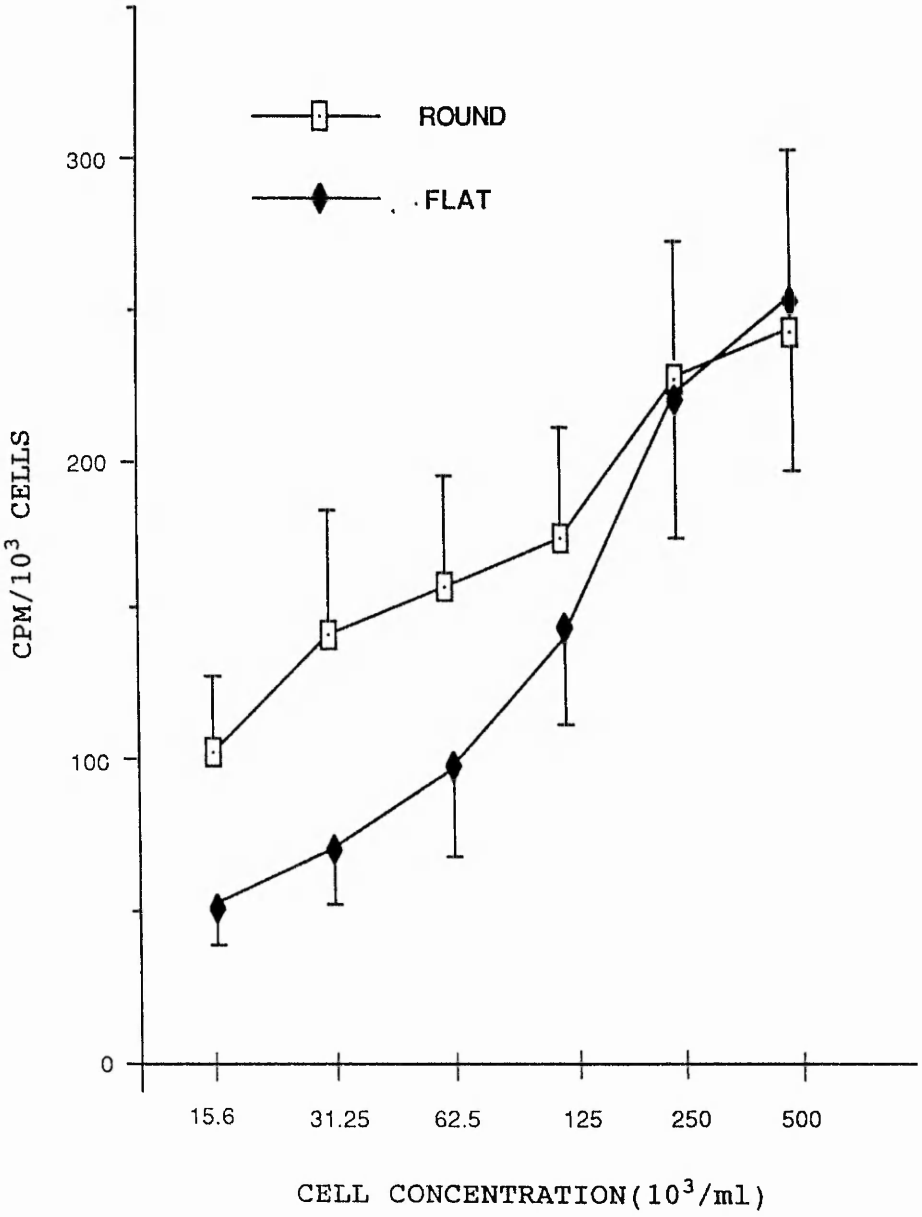


Figure 3.5

DNA synthesis ([^{3}H]TdR incorporation) of unstimulated AML blast cells cultured at specified concentrations in round bottomed and flat bottomed wells. At concentrations $\leq$ 62.5x10^3/ml DNA synthesis was significantly greater ($p \leq 0.05$) in round bottomed as compared to flat bottomed wells.

Results are the mean$\pm$SEM of 7 patients.

incorporated into stimulated cultures at the two lowest cell concentrations $15.6x10^3$/ml (figure 3.6) and $31.25x10^3$/ml (figure 3.7) at ratios of 1:40 and 1:100 cell:beads. A significant reduction in DNA synthesis was seen at both cell concentrations in round bottomed wells at both cell:bead ratios ($p=0.05$ in each case), whereas no effect was seen when beads were added to flat bottomed wells. In fact the addition of latex beads reduced the DNA synthesis in round bottomed wells to the level of that in flat bottomed wells.

These latex bead results indicate that direct cell to cell contact is an important element of the mechanisms responsible for the crowding effect seen at low cell concentrations.

It was also necessary to verify that the differences in cell proliferation levels between round bottomed wells and flat bottomed wells at low cell concentration were in fact consistent with production of leukaemic progenitor (clonogenic) cells.

Stimulated leukaemic blast cells were cultured at $31.25x10^3$/ml and $250x10^3$/ml in both round bottomed and flat bottomed wells. After 48 hours cells were harvested and plated in a blast colony assay under stimulated conditions (figure 3.8). In all cases colony numbers increased in comparison to control cultures in which no period of suspension culture had been performed, the cells being placed directly into a blast cell colony assay. Results indicate that following a short term suspension culture there is a rise in

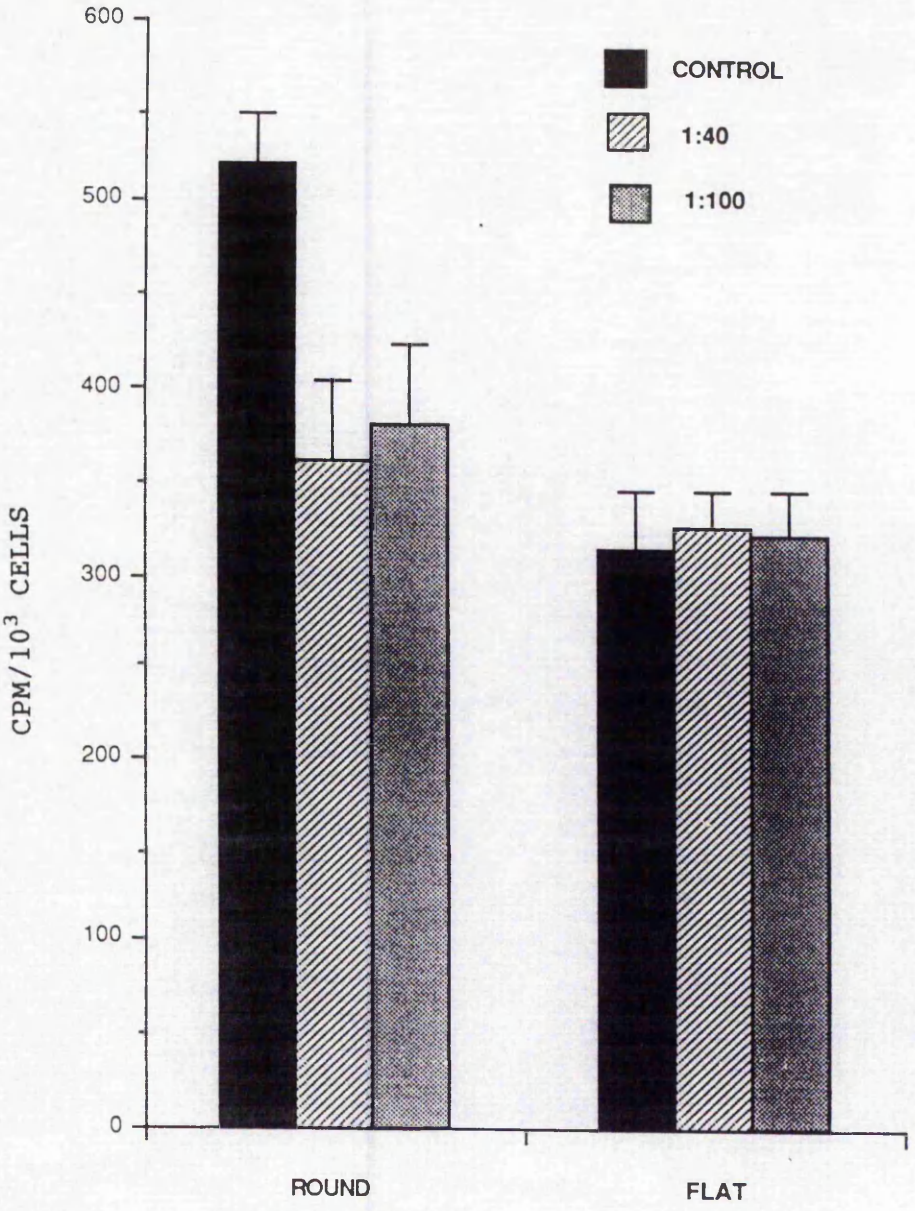


Figure 3.6

DNA synthesis ([^{3}H]TdR incorporation) of AML blast cells cultured at 15.6x10^3/ml without latex beads (control) or with beads present at two particular cell to bead ratios (1:40 and 1:100) in both round and flat bottomed wells. Significant reductions in DNA synthesis were found only on addition of latex beads to round bottom wells. (In both cases p=0.05)

Results are the mean±SEM of 4 patients.

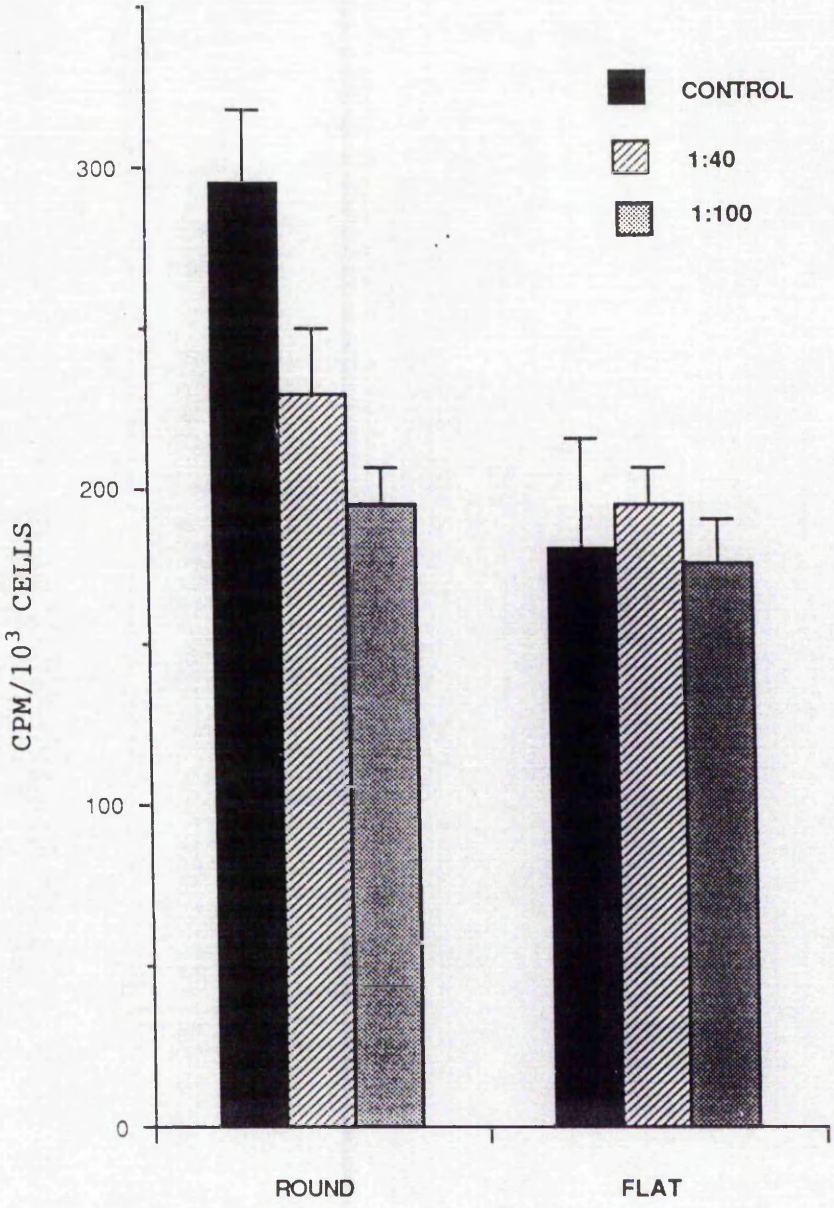


Figure 3.7

DNA synthesis ([^{3}H]TdR incorporation) of AML blast cells cultured at 31.25x10^3/ml without latex beads (control) or with beads present at two particular cell to bead ratios (1:40 and 1:100) in both round and flat bottomed wells. Significant reductions in DNA synthesis were found only on addition of latex beads to round bottomed wells. (In both cases p=0.05)

Results are the mean±SEM of 4 patients.

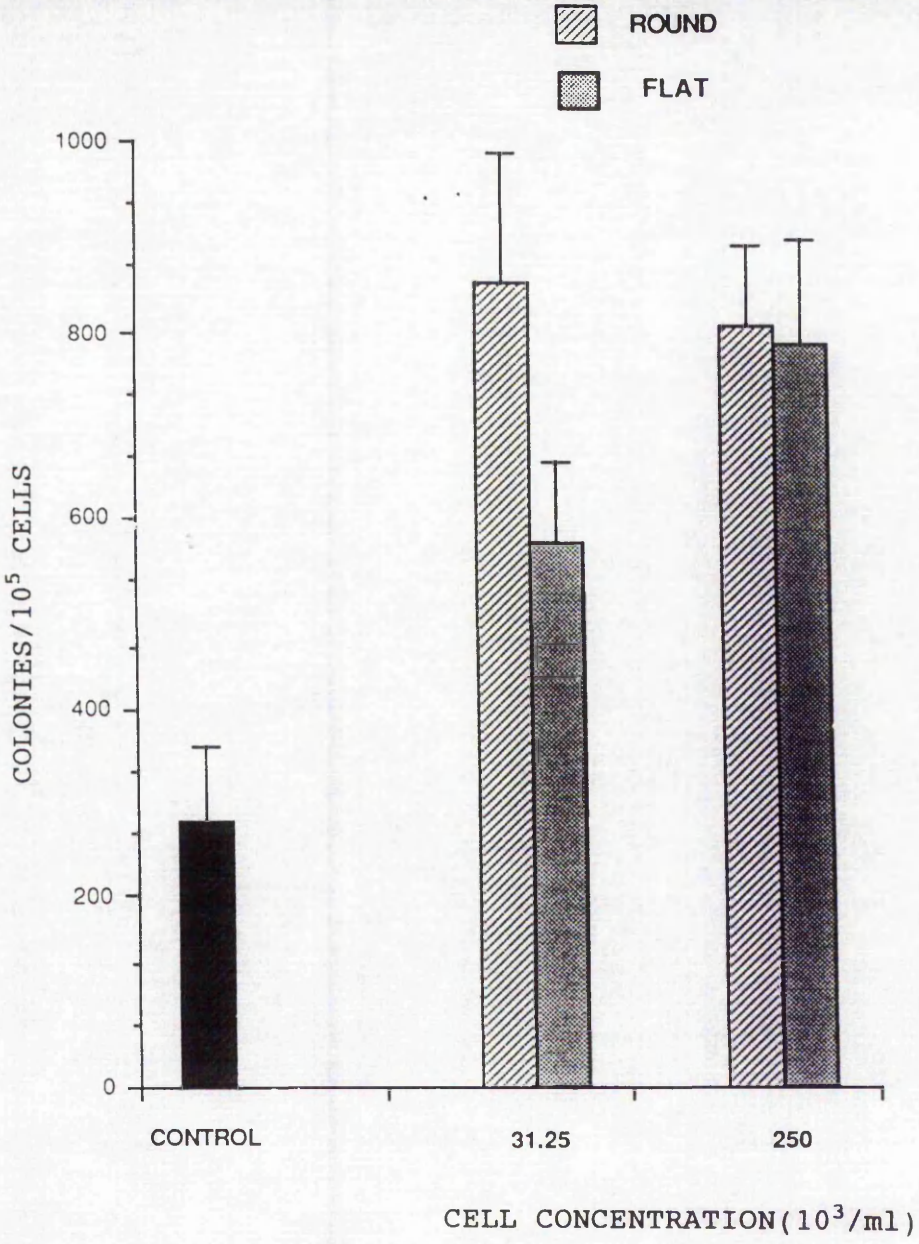


Figure 3.8

Following GCT-CM stimulation for 48 hours at two cell concentrations in round and flat bottomed wells AML blast cells were subsequently harvested and cultured in a blast colony assay with GCT-CM at 10^5/ml.

Controls were conducted with cells receiving no culture prior to blast colony assay. Only following culture at 31.25×10^3/ml in round wells were significantly greater numbers of blast colonies produced when compared to the flat ($p=0.05$).

leukaemic progenitor cell numbers, but only following suspension culture at the low cell concentration ($31.25x10^3$/ml) was there a significant increase ($p=0.05$) in subsequent colony numbers arising from round bottomed wells when compared to flat bottomed wells. This result confirms the fact that the increase in DNA synthesis seen in crowded cultures at low cell concentration is directly related to an increase in production of leukaemic progenitor cells.

It is clear that crowding increases leukaemic blast cell proliferation at low cell concentrations and direct cell to cell contact plays an important part in this process.

The fact that this crowding effect is abrogated once cells are cultured at high concentrations ($250x10^3$/ml and $500x10^3$/ml) leads to speculation that other factors, apart from crowding, may control leukaemic blast cell proliferation. Speculation was also heightened by the observation that with increasing cell concentrations leukaemic blast cells progressively lose their dependence on exogenous stimulation for proliferation. Studies were subsequently carried out to analyse other characteristics of leukaemic blast cell proliferation, in particular the concept of autonomous proliferation.

3.5 Proliferation Characteristics of AML Blast Cells

Stimulated and unstimulated blast colony assay

results, as well as FAB types, of twenty-five patients are shown in Table VI. The proliferation characteristics of these patients' blast cells were described in terms of an ASI (see 2.13). On the basis of their ASI the patients were assigned to particular groups. Group 1 patients failed to grow on repeated testing either autonomously or in response to stimulation. Group 2 patients had ASIs<0.1 which indicated that their blast cells were totally dependent on exogenous stimulation for proliferation. Group 3 patients had ASIs between 0.1 and 0.8 demonstrating that their blast cells display some ability for autonomous proliferation but exogenous stimulation is required for maximum colony growth. Group 4 patients had ASIs>0.8 indicating the high level of autonomous proliferation shown by the patients blast cells. In fact addition of exogenous stimulation to Group 4 patients increased blast cell proliferation in only two cases with the rise in colony numbers being <20% improvement on the unstimulated cultures. In the remaining five Group 4 patients identical blast colony numbers were seen in both stimulated and unstimulated cultures.

These results indicate that the proliferation characteristics of AML blast cells from the twenty-five patients studied were extremely heterogeneous. As Group 3 and Group 4 patients blast cells were the ones showing varying degrees of autonomous proliferation they were deemed most suitable for further examination.

Patients	FAB type	No. of colonies/ 10^5 cells		Autostimulatory index *
		No.CSF	5637-CM	
Group 1				
1	M2	0	0	-
2	M2	0	0	-
3	M4	0	0	-
Group 2				
4	M2	0	115	0
5	M3	0	35	0
6	M5a	0	235	0
Group 3				
7	M1	280	520	0.54
8	M1	220	900	0.24
9	M1	270	420	0.64
10	M2	15	100	0.15
11	M3	23	93	0.25
12	M4	25	95	0.26
13	M4	30	44	0.68
14	M4	20	40	0.50
15	M4	160	300	0.53
16	M4	105	265	0.40
17	M5b	285	555	0.51
18	M5b	95	785	0.12
Group 4				
19	M1	325	293	1.10
20	M4	260	290	0.90
21	M4	290	270	1.07
22	M4	440	370	1.19
23	M4	570	510	1.12
24	M5b	345	440	0.78
25	UNC	460	460	1.00

Table VI.

Autonomous growth of AML blasts: colony formation and the autostimulatory index (ASI).

* See methods
UNC = Unclassified

3.6 Comparison of Group 3 and Group 4 Patients

Results indicating the proliferation of Group 3 and Group 4 patients blast cells in suspension cultures at a series of cell concentrations with and without exogenous stimulation are shown in Figures 3.9 and 3.10.

In the Group 3 patients studied increasing cell concentration lead to a gradual reduction in the dependence on exogenous stimulation for maximum cell proliferation. With cells at concentrations $\leq 125 \times 10^3$/ml addition of exogenous stimulation produced a significant increase in their level of DNA synthesis. Whilst at 250×10^3/ml there was no significant difference between the stimulated and unstimulated cultures.

In contrast to Group 3 patients the Group 4 patients blast cells at all concentrations showed no significant differences in DNA synthesis when cultured in the presence or absence of exogenous stimulation.

These suspension cultures were consistent with results from the blast cell colony assays and consequently support the theory of autocrine growth of blast cells in Group 3 and Group 4 patients.

Only at 250×10^3/ml were Group 3 blast cells completely autonomous, whereas in Group 4 this phenomenon was seen at all concentrations tested suggesting that these two groups may have different mechanisms for maintaining autocrine growth. Further investigations were carried out to ascertain whether this difference between the Group 3 and Group 4 patients

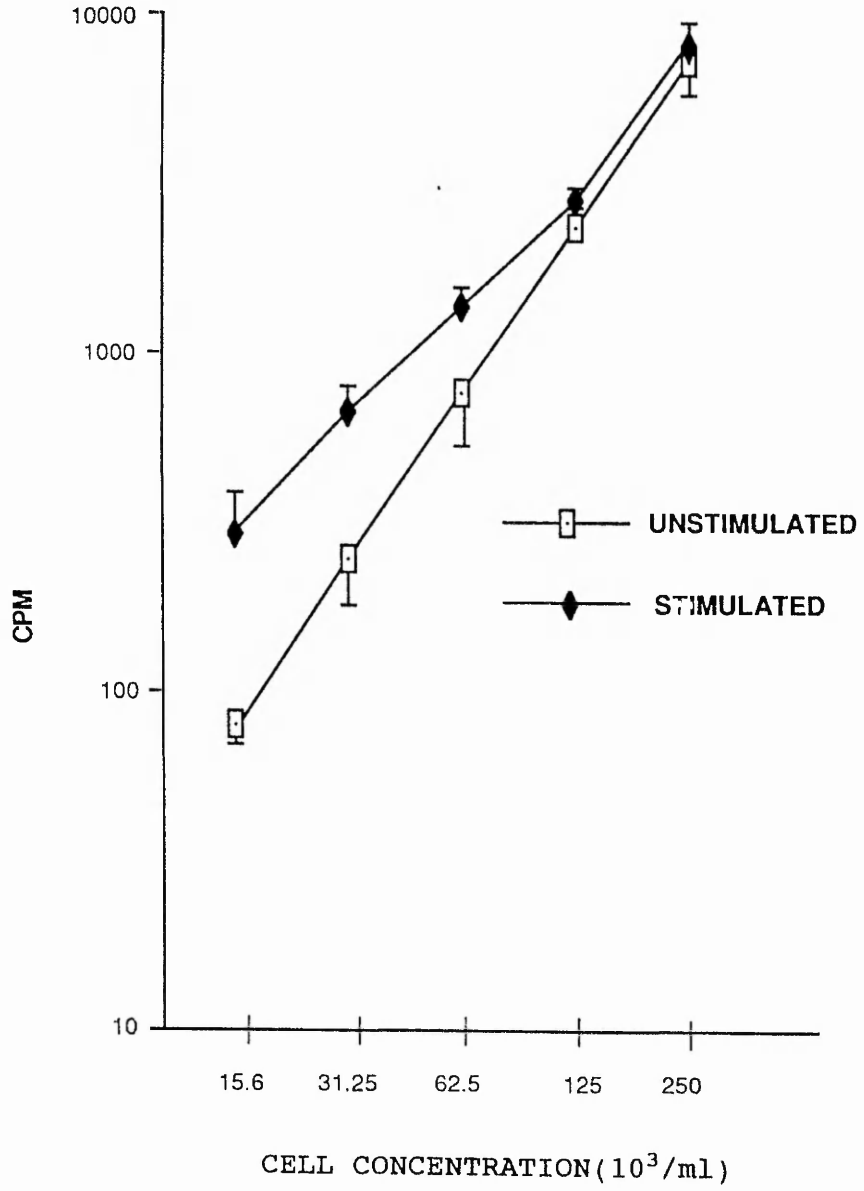


Figure 3.9

DNA synthesis ([^{3}H]TdR incorporation) in unstimulated and stimulated (5637-CM or GCT-CM) AML blast cells from group 3 patients cultured at a series of concentrations in flat bottomed wells.

At concentrations $\leq 125x10^3$/ml the p values were all $\leq$ 0.05.

Results are the mean$\pm$SEM of 4 patients.

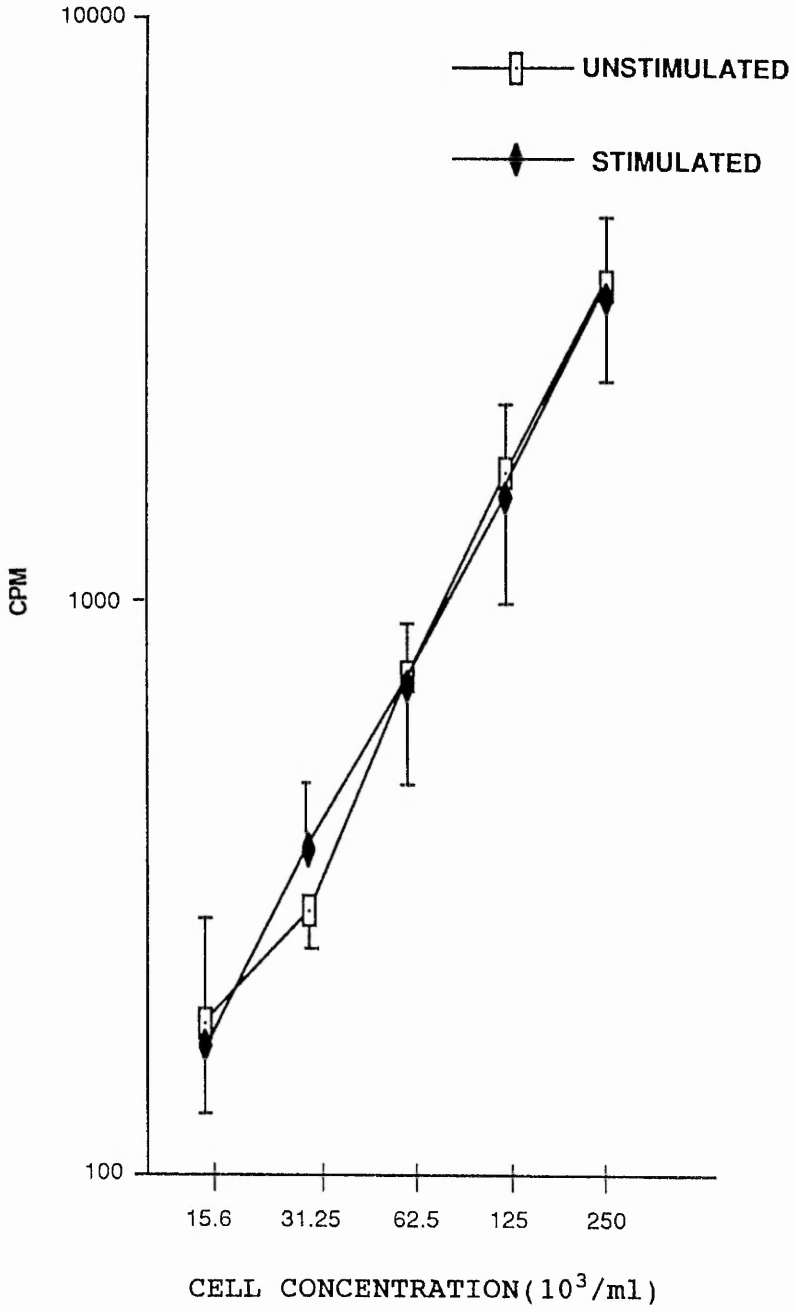


Figure 3.10

DNA synthesis ([^{3}H]TdR incorporation) in unstimulated and stimulated (5637-CM or GCT-CM) AML blast cells from group 4 patients cultured at a series of concentrations in flat bottomed wells. No significant differences were shown at any of the concentrations tested.
Results are the mean±SEM of 3 patients.

was due to growth stimulatory factors produced by the AML blast cells themselves. The secretion of growth factors by the blast cells from Group 3 and Group 4 patients was tested by assaying their BCCMs ability to stimulate autologous and homologous cell proliferation .

3.7 Effect of Autologous BCCM on AML Blast Cell Proliferation

Figures 3.11 and 3.12 show the effect of 20% autologous BCCM on Group 3 and Group 4 patients blast cells when cultured at a low cell concentration ($31.25x10^3$/ml).

In all four Group 3 patients tested the addition of autologous BCCM increased DNA synthesis in comparison to that seen in unstimulated cultures. The mean ±SEM for the four patients being 123±41 for unstimulated cells and 462±121 for cells stimulated with autologous BCCM.

In contrast to Group 3 patients all four Group 4 patients showed no difference in DNA synthesis between unstimulated and autologous BCCM stimulated cultures.

These results indicated that the four Group 3 patients studied did in fact secrete a growth stimulatory factor when cultured at a high concentration (10^6/ml) which was capable of increasing DNA synthesis of autologous cells cultured at a low concentration ($31.25x10^3$/ml). However growth stimulatory factor secretion by Group 4 patients remained unclear as addition of autologous BCCM to these cells had no

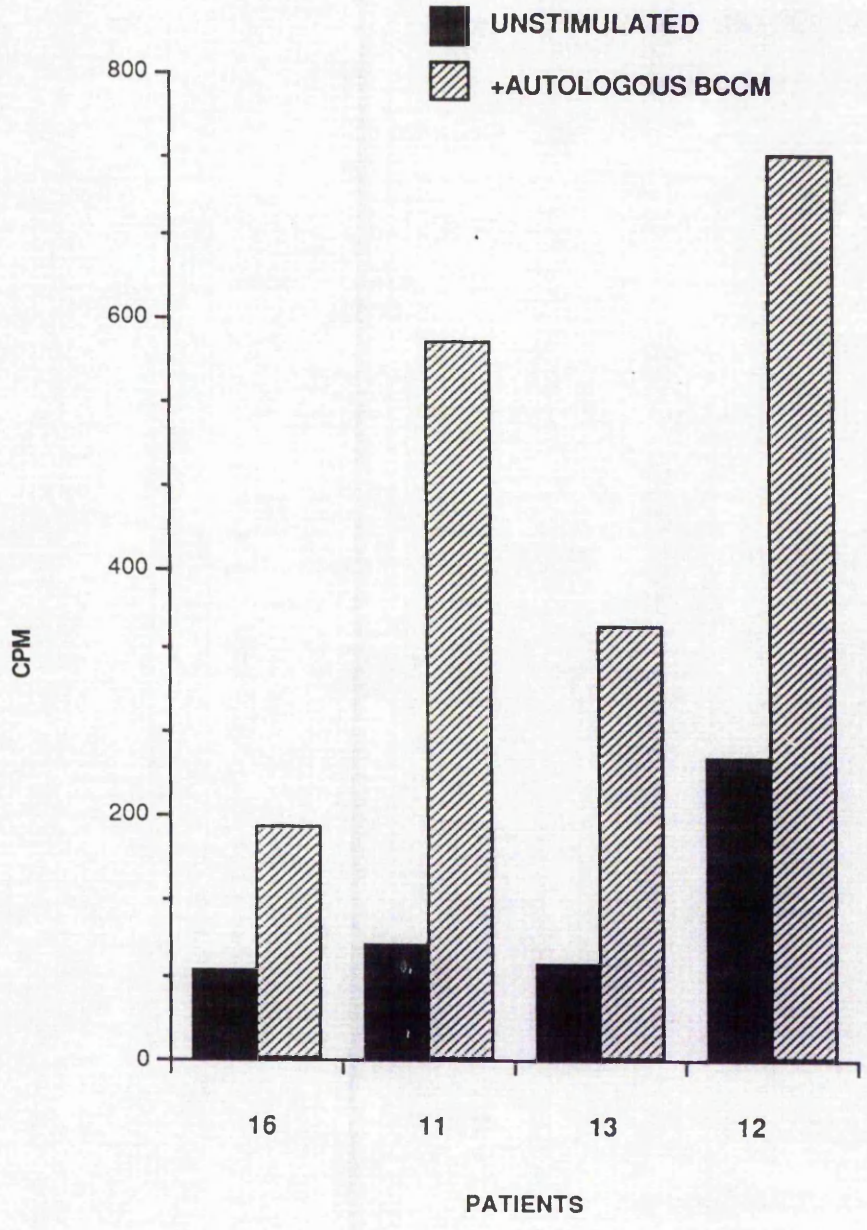


Figure 3.11

Effect of autologous BCCM on the DNA synthesis ([^{3}H]TdR incorporation) of AML blast cells from group 3 patients cultured at 31.25x10^3/ml in flat bottomed wells.
DNA synthesis is significantly increased by addition of autologous BCCM (p=0.05).

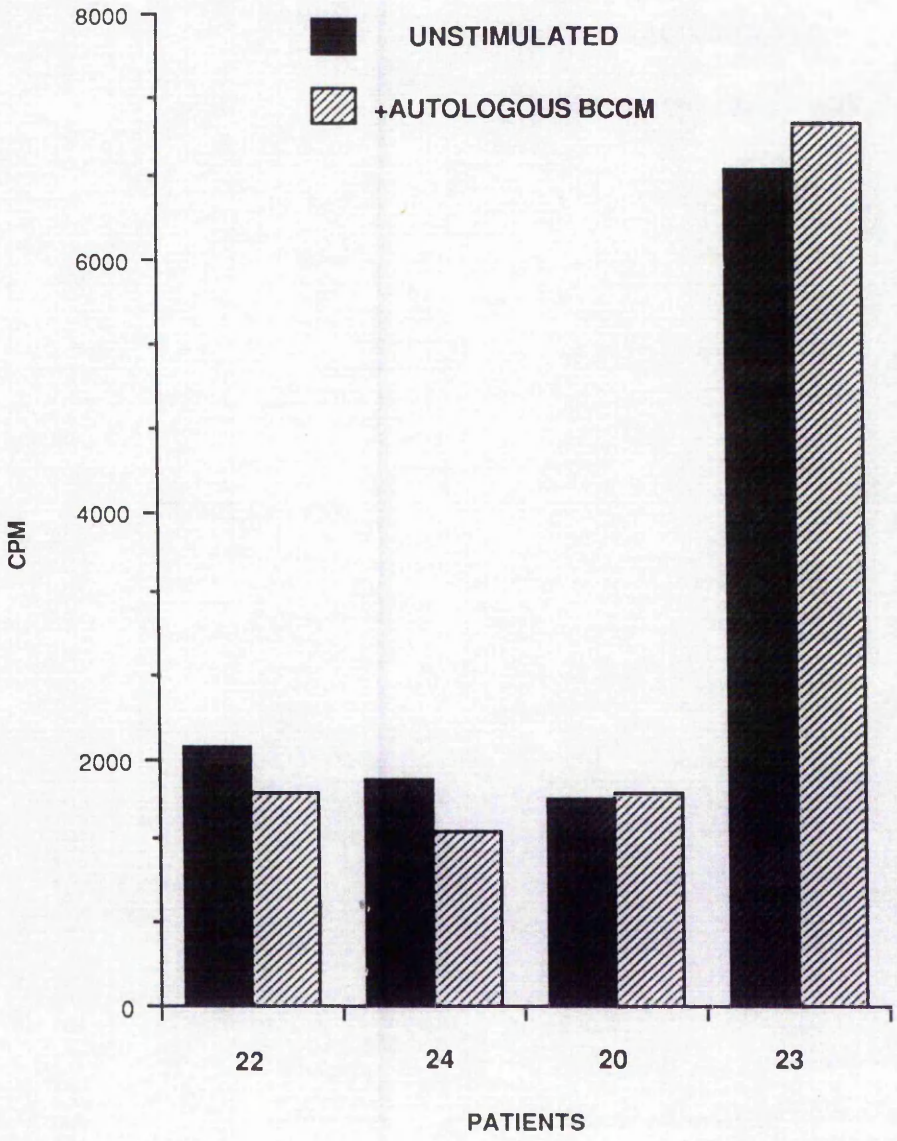


Figure 3.12

Effect of autologous BCCM on the DNA synthesis ([^{3}H]TdR incorporation) of AML blast cells from group 4 patients cultured at 31.25x10^3/ml in flat bottomed wells.

In these 4 patients no significant change in DNA synthesis occurs following addition of autologous BCCM.

effect. This may be due to the fact that even when at low cell concentrations Group 4s are totally autonomous and consequently reach a point of optimal proliferation even in unstimulated conditions.

One cytokine that had been implicated as a possible autocrine growth factor was granulocyte-macrophage colony stimulating factor (GM-CSF). Following acquisition of a monoclonal anti-GM-CSF antibody (anti-GM-CSF), with the capacity to block GM-CSF binding to it's receptor, the role of this growth factor in the proliferation of Group 3 and Group 4 blast cells was investigated.

3.8 Effect of Anti-GM-CSF on Autonomous Proliferation

The effect of anti-GM-CSF on autonomous proliferation of unstimulated blast cells from a Group 3 patient was assessed. Cells cultured at a high concentration ($250x10^3$/ml) were treated with the antibody at a series of concentrations ranging from 0.1 to 10μg/ml (Figure 3.13).

From the results it is clear that in this Group 3 patient anti-GM-CSF caused a dose dependent inhibition of the autonomous blast cell proliferation. As the plateau for maximum inhibition was achieved at an antibody concentration of 5μg/ml this concentration was used in all subsequent experiments.

Due to the encouraging result of this first anti-GM-CSF experiment more Group 3 patients, as well as a number of Group 4 patients, blast cells were treated

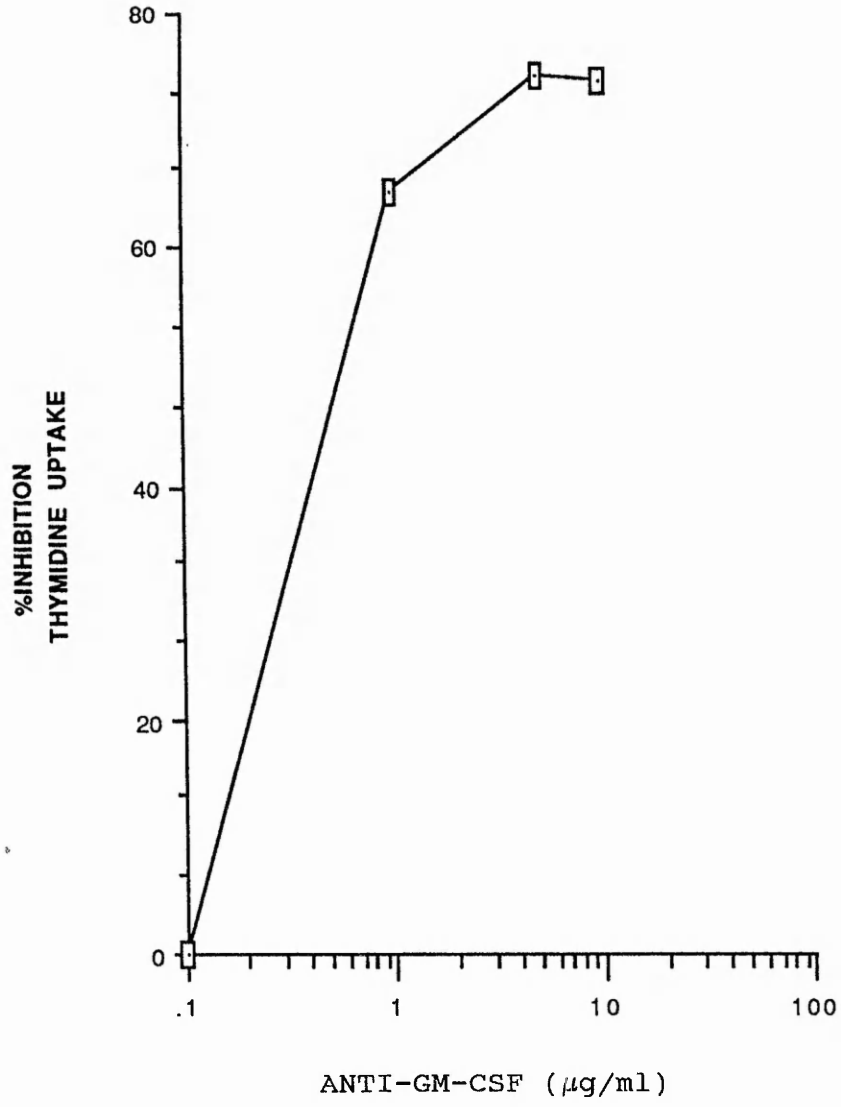


Figure 3.13

Inhibition of DNA synthesis ([^{3}H]TdR incorporation) by an anti-GM-CSF antibody was tested using unstimulated AML blast cells from a group 3 patient (No. 16) cultured at 250x10^3/ml in flat bottomed wells.

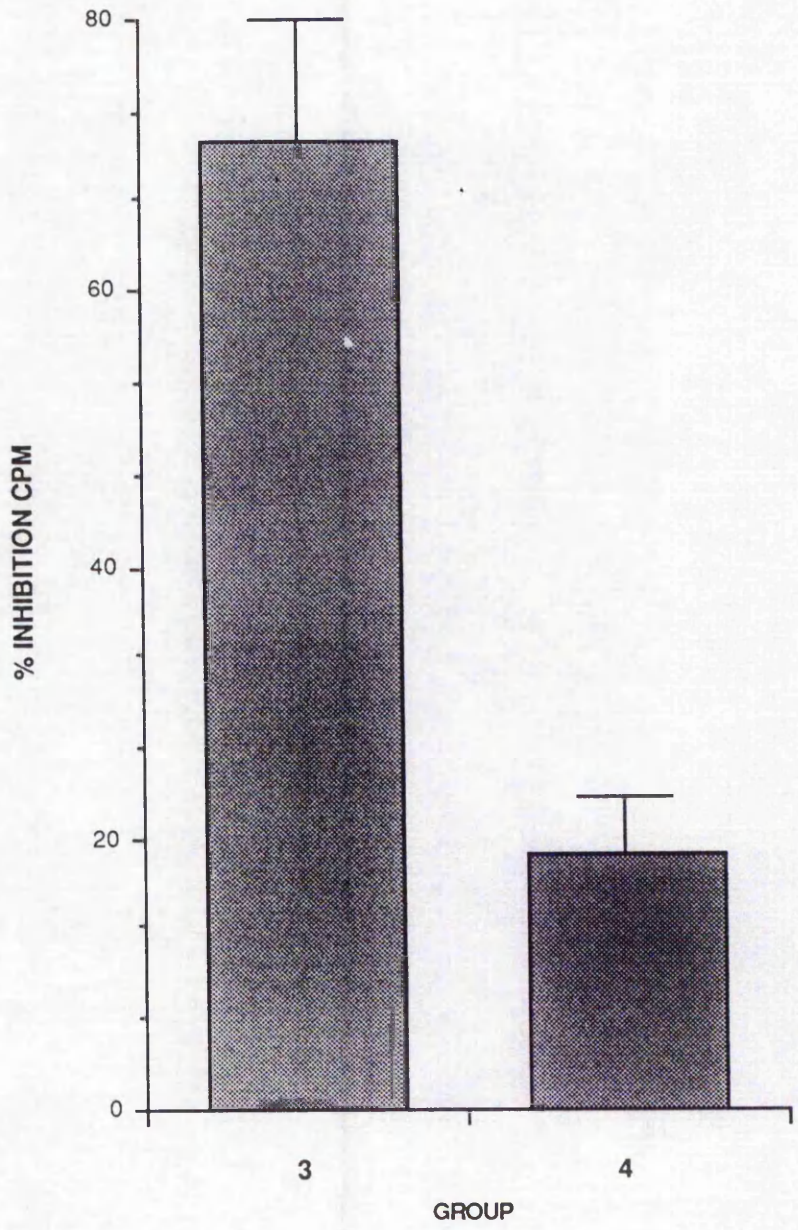


Figure 3.14

Inhibition of DNA synthesis ([^{3}H]TdR incorporation) on addition of anti GM-CSF antibody (5µg/ml) to unstimulated AML blast cells from group 3 and group 4 patients cultured at 250x10^3/ml in flat bottomed wells. Results are the mean±SEM of 5 patients from each group.

with this antibody (Figure 3.14).

The autonomous blast cell proliferation of all five Group 3 patients studied was substantially reduced by the addition of anti-GM-CSF. In contrast the inhibition of autonomous blast cell proliferation in the five Group 4 patients was negligible and remained so even when the anti-GM-CSF concentration was increased to 20μg/ml. There is in fact a significant difference in the level of inhibition between the two groups ($p=0.0295$).

The findings that anti-GM-CSF almost completely abrogated the autonomous proliferation of Group 3 patients blast cells but had little effect on Group 4 patients blast cells suggests heterogeneous mechanisms may exist for controlling autonomous blast cell proliferation.

Such heterogeneous mechanism had already been observed in autologous BCCM experiments (figures 3.11 and 3.12). BCCM from Group 3 patients contained a growth stimulatory factor which, given the anti-GM-CSF inhibition results, was most probably GM-CSF or a GM-CSF like molecule. In contrast the autonomous proliferation of Group 4 patients blast cells showed no increase following the addition of autologous BCCM and was not inhibited by anti-GM-CSF. This difference between the autonomous proliferation characteristics of Group 3 and Group 4 patients was investigated with regard to growth factor production by testing the ability of two groups BCCMs to stimulate the proliferation of normal marrow

progenitors and homologous AML blast cells.

3.9 Effect of BCCM on Normal Marrow CFU-GM

As shown in Table VII all the BCCMs tested stimulated the growth of CFU-GM from normal marrow. This stimulation in all cases was markedly reduced in the presence of anti-GM-CSF.

These results show that a growth factor capable of stimulating normal CFU-GM is present in the BCCMs of both Group 3 and Group 4 patients. The inhibition of this stimulatory activity by anti-GM-CSF again suggests that the factor involved could well be GM-CSF.

3.10 Effect of BCCM on Homologous AML Blasts

Figure 3.15 shows a preliminary experiment in which a Group 2 patient's (No 6) cells were cultured in a blast colony assay to assess the stimulatory effect of a number of homologous BCCMs. Controls were carried out to measure unstimulated (NCM) and stimulated (5637-CM) colony formation. The number of colonies/10^4 cells for the unstimulated and stimulated controls were 1 and 69 respectively, whilst the BCCM stimulated colonies numbers varied between 45 and 87.

These results show that BCCM from all patients tested stimulated proliferation of blast cells from patient No 6. In order to confirm these blast cell colony results suspension cultures were carried out with these patients cells.

Although patient No 6 had been shown to be totally

	CFU-GM/10^5	
Patients	BCCM	BCCM +anti GM-CSF(5μg/ml)
Group 3		
11	7	0
12	15	2
13	4	0
16	18	2
Group 4		
20	9	3
22	6	1
23	11	1

Table VII.

Effect of BCCM on CFU-GM from normal marrow

Control colony growth in the absence of CSF was 0 CFU-GM/10^5 cells whereas maximal stimulation with 10% 5637-CM produced 46 CFU-GM/10^5 cells.

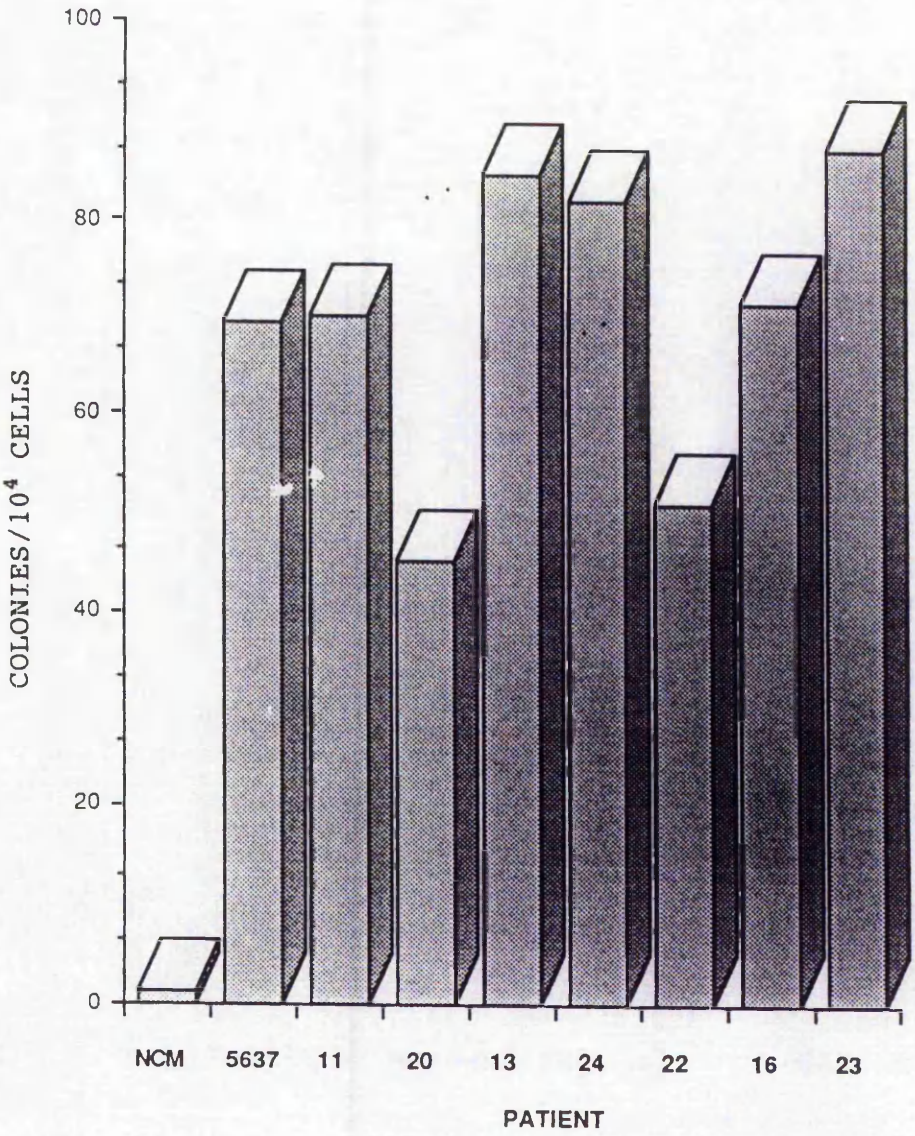


Figure 3.15

AML blast cells from a group 2 patient (No. 6) were cultured at $2x10^5$/ml in a blast cell colony assay to assess the stimulatory activity of homologous patients BCCMs.

Controls were performed to indicate the stimulated (5637-CM) and unstimulated (NCM) levels of colony formation.

dependent on exogenous stimulation for proliferation in the blast cell colony assay whether a similar response occurred in suspension cultures had not yet been confirmed.

In Figure 3.16 the mean ± SEM of eight suspension culture experiments are shown in which the proliferation of stimulated and unstimulated blast cells from patient No 6 were compared.

As these experiments demonstrated that blast cells from patient No 6 require exogenous stimulation for proliferation in suspension culture they confirmed the suitability of these cells for testing the stimulatory activity of homologous BCCMs.

Results in Figure 3.17 indicate that all six homologous BCCMs tested stimulated proliferation of patient No 6's blast cells in suspension culture. The mean ± SEM for the six BCCM stimulated cultures being 355 ± 33 CPM/10^3 cells. The presence of anti-GM-CSF in BCCM treated cultures significantly inhibited the stimulation in all cases ($p=0.0180$) with the mean ± SEM reduced to 157 ± 251.

The proliferation of normal CFU-GM and blast cells from patient No 6 in response to stimulation with Group 3 and Group 4 patients BCCMs indicates that a growth stimulatory factor is in fact produced and secreted by both groups.

Autonomous proliferation of blast cells from Group 3 patients as well as the stimulatory activity shown to

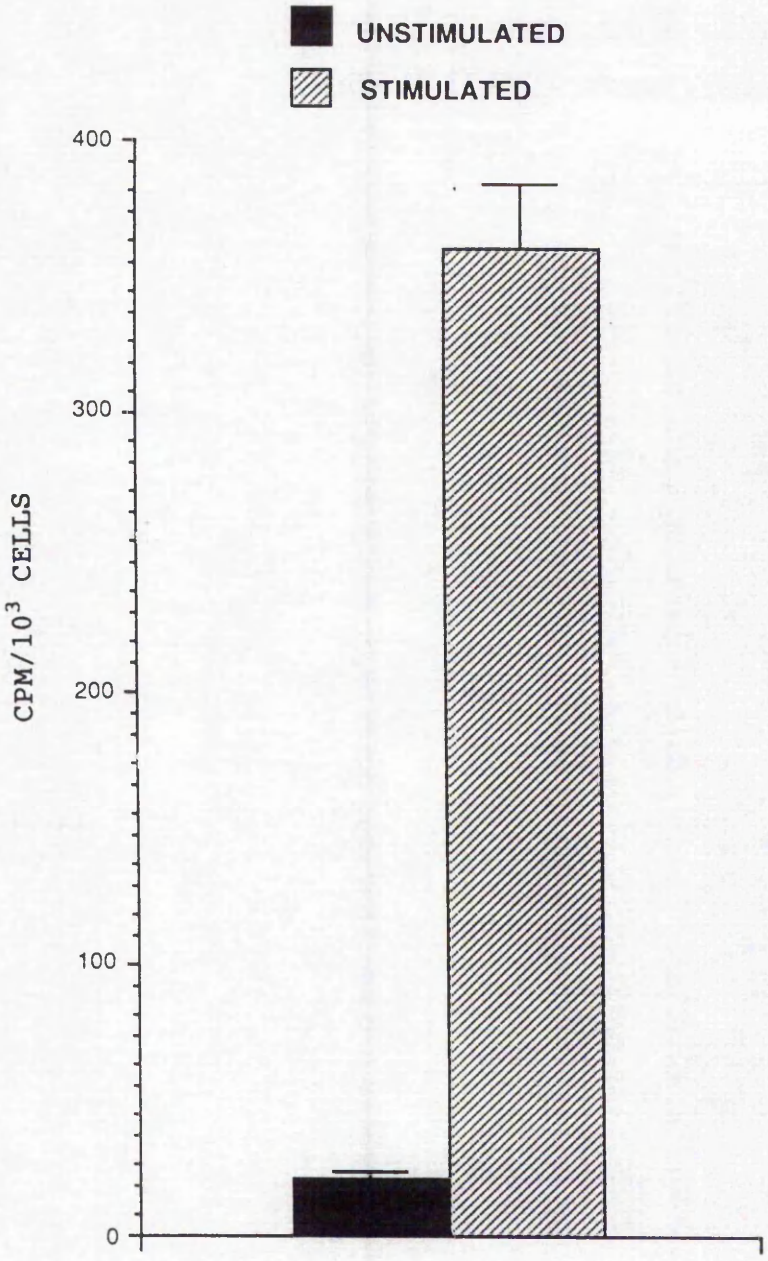


Figure 3.16

DNA synthesis ([^{3}H]TdR incorporation) in unstimulated and stimulated (5637-CM) AML blast cells from a group 2 patient (No. 6) cultured at 250x10^3/ml in flat bottomed wells.

Results are the mean+SEM of 8 experiments.

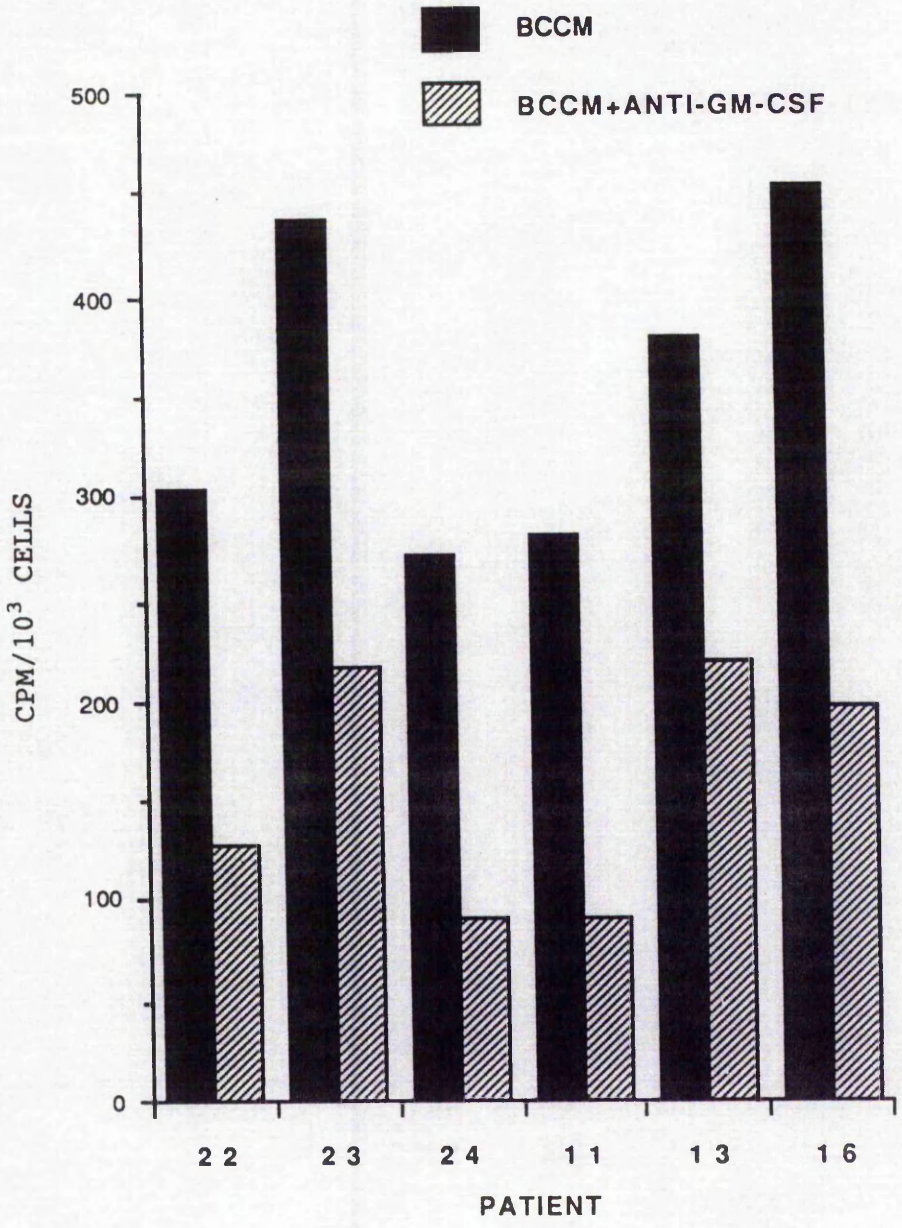


Figure 3.17

DNA synthesis ([^{3}H]TdR incorporation) of AML blast cells from a group 2 patient (No. 6) cultured at 250x10^3/ml in flat bottomed wells when stimulated with homologous patients BCCMs. Presence of GM-CSF in the BCCMs was investigated using polyclonal anti-GM-CSF antibody (5µg/ml).

be present in this group's BCCMs are both reduced by the addition of anti-GM-CSF. In contrast although the addition of anti-GM-CSF significantly inhibited the stimulatory activity of the Group 4 patients BCCMs it had very little effect on the autonomous proliferation of these patients blast cells.

These results suggest that the stimulatory factor secreted by the Group 3 patients serves as part of an autocrine loop which maintains autonomous proliferation of their blast cells. Whilst in Group 4's although this factor is secreted it does not appear to be responsible for the autonomous proliferation of these patients blast cells. This difference in autonomous proliferation was studied by analysing subcellular fractions from both groups.

3.11 Subcellular Fraction Analysis

Membrane and cytosolic fractions prepared from the blast cells of two patients from Group 3 and two from Group 4 were tested for stimulatory activity in suspension culture using a Group 2 patients (No 6) blast cells (Table VIII). Controls were performed to measure unstimulated, stimulated with and without addition of anti-GM-CSF and stimulated in the presence of an irrelevant antibody (anti-CD3). These controls indicated, once again, dependence on exogenous stimulation for proliferation of patient No 6's blast cells whilst showing the specific inhibition displayed

		DNA synthesis (^{3}H-TdR,cpm)
Controls		
No. csf/subcellular fraction		694
10% 5637-CM		10064
10% 5637-CM+anti-GM-CSF(5μg/ml)		3549
10% 5637-CM+OKT 3 *		9531
Group 3		
Membranes	10% vol:vol	488±9
Cytosol	10% vol:vol	620±7
Group 4		
Membranes	10% vol:vol	410±177
Cytosol	10% vol:vol	1478±266
Cytosol	10% vol:vol + anti-GM-CSF(5μg/ml)	860±232

Table VIII.

Effect of AML blast subcellular fractions from Group 3 and Group 4 patients on the proliferation of homologous blast cells.

Values are mean±SEM of experiments carried out with subcellular fractions from two patients in both Group 3 and Group 4 used to assess the stimulation of DNA synthesis in CSF dependent blasts from patient 6.

* Non-specific antibody control.

by anti-GM-CSF.

The membrane and cytosol fractions from Group 3 patients showed no evidence of stimulatory activity. Group 4 patients also showed no stimulatory activity in the membrane fraction but some was present in the cytosol fraction, with this activity being inhibited by anti-GM-CSF. These results suggest that only in Group 4 patients is there intracellular retention of a stimulatory growth factor. This intracellular growth factor could be responsible for autonomous proliferation and may explain why the anti-GM-CSF effect is so negligible.

DISCUSSION

4. DISCUSSION

A number of different experiments were carried out in this study to analyse the proliferation of AML blast cells. Initially TfR expression of these cells was compared to their proliferation characteristics.

Following labelling of the TfRs, either directly with anti-CD71 antibody or indirectly with anti-Tf antibody, fluorescent microscopy and flow cytometric analysis indicated that TfR expression occurred in a high percentage of the leukaemic blast cell population in all the AML patients tested. These results were in accord with those of Larrick et al [284], Omary et al [285] and Taetle et al [286] who utilized either iodinated (I^{125}) transferrin or the anti-CD71 antibodies 42/6 and B3/25 to label the TfR. Sutherland et al [60] used the same anti-CD71 antibody as we had employed (OKT9) and again obtained results which concurred with ours. The low TfR expression which we detected on normal peripheral blood mononuclear cells was also similar to the levels reported by Sieff et al [59] and Sutherland et al [60].

Following confirmation that TfRs occur on the majority of leukaemic blast cells the next step was to confirm whether TfR expression was proportional to blast cell proliferation.

After exposure to short term suspension culture blast cells from six AML patients were analysed for TfR expression and cell proliferation. TfRs were again

labelled with anti-CD71 as well as fluorescently conjugated transferrin (FITC-Tf) which had been previously tested for its specificity by competitive binding studies with 100 fold excess diferric transferrin. Cell proliferation was assessed by measuring DNA synthesis.

Blast cells from five of the patients displayed a significant increase in their TfR expression when subjected to short term suspension culture. The percentage increases in TfR expression were almost identical when assessed by either anti-CD71 or FITC-Tf. DNA synthesis in the same five patients' blast cells was also enhanced over the short term culture period. In the one patient where no increase in TfR expression occurred there was also no change seen in the level of DNA synthesis.

These findings are similar to results obtained with leukaemic cell lines, such as HL60, which indicate that TfR expression is directly related to proliferation and show that de novo production is the cause of the increasing numbers of TfRs expressed during exponential growth [287]. It has also been shown that the decline in proliferation of cell lines which occurs following treatment with factors known to cause differentiation, such as dimethyl sulphoxide, is accompanied by a similar reduction in TfR expression [60, 287]. From our results it is clear the level of TfR expression by AML patients blast cell population is a measure of their rate of proliferation.

These proliferation studies indicated the relationship between TfR expression and proliferation of the entire blast cell population but had shed no light on these characteristics with respect to individual blast cells.

The flow cytometric analyses carried out on the FACS had shown that heterogeneous TfR expression was displayed within a leukaemic blast cell population. This fact, coupled with the ability of the FACS to isolate cells according to their level of fluorescent intensity, was then exploited in an effort to divide a patient's leukaemic blast population on the basis of TfR expression.

With this technique leukaemic blast cell populations in four patients were divided into TfR positive and TfR negative fraction. In another three patients the extremely heterogeneous nature of TfR expression displayed by the blast cell population made it impossible to isolate a purely negative fraction. In these three patients separation of the blast cell population was performed by collecting TfR strongly positive and TfR weakly positive fractions. Following collection fractions were cultured in blast cell colony assays to detect which contained the AML-CFU.

In all patients the vast majority of AML-CFU were found in the TfR positive and TfR strongly positive fractions. In two patients a small number of AML-CFU

were present in the TfR weakly positive fraction. Further analysis of these two patients showed that the colonies cultured from the TfR weakly positive fraction were much smaller in size than the ones obtained from the TfR strongly positive fractions. This result suggested that the TfR strongly positive cells have a much greater proliferative capacity when compared to the TfR weakly positive cells.

Results from these blast cell colony assays are evidence that the AML-CFU were present in the highly TfR expressing fractions of the leukaemic blast cell population. Our findings that AML-CFU are highly TfR expressing cells are supported by data published by Clarkson et al [235] and Minden et al [236] who found that AML-CFU remain in the active S phase of the cell cycle and subsequently display no resting Go phase.

In conclusion although TfRs are found on a large proportion of leukaemic blast cell they also display heterogeneous expression within the blast cell population and the fraction of the population with the highest expression was shown to contain the AML-CFU. These findings support the theory of a LSC which has recently been discussed by Griffin [227] and McCulloch [34] in two excellent reviews. The discovery that AML-CFU have high levels of TfR expression is not only important to our understanding of AML biology but also suggests a fundamental difference between normal haemopoietic progenitors and the cells responsible for maintaining the entire leukaemic cell population.

In contrast to AML-CFU the majority of early haemopoietic progenitors, such as CFU-GEMM, have been shown, through cell cycle studies, to remain quiescent during basal haemopoiesis which is also evident by the fact that only very low percentages of early progenitor cell populations express TfR [52, 59, 61-64]. This difference in TfR expression could be important to the development of a technique for the specific elimination of the AML-CFU.

At present there are no known cell surface antigens specific to AML blasts. In fact AML blasts display aberrant expression of the cell surface antigens usually found on normal haemopoietic progenitors [232, 233]. The TfRs are then the only cell surface antigens presently available on AML-CFU that could be exploited for specific cytotoxic targetting. Development of cytotoxic conjugates that are specific for the TfRs may lead to the eradication of the AML-CFU whilst leaving the majority of the normal haemopoietic progenitor cell population unaffected. As TfRs are known to enter the intracellular environment, cytotoxic agents linked to either anti-CD71 antibodies or transferrin itself would be an excellent way to deliver the agent directly into the target cells. Recently a number of cytotoxic agents, including diphtheria toxin, ricin toxin, adriamycin and the ribosomal-inactivating protein S0-6, have been bound to either transferrin or the anti-CD71 antibody B3/25 [288-292]. All of these conjugates show

significant cytotoxicity against various malignant cell lines which are known to express high levels of TfRs. Therefore a study to assess the effect of these cytotoxic conjugates on the in vitro proliferation of AML-CFU and normal haemopoietic progenitors is a path worthy of pursuit.

Whilst studying the proliferation characteristics of AML blast cell with respect to TfR expression it was observed that cell density may be another factor which regulates the proliferation of AML blast cells. Cell concentration had already been shown as a crucial factor in the proliferation of normal B lymphocytes and the leukaemic cell line HL60 [293, 294]. One study also found that AML blast cells would only proliferate in suspension cultures plated in 35mm petri dishes when the cell concentration was 10^6/ml or higher [238]. These findings suggested a possible association between cell concentration and cell proliferation which led us to conduct a series of experiments where the role of cell to cell contact was examined with regard to it's effect on AML blast cell proliferation.

Stimulated and unstimulated AML blast cells were cultured in round and flat bottomed microtitre plate wells at a series of cell concentrations. Under these conditions maximum cell to cell contact was maintained in the round bottomed wells at all cell concentrations whilst in flat bottomed wells a critical cell concentration was required to achieve cell to cell contact below which contact would diminish.

These experiments clearly demonstrated that at low cell concentrations ($\leq 62.5 \times 10^3$/ml) AML blast cell proliferation was significantly enhanced in round bottomed wells when compared to that found in flat bottomed wells and that this cell to cell contact effect occurred in both maximally stimulated and unstimulated conditions. It was also found that in flat bottomed wells the critical cell concentration required to achieve the level of cell to cell contact at which cell proliferation became equivalent to that seen in round bottomed wells was 250×10^3/ml.

Other experiments were conducted to determine whether direct cell membrane contact was responsible for the differences in AML blast cell proliferation observed between flat and round bottomed wells at low cell concentrations ($\leq 31.25 \times 10^3$/ml). To prevent direct cell to cell contact latex beads with approximately the same diameter as the AML blast cells were incorporated into cultures in both flat and round bottomed wells. The latex beads had no effect on proliferation in the flat bottomed wells but reduced proliferation in the round bottomed wells to such an extent that it abolished the difference between cultures in round and flat bottomed wells.

These cell crowding experiments clearly indicated that cell contact had an influence on AML blast cell proliferation but whether this was reflected by an increase in AML-CFU numbers had not yet been shown. The

effect on AML-CFU was examined by placing AML blast cells in short term suspension cultures at two cell concentrations, one where a difference existed between the levels of cell proliferation in round and flat bottomed wells ($31.25x10^3$/ml) and another where there appeared to be none ($250x10^3$/ml), after which the cells were removed and grown in a blast cell colony assay.

Only at the lower cell concentration ($31.25x10^3$/ml) was there a significant difference in the numbers of AML-CFU recovered from the round bottomed wells when compared to the flat bottomed wells. The increase in AML-CFU numbers under conditions where maximum cell to cell contact was achieved indicates that this physical property can stimulate AML-CFU proliferation.

The results obtained from these experiments are consistent with the theory that cell crowding is involved in the regulation of AML blast cell proliferation but the mechanisms responsible are still a matter of conjecture. An insight into the possible mechanisms involved was obtained from the latex bead experiments which, whilst severely limiting direct cell membrane to membrane contact, did not restict the close proximity of the cells. The latex beads results were therefore a strong indication that the increase in blast cell proliferation seen in round bottomed wells was not simply due to the close proximity of cells but that direct cell membrane to membrane contact is the crucial factor in this phenomenon. The role of cell membrane to membrane contact in AML blast cell proliferation is

interesting due to the fact that cell contact effects are also known to influence normal cellular events through a variety of cell surface antigens.Normal cellular events which are influenced by cell contact include the retention of haemopoietic progenitor cells within the BMM and the regulation of lymphopoiesis through interactions between macrophages and lymphocytes [39, 295-297]. Whether similar factors are responsible for the cell contact effect seen in AML blast cell proliferation is still unclear.

It has been proposed by Metcalf that oncogene products, by acting as intermediate elements of the cytokine pathways, may be responsible for stimulating cell proliferation through intracellular mechanisms [298]. The finding that some of these oncogene products, such as the abl protein in chronic myeloid leukaemia, are localised within cell membranes leads to speculation that direct cell membrane to membrane contact may influence the way in which these proteins control intracellular processes [299, 300]. If similar abnormal oncogene proteins are present on AML blast cell membranes it is possible that the enhanced proliferation of AML blast cells due to cell contact may be caused by the activation of these proteins by direct membrane interactions.

Although the discovery that cell membranes, when isolated from AML blasts, were able to augment blast cell proliferation may support the oncogene protein

theory recent suggestions that certain cytokines remain membrane bound could also explain why isolated membranes appear stimulatory [301-303]. In fact membrane bound cytokines by exerting their effects only when direct cell to cell contact occurs could also be responsible for the enhanced proliferation seen in crowded cultures. Studies on the proliferation characteristics of AML blast cells are still an important area of research which may yet uncover the mechanism through which cell contact regulates AML blast cell proliferation. It is also important to remember that these AML blasts are after all aberrant cells and may therefore possess unique characteristics which are absent in normal haemopoietic cells.

One example of how these aberrant AML blasts differ with respect to normal haemopoietic progenitor cells has only recently been discovered. Blast cells from a number of AML patients have been shown to express mRNA for a variety of cytokines including GM-CSF, G-CSF, M-CSF, IL-1, IL-6 and TNF [251, 256, 257, 263-265]. The discovery that in some of these AML blast cells this mRNA is translated leading to the release of functional cytokines [145, 256-260, 261, 266] indicates a fundamental difference between these cells and normal haemopoietic progenitors which do not secrete cytokines. The release of cytokines by leukaemic blast cells raises an important issue regarding the biology of AML. It has been proposed that AML blast cell proliferation may be sustained by this endogeneous cytokine production

thereby obviating their need for exogeneous stimulation which again contrasts with normal haemopoietic progenitors which are entirely dependent on exogeneous stimulation for proliferation [298]. As the effects of cytokines on various mature cells is often to cause the release of more cytokines these paracrine loops may provide an avenue through which the endogeneous secretion of cytokines in AML increases the proliferation of leukaemic blast cells [304].

Evidence for these paracrine loops has recently been obtained from a study by Griffin et al in which the endogeneous release of IL-1 by AML blast cells stimulated endothelial cells to release GM-CSF and G-CSF [265]. There is also a notion that the stimulation of blast cell proliferation brought about by endogeneous secretion of cytokines in some AML patients is due to autocrine loops [300]. This theory of autocrine loops is speculative at present but is supported by reports that some AML patients whose leukaemic blast cells demonstrate endogeneous cytokine production also exhibit autonomous proliferation characteristics [256,260,263].

Whilst studying cell to cell contact mechanisms we noted that AML blast cell proliferation appeared independent of exogeneous stimulation when cells were cultured at high concentration ($\geq 250 \times 10^3$/ml). These preliminary findings supported the autonomous proliferation theory and subsequently prompted the examination of a much larger number of AML patients.

Blast cells from twenty-five patients were cultured in blast cell colony assays in both stimulated and unstimulated conditions in order to assess their degree of autonomy. It was apparent from the results that a great deal of heterogeneity existed within this group of patients with regard to their proliferation characteristics. While some patients were entirely dependent on exogeneous stimulation for proliferation others displayed totally autonomous proliferation.

Having found such a heterogeneous pattern of AML blast cell proliferation a system was devised for classifying patients on the basis of their growth characteristics in blast cell colony assays. By simply dividing the number of colonies obtained from unstimulated cultures by those measured in stimulated conditions was to provide a figure used for evaluating the degree of autonomous proliferation displayed by AML blast cells. This figure became known as the autostimulatory index (ASI) and was used to divide patients into four distinct groups [281]. A small number of patients blast cells displayed no growth in either stimulated or unstimulated conditions and were classified as Group 1. Patients whose blast cells proliferated only when stimulated were placed in Group 2. The patients in Group 3 had blast cells which displayed partially autonomous proliferation but still remained responsive to exogeneous stimulation. No difference in stimulated and unstimulated blast cell colony growth was observed in Group 4 patients which

indicated the totally autonomous nature of their blast cells. Similar heterogeneous proliferation characteristics have also been observed in studies carried out with the murine haemopoietic cell line FDC-P1 [305]. Although FDC-P1 is usually dependent on GM-CSF for proliferation following transformation of this cell line with a retrovirus encoding polyoma middle T antigen some of the clones which developed exhibited autonomous proliferation characteristics [306]. Further investigation of the cell lines which developed from the FDC-P1 cell line as a result of viral transformation revealed that they could be placed into three different groups according to their proliferation characteristics [306]. These patterns were similar to those described for the Group 2, Group 3 and Group 4 patients which had been observed in our autonomous proliferation studies [281, 306].

These blast cell colony results are in agreement with the data obtained from a number of other centres which show that a small percentage of AML patients have blast cells which possess the capacity for autonomous proliferation [256, 260, 263]. These results contradict many earlier reports which stated that exogeneous stimulation was essential for in vitro growth of AML blast cells. Since the autonomous proliferation recorded by the AML blast cells in other groups was thought to be due to autocrine loops investigations were carried out to verify that these loops were operating in

Group 3 and Group 4 patients blast cells. As cell contact has previously been shown to affect blast cell proliferation the association between this and autocrine loops in Group 3 and Group 4 patients was also scrutinised [307].

The autonomous proliferation of Group 3 patients blast cells was dependent on cell concentration, whilst this proliferation in Group 4 patients blast cells was entirely independent of cell concentration. These results indicate that cell contact may not be the only mechanism controlling autonomous proliferation in AML blast cells. As autocrine secretion of various cytokines had already been postulated as a factor which may influence autonomous proliferation it was therefore considered with regard to Group 3 and Group 4 patients.

Following the unstimulated suspension culture of Group 3 and Group 4 patients blast cells at a high cell concentration (10^6/ml) the cells were removed and the blast cell conditioned medium (BCCM) was assayed for presence of growth stimulatory activity against a variety of cells.

BCCM from Group 3 patients was found to possess significant autostimulatory activity. Whether this autostimulatory activity was due to a unique factor which was active only on Group 3 patients blast cells or whether it is one of the recognised cytokines which are already thought to have a possible autocrine role was determined by testing the BCCM against normal marrow progenitor cells as well as homologous blast cells from

a growth factor dependent Group 2 patient.

When the BCCM from four Group 3 patients was tested against normal marrow progenitors stimulation of CFU-GM growth was shown in all four, but numbers were low ($\leq$40%) in comparison to that achieved in the maximally stimulated control. Three of the four BCCMs also caused significant stimulation of homologous AML blast cells in the blast cell colony assay as well as substantially increasing proliferation in suspension culture when measured by DNA synthesis.

Group 3 patients BCCMs, by demonstrating stimulatory activity against normal CFU-GM and homologous AML blast cells, appear to contain a growth factor with properties similar to many known cytokines. GM-CSF was a likely candidate as it had already been implicated as one of the cytokines released by AML blast cells [256, 263]. To confirm the presence of GM-CSF in Group 3 patients BCCMs an anti-GM-CSF antibody known to have the ability to block the binding of this cytokine to its receptor was incorporated into some of the assays.

The effects of the Group 3 patients BCCMs on homologous AML blast cell proliferation and normal CFU-GM growth were significantly reduced in the presence of anti-GM-CSF. The autonomous proliferation of Group 3 patients blast cells at high concentration ($250x10^3$/ml) was also inhibited, by approximately 70%, in the presence of anti-GM-CSF. These anti-GM-CSF results

indicate that the major stimulatory activity present in Group 3 patients BCCMs is due to GM-CSF and that it has a significant role in the autonomous proliferation of Group 3 patients blast cells. The observations that anti-GM-CSF did not completely neutralise the effect of Group 3 patients BCCMs on homologous AML blast cells and normal CFU-GM nor totally abrogate the autonomous proliferation of Group 3 patients blast cells were indications that there may be other cytokines present in the BCCMs apart from GM-CSF.

The stimulatory activity of Group 4 patients BCCMs was also assessed by investigating its effect on autologous and homologous AML blast cells as well as normal CFU-GM. Group 4 patients BCCMs showed similar effects on the proliferation of homologous AML blast cells and normal CFU-GM as had been described with Group 3 patients BCCMs. Anti-GM-CSF again significantly reduced these effects indicating that GM-CSF was responsible for most of the stimulatory activity present in the Group 4 patients BCCMs.

In contrast to Group 3 patients the autonomous blast cell proliferation seen in Group 4 patients was not inhibited by anti-GM-CSF and could not be enhanced by addition of autologous BCCMs. These results compare well with earlier studies in which the autonomous proliferation of Group 4 patients blast cells displayed no requirement for exogenous stimulation whereas the proliferation of Group 3 patients blast cells was clearly affected by cytokines.

Therefore although GM-CSF is secreted by both Group 3 and Group 4 patients the way in which it affects the autonomous proliferation of these patients blast cells clearly differs between the two groups. Group 3 patients appear to possess an autocrine loop through which GM-CSF is able to sustain the proliferation of these patients blast cells. Group 4 patients seem to have acquired a rather novel mechanism for maintaining autonomous proliferation because although their blast cells secrete significant amounts of GM-CSF it does not appear to be required for autonomous proliferation.

Recent studies have given an indication as to the type of mechanism which may be operating in the Group 4 patients. A number of growth factors, including platelet derived growth factor, IFN-γ, GM-CSF and IL-3, are all thought to cause cell proliferation via intracellular mechanisms in a number of transformed cell lines [308-312]. It is possible that such an intracellular loop involving GM-CSF may be operating in Group 4 because in keeping with these cell lines these patients' blast cells are independent of exogeneous stimulation and are unable to be inhibited by specific antibodies.

If such an intracellular loop exists in Group 4 patients then it is possible that active GM-CSF is retained within the cytosol whereas in Group 3 there may be none. Preliminary experiments to test for intracellular GM-CSF were conducted on two patients from

Group 3 and two from Group 4. Cytosol and membrane fractions were assayed for stimulatory activity against homologous AML blast cells from a Group 2 patient.

Stimulatory activity was only found to be present in the cytosol fraction obtained from the Group 4 patients blast cells. The fact that this activity was inhibited in the presence of anti-GM-CSF was a strong indication that it was due to the presence of GM-CSF.

These results, although only obtained on a limited number of patients, show that active intracellular GM-CSF is only found in Group 4 patients blast cells and suggest that an intracellular loop may be responsible for the autonomous proliferation of these blast cells.

In contrast to a study by Nara et al [301] which showed AML blast cell membranes possessed stimulatory activity no such activity was detected in the four patients examined in our study. These differences in the stimulatory activities displayed by AML blast cell membranes are not surprising given the heterogeneous nature of AML blast cell proliferation characteristics.

CONCLUSION

5. CONCLUSION

This study has revealed a number of AML blast cell characteristics which are important with regard to the physiology of the disease. TfR, although found to be expressed on a high percentage of AML blast cells, displayed heterogeneity with respect to the degree of expression shown by individual blast cells within a given population. It was found that the blast cells which displayed the highest TfR expression were the ones capable of forming colonies in vitro (AML-CFU). This finding supported the idea that only a small percentage of the leukaemic blast cell population is highly proliferative and that these AML-CFU act as leukaemic stem cells.

This high TfR expression by AML-CFU is important because of the recent development of anti-TfR cytotoxic conjugates. If these conjugates are specific they should destroy AML-CFU but have no effect on the early haemopoietic progenitor cells, such as CFU-BLAST and CFU-GEMM, because the majority of these cells do not express TfR. Studies are therefore required to test the toxicity of these conjugates on both AML blast cells and normal haemopoietic progenitor cells in an effort to assess their suitability for future treatment regimes.

Factors thought to regulate the proliferation of AML blast cells have also been investigated in this study. It has been shown that AML blast cell proliferation is significantly enhanced at low cell concentrations ($\leq 62.5 \times 10^3$/ml) when the cultures were performed in round

bottomed wells. This enhanced proliferation of AML blast cells under crowded conditions occurred irrespective of whether the cells were stimulated or unstimulated. It was found that this crowding phenomenon depended on direct cell to cell contact which suggests that membrane bound elements may have an important role in this process but as yet no such factors have been identified. Therefore further investigation of this crowding phenomenon is necessary to reveal the mechanisms which influence AML blast cell proliferation as a result of direct cell to cell contact.

A number of the AML patients blast cells investigated in this study have clearly displayed the ability to proliferate autonomously. For this reason a system was devised for grouping patients according to their in vitro proliferation characteristics. Dividing the number of blast cell colonies obtained from unstimulated cultures by those seen in stimulated cultures gave a figure between 0 and 1, which was referred to as the Autostimulatory Index, or ASI. The ASI was then used to assign the patients into four seperate groups. Group 1 patients were termed non growers because whether stimulated or not their blast cells did not form colonies. Group 2 patients were totally dependent on exogeneous growth factors for colony formation (ASI <0.1). Group 3 patients blast cells displayed some degree of autonomous proliferation

but colony growth could be enhanced by addition of exogeneous growth factor (ASI 0.1-0.8). Group 4 patients blast cells showed totally autonomous colony growth which remained unaffected by exogeneous stimulation (ASI>0.8).

The importance of this ASI became obvious when it was found that different mechanisms were responsible for the autonomous proliferation characteristics of the Group 3 and 4 patients blast cells. It was found that the BCCMs prepared from Group 3 and Group 4 patients blast cells stimulated the proliferation of both homologous AML blast cells and normal haemopoietic progenitor cells. The use of a specific anti-GM-CSF antibody confirmed that the mojority of BCCM stimulatory activity was due to the presence of GM-CSF. It was also found that the anti-GM-CSF antibody significantly inhibited the autonomous proliferation of the Group 3 patients blast cells which suggests that the endogeneously secreted GM-CSF was acting via an autocrine loop.

In contrast the GM-CSF secreted by the Group 4 patients blast cells did not appear to support their proliferation in an autocrine manner. On further examination Group 4 patients blast cells were found to contain active GM-CSF in their cytosol whereas none could be detected in the cytosol of the Group 3 patients blast cells. Whether this GM-CSF is responsible for the autonomous proliferation seen in Group 4 patients blast

cells via an intracellular loop is a question which remains unanswered but one which may be extremely important in terms of understanding leukaemic blast cell proliferation.

In an effort to confirm the role of endogeneous GM-CSF in the autonomous proliferation of AML blast cells further studies are being undertaken in our laboratories. These studies are involved in testing the ability of certain oligonucleotides to block the translation of GM-CSF.mRNA into the active protein thereby inhibiting the autonomous proliferation of the Group 3 and Group 4 patients blast cells. It is important to note that the intracellular mechanisms which may be operating in Group 4 patients are not necessarily confined to just leukaemic blast cells. This fact may be confirmed by the numerous studies presently being conducted to assess whether the proliferation of other malignant cells is also dependent on specific intracellular events.

Understanding the physiology of malignant cell proliferation is extremely important as it may lead to the development of therapeutic measures which could directly inhibit the mechanisms responsible for the uncontrolled proliferation of malignant cells. Such therapeutic measures would therefore be vital in the treatment of patients because a major goal in their clinical management is to prevent the uncontrolled proliferation of malignant cells.

APPENDIX

APPENDIX. Reagents, Tissue Culture Disposables, Equipment and Suppliers.

Reagents

All general reagents were obtained from the Sigma Chemical Company.

Reagent	Supplier
Antibodies - Rabbit anti-mouse $(FAB)_2$ FITC conjugated	Dako
- Rabbit anti-human transferrin	Dako
- Goat anti-rabbit IgG FITC conjugated	Pharmacia
- OKT3 (anti-CD3)	Ortho
- OKT9 (anti-CD71)	Ortho
Foetal Calf Serum	Sera Lab
Giant Cell Tumour Conditioned Medium	Gibco
Glass Fibre Filter Papers	Whatman
Heparin (preservative free)	Leo Labs
Iscoves Modified Dulbeccos Medium	ICN Flow
Latex Beads (15.8μm)	Sigma
L-Glutamine	Sera Lab
Lymphocyte Separation Medium	ICN Flow
Magnetic Beads (anti-mouse IgG coated)	Dynal
Magnetic Beads (CD2 coated)	Dynal
Penicillin/Streptomycin	Sera Lab
Ready Protein Scintillation Cocktail	Beckman
RPMI 1640 Tissue Culture Medium	Sera Lab
Transferrin (human)	Behring
Thymidine (tritiated)	Amersham

Plastic Tissue Culture Disposables	Supplier
Cryotubes Nunc (1ml)	Gibco
Microtitre Plates (96 well flat bottomed)	Northumbria
Microtitre Plates (96 well round bottomed)	Northumbria
Petri Dishes (35mm gridded)	ICN Flow
Sterile Filters (0.2μm)	Anderman

Equipment	Supplier
Automash Cell Harvester	Dynateck
CO_2 Incubator	LEEC
FACS	Becton Dickinson
Kryo 10 Cell Freezer	Planar
Laminar Flow Cabinets	Gelman
Microscopes - Fluorescent	Leitz
- Diavert	Leitz

Suppliers

Amersham PLC., Aylesbury, Bucks. (0296) 395222

Anderman & Co., Surrey. (01) 541 0035

Beckman Ltd., High Wycombe, Bucks. (0494) 441181

Becton Dickinson Ltd., Plymouth. (0752) 701281

Behring (Hoechst Ltd.), Middlesex. (01) 5707712

Dako Ltd., High Wycombe, Bucks. (0494) 452016

Dynal (UK) Ltd., New Ferry, Wirral. (051) 6446555

Dynatech Ltd., West Sussex. (0403) 783381

Gelman Sciences Ltd., Northhampton. (0604) 765141

Gibco Ltd., Paisley, Scotland. (041) 8896100

ICN Flow, High Wycombe, Bucks. (0494) 443826

LEEC, Nottingham. (0602) 616222

Leitz (LEICA) Ltd., Milton Keynes. (0908) 666663

Leo Labs. Ltd., Princes Risborough, Bucks.

Northumbria Biol. Ltd., Northumbria. (0670) 732992

Ortho Ltd., Loudwater, Bucks. (0494) 442211

Pharmacia Ltd., Milton Keynes. (0908) 661101

Planar, Sunbury-on-Thames, Middlesex. (0932) 786262

Sera Lab. Ltd., Sussex. (0342) 716366

Sigma Chemical Co., Poole, Dorset. (0202) 733114

Whatman Ltd., Maidstone, Kent. (0622) 674821

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PUBLICATIONS ARISING FROM THIS WORK

Kozlowski, R.E., Reilly, I.A.G., Sowter, D., Robins, R.A. and Russell, N.H.
Transferrin Receptor Expression on AML Blasts is Related to Their Proliferative Potential.
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