

DIFFERENTIATION INDUCTION AND GROWTH  
FACTOR RESPONSES OF MURINE MYELOID  
LEUKEMIAS

Helen Kavnoudias

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FACTOR RESPONSES OF MURINE MYELOID  
LEUKAEMIAS**

BY  
HELEN KAVNOUDIAS

A thesis submitted  
for the degree of  
Doctor of Philosophy

Department of Biology and Preclinical Medicine  
University of St. Andrews  
St. Andrews, Fife  
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## **DECLARATION**

I hereby declare that this thesis has been composed by myself, that it is a record of my own work, and that it has not previously been presented for a higher degree.

This research was carried out in the Department of Biology and Preclinical Medicine, in the University of St. Andrews under the supervision of Professor D. Brynmor Thomas.

Helen Kavnoudias

## **CERTIFICATE**

I hereby certify that the candidate, Helen Kavnoudias, has fulfilled the conditions of the Resolution and Regulations of the University of St. Andrews appropriate to the degree of Ph.D.

Professor D. Brynmor Thomas  
Research Supervisor

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## LIST OF PUBLICATIONS

### *From the Thesis*

**Riches AC and Kavnoudias H (1988)** In vitro proliferative effects of haemopoietic growth factors on murine myeloid leukaemias. Poster and Abstract at the British Royal Society of Cell Biology Conference

### *Others*

**Kavnoudias H, Jackson H, Ettlinger K, Bertoncetto I, McNiece I, Williams N (1992)** Interleukin-3 directly stimulates both megakaryocyte progenitor cells and immature megakaryocytes *Experimental Hematology* 20 43-46

**Williams N, Bertoncetto I, Kavnoudias H, Zsebo K, McNiece I (1992)** Recombinant rat stem cell factor stimulates the amplification and differentiation of fractionated mouse stem cell populations *Blood* 79 58-64

**Williams N, Bertoncetto I, Jackson H, Arnold J, Kavnoudias (1992)** The role of interleukin-6 in megakaryocyte formation, megakaryocyte development and platelet production *CIBA Foundation Symposia* Vol 167 160-173

**Williams N, Bertoncetto I, Kavnoudias H, Zsebo K and McNiece I (1991)** Recombinant rat stem cell factor stimulates amplification and differentiation of purified mouse haemopoietic stem cells *Experimental Hematology* (abstract) 19 501

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Presented at XXth annual meeting of the International Society for Experimental Hematology. 21-25 July, 1991

Αφιερωμένο με σεβασμό στον πατέρα μου Στυλιανό και την μητέρα μου Αθανασία για την ανεκτίμητη υποστήριξη και αγάπη τους.

(Dedicated with respect to my father Stiliano and my mother Athanasia for their invaluable support and love)

*" : during normal blood cell production the cells differentiate into specific types. In a pathological situation the differentiation into specific cells is blocked. This disturbance of normal differentiation - so called leukaemia - is a disease sui generis. We know the sequelae of this disease, we do not know its origin."*

Virchow R. 1856

## ABSTRACT

Current therapeutic regimes for the treatment of acute myeloid leukaemia employ aggressive cytotoxic strategies which have limited success and are not suitable for all patients. Manipulating myeloid leukaemic cells according to their responses to pharmacologically tolerable agents rather than employing aggressive non specific cell kill would be desirable. Differentiation induction of myeloid leukaemic cells as an alternative therapeutic regime was evaluated.

To elucidate the growth and differentiation abnormalities of myeloid leukaemic cells the differentiation induction and growth factor responses of bone marrow from three myeloid leukaemic models (SA2, SA7, SA8) and a leukaemic cell line (SA2 CL) were investigated in microtitre suspension cultures and compared with normal. The regulators of normal haemopoiesis occupy a key position in attempts to understand the nature of the abnormal state existing in myeloid leukaemia and could potentially play an important role therapeutically by suppressing myeloid leukaemic populations. To evaluate differentiation induction as an alternative therapeutic regime for the treatment of myeloid leukaemia both the physiological regulators and the differentiation inducer  $\beta$ -all *trans* retinoic acid ( $\beta$ atRA) alone and in combination with ara-C were investigated. The effects of these agents directly on the leukaemic clonogenic cell populations *in vitro* were measured following *in vivo* transplantation.

The proliferative effects of WEHI-3B CM, as a source of IL-3, L929 CM as a source of M-CSF, and recombinant murine GM-CSF alone and in combination were investigated. Bone marrow cells from the SA7 and SA8 transplanted leukaemias were growth factor dependent for proliferation *in vitro*. The SA2 transplanted leukaemia proliferated autonomously at low passage numbers, the dominant leukaemic clone changed characteristics with progressive transplantation and became growth factor dependent at high passage numbers. A cell line (SA2 CL) was derived from leukaemic bone marrow cells of a low passage number of the SA2 leukaemia and proliferated at a high rate autonomously with minimal spontaneous differentiation in culture.

The growth factor dependent leukaemias were induced to proliferate with each of the growth factors. No differences were observed in the dose response relationships between normal and leukaemic cells. Differences were observed in the proliferative rates, the day to which exponential cell growth was sustained in culture and the growth factor which induced maximal proliferation. WEHI-3B CM induced the maximal proliferative response of normal bone marrow cells and

two of the leukaemias whereas rGM-CSF induced a maximal response of the third leukaemia. Leukaemic cells proliferated at a higher rate than normal cells in the first two to three days in culture but were not sustained in culture for as long as normal bone marrow cells. It appeared therefore, that in the case of some leukaemias the haemopoietic growth factors were effective at inducing a high initial but short term proliferative response of leukaemic cells and were more effective in sustaining normal bone marrow cells *in vitro*. L929 CM induced a lower proliferative response of both normal and leukaemic cells compared to WEHI-3B CM and rGM-CSF. The regulators of normal haemopoiesis have been shown to exert stimulating effects upon the same target cells. Different subsets of leukaemic cells were observed according to their proliferative response to combinations of growth factors. Variable responses were obtained, different combinations of factors induced synergistic responses with the different leukaemias. A systematic approach of this type of study may be useful for the reclassification of the heterogeneous human leukaemic subtypes and may be of prognostic value.

The three growth factors studied induced differentiation of the transplanted leukaemic cells, L929 CM was the most effective. Higher numbers of immature cells combined with a differentiation effect were observed with the other two growth factors. A 10 and 100 fold reduction in the number of leukaemic clonogenic cells was observed with WEHI-3B and L929 CM cultured cells respectively as assessed in the transplantation study. The differentiation effect was not enhanced with the combinations of growth factors. Generally higher numbers of immature cells were sustained in culture compared to at least one of the growth factors used singularly. The addition of growth factors to the autonomously proliferating SA2 CL did not alter the proliferative or differentiative responses observed in foetal calf serum (FCS) alone.

The addition of  $\beta$ atRA to growth factor stimulated cultures enhanced the differentiation response of the transplanted leukaemic cells and induced an inhibitory response without evidence of differentiation of the SA2 CL. A decrease in the number of clonogenic cells compared to growth factor alone was observed with the combination of  $\beta$ atRA plus L929 CM but not with WEHI-3B CM indicating that a combination of differentiation inducers further decreased the number of leukaemic clonogenic cells.

It has been postulated that two agents having different modes of action, one of DNA synthesis inhibition and a differentiation inducer would enhance the

differentiation and inhibitory effect of these agent on leukaemic cells, allowing for the use of lower concentrations of the more toxic agent. We tested this hypothesis on the murine myeloid leukaemic models. The combination of  $\beta$ atRA and ara-C induced varied proliferative responses on the leukaemic bone marrow cells. A stimulatory response was observed with normal bone marrow and the SA7 leukaemia, a decrease in the proliferative response compared to ara-C alone was observed with the SA2 CL and synergistic decreases were observed with the SA2 and SA8 leukaemias. With the synergistic responses, the proliferative responses observed with low doses of ara-C in combination with  $\beta$ atRA were similar to the responses observed with 10 or 100 times greater concentration of ara-C alone. The concentration of each agent required to induce a synergistic response for each leukaemia was observed to be critical.

The murine transplanted myeloid leukaemias were shown to be suitable models for the study of myeloid leukaemia. The heterogeneity of responses observed were comparable to studies with fresh human leukaemic bone marrow cells. These models facilitate the study of the effect of therapeutic agents on leukaemic clonogenic cell populations which sustain the leukaemic condition *in vivo*.

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# INTRODUCTION

## NORMAL HAEMOPOIESIS

Normal haemopoiesis is a highly dynamic process and is continuous throughout life. The number of new blood cells that the haemopoietic tissues must produce to replace effete cells is extremely large. In an adult human approximately  $10^{10}$  red cells and  $4 \times 10^8$  white cells are produced per hour. In normal health, levels of the various mature cells in the blood are maintained within quite narrow limits, yet in response to emergencies such as blood loss or infection, the haemopoietic tissues are able to respond rapidly by increasing production (Metcalf, 1988).

It is now well established that all the functionally diverse mature blood cells arise originally from a common pluripotential stem cell (Sieff, 1988). There is a hierarchial and clonal progression from pluripotent stem cells, to transit cells (progenitor cells with high proliferative potential, to morphologically recognisable proliferative cells) and finally maturation to fully functional non proliferative blood cells. Stem cells, under normal conditions are quiescent, relatively few in number ( $10^6$  to  $10^7$  cells in the adult) and through a stochastic mechanism which is still undefined they are capable of self-renewal and producing daughter cells which undergo initial differentiation to progenitor cells (for review see Dexter et al, 1987). As proliferation and differentiation continues the progenitor cells become more restricted and eventually committed to specific cell lineages. Early progenitor cells have a high proliferative potential and can generate clones of up to  $10^5$  lineage restricted cells which finally mature into blood cells and it is from this cell compartment that haemopoiesis is sustained under normal conditions. Hence the hierarchial system can be described as a clonal progression from a quiescent pluripotent stem cell which is capable of self

renewal to progenitor cells which sustain the body's haemopoietic requirements, to finally bi- or uni- potential progenitors which give rise to the specific lineage, mature, non proliferative functional blood cells.

This systematic progression of proliferation and differentiation of haemopoietic cell is partly regulated by a group of glycoprotein regulators. The development of *in vitro* culture systems for supporting haemopoietic cell growth (Plutznik and Sachs, 1965; Bradley and Metcalf, 1966) and the discovery of these regulators in cell culture supernatants (Lotem and Sachs, 1966; Ichikawa et al, 1966) has led to the isolation, identification and molecular cloning of these growth factors. The first four regulators to be identified in these systems were the colony stimulating factors, (CSF's), a term derived from the *in vitro* observation that they stimulate progenitor cells to form colonies of recognizable maturing cells; prefixes are used to denote the cell type in the mature colonies, ie GM-CSF granulocyte and macrophage-CSF, M-CSF macrophage-CSF, G-CSF granulocyte-CSF and multi-CSF also named interleukin-3 (IL-3) which produces a mixture of cell types (Metcalf, 1988).

The mode of action of the CSF's is by binding to specific receptors on the cell surface. When a stem cell becomes committed to differentiation it produces receptors on its surface that respond to specific regulator signals. Those signals in turn push the cell further down the pathway toward ultimate specialization (Golde and Gasson 1988; Nicola, 1987).

The regulation of normal haemopoiesis is a complex process of which the CSF's form part of the total system. The haemopoietic bone marrow microenvironment is essential and numerous other regulatory processes are also involved. As well as

the CSFs a series of interleukins, 17 to date, various inhibitors (Lord, 1976) and other immune modulators such as interferon (Resnitzky et al, 1986), tumour necrosis factor and small peptides are involved.

### **The Haemopoietic Microenvironment**

The role of the microenvironment is not well understood. Sustained *in vitro* haemopoiesis requires an adherent stromal layer (Dexter et al, 1977) that consists of fibroblastoid cells, adipocytes, 'blanket cells', macrophages and possibly endothelial cells (Dexter et al, 1984). These cells secrete components of a complex extracellular matrix that include fibronectin, laminin, collagen and glycosaminoglycans (Sieff, 1988). The cellular components have been shown to synthesize CSF (Heard et al, 1982) and factors that affect stem cell cycling (Toksoz et al, 1980). Furthermore, synthesis of proteins such as fibronectin and haemonection may provide essential substrates to which developing erythroid and myeloid progenitors adhere during maturation (Tsai et al, 1986; Campbell et al, 1987).

## **THE COLONY STIMULATING FACTORS**

### **Interleukin-3 (IL-3)**

IL-3 is produced by activated T-lymphocytes (Andreeff and Welte, 1989). Murine IL-3 was first purified by Ihle et al (1982) and was consequently shown to support the formation of multilineage colonies *in vitro* (Prystowsky et al, 1984). It was shown that IL-3 was capable of generating colonies of all three granulocytic cell types, neutrophils, eosinophils and basophils, macrophage colonies, megakaryocyte colonies and early erythroid colonies in the presence of erythropoietin (Epo). Because of its regulatory role in all the myelopoietic

lineages IL-3 has also been called multi-CSF. The precise role of IL-3 in the regulation of self renewal, proliferation and differentiation of the pluripotent stem cell remains obscure.

Gibbon, human (Yang et al, 1986) and murine (Fung et al, 1984) IL-3 have also been cloned. IL-3 was found to be a complex glycoprotein, ranging in size from 14 to 28 kilodaltons (kD). The expected size of the active polypeptide is 14 to 15 kD. The sequence homology of gibbon and human IL-3 was found to be 96%, and human and murine IL-3 only 49%. The gene encoding human IL-3 is located on the long arm of chromosome 5, in close vicinity to the GM-CSF gene (Le Beau et al, 1987).

Recombinant gibbon IL-3 has been tested on enriched populations of human progenitors (Leary et al 1987). It supported the formation of various types of single lineage, as well as multilineage colonies in the presence of Epo. In addition, IL-3 supported, and more effectively than GM-CSF, the formation of blast cell colonies with a high replating capability. These data suggested that IL-3 was the least restricted of the CSFs with regard to cell lineage induction and had the greatest variety of action with regard to target cells. Also, there is evidence that IL-3 supports stem cell self renewal (Testa and Dexter, 1992).

The *in vivo* action of IL-3 have been investigated in simian studies (Donahue et al 1987; Krumwieh et al, 1988 ;Mayer et al 1988). It was shown that IL-3 was a poor haemopoietic stimulus when given alone, however, it primed the haemopoietic system to the effects of GM-CSF. It was suggested that IL-3 is capable of acting on an immature population of progenitor cells, which in turn can be stimulated to proliferate and terminally differentiate in the presence of a second haemopoietic growth factor such as GM-CSF. These studies have also shown that IL-3 is a

differentiation factor for eosinophils and basophils.

These *in vivo* studies have also been supported by *in vitro* studies using fractionated stem and early progenitor cell populations (Iscove et al, 1989; McNiece et al 1987; Saeland et al 1988; Kavnoudias et al, 1992; Williams et al, 1992). IL-3 was shown to directly induce colony formation and/or proliferation of murine and human stem or early progenitor cells and be more effective in supporting formation and/or proliferation of early progenitor cells compared to other myelopoietic growth factors.

It has been shown that IL-3 binds to high or low affinity binding sites. In the murine system, the low affinity receptor gene has been cloned and apparently represents one member of a gene family which includes other interleukins (IL-2, -4, and -6). (Itoh et al, 1990). More recently, it has been suggested that the specific receptors for human IL-3 and GM-CSF share an identical subunit (Park et al, 1989).

### **Macrophage-Colony Stimulating Factor (M-CSF)**

M-CSF characteristically produces macrophages *in vitro*. Activated monocytes have been shown to contain high levels of mRNA for M-CSF. M-CSF has been shown to be a potent stimulator of murine macrophage colony formation, but both murine and human forms which have been biosynthesized were less active with human target cell (Andreeff and Welte, 1989). This however may be due to limitations of human haemopoietic cell culture techniques.

Murine M-CSF was the first of the CSF's to be identified in the colony assay however the cloning of M-CSF was the most complex of all the CSF's. M-CSF was found to be a homodimer of two identical subunits connected by an interchain

disulphide bond and highly glycosylated. The system was complicated because at least two differentially spliced messenger ribonucleic acids (mRNA's) were expressed from a common gene, and each of these encoded different M-CSF precursors. Additional processing and glycosylation resulted in at least two forms of M-CSF; a 10 to 90 kD glycoprotein comprising a dimer of 35 to 45 kD subunit, and a smaller 40 to 50 kD glycoprotein comprising of a dimer of a 20 to 25 kD subunit (Kawasaki et al 1985; Wong et al, 1987). The gene of human M-CSF has been localized on chromosome 5q23-q31, and the receptor for M-CSF is encoded by the *fms* protooncogene (LeBeau et al, 1986).

M-CSF has been shown to activate mature macrophages, and the larger form of recombinant M-CSF strongly potentiates human macrophage cytotoxicity (Clark and Karmen, 1987). The responsiveness of M-CSF has been shown to be greatly enhanced by GM-CSF and to participate in maintaining a reserve of progenitor cells for neutrophils (Rothstein et al, 1988). It has also been shown that GM-CSF in very small concentrations enhanced the responsiveness of marrow progenitors to M-CSF (Caracciolo et al, 1987). Clinical trials of M-CSF are currently in progress (Andreeff and Welte, 1989)

### **Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)**

Murine GM-CSF was the first of the CSF's to be purified (Burgess, 1980) and cloned (Gough et al, 1984). Compared to IL-3, GM-CSF has been shown to be more restricted in its activities. Several cell types have been shown to express mRNA for GM-CSF following activation. These include T lymphocytes, and endothelial cells and fibroblasts present in the bone marrow stroma (Herrman et al, 1986). Vascular endothelial cells produce mRNA and release both G- and GM-CSF upon stimulation with interleukin-1 (IL-1) (Zsebo et al, 1988).

Recombinant human GM-CSF (rhGM-CSF) has been shown to be highly variable in size with molecular species in the range of 14 to 30 kD. The expected size of the mature polypeptide was 14 to 15 kD therefore, the carbohydrate content of the molecule can reach 50% of the total mass. The most highly glycosylated GM-CSF was less than one tenth as active as the smallest form of the molecule (Clark and Karmen, 1987).

GM-CSF has been shown to have a wide range of activities. It supports the proliferation of neutrophil, macrophage, and eosinophil colonies and affects numerous functions in these end cells (Lopez et al, 1986; DePersio et al, 1988). rhGM-CSF acts directly or in combination with Epo or IL-3 on erythroid progenitors. GM-CSF produces monocyte-macrophage colonies and also controls specific monocyte functions through the induction of M-CSF transcripts in monocytes (Horiguchi et al, 1987) and the stimulation of Fc-dependent phagocytosis of tissue derived macrophages (Coleman et al, 1988). GM-CSF and IL-3 induces the production of tumour necrosis factor from monocytes and significantly enhances monocyte killing in response to endotoxin (Cannistra et al, 1988). GM-CSF also has megakaryocyte colony stimulating activity and augments the effect of IL-3 on megakaryocyte colony formation (Robinson et al, 1987). GM-CSF has been shown to have a direct effect on multipotential progenitor cells from 5-fluorouracil treated mice (Koike et al, 1987), however normal human bone marrow cells obtained by fluorescence-activated cell sorting did not proliferate after a single pulse stimulation with GM-CSF (Begley et al, 1988).

*In vivo* studies of the effects of GM-CSF have been studied in simians and humans. In simian studies rhGM-CSF induces a prompt five fold increase in granulocytes, a two to four fold increase in lymphocytes and a three to four fold

increase in monocytes (Donahue et al, 1986). It also primes circulating granulocytes for enhanced oxidative metabolic activity and for killing of *E coli* (Mayer et al, 1987). In another study by Nienhuis et al (1987) GM-CSF was administered before and after total body irradiation and autologous bone marrow transplantation. GM-CSF shortened the period of neutropenia by 10 days and produced accelerated production of platelets. Clinical studies of GM-CSF have also been carried out on patients with aplastic anaemia (Vadhan-Raj et al, 1988) and AIDS (Groopman et al, 1987) with favourable results.

### **Granulocyte-Colony Stimulating Factor (G-CSF)**

G-CSF induces bone marrow progenitor cells to form colonies of granulocytes *in vitro* (Ottman et al, 1987). G-CSF has also been shown to have an effect on the functional activity of peripheral granulocytes (Platzer et al, 1987). Two G-CSF cDNA clones were isolated from different tumour cell lines. The first clone encoded a 207 amino acid protein, of which 177 amino acids comprised mature G-CSF, while 30 hydrophobic amino acids determine the probable leader sequence (Nagata et al, 1986). The second clone was identical apart from a deletion of three amino acids. The predicted molecular masses of the two proteins have been 19 and 18.6 kD. Monocytes, endothelial cells, and fibroblasts have been identified as G-CSF producers (Herrman et al 1986; Zsebo et al, 1988; Kaushansky et al, 1988).

The *in vivo* effects of G-CSF have been studied in the rhesus monkey. Dose dependent increases in the total white blood cell count, mainly of neutrophils were observed (Welte et al, 1987). The effects of G-CSF on chemotherapeutically-induced cytopenias were investigated in this study. The administration of G-CSF dramatically increased the peripheral white cell count,

however there was no decrease in the duration of the cytopenia. The effects of G-CSF after autologous bone marrow transfusion were also investigated (Bonilla et al, 1987). With the administration of G-CSF the white blood cell counts were six times greater than the controls. Chemotherapeutic regimes for various malignancies result in significant decreases in white blood cell numbers resulting in high risks of fatal infections. Clinical trials of G-CSF on patients receiving chemotherapy for bladder cell carcinoma have shown favourable results with at least three fold increases in the number of granulocytes (Gabilove et al, 1988). Favourable results have also been obtained with the administration of G-CSF to children with severe congenital neutropenia (Welte et al, 1988).

## LEUKAEMIA

Leukaemias are a very heterogeneous group of malignant diseases of varying aetiology, pathogenesis and prognosis. In most leukaemic cases the aetiology is unknown. As with other malignancies, known causative agents are radiation, viruses and toxic chemicals. The present most widely used system of classification of the leukaemias is the FAB (French, American, British) system. Currently, the MIC (Morphologic-Immunologic-Cytogenetic) classification system is also being used (Bain, 1988). However, heterogeneity of the disease is observed within these classification groups.

The clinical manifestations of the leukaemias are due directly or indirectly to the proliferation of leukaemic cells and their infiltration into normal tissues. Increased cell proliferation has metabolic consequences and infiltrating cells also disturb tissue function. Anaemia, neutropenia and thrombocytopenia are important consequences of infiltration of the bone marrow which can lead to

infection and haemorrhage (Bain, 1988).

Genetic alterations leading to leukaemic transformations of a cell were often associated with major alterations of chromosomes which could be detected by studying cells of the leukaemic clone in mitosis. Particular chromosomal aberrations were often associated with a particular leukaemia and have been shown to have prognostic value (Bain, 1988). These microscopically detectable abnormalities have helped identify the chromosomes where significant alterations have occurred at a molecular level in relation to haemopoietic regulators (Le Beau et al, 1986; de The et al, 1991).

### **Clonal Development and Stem Cell Origin of leukaemia**

Myeloid leukaemia has been described as a clonal neoplasm involving a progressive expansion of proliferative myeloid cells with evidence of a differentiation block or abnormal maturation (Sachs, 1978, 1986; Francis et al 1982; Metcalf, 1988). There was convincing evidence to show that acute leukaemia begins in a single cell that undergoes clonal proliferation to give rise to a recognizable population of leukaemic cells. The nature of this change was central to theories concerning the pathogenesis of acute leukaemia (Adamson 1984).

Although the etiology of human leukaemia is unknown, it was clear that in experimental systems, where leukaemogenesis can be studied, the responsible agents were capable of modifying nuclear DNA, and their actions were enhanced if certain genetic constitutions were present (Metcalf, 1988). The growing body of data showing associations between nonrandom chromosome abnormalities particularly translocations and activation, amplification, or mutation of cellular proto-oncogenes strengthened the view that leukaemia begins as a somatic

mutation from which an abnormal clone arises (Fialkow, 1990).

The observation that nonrandom cytogenetic abnormalities characterised haemopoietic neoplasias, prompted the 'clonal' theory. Evidence on the clonality of leukaemia was obtained when studies were carried out on cells from females who were heterozygous for the enzyme glucose-6-phosphate dehydrogenase (G<sub>6</sub>PD). Tumour cells from these females all contained the same allele of the G<sub>6</sub>PD gene. Normal cells contain either one or the other allele at a 50% distribution (Fialkow 1970). Another marker system based on X chromosome inactivation mosaicism which has provided evidence of the clonality of leukaemia was the analysis of DNA polymorphisms of restriction fragment length (Vogelstein et al, 1985).

The most widely accepted theory on the development of leukaemia is that leukaemias are clonal disorders; however, two arguments arose from this observation:-

- i) that an agent involved in the cause of disease may have specific affinity for certain chromosomal regions, therefore a common karyotypic change would not necessarily indicate clonal development and
- ii) that the chromosomal marker identified an evolved subclone and that cells lacking the cytogenetic abnormality may have been clonal as well (Dameshek and Gunz, 1990)

### **Target Cells for Leukaemogenesis**

The location in the haemopoietic hierarchy of the clonogenic cell that initiates what is clinically recognized as myeloid leukaemia can vary. In chronic myeloid leukaemia all the cell lineages contain the chromosomal aberrations hence the

initiating cell was expected to be from the pluripotent stem cell compartment (Metcalf, 1988). In acute myeloid leukaemia (AML) there is evidence that the initiating cell in some cases may be from the pluripotent stem cell compartment and in others, from the granulocyte macrophage progenitor cell compartment. In one patient with AML it has been shown that B lymphocytes had arisen from the leukaemic clone. By contrast, the leukaemic population in younger AML patients appeared to arise from a stem cell restricted to granulocyte macrophage progenitor cell compartment, as other cell lineages appear to arise from non-clonal progenitors. (Fialkow, 1981).

### **Multistep Pathogenesis**

The theory of multistep, rather than single step pathogenesis of leukaemia and myelodysplastic syndromes (Raskind et al, 1984) has evolved from studies utilising G6PD/X chromosome inactivation mosaicism and cytogenetic studies of Epstein-Barr virus (EBV) transformed B lymphoid cell lines. The nonrandom cytogenetic abnormalities observed in myeloid leukaemia were more likely to be part of the progression or manifestation of the disease rather than the cause. This process has been described as a stepwise series of events rather than a single genetic alteration (multistep pathogenesis). It has been postulated that, at least, a two step process is involved in leukaemogenesis. The first causing growth of a leukaemic clone of pluripotent haemopoietic stem cells and the second inducing the nonrandom chromosomal aberration in descendants of those progenitors (Fialkow, 1985).

### **Suppression of Normal Haemopoiesis**

Failure of normal haemopoiesis is the most serious pathophysiological consequence of acute leukaemia. This pathogenesis is poorly understood however,

a number of observations have been made. It has been shown that normal haemopoietic progenitors [Colony Forming Units -GM (CFU-GM)] fail to proliferate normally if cultured *in vitro* with leukaemic cells (Morris et al, 1975; Nara et al., 1984), with leukaemic extracts or medium conditioned by incubation with leukaemic cells (Broxmeyer et al, 1978 and 1979; Olofsson and Olsson 1980; Olofsson et al, 1984). Two distinctly different inhibitory activities have been isolated. Leukemia cell-derived inhibitory factor (LIA) (Broxmeyer et al., 1978) and leukemia-associated inhibitor (LAI) (Olofsson and Olsson 1980). The first was produced by lymphoid like cells from patients with acute and chronic myeloid leukaemia and chronic lymphocytic leukaemia and was attributed to being an acidic isoferritin, however other workers regard this as incompatible with available information of ferritin biochemistry (Sala et al, 1986). LAI differed physically and chemically from LIA but had the same effect of reducing the proliferation of CFU-GM. The cells producing LAI were large non-T non-B, Fc receptor positive mononuclear cells, different from those producing LIA (Olofsson et al, 1984). LIA acted selectively on S-phase cells. Inhibition of normal CFU-GM by an inhibitory factor could explain the suppression of normal haemopoiesis in the leukaemic state.

Other observations involved the haemopoietic microenvironment. Another explanation for depression of normal haemopoiesis in AML, based on studies in an animal model, was that AML cells specifically localized to the subendosteal region of the bone marrow, where the microenvironment is optimal for normal stem cell proliferation (Van Bekkum et al, 1981). Hence, there would be displacement of normal stem cells from optimal bone marrow sites. Leukaemic cells have also been shown to suppress the growth of fibroblast colonies from normal marrow (Nagao et al, 1983). It has also been shown that stromal cells in long term culture

from four of five AML patients studied by Singer et al were derived from the same progenitors as the leukemic clone (Singer et al, 1984). In view of the dependence of haemopoietic stem cells on an intact functional stromal layer, this might contribute to the failure of normal haemopoiesis in acute leukaemia. From studies on murine leukaemias in our own laboratory (Michaelis and Riches, unpublished data) it was noted in a study on the histology of the marrow that the bone marrow becomes progressively necrotic with progression of the disease. Bone marrow necrosis has also been observed in leukaemic patients (Maisel et al, 1988)

Normal haemopoietic stem cells are almost certainly present, albeit suppressed, in the marrow of many patients when they present with acute leukaemia. AML patients in very early remission produced a wave of multipotent stem cells in the blood whose presence could be inferred from the successful trilineage engraftment when the blood was subsequently reinfused after ablative chemotherapy (Juttner et al, 1985) and by greatly increased numbers of circulating CFU-GM which grew normally in culture (Tilly et al, 1986). Also, normal stem cells capable of engraftment following ablative chemoradiotherapy have been grown from long term culture of bone marrow of patients with AML (Chang et al, 1986). By contrast however residual leukaemic stem cells have been shown to persist in the blood and marrow of patients with AML in chemotherapy induced remission (Consolini et al, 1986; Estrov et al, 1986; Jasmin et al 1991).

### **Possible Theories on Leukaemopoiesis**

Central to the discussion of the leukaemogenic event was the fact that the majority of normal pluripotent stem cells have been inferred to be in a quiescent non cycling or Go state. In one hypothesis, it was suggested that damage (i.e. a potentially leukaemogenic event) occurring in a Go stem cell produced leukaemia

only if that cell was subsequently recruited into cell cycle (Killman SA, 1968). An alternative hypothesis proposed that the leukaemogenic event altered the responsiveness of a G<sub>0</sub> stem cell to normal control mechanisms, increased its cycling rate, and decreased the time in G<sub>0</sub> (Lajtha, 1981). If G<sub>0</sub> is a phase during which the cell undertakes "genetic housekeeping" to keep the genome error-free, shortening the time spent in G<sub>0</sub> would carry with it risk of accumulating additional errors.

The extent to which input from a stem cell compartment serves to maintain a leukaemic cell population once the disease is established is not known with certainty. The relationship between the stem cell in which acute leukaemia originates and the putative leukaemic stem cell present at the time the disease becomes clinically evident has been vigorously debated.

Most acute leukaemias at presentation show a population of blasts in the marrow and blood which is relatively homogeneous, which may show morphologic and phenotypic evidence suggestive of maturation (eg promyelocytes in AML-3), and which is capable of reentering the cell cycle. Although large numbers of leukaemic cells normally die, the fraction reentering division is sufficient to ensure that the population continues to expand, and it could be argued that acute leukaemia will persist without any continued input from a stem cell compartment. This interpretation is strengthened by experience with cell lines such as the HL-60, in which a population of mature-looking promyelocytes proliferates indefinitely *in vitro*. On the other hand, predictions based on the response of acute leukaemias to chemotherapy and the identification *in vitro* of small subpopulations of leukemic cells with self renewal capacity and high proliferative activity suggest that maintenance of the leukaemic process might

require a continued inflow of cells from a stem cell compartment (Fialkow, 1985).

### **Cell Surface Markers and Acute Myeloid Leukaemia**

The surface phenotype markers of AML have been less well defined than is the case for ALL, but antigens present on blast and colony forming cells have been identified using monoclonal antibodies (Lange et al, 1984; Lowenberg and Bauman, 1985).

Of particular interest is the observation that fucosylglycopeptides, normally found only on mature marrow neutrophils, are also found on the surface of blasts in AML. These substances are postulated to play a role in the egress of mature neutrophils from the marrow into the blood, and their presence on AML could account for the premature release of these cells from the marrow (Van Beek et al, 1984).

### **Oncogenes and Acute Myeloid Leukaemia**

In normal cellular systems a balance needs to be maintained between cellular proliferation, cellular differentiation, cellular function and cell death. Oncogenes have been implicated in malignancy and are involved in all of these four areas. Oncogenes are the genes carried by retroviruses which produce cancer in experimental animals and which are now known to be normal components of human chromosomes (Garson, 1990). Nearly 50 cellular proto-oncogenes have been identified and are classified into seven main groups according to their involvement in cellular function i) growth factor, ii) cell surface receptors, iii) membrane associated tyrosine kinase, iv) G-proteins, v) cytoplasmic serine/threonine kinases vi) nuclear proteins and vii) putative or unclassified (Demczuk S, 1991). Activation of oncogenes is often a result of structural

chromosome rearrangement, a common feature of leukaemia, and is involved in the development of malignancy (Garson, 1990).

Cellular oncogenes seem to play a role in haemopoietic growth stimulation. Anti-oncogenes, which have a suppressive effect on oncogene products, may be directly linked to terminal differentiation. The maturation arrest in leukaemia may result from both constitutive expression of oncogenes and lack of expression of anti-oncogenes (Olsson et al, 1988).

To date none of the translocations described in human AML have been proven to involve oncogene rearrangement although *c erts* may be altered in the 11q abnormalities, *c mos* is close to the breakpoint on Chromosome 8 in the t(8;21), *erb A* on chromosome 17 may be involved in the t(15;17) (Garson, 1990) and the *c-fms* oncogene product is related to the M-CSF receptor (Sherr et al, 1985). Point mutations of the gene *N-ras* in codons 12, 13,61 have been shown to occur in preleukaemia and such patients progress to overt leukaemia with the same mutation (Garson, 1990).

### **Classification and the Heterogeneity of Acute Myeloid Leukaemia**

The AML's appear to be heterogeneous in at least two important respects:-

- The pattern of differentiative expression of the involved stem cells and
- The nature of the remission.

The heterogeneity of AML imposes difficult problems for the prognosis and treatment of the disease. The FAB classification system classifies the acute myeloid leukaemias according to the predominant proliferative morphologically recognizable myeloid leukaemic cell. However, heterogeneity within these subclasses is observed when conventional chemotherapeutic regimes are employed

for the treatment of the disease. Immunological classification with cell surface markers has been limited with AML and identification of specific chromosomal abnormalities within the subgroups has been shown to have some limitations for prognosis (Bain, 1988). In *in vitro* studies of human AML a marked heterogeneity in response to growth factors has also been observed. It has been postulated that the pattern of the *in vitro* proliferative response to growth factors of AML cells may be of prognostic significance (Moore et al, 1974).

The purpose of any pathological classification of disease is to bring together cases which have fundamental biological similarities and which are likely to share features of causation, pathogenesis and natural history. The recognition of more homogeneous subgroups of biologically similar cases is important as it permits an improved understanding of the leukaemic process and increases the possibility of causative factors being recognized. Since such subgroups may differ from one another in the cell lineage affected and in their natural history and prognosis, it is likely that the identification of biologically different subgroups will lead ultimately to a different therapeutic approach in some subgroups and to an overall improvement in the prognosis of patients with acute leukaemia.

### **THE COLONY STIMULATING FACTORS AND ACUTE MYELOID LEUKAEMIA**

The most accepted theoretical postulate on the proliferation and differentiation mechanisms in haemopoiesis is that of asymmetrical or stochastic events (Metcalf, 1988). If this is correct, for the understanding and manipulation of leukaemia, it becomes fundamentally important to establish whether abnormally high levels of self generation characteristic of the disease can be suppressed by manipulating

extrinsic signals impinging on the cell.

The CSF's are active on normal multipotential and stem cells hence it appeared obvious that the role of these factors in AML needed to be elucidated. It has been stated that '*it may be a serious oversimplification to regard the myeloid leukaemias merely as neoplasms of CSF-dependent populations. How much of the information we are generating in our culture techniques is relevant to the leukaemic cell is difficult to assess?*' (Metcalf, 1988). However, the roles played by the CSF's in the progression and maintenance of the disease are fundamental to our understanding of the disorder. The proliferative capability of myeloid leukaemic cells is not completely understood. What has become evident from previous studies is the heterogeneity of the leukaemic response to normal haemopoietic regulators (Moore et al, 1974). Also it has become evident that leukaemic cell populations can be manipulated to alter their responses *in vitro* with physiological regulators (Lotem and Sachs, 1974, 1977, 1980, 1982, 1983).

In view of the current concepts of myeloid leukaemia we have attempted to study the responses of myeloid leukaemic cells to three of the CSF's. The first part of our study concentrated on documenting *in vitro* proliferative responses to single and combinations of growth factors. With the use of combinations of growth factors we attempted to identify possible subgroups of myeloid leukaemia according to or lack of synergistic responses to growth factors as compared to normal bone marrow cells.

## **DIFFERENTIATION INDUCTION AS AN ALTERNATIVE THERAPEUTIC REGIME FOR THE TREATMENT OF ACUTE MYELOID LEUKAEMIA**

In 1856 Virchow gave the following description of leukaemia:-

*"In a pathological situation the differentiation into specific cells is blocked. This disturbance of normal differentiation - so called leukaemia - is a disease 'sui generis". We know the sequelae of this disease, we do not know its origin.*

During the 1970's Lotem and Sachs gave attention to the concept that a block in the differentiation process was inherent to the leukaemic state (Lotem and Sachs, 1978). Their experimental work with murine myeloid leukaemias and work on myeloid leukaemic cell lines showed that the differentiation block in some leukaemias could be bypassed with regulators of normal haemopoiesis (CSF's). Guimares et al (1984) have described the imbalance between proliferation and differentiation in myeloid leukaemia as observed in CSF stimulated cultures as, *'merely a profound shift in the differentiation probability of one or more cell stages rather than a particular stage which cannot be achieved'*.

Current therapeutic regimes for the treatment of myeloid leukaemia rely upon non-specific cytotoxicity and although a number of advances have been made towards cure and palliation of the disorder their beneficial effects are usually accompanied by significant morbidity. With increasing evidence on the differentiability of a number of malignancies including myeloid leukaemia, studies on an alternative approach for the treatment of myeloid leukaemia utilizing differentiation induction appeared warranted. The assumption with this approach is that malignancy is not an irreversible state and that malignant cells can be induced to differentiate into mature benign forms with no proliferative potential.

Spontaneous differentiation of leukaemic cells *in vitro* has been noted since 1960 (Nowell, 1960). During the 1970's in a series of experiments utilizing a spontaneous murine leukaemia in a SL mouse, radiation induced murine leukaemias in various mice strains and in murine leukaemic cell lines, including WEHI-3B, Sachs and colleagues isolated various 'differentiation positive' leukaemic clones. 'Differentiation negative' clones were also isolated. It was shown that physiological regulators of haemopoiesis were able to induce differentiation in some leukaemic clones (Sachs, 1978a,1978b, 1980, 1982; Lotem and Sachs, 1985; Shabo and Sachs, 1988; Lotem et al, 1988). The *in vitro* studies were supported with evidence from *in vivo* studies in murine leukaemic models (Lotem and Sachs, 1981, 1984, 1988). Studies using leukaemic cell lines of homogeneous undifferentiated populations of leukaemic cells provided strong evidence that differentiation induction of murine leukaemic cells could be achieved *in vitro*. During the early 1980's Collins et al induced differentiation in the human promyelocytic cell line HL60 (Collins et al, 1980).

Various physiological and non physiological compounds have been shown to induce differentiation in leukaemic cells. These agents can be classified into four main groups:-

1. The regulators of normal haemopoiesis, CSF's and the CSF's in combination with the immunomodulators interferon and tumour necrosis factor- $\beta$ .
2. Steroids, the vitamin A derivatives retinoic acid, 13-*cis* and  $\beta$ atRA, and the vitamin D<sub>3</sub> derivative 1,25 dihydroxycholecalciferol.
3. Low doses of some antimetabolite agents eg ara-C, aclacinomycin A or other cytotoxic agents.
4. Polar compounds such as dimethylsulphoxide (DMSO) and hexylmethylbes-anthracene or phorbol esters (Chomienne, 1991).

Of the CSF's G-CSF has been shown to be the most effective in the induction of differentiation of murine leukaemic cells *in vitro* (Metcalf and Nicola, 1985). However, the inclusion of the physiological growth factors in therapeutic regimes for myeloid leukaemia have been treated with caution because they have been shown to have proliferative effects on leukaemic cells *in vitro* (Jones and Millar, 1989). The relationship of the CSF's to normal and leukaemic cells in the leukaemic state still needs to be elucidated (Jones and Millar, 1989). Current *in vitro* data suggests that the CSF's may be able to play a role in differentiation induction regimes for the treatment or maintenance of myeloid leukaemia. We aim to study the *in vitro* differentiation effects of WEHI-3B CM (as a source of IL-3), L929 CM (as a source of M-CSF) and rGM-CSF, the interrelationships and synergy of the growth factors in relation to differentiation induction on myeloid leukaemic cells and to compare these to the responses of normal bone marrow. More specifically, we aim to determine the effect of the growth factors on the clonogenic leukaemic cell fraction utilizing an *in vivo* clonogenic cell assay.

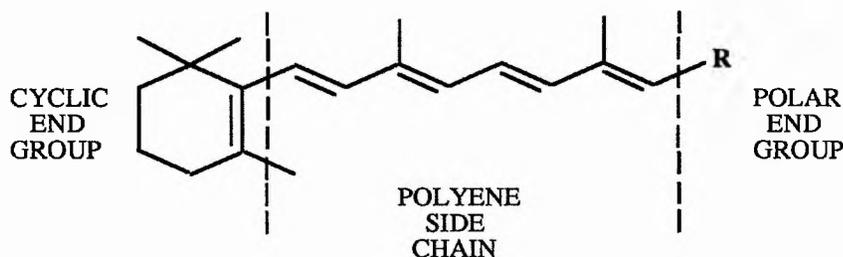
## **RETINOIC ACID AND DIFFERENTIATION INDUCTION OF MYELOID LEUKAEMIA**

Retinoids are a class of chemical compounds structurally related to Vitamin A (Vit A) and are comprised of natural and synthetic analogs. A correlation between Vit A and cancer was first noted with the classic work of Wolbach and Howe (1925) when experimentally-induced Vit A deficiency was shown to lead to hyperplastic, metaplastic and dysplastic tissue changes. In the forties Abels et al (1941) conducted the first studies which associated vitamin A deficiency with human malignancy. At this time success rates in clinical trials of Vit A were not promising as very high concentrations of the vitamin were required to induce a

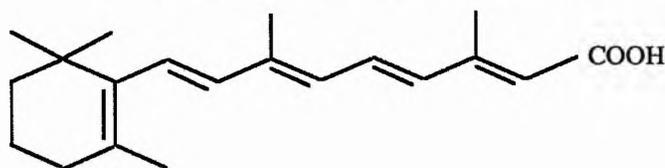
response resulting in undesirable side effects of hypervitaminosis A. Since the mid fifties derivatives were developed in an attempt to produce drugs with an improved therapeutic ratio and now over 2000 analogs have been synthesized (for reviews see Lippman et al, 1987 part I; Bollag and Holdener, 1992). During the fifties and sixties a preventative effect of Vit A on the development of chemically induced tumours was demonstrated in animal models (Chu and Malmgren, 1965) By the seventies the therapeutic effects of Vit A and Vit A acid were demonstrated experimentally on epithelial and skin tumours (Bollag 1970, 1971).

Differentiation induction with the use of retinoids was achieved in certain malignant cell lines in mice and from humans, such as, neuroblastoma (Schubert et al, 1971; Sidell 1982), teratocarcinoma (Pierce and Wallace, 1971; Strickland and Mahdavi, 1978), squamous cell carcinoma (Pierce and Wallace, 1971), adenocarcinomas of the breast (Decosse et al, 1973), embryonal carcinoma (Jetten et al, 1979) and on human myeloid leukaemic cell lines HL60 (acute promyelocytic), the histiocytic lymphoma U-937 and THP-1 (Metcalf et al, 1969; Breitman et al, 1980, 1983; Gootwine et al, 1982). During the early to mid eighties numerous observations were made on the differentiation inducing or antiproliferative properties of RA on human acute myeloid leukaemic cells (for review see Lippman et al, 1987 part II) which warranted further studies on the efficacy of RA in the treatment of AML. Differentiation induction was achieved only on fresh human promyelocytic leukaemic cells in primary culture (Breitman et al, 1981; 1983). Antiproliferative effects have been observed with other AML subtypes (Findley et al, 1984; Lawrence et al, 1987). Only the two naturally occurring isomers of RA,  $\beta$ -all *trans* and 13-*cis* RA with a free carboxyl end group (Fig. 1) have been shown to be active inducers of differentiation in human

## RETINOIC ACIDS



### $\beta$ -ALL TRANS RETINOIC ACID



### 13-CIS RETINOIC ACID

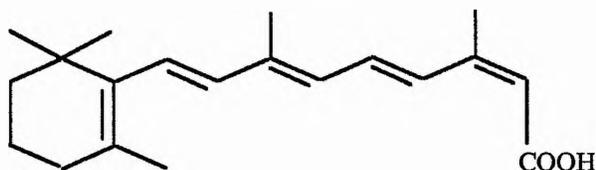


Figure 1. The basic structure of the retinoid molecule consists of a cyclic end group, a polyene side chain and a polar end group. By modifying any part, different therapeutic ratios can be achieved. Chemical manipulation of the polar end group and the polyene side chain produced retinyl esters, amides, aldehydes, ethers, amines and acids. These were the first analogues to be developed and were termed first generation retinoids. Second generation retinoids were produced by altering the cyclic end group, and cyclization of the polyene side chain has produced potent and less toxic third generation retinoids. The two naturally occurring isomers of RA, 13-cis RA and especially  $\beta$ -all trans RA have been shown to have differentiation inducing properties in acute myeloid leukaemias.

myeloid leukaemic cell lines and on fresh human APL cells. Although other synthetic analogues may be more potent some like etretinate have no effect on leukemic cell differentiation (Castaigne et al, 1990). Until the late eighties studies concentrated on the use of 13-*cis* RA. The aim of our experiments was to evaluate the efficacy of  $\beta$ atRA to differentiate myeloid leukaemic cells. Bone marrow cells from three out of the four transplanted murine myeloid leukaemias studied were induced to differentiate, an antiproliferative effect was shown with the fourth and with a highly proliferative SA2 CL. In recent studies on structure-function relationships of RA on APL cells (Chomienne et al, 1989; 1990) and in clinical trials on patients with APL (Huang Menger et al, 1988; Castagne et al, 1990)  $\beta$ atRA has been shown to be the more effective of the two isomers for differentiation induction.

It had been postulated that the anti-tumour activity of retinoids was at least partially due to either induction of differentiation (Breitman et al 1983; Chomienne et al, 1986) and/or inhibition of proliferation (Roberts and Sporn, 1984). Although the experimental evidence was not directly conclusive the use of homogeneous cell lines strongly suggested that the mode of action of retinoids, at least in these cell lines, was via differentiation induction. Definitive evidence on the differentiation inducing properties of RA has been provided very recently by Lo Coco et al (1991). Utilizing molecular evaluations of genetic rearrangements characteristic of promyelocytes from APL patients they showed that these rearrangements were present in mature granulocytes after patients were treated with  $\beta$ atRA.

## RETINOIC ACID IN COMBINATION WITH PHYSIOLOGICAL REGULATORS OF HAEMOPOIESIS

In 1984 Olsson et al, reported on synergistic differentiation effects on the HL-60 cell line with RA in combination with T-lymphocyte derived differentiation inducing factor. This was followed by a study by Ho (1985) who reported synergistic anticellular effects with the combination of RA and  $\beta$ -interferon against U-937 cells. In our studies we aimed to evaluate the combination of  $\beta$ tRA with regulators of normal haemopoietic proliferation and differentiation for synergistic differentiation effects on myeloid leukaemic cells. As a source of IL-3 we evaluated WEHI-3B CM which we showed maintained immature leukaemic cells in culture, and as a source of M-CSF we evaluated L929 CM which we showed predominantly induced differentiation of leukaemic cells in culture. We were not able to make comparisons with RA treated cells in growth factor free cultures however, we showed that the combination of  $\beta$ tRA with L929 CM induced a more enhanced differentiation response than with WEHI-3B CM. In the preceding years a number of cytokines which had previously been shown not to have differentiation inducing properties on transformed cell lines when used alone, were shown to have synergistic effects when used in combination with RA. These cytokines include IL-1 a and b, IL-2, IL-4, interferon- $\alpha$ , - $\beta$  and - $\gamma$ , tumour necrosis factor-a, G-CSF, epidermal growth factor, transforming growth factor- $\beta$ 1 and - $\beta$ 2 (Hemmi and Breitman, 1987; Kohlhepp et al, 1987; Trinchieri et al, 1987; Nakamaki et al, 1989; Santini et al, 1989; Peck and Bollag, 1991)

## THE COMBINATION OF A DNA SYNTHESIS INHIBITOR, CYTOSINE ARABINOSIDE, AND A DIFFERENTIATION INDUCER, $\beta$ -ALL TRANS RETINOIC ACID, FOR THE TREATMENT OF MYELOID LEUKAEMIA

Vigorous chemotherapeutic regimes have been shown to:-

- 1) be ineffective in some AML patients as they are refractory to such treatment
- 2) be undesirable as they result in high mortality rates in elderly AML patients who cannot tolerate aggressive chemotherapy and in patients with MDS whose cytopenias are exacerbated with cytotoxic protocols and
- 3) following successful initial induction of complete remission, not to eliminate minimal residual disease resulting in relapse and often the emergence of dominant resistant leukaemic clones.

Less toxic and more effective therapeutic regimes would be desirable for such patients.

Combinations of differentiation agents and DNA synthesis inhibitors have been shown to have synergistic differentiation effects on bone marrow cells from patients with AML and MDS (Francis et al, 1985, 1987; Ho et al, 1987; Hoffman et al, 1985; Hassan and Rees, 1988; Hellstrom et al, 1987). The resultant responses observed *in vitro* suggested that with the combination of agents which use different modes of action, inhibition or slowing down of proliferation and differentiation induction; lower, less toxic doses of each agent can be used to achieve decreases in the proliferation of the malignant clones comparable to decreases observed with higher doses of single agents (Francis et al, 1985).

Studies on the combination of these agents have been mainly limited to bone marrow cells from patients with MDS (Francis et al, 1985; Francis et al, 1987; Ho et al, 1987). Studies on bone marrow cells from patients with AML have been limited (Hoffman et al, 1985; Hassan and Rees, 1988; Hellstrom et al, 1987) and

have focused on the use of 13-*cis* RA or have not specified which RA was employed.

We aimed to evaluate the combination of  $\beta$ tatRA and ara-C in the treatment of myeloid leukaemic cells *in vitro*. We monitored for synergistic decreases in the proliferative response and found that synergistic decreases in proliferative response were observed with two myeloid leukaemias tested and an enhanced stimulatory response was observed with one. An enhanced stimulatory effect with the combination of agents was also observed with normal bone marrow cells. One of the transplanted leukaemic cell lines, in which a synergistic response was observed, was progressively undergoing changes in respect to the dominant leukaemic clone. With progressive transplantation the characteristics of the dominant leukaemic were observed to change from being growth factor independent *in vitro* to growth factor dependent. We show in these studies that with progressive transplantation the leukaemic clones became less sensitive to both differentiation induction and to the cytotoxic effects of ara-C.

### **LOW DOSE CYTOSINE ARABINOSIDE AND LEUKAEMIC CELL DIFFERENTIATION**

Ara-C is the chemotherapeutic agent of choice for the treatment of acute myeloid leukaemia. It is a naturally occurring antimetabolite. A number of other antimetabolites are used in conjunction with ara-C for the treatment of AML and these have been deliberately designed. Ara-C is a pyrimidine antimetabolite, a close structural analogue of the normal nucleoside metabolite, deoxycytidine (Capizzi et al, 1991).

Baccarani and Tura (1983) and Housett et al, (1982) showed that low doses of ara-C were also effective in the treatment of AML. It was postulated that the mode of action of ara-C may be via differentiation of the leukaemic clone. Ara-C was also shown to induce differentiation in leukaemic cell lines. 100% differentiation was observed with the monoblastic cell line U-937 and a 40% differentiation effect was observed with the HL-60 cell line (Griffin et al, 1982; Takeda et al, 1982).

There was disagreement in the literature as to whether low doses of chemotherapeutic agents acted principally as cytotoxic agents or differentiation inducers. The most widely accepted theory was that they primarily slow down DNA synthesis. Studies on differentiation commitment of leukaemic cells using chemical inducers of differentiation such as butyric acid and phorbol esters suggested that there is a decision point in the cell cycle regarding commitment late in the cell cycle or in G1 (Boyd and Metcalf 1984). This has been referred to as the differentiation responsive "window" (Francis et al, 1985). By slowing DNA synthesis with a chemotherapeutic agent this phase of the cell cycle may be lengthened and/or the cell may be rendered more responsive to differentiation induction. Hence, it has been suggested that the inhibitory effect of chemotherapeutic agents on leukaemic cell proliferation was an initiating factor for differentiation induction, by maintaining the cells in a cell-cycle phase responsive to endogenous or exogenous differentiation agents (Francis et al, 1985).

### **A MODEL OF LEUKAEMIA: RADIATION-INDUCED TRANSPLANTED MURINE MYELOID LEUKAEMIAS**

The high frequency of acute leukaemia witnessed after the dropping of the atomic bomb on Hiroshima and in patients treated with ionizing radiation for ankylosing

spondylitis first established the connection between ionizing radiation and leukaemogenesis (Mole, 1990). The myeloid leukaemic models used for these studies were radiation induced murine myeloid leukaemias established in our laboratory on the criteria established by Major and Mole (1978). CBA/H mice, in which no spontaneous leukaemia has been observed, have been used for the establishment of these cell lines. Major and Mole (1978) showed that with 300Gy total body irradiation approximately 20% of the mice developed myeloid leukaemia over their lifetime. This has been the experience in our laboratory (Riches, unpublished data). Transplantation and morphological studies of the primary and passaged leukaemias have been reported (Hepburn et al, 1987). Differing radiation doses induced lymphocytic leukaemias (Major and Mole, 1978).

It is important in the establishment of leukaemic cell lines that their characteristics parallel those of the human form of the disease. The models need to reflect the hierarchial organisation of the leukaemic population into stem cell, progenitor cell and transit cell compartments (Hepburn et al, 1987). The *in vitro* responses of the myeloid leukaemic cells in this study closely paralleled the responses observed with human myeloid leukaemic cells *in vitro* and are discussed.

### **IN VITRO ASSAYS FOR THE STUDY OF NORMAL AND LEUKAEMIC HAEMOPOIESIS**

Currently, two types of culture systems are used for the study of haemopoietic cells. Consequent cell growth in these cultures is dependent upon the initial cell population cultured and the growth factor used. The colony assay, or semisolid

culture system, permits the detection of haemopoietic progenitor cells by their ability to form colonies. Hence, progenitor cell numbers can be enumerated and specific growth factors can be assayed (Plutznik and Sachs, 1965; Bradley and Metcalf, 1966). Suspension cultures can also be used for the growth of haemopoietic cells. Lusi and Koeffler (1980) developed a rapid microtitre suspension culture assay for human colony stimulating activity based on measurements of growth factor dependent tritiated thymidine ( $^3\text{H-TdR}$ ) uptake in the human acute myelogenous leukaemia cell line (KG-1). In 1983 Horak et al extended this assay for the determination of dose response relationships of haemopoietic cells and mouse lung conditioned medium. This assay reflected the number of progenitor cells and the proliferative capacity within each colony.

Both these culture techniques have limitations. In the study of leukaemic cells the problems are further accentuated as it is not possible to determine directly the effects of various stimuli or inhibitors on the leukaemic clonogenic cell. It has been reported that malignant proliferation can occur even without the presence of the clonogenic cells as defined by the colony assays performed in semisolid media (Buick and Pollark, 1984). Both techniques favour the growth and survival of leukaemic progenitor cells however the cells and parameters studied may not be the same (Chomienne, 1991). We have chosen to use microtitre suspension cultures as they were more rapid assays for determining the proliferative responses of cultured cells, differentiative induction was easier to assess by preparing cytopins and we wanted to quantify the response of the leukaemic bone marrow as a whole.

## AIMS

The aims of these studies were:-

- a). to investigate the *in vitro* responses of myeloid leukaemic cell populations to regulators of normal haemopoiesis and
- b). to evaluate differentiation induction as an alternative therapeutic regime for the treatment of acute myeloid leukaemia.

Three radiation induced, serially transplanted murine myeloid leukaemic models developed in our laboratory (Riches et al, unpublished data) (SA2, SA7, and SA8) and a cell line derived from the SA2 transplanted murine leukaemia (SA2 CL) were used for these studies. The physiological characteristics of the transplanted leukaemic cell lines are described in Chapter One.

The initial classical *in vivo* studies on normal haemopoiesis by Till and McCulloch (1961), the establishment of *in vitro* culture systems (Lotem and Sachs, 1965; Bradley and Metcalf, 1966; Dexter et al, 1977; Lusa and Koeffler, 1980) for the study of normal haemopoiesis, the consequent identification of regulators of normal haemopoiesis, experimental techniques for maintaining leukaemic cell lines *in vitro* and the establishment of leukaemic models in rodents (Major and Mole, 1981; Hagenbeek and Van Bekkum, 1977) have provided the foundations and tools for the study of acute myeloid leukaemia.

In the first *in vitro* study (Chapter Two) the proliferative responses of bone marrow cells from the leukaemic models were characterised using regulators of normal haemopoiesis (growth factors) and compared to normal bone marrow. The regulators of normal haemopoiesis occupy a key position in attempts to understand the nature of the abnormal state existing in myeloid leukaemic cells and potentially could play an important role in therapeutic attempts to suppress

myeloid leukaemic populations. The growth factors studied were WEHI-3B CM as a source of interleukin-3 (IL-3), L929 CM as a source of macrophage-colony stimulating factor (M-CSF) and recombinant murine granulocyte-macrophage colony stimulating factor (rGM-CSF)

The regulators of normal haemopoiesis have been shown to affect overlapping stages of the haemopoietic pathways, that is, they exert stimulating effects upon the same target cells (Metcalf, 1988). They have also been shown to have synergistic proliferative effects on the same target cell when used in combination (McNeice et al, 1988 ). To further characterise the various leukaemic populations the interactions of the growth factors in controlling myeloid leukaemic cells by using combinations of growth factors *in vitro* were studied. With the use of combinations of growth factors we have attempted to identify aberrations of synergistic responses compared to normal bone marrow cells and to possibly identify specific subsets of myeloid leukaemic cells according to these responses. The comparative variations in response between the leukaemias and compared to normal myeloid cells may provide indicators of classifying leukaemic cell populations with biologically different disease and may prove to be of prognostic value.

Experimental observations have shown that malignant cells could be induced to differentiate with both physiological and non physiological agents (Fibach and Sachs, 1976; Boyd and Metcalf,1984). In Chapter Three the differentiation inducing properties of WEHI-3B CM, L929 CM and rGM-CSF on the leukaemic cell populations were investigated. To determine the effect of the differentiation response of the growth factors specifically on the leukaemic clonogenic cell fraction we employed an *in vivo* leukaemic clonogenic cell assay.

In Chapter Four the differentiation effects of  $\beta$ -all *trans* retinoic acid ( $\beta$ atRA) on leukaemic cell populations was investigated. The acute promyelocytic cell line HL60 (Collins et al, 1980) and bone marrow cells from patients with acute promyelocytic leukaemia have been shown to differentiate *in vivo* when treated with 13-*cis* retinoic acid (13-*cis* RA) (Flynn et al, 1983). In this study we aimed to evaluate the efficacy of  $\beta$ atRA for the differentiation induction of leukaemic cell populations. The effects of the combination of  $\beta$ atRA and growth factors on leukaemia cell differentiation were also investigated. We evaluated the effects of  $\beta$ atRA on the leukaemic clonogenic cell fraction using the *in vivo* leukaemic clonogenic cell assay.

It has been postulated that combinations of factors which act with different modes of action, inhibition or slowing down of proliferation and differentiation induction, lower, less toxic doses of each agent can be used to achieve increases in the proliferation of the malignant clones comparable to decreases observed with higher doses of single agents (Francis et al, 1985). In Chapter five the effects of the combination of the differentiation inducer,  $\beta$ atRA, and the DNA synthesis inhibitor, ara-C on the proliferation of myeloid leukaemic cells was investigated.

## MATERIALS AND METHODS

### **Experimental Animals**

Syngeneic CBA/H mice, derived from the breeding nucleus at the Medical Research Council radiobiology unit (Harwell, UK) were used for the murine myeloid leukaemia models and normal controls. All mice used for these experiments were bred and housed in the Bute animal house, University of St. Andrews. They were fed on R and M No. 1 food pellets and chlorinated water (15ppm chloride) ad libitum.

### **Culture Medium, Supplements, Serum and Antibiotics**

Fisher's modified medium, Dulbecco's medium and horse serum were obtained from Gibco Bioculture Ltd.

RPMI 1640 and glutamine were obtained from Flow laboratories Ltd.

Foetal calf serum was obtained from Northumbria Biological Ltd.

Penicillin was obtained from Glaxo and streptomycin from Evans.

For cell culturing RPMI 1640 medium was supplemented with 50 IU penicillin per ml, 50 mg streptomycin per ml, 2mM glutamine and 10% foetal calf serum. Fisher's modified medium and Dulbecco's medium were supplemented with 50 IU penicillin per ml, 50 mg streptomycin per ml, 2 mM glutamine and 20% horse serum.

### **Murine Radiation Induced Transplanted Myeloid Leukaemia**

Primary murine myeloid leukaemias were induced in CBA/H male mice aged  $100 \pm 10$  days following exposure to whole body doses of 3 Gray X irradiation. There has been no evidence of spontaneous incidence of leukaemia in CBA/H mice.

The various leukaemias induced after irradiation were characterised and numbered. Three primary leukaemias, numbers 2, 7 and 8, were maintained by serial transplantation of splenic single cell suspensions (see Splenic Single Cell Suspensions) from leukaemic animals into syngeneic recipients, injecting either ip or iv,  $10^4$  cells were required for 100% leukaemia take in the recipients. The transplanted leukaemias used for this study were maintained by serially passaging high doses of splenic cells ( $10^6$  total cells in 0.5ml unsupplemented medium). With the injection of  $10^6$  cells leukaemia was manifest by day seven. The transplantation number was serially recorded and the cell lines were coded as SA(X)T(Y):-

SA = St. Andrews

X = The number of the primary leukaemia (2, 7 and 8 were used for this study) T(Y) = Transplantation Number

These leukaemias had been rederived from frozen stocks of early passages. The leukaemic bone marrows used from each cell line for these experiments were between the following transplantation numbers:-

SA2 - T34 to T77

SA7 - T83 to T185

SA8 - T70 to T162

The SA2 leukaemic cells from spleen and bone marrow were found to proliferate in liquid culture without the addition of an exogenous source of growth factor. They were maintained and passaged in liquid suspension cultures and consequently labelled SA2 cell line (SA2 CL). Fisher's medium was gassed with 5% CO<sub>2</sub> in air for two minutes and the supplements were added. 10ml of  $10^5$  cells/ml single cell suspension was

seeded in 25 mm plastic culture flasks (Sterilin), gassed for 15 seconds with 5% CO<sub>2</sub> in air and incubated at 37<sup>o</sup> C. Cultures were refed with fresh medium every three to four days.

### **The HL-60 Cell Line**

The HL-60 cell line was used as a control for determining the activity of the differentiation inducing agents eg. retinoic acid.

The HL-60 cell line derived from a patient with acute promyelocytic leukaemia (Collins et al, 1977) was kindly provided by Dr. Eric Wright (St. Andrews University).

2 ml aliquots of  $2.77 \times 10^6$  cells/ml in 20% FCS RPMI were stored in liquid nitrogen. Cells were rederived and passaged in liquid suspension cultures (supplemented RPMI) for at least one week prior to experiments and not for longer than four weeks.

Cells were thawed quickly in a 37<sup>o</sup> C waterbath and transferred to a 30 ml plastic universal tube. Approximately 15 ml of medium was added dropwise while the cells were continuously mixed. The cell suspension was centrifuged at 1000 rmp for 10 minutes in a MSE chillspin centrifuge. The supernatant was discarded, the cells were resuspended in 20 ml of medium and pipetted into a 75 mm plastic tissue culture flask (Sterilin), gassed with 5% CO<sub>2</sub> in air for 1 minute and incubated at 37<sup>o</sup> C. The culture was refed by aspirating several times into a 10 ml glass pipette, 10 ml of cell suspension removed and replaced with 10 ml of fresh medium.

## **Growth Factors**

### **WEHI-3B Conditioned Medium**

WEHI-3B CM was used as an unpurified source of IL-3. WEHI-3B cells were kindly provided by Professor M. Dexter (Patterson Laboratories Manchester, UK) and were stored in liquid nitrogen in 1 ml aliquots of  $2$  to  $3 \times 10^6$  cells per ml. The cells were thawed quickly in a  $37^{\circ}$  C waterbath and transferred to a 30 ml plastic universal tube. Approximately 10 ml of Dulbecco's medium was added dropwise while the cells were continuously mixed. The cell suspension was centrifuged at 1000 rpm for 10 minutes and the supernatant discarded. The cells were resuspended in 25 ml of medium and pipetted into a 75 mm plastic flask (Sterilin) gassed with 5% CO<sub>2</sub> in air for 30 seconds and incubated at  $37^{\circ}$  C. Every three days 15 ml of cell suspension was removed and centrifuged as above. The supernatant (conditioned medium) was progressively collected in a two litre bottle and stored at  $-20^{\circ}$  C. 15ml of fresh medium was added to the original culture flask, it was gassed and reincubated as above. Cultures were maintained for up to six weeks. The collected supernatant was thawed, filtered through a 0.2 micron millipore filter (Millipore), aliquoted into 5 ml plastic tubes (Titertek) and stored at  $-20^{\circ}$  C. Each batch of conditioned medium (CM) was titrated using normal bone marrow in the microtitre and clonogenic assay (see below) to determine the specific activity.

### **L929 Conditioned Medium**

L929 CM was used as a source of M-CSF. The cells were kindly provided by Professor M. Dexter (Paterson Laboratories Manchester, UK). L cells (C3H lung fibroblasts, stain L60T) stored in liquid nitrogen and thawed with the same procedure as the WEHI-3B cells. Cells were grown to confluence in supplemented Dulbecco's medium

(10% FCS, penicillin, streptomycin, glutamine). Cells were trypsinized and seeded at  $3.3 \times 10^6$  cells/20ml medium in flat bottomed flasks, incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for five to six days. The supernatant (CM) was collected and the cells were trypsinized and recultured for two further growth cycles and then discarded.

### **Recombinant Murine Granulocyte-Macrophage Colony Stimulating Factor (rGM-CSF)**

Recombinant murine granulocyte-macrophage colony stimulating factor (rGM-CSF) was kindly provided by the Immunex Corporation (Seattle, Washington, USA). A 250 mg lyophilized sample in 24 mg mannitol, 6 mg sucrose in 728 mg tris pH 7.4 buffer was diluted in 10 ml Dulbecco's medium plus 10% FCS and 100 $\mu$ l aliquoted into 1ml eppendorf tubes and stored at -20°C. Prior to use rGM-CSF was diluted to the required concentration in the appropriate medium.

### **$\beta$ -all *Trans* Retinoic Acid**

All handling of  $\beta$ -all *trans* retinoic acid ( $\beta$ atRA) was carried out in subdued light conditions.  $\beta$ atRA (Sigma Chemical Co.) was obtained in 50 mg vials, diluted to  $10^{-2}$  molar concentration in aristor alcohol (BDH), aliquoted into small bottles and stored at -20°C. Immediately prior to each experiment it was serially diluted in the appropriate medium to the required concentration for addition to cultures. The final concentration of aristor alcohol in culture was always < 0.1% and control runs on each leukaemic cell line and normal marrow showed it had no significant effect on cell growth and differentiation at that concentration.

### **Cytosine Arabinoside (ara-C)**

Cytosine Arabinoside (ara-C) (Sigma Chemical Co.) was preweighed into 5ml plastic titertek tubes and stored at 4<sup>o</sup> C with a dessicant. For use in experiments it was serially diluted in the appropriate medium to the required concentration and volume immediately prior to addition to cultures.

### **Tritiated Thymidine**

Methyl - tritiated thymidine (<sup>3</sup>H-TdR) was obtained from Amersham International in 1ml vials at a radioactive concentration of 37.0 MBq/ml, specific activity of 185 GBq/mmol, purity 98.5% and thymine content of < 0.1 %. This was diluted to 300 kBq/ml in RPMI, aliquoted into 5ml plastic teklab tubes and stored at 4<sup>o</sup> C. A final concentration of 7.5 kBq in 25ml was added per culture well. This level for the addition to cultures had been predetermined in our laboratory. <sup>3</sup>H-TdR of the same batch number was used within each experimental group. Use and disposal of the material was recorded with accordance to the radioactive safety regulations of the ICPR and the University of St.Andrews local regulations.

### **Bone Marrow and Spleen Single Cell Suspensions**

Mice were sacrificed with an overdose of ether anaesthesia. The skin was cleansed with 70% ethanol and using sterile instruments the femurs and/or spleen were removed and placed into a sterile petri dish. For the diagnosis of leukaemia , each animal was observed for splenomegaly, and splenic imprints were prepared, stained with Diff-Quik (Merz-Dade) and observed for leukaemic blast cell infiltration.

Under sterile conditions in a laminar flow hood the femurs were dissected free of fat and muscle, the proximal end cut off with a scalpel blade and the bone marrow was expelled from the diaphysis into a 10ml plastic tube with 1ml of the appropriate medium using a 1ml syringe and number 23 gauge needle. The volume in the 10ml tube was made up to 5 or 10ml with medium and the bone marrow plugs were dispensed gently to a single cell suspension by aspirating several times into a 10ml glass pipette. The spleen was dissected free of fat, cut into small pieces with a scalpel blade, placed in a 10 ml plastic tube containing approximately 5 ml of appropriate medium and aspirated several times into a 1 ml syringe. The large clumps were allowed to settle and the supernatant was dispersed gently to a single cell suspension by aspirating several times into a 10ml glass pipette.

The cells were counted on a Coulter Cell Counter model B (Coulter Electronics) and diluted in the appropriate medium to the required cellular concentration and volume. Two cytopins (Shandon) were made of every single cell suspension prepared for experimentation, and were stained with Jenner Giemsa (BDH/Gurr) haematological stain and differentials on at least 300 cells per slide were counted to further confirm the diagnosis of leukaemia.

### **Preparation of Cytospins**

Cells required for the preparation of cytopins were diluted in medium containing 10% FCS. 100,000 cells were required per slide in  $\leq 500\mu\text{l}$  of medium.

### **Haematological Cytochemical Staining**

**Sudan Black B** Cytospins were prepared allowed to dry and fixed for 10 seconds in fixative containing 95% ethanol (BDH) and 40% formaldehyde (BDH), rinsed in

water and then allowed to dry. They were then stained for 30 minutes in Sudan Black B solution at 37° C. The solution was drained off the slides and the slides were counterstained with Jenner/Giemsa (BDH/Gurr). Strong positive staining was observed in the phospholipid containing granules of the myeloid cells and blast cells of the myeloid leukaemias stained blue black.

**Myeloperoxidase** Cytospins were prepared allowed to dry and fixed for 15 seconds in fixative containing 95% ethanol (BDH) and 40% formaldehyde (BDH), and rinsed in water. The slides were then stained for 30 seconds at 20° C in a myeloperoxidase staining solution [30% ethanol, 100ml, benzidine dihydrochloride 0.3g, zinc sulphate (38g/l  $ZnSO_4 \cdot 7H_2O$ ) 1ml, sodium acetate ( $NaC_2H_3O_2 \cdot 3H_2O$ ) 1g, N-sodium hydroxide 1.5ml, 3% hydrogen peroxide (10 vol) 0.7ml. The slides were then rinsed in tap water and counterstained with dilute Giemsa stain for 10 minutes, rinsed and allowed to dry. A strong positive reaction was observed in the azurophilic granules of developing granulocytes and in the myeloid leukaemic blasts.

### **The Microtitre Assay**

Ninety-six well flat bottomed plastic microtitre (Nunc) were used for these assays. Growth factors and bone marrow single cell suspensions were diluted in RPMI. Each test condition was carried out in quadruplicate. The cultures were incubated at 37° C in a fully humidified atmosphere of 5%  $CO_2$  in air for the specified number of days

a). **For cell dose studies.** 100  $\mu$ l of the required concentration of bone marrow single cell suspension was added to 100  $\mu$ l of RPMI containing optimal concentrations of WEHI-3B CM (10-15%) and L929 CM (10-15%).

**b) For growth factor titrations.** 100  $\mu$ l of  $7 \times 10^4$  cell/ml bone marrow single cell suspension was added to 100  $\mu$ l of the required concentrations of growth factor.

**c) For studies on the effects of  $\beta$ atRA and ara-C.** 100  $\mu$ l of  $7 \times 10^4$  cell/ml bone marrow single cell suspension was added to 100  $\mu$ l of growth factor diluted to optimal concentrations for cellular proliferation. A total volume of 50  $\mu$ l of  $\beta$ atRA and ara-C were added either alone or in combination at the required concentrations. 50  $\mu$ l of RPMI was added to the control wells.

**For proliferation studies** On the appropriate day of culture the cells were labelled with  $^3\text{H-TdR}$  for eight hours, collected using a 12 channel cell harvester (Titertek) on to glass microfibre filter paper (Titertek) using 9% saline. These were allowed to dry for at least 18 hours in a drying oven and the disks were added to plastic tubes containing 2 ml of Optiphase Safe scintillation fluid. The radioactivity incorporated by the cells was measured as counts per minute (CPM) on a LKB 1214 scintillation counter.

**For determining the total cell count per cell** The cells were removed from the wells with a pasteur pipette and immediately transferred to 20ml of Isoton (Coulter) and the cells were counted on a Coulter Cell Counter model B (Coulter Electronics)

**For differential cell counts** Concurrently with  $^3\text{H-TdR}$  uptake, and cell count studies, cultures were set up for the preparation of cytopsins. Cells were removed from the wells with a pasteur pipette placed into a 5ml titertek tube and the total cell count was determined, at least 3 cytopsins were prepared with approximately 100,000

cells in no more than 500µl of medium, stained in Jenner/Giemsa (BDH/Gurr) and 500 cells were counted per slide. Differentials were reported in both percentage and absolute cell numbers per slide.

**For the determination of the percentage of phagocytic cells** Cells were cultured in the microtitre assay with appropriate growth factors and/or differentiating agents as above. On the appropriate day the cultures were incubated with 25 µl of a 0.04% solution of polystyrene 0.8µ particles size (Sigma) for eight hours. Cytospins were prepared and stained as above, the percentage of phagocytic cells was determined microscopically, at least 1000 cells were counted per slide. Cultures for the determination of cell number per well were set up concurrently to determine the absolute number of phagocytic cells.

### **The Colony Assay**

A single layer agar technique was used. 3% agar (Bactoagar Difco Laboratories) w/v in double distilled water was pre heated and sterilised in boiling water for at least half an hour. 8ml aliquots of Dulbecco's medium and single cell suspension of  $5 \times 10^5$  cells/ml were warmed to 37° C in a water bath just prior to use. 1ml of agar was added to the 8mls of medium followed by 1ml of single cell suspension and thorough mixing was insured by inversion. For plating 1ml of suspension was added to 35 ml plastic petri dishes (Sterilin) containing 100µl of the required growth factor. The cultures were mixed gently, the agar was allowed to set at room temperature for 10 minutes, and incubated at 37° C in a fully humidified atmosphere of 5% CO<sub>2</sub> in air. After a seven day incubation period the number of colonies (>50 cells) and clusters (< 50 cells) were counted using a Kyowa dissecting microscope set-up for dark field illumination.

### **The *In Vivo* Leukaemic Clonogenic Cell Assay**

Single cell suspension of SA7 leukaemic bone marrow was prepared in non supplemented RPMI and injected into syngeneic recipients at a total cell dose of  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$  and 10 cells in 0.5ml of medium. Five mice were injected per test group and leukaemia free survival was monitored over 90 days. A sample of the cells was stained with fast green and cytopspins were prepared for determining percentage cell viability prior to injecting. Microtitre cultures were set up from the same single cell suspension in appropriate growth factors and with or without  $\beta$ atRA. On day four of culture the cells were harvested from the wells with a pasteur pipette and cell number and viability determined. The cultured cells were then injected into syngeneic recipients at the same concentrations as above and leukaemia free survival was monitored for 90 days to determine whether the *in vitro* treatment altered the number of leukaemic clonogenic cells. The mice were sacrificed when moribund and leukaemia confirmed by spleen weight and microscopic examination of splenic imprints.

### **Statistical Analysis**

The Students t-test was used as the statistical analysis.

## CHAPTER ONE

### CHARACTERISTICS OF THE MURINE, RADIATION-INDUCED, TRANSPLANTED MYELOID LEUKAEMIAS

The myeloid leukaemic models used for these studies were CBA/H murine, radiation-induced, transplanted myeloid leukaemias developed in our laboratory on the criteria established by Major and Mole (1978). Three transplanted leukaemias the SA2, SA7 and SA8 and a cell line established from the SA2 leukaemia (SA2 CL) were studied. The characteristics of the SA2 transplanted leukaemia changed with progressive transplantation hence, this leukaemia was described at low passage (LP) and high passage (HP) numbers. Experiments for this study were carried out on the transplanted cell lines between the transplantation numbers listed under Materials and Methods. The physiological changes, including blood and bone marrow cellular changes, of the transplanted leukaemias will be described and compared to normal. The parameters measured were:-

1. The gross physiological characteristics of body weight, spleen weight, haemoglobin (Hb), haematocrit, the nucleated cell count of the blood and the bone marrow nucleated cell count per femur.
2. The peripheral blood leukocyte differentials in percentage and absolute cell numbers
3. Bone marrow nucleated cell differential counts in percentage and absolute number per femur.
4. Cytochemical staining of bone marrow cells

The characteristics of the SA2 CL were also studied with differential cell counts and cytochemical staining.

## **1.1 EXPERIMENTAL PROCEDURE**

- a). Six week old mice were used for these studies.
- b). Moribund animals were weighed, blood was taken for blood nucleated cell counts, preparation of blood films and haematocrit. Blood films were stained with Jenner Giemsa and differentials counted (100 cell counts). Percentage and absolute numbers of blood cells were determined.
- c). Spleens were removed, weighed and imprints were prepared on glass slides, and stained with Diff-Quik to confirm leukaemic cell infiltration.
- d). Bone marrow single cell suspensions were prepared in RPMI, the number of nucleated cells per femur were counted, cytopspins were prepared, stained with Jenner Giemsa and differentials counted (500 cell counts). Percentage and absolute nucleated cell counts per femur were determined.
- e). Cytopspins of the bone marrow single cell suspensions were prepared for haematological cytochemical staining. Myeloperoxidase and Sudan Black B stains were performed in this laboratory, and slides were sent to Cytocolor Inc. Laboratories (Ohio, USA) for further staining.
- f). Cytopspins of the SA2 CL were prepared and stained with Jenner Giemsa for differential cell counts and with cytochemical stains as described above.

## **1.2 RESULTS**

### **1.2.1 The Physiological Characteristics of the Leukaemic Models**

Comparative studies were made between normal and leukaemic mice for body weight, spleen weight, haemoglobin, haematocrit and on the blood and bone marrow nucleated cell counts (Table 1.1). There was evidence of weight loss in the leukaemic animals. There was a six to eight fold increase in spleen weight mainly due to myeloid cell infiltration, as observed microscopically on splenic imprints and

	NORMAL	SA7	SA8	SA2 LP	SA2 HP
<b>Body Weight (g)</b>	27.00 ± 0.20	20.40 ± 0.31	21.5 ± 0.41	23.63 ± 0.25	22.00 ± 0.29
<b>Spleen Weight (mg)</b>	66.53 ± 5.85	546.23 ± 6.42	576.81 ± 4.32	368.4 ± 5.83	502.5 ± 6.17
<b>Haemoglobin (g/l)</b>	154.3 ± 2.1	54.6 ± 3.0	61.3 ± 2.8	74.5 ± 2.9	67.3 ± 2.4
<b>Haematocrit (l/l)</b>	0.43 ± 0.01	0.17 ± 0.03	0.18 ± 0.02	0.27 ± 0.01	0.20 ± 0.03
<b>Blood NCC/l x 10<sup>9</sup></b>	5.13 ± 0.03	5.13 ± 0.03	31.22 ± 1.22	20.31 ± 1.42	28.68 ± 1.18
<b>BM NCC/femur x 10<sup>6</sup></b>	14.21 ± 3.8	14.21 ± 3.8	7.93 ± 2.4	13.96 ± 3.6	9.44 ± 2.3

**Table 1.1** The physiological characteristics of six week old CBA/H murine, radiation induced, transplanted leukaemias compared to normal.

cytospins. The leukaemic mice were grossly anaemic. Haemoglobin levels were between  $54.6 \pm 3.0$  to  $74.5 \pm 2.9$  g/l; normal levels were  $154.3 \pm 2.1$  g/l. Haematocrits ranged between  $0.17 \pm 0.03$  to  $0.27 \pm 0.01$  l/l; normal levels were  $0.43 \pm 0.01$  l/l. The total nucleated cell count of the peripheral blood of the leukaemic mice was increased between four to six fold. The nucleated cell count of the femur was decreased in the case of the SA7, the SA8 and the SA2 HP leukaemias. The nucleated cell count of the femur of the SA2 LP leukaemia was equivalent to that of normal.

### **1.2.2 Peripheral Blood Leucocyte Differentials**

Leucocyte differentials were determined on the peripheral blood, the results are listed as percentages (Table 1.2) and absolute cell numbers (Table 1.3). The predominant cells in the normal CBA/H murine peripheral blood leukocyte differential were lymphocytes, 73.5%, followed by 16.0% segmented cells, 10.5% monocytes and 0.5 % bands.

The four to six fold increase in the nucleated blood cell counts of the leukaemic mice, as described above, were due to leukaemic myeloid cell infiltration. The peripheral bloods of the leukaemic transplanted cell lines were infiltrated with numerous immature myeloid cells, and mature myeloid cells monocytes or macrophages. Absolute lymphocyte numbers were equivalent to that of normal. Without a specific marker it is difficult to determine whether the mature myeloid cells observed in the blood of the leukaemic animals were from the leukaemic clone or due to a host reaction. The absolute numbers of mature myeloid cells were higher in the leukaemic animals than in the normal and the differential pattern of the mature cells was not the same with each of the leukaemias. Macrophages and monocytes were the predominant mature cells of

CELL	PERCENTAGE CELL NUMBER $\pm$ STANDARD ERROR									
	NBM		SA7 HD		SA8 HD		SA2 HD LP		SA2 HD HP	
Blasts	0	0	10.3	2.2	14.4	2.8	8.8	2.2	16.8	3.2
Promyel	0	0	13.5	2.5	6.7	1.7	13.8	3.1	15.2	3.5
Myel	0	0	5.2	0.8	2.0	0.7	5.4	1.5	5.4	2.1
Metamyel	0	0	5.7	1.2	1.5	1.0	4.5	1.5	4.1	2.0
Bands	0.5	0.1	7.7	2.6	11.9	3.4	7.2	2.1	6.4	1.5
Grans	15.5	0.5	7.0	2.1	35.3	3.8	26.5	4.1	25.8	4.4
Monoc	10.5	0.5	11.5	1.6	9.0	2.6	11.5	4.0	11.5	3.1
Mf	0	0	21.7	3.6	2.0	1.2	0	0	2.0	1.5
Lymph	73.5	1.0	17.2	4.2	11.3	3.0	22.3	4.5	12.8	3.8

Table 1.2 Differential leukocyte count of normal and leukaemic blood.

CELL	ABSOLUTE CELL NUMBER $\times 10^9 / L \pm$ STANDARD ERROR									
	NBM		SA7 HD		SA8 HD		SA2 HD LP		SA2 HD HP	
Blasts	0	0	2.99	0.16	4.50	0.88	1.78	0.45	4.82	0.92
Promyel	0	0	3.92	0.73	2.09	0.53	2.80	0.63	4.36	1.00
Myel	0	0	1.51	0.23	0.62	0.22	1.10	0.31	1.55	0.60
Metamyel	0	0	1.66	0.35	0.47	0.31	0.91	0.30	1.18	0.58
Bands	0.03	0.01	2.24	0.76	3.72	1.06	1.46	0.43	1.84	0.43
Segs	0.80	0.04	2.03	0.61	11.02	1.19	5.38	0.83	7.40	1.26
Monoc	0.54	0.03	3.34	0.46	2.81	0.81	2.33	0.81	3.30	0.89
Mf	0	0	6.03	1.00	0.62	0.04	0	0	0.57	4.28
Lymph	3.77	0.05	4.99	1.22	3.53	0.94	4.53	0.91	3.67	0.80

Table 1.3 Absolute cell numbers of peripheral blood differentials. Absolute cell number = total cell number / L  $\times$  percentage cell number (see Table 1.1 for total peripheral blood nucleated cell count).

Promyel=Promyelocytes, Myel=Myelocytes, Metamyel=Metamyelocytes  
Grans=Granulocytes, Mf=Macrophages, Lymphs=Lymphocytes

the SA7 leukaemia whereas with the granulocytes were the predominant mature cells of the SA8 and SA2 leukaemias. It may be predicted from these two observations that the full range of myeloid cells (immature to mature) observed in the leukaemic animals were from the leukaemic clones.

Both high numbers of blasts and promyelocytes were observed in the leukaemic blood samples. The SA7 leukaemia had a higher absolute number of promyelocytes than blast cells and a higher number of macrophages compared to the other leukaemias. The SA8 leukaemia had a higher number of blasts than promyelocytes, and a higher number of granulocytes compared to the other leukaemias. The SA2 LP leukaemia had a higher number of promyelocytes than blasts and the SA2 HP leukaemia had equal numbers of blasts and promyelocytes. The SA2 HP leukaemia had the greatest number of infiltrating blast cells and promyelocytes.

### **1.2.3 Bone Marrow Differentials**

The bone marrow differentials were listed as both cell percentages (Table 1.4) and absolute cell number per femur (Table 1.5). Even though the femur cellularity of the leukaemic mice was generally less than that of normal, absolute blast cell numbers were higher or of the same order as that of normal. The bone marrow differential of normal CBA/H mice was 9.5% blasts, 9.0% promyelocytes, 14.4% myelocytes, 63.3% metamyelocytes, 20.6% bands, and 0.7% of both segmented cells and monocytes.

#### **1.2.3.1 The SA7 Leukaemia**

Blast cell numbers constituted nearly 50% and promyelocytes 31% of the cells of the SA7 bone marrow; intermediate cell (myelocytes, metamyelocyte and band

CELL	PERCENTAGE ± STANDARD ERROR									
	NBM		SA7 HD		SA8 HD		SA2 HD LP		SA2 HD HP	
Blasts	9.5	0.2	49.0	2.14	17.2	0.9	36.0	1.7	19.3	0.4
Promyel	9.0	0.4	31.0	0.9	18.5	0.9	7.7	0.1	18.7	0.7
Myel	14.4	0.4	9.5	0.4	8.6	0.3	4.6	1.5	5.5	0.1
Metamyel	63.6	0.4	19.1	1.3	42.9	1.7	49.0	4.5	49.0	1.6
Bands	20.6	0.2	0	0	2.8	0.4	1.3	0.2	2.8	0.1
Grans	0.7	0.4	1.4	0.1	0.8	0.4	1.3	0.2	3.4	0.1
Monoc	0.7	0.4	2.0	0.6	8.6	0.3	0	0	13.0	0.2
Mf	0	0	1.4	0.1	0.8	0.6	0	0	0	0

**Table 1.4** Differential nucleated cell count of normal and leukaemic bone marrow as percentage.

CELL	ABSOLUTE CELL No. PER FEMUR ± STANDARD ERROR									
	NBM		SA7 HD		SA8 HD		SA2 HD LP		SA2 HD HP	
Blasts	1.35	0.42	3.79	1.05	1.36	0.45	5.02	1.20	1.81	0.51
Promyel	1.28	0.40	2.40	0.96	1.47	0.42	1.07	0.85	1.76	0.52
Myel	2.05	0.80	0.74	0.18	0.68	0.22	0.64	0.02	0.52	0.06
Metamyel	9.03	2.12	1.48	0.57	3.41	1.05	6.84	1.55	4.61	0.88
Bands	2.93	0.96	0	0	0.22	0.08	0.18	0.05	0.26	0.03
Grans	0.10	0.06	0.11	0.08	0.06	0.03	0.18	0.03	0.32	0.03
Monoc	0.10	0.06	0.15	0.08	0.68	0.21	0	0	1.22	0.036
Mf	0	0	0.11	0.08	0.06	0.03	0	0	0	0

**Table 1.5** Differential nucleated cell count of normal and leukaemic marrow as absolute cell number per femur. Absolute cell number per femur = total cell number per femur x percentage cell number (see Table 1.1 for total number of nucleated cells per femur).

Promyel=Promyelocytes, Myel=Myelocytes, Metamyel=Metamyelocytes  
 Segs=Segmented cells, Mf=Macrophages, Lymphs=Lymphocytes

cells) numbers were reduced to one third of normal a level lower than observed with the other leukaemias; the percentages of granulocytes and macrophages were mildly higher than normal. The femur cellularity of the SA7 leukaemia was half that of normal however, absolute blast cell numbers were three times higher, absolute numbers of intermediate cells were significantly decreased and mature cell numbers equivalent to normal.

#### **1.2.3.2 The SA8 Leukaemia**

An equal percentage of blasts and promyelocytes were observed in the bone marrow of the SA8 leukaemia (approximately 18%); intermediate cell numbers were reduced to two thirds of normal; a high percentage of monocytes was observed and granulocyte numbers were similar to normal.. The absolute number of blasts and promyelocytes of the SA8 leukaemia were equivalent to that of normal, however the absolute numbers of the intermediate cell numbers were significantly reduced; granulocyte numbers were equivalent to normal however, the absolute number of macrophages was significantly higher than normal.

#### **1.2.3.3 The SA2 LP Leukaemia**

High percentage numbers of promyelocytes were not observed with the SA2 LP leukaemia; 36% blasts were observed and metamyelocyte and band cell numbers were reduced to two thirds of normal; granulocytes and macrophage numbers were similar to normal. The femur cellularity of the SA2 LP leukaemia was equivalent to that of normal however, absolute numbers of blast cell were four times higher than normal; absolute numbers of intermediate cells were reduced and absolute mature cell numbers were similar to normal.

#### **1.2.3.4 The SA2 HP Leukaemia**

The bone marrow differential of the SA2 HP leukaemia was similar to that of the SA8 leukaemia however, few similarities were observed between these leukaemias in relation to their *in vitro* responses (see Chapters Two to Five). The SA2 HP leukaemia had higher percentage and absolute numbers of macrophages than the SA8.

#### **1.2.4 The SA2 CL**

Cytospins were prepared from suspension cultures of the SA2 CL. A fairly homogeneous cell population was observed with 99% blasts and 1% blastic cells containing a few azurophilic granules; the latter were classified as pro-myelocytes.

#### **1.2.5 Haematological Cytochemical Staining**

The bone marrow cells from the four transplanted leukaemias and the SA2 cell line stained strongly positive for myeloperoxidase and Sudan Black B and negative for PAS and acid phosphatase which is characteristic of myeloid leukaemia. Staining for chloroacetate and naphthol acetate were difficult to assess in our laboratory. A full panel of cytochemical staining was done for us by Cytocholor Incoporated (Ohio USA) and their results indicated myeloid leukaemia.

### **1.3 DISCUSSION**

The physiological characteristics of the transplanted cell lines were examined and found to indicate myeloid leukaemia. The leukaemic animals showed evidence of weight loss, splenic enlargement with leukaemic cell infiltration and

gross anaemia. The blood nucleated cell counts were four to six times greater than normal. Abnormally high percentages of immature and mature myeloid cells were observed in the blood. It could not be inferred whether the increased number of mature myeloid cells observed in the blood of the leukaemic animals were part of the leukaemic clone or due to a host reaction.

A decreased femur cellularity was observed with the SA7, SA8 and SA2 HP leukaemias; the femur cellularity of the SA2 LP leukaemia was similar to normal. The absolute numbers of blast cells observed in the leukaemic femurs however, were greater than or equivalent to normal. Intermediate cell numbers in the bone marrow were reduced in each case, indicating abnormal maturation. The SA8 and SA2 HP leukaemias had abnormally high numbers of macrophages in their femurs, higher than normal and higher than the other two leukaemias. Differential counts on the bone marrow confirmed a heterogeneous cellular composition of myeloid leukaemic cells with each of the transplanted cell lines which is also characteristic of human AML cell populations (Griffin and Lowenberg, 1986). The cells of the SA2 CL appeared homogeneous.

Haematological cytochemical staining confirmed that the four transplanted leukaemias and the SA2 CL were myeloid.

## CHAPTER TWO

### ***IN VITRO* PROLIFERATIVE EFFECTS OF WEHI-3B CM L929 CM AND rGM-CSF ON LEUKAEMIC AND NORMAL BONE MARROW CELLS**

The *in vitro* proliferative responses of the transplanted leukaemic cell lines and the SA2 CL to WEHI-3B CM ( as a source of IL-3), L929 CM (as a source of M-CSF) and recombinant murine GM-CSF were determined and compared to normal. The regulatory processes affecting leukaemic cells are not fully understood. What became evident from previous studies was the heterogeneity of the leukaemic response to normal haemopoietic regulators (Moore et al, 1974). CSF's are active on normal multipotential and stem cells (Metcalf, 1988) hence it appeared obvious that the role of these factors in AML needed to be elucidated. The roles played by the CSFs in the progression and maintenance of the disease appeared fundamental to our understanding of the disorder. The regulators of normal haemopoiesis may occupy a key position in attempts to understand the nature of the abnormal state existing in myeloid leukaemic cells and potentially could play an important role in therapeutic attempts to suppress myeloid leukaemic populations either by reducing the leukaemic cell population or by preferentially enhancing normal haemopoiesis. In the first study we investigated the dose-response relationships between the leukaemic cell populations and the individual CSF's. This was followed by a time study of proliferative response of the leukaemic populations to optimal concentrations of CSF's and compared to normal. The proliferative response of the leukaemic cells to individual CSF's were studied daily between day one and day seven.

The regulators of normal haemopoiesis affect overlapping stages of the normal haematopoietic pathways, that is, they exert stimulating effects upon the same

target cells (Metcalf, 1988). They have also been shown to have synergistic proliferative effects on the same target cell when used in combination (McNeice et al, 1988). To further characterise the various leukaemic populations we investigated the interactions of the CSFs in controlling myeloid leukaemic cells by using combinations of growth factors *in vitro*. With the use of combinations of growth factors we have attempted to identify aberrations of synergistic responses observed with normal bone marrow cells and to possibly identify specific subsets of myeloid leukaemic cells according to these responses. The comparative variations in response between the leukaemias and compared to normal myeloid cells may provide indicators of classifying leukaemic cell populations with biologically different disease and may prove to be of prognostic value. The proliferative responses of the leukaemic cells to combinations of CSF's were studied daily over seven days.

The leukaemic bone marrow cell populations were studied as a whole in microtitre suspension cultures over the time periods specified and proliferation was measured by utilizing  $^3\text{H-TdR}$  uptake. Colony assays in semi-solid medias were run in parrallel and the results obtained with the two culture techniques were compared although, the cells and parameters studied in each system may not be the same. The first experiments in this study determined the optimal number of cells for the the microtitre assay. Optimal cell numbers for the colony assay were determined by others in our laboratory (Briscoe and Riches unpublished data).

## **2.1 CELL DOSE STUDIES OF LEUKAEMIC AND NORMAL BONE MARROW CELLS IN THE MICROTITRE ASSAY**

These experiments were designed to determine the optimal cell concentrations required for optimal cellular proliferation of normal and leukaemic bone marrow cells in the microtitre assay.

### **2.1.1 Experimental Procedure**

a) Optimal levels of WEHI-3B CM and L929 CM for stimulating normal bone marrow murine haemopoietic cells were predetermined in the laboratory using a standard procedure in the colony assay. Optimal levels were usually found to be between 10 and 15% v/v. The rmGM-CSF was also initially titrated in this assay and was found to give optimal stimulation between 40 to 100 IU/ml.

b) The predetermined levels for optimal stimulation of normal bone marrow were used to determine optimal cell doses for normal and leukaemic bone marrow in the microtitre assay. Cells at varying cell concentrations (1 to  $10 \times 10^4$  cells per well) were cultured for four days in microtitre cultures with optimal levels of WEHI-3B or L929 CM and cellular proliferation was determined by measuring  $^3\text{H-TdR}$  uptake.

### **2.1.2 Results**

Optimal cell concentration for normal and leukaemic marrow from the transplanted cell lines in the microtitre assay was found to be  $7 \times 10^4$  cells per well in a total volume of 200ml (Fig. 2.1 and Appendix Table A2.1). The SA2 CL differed; proliferation increased exponentially to  $10^5$  cells per well, the upper limit tested. For uniformity  $7 \times 10^4$  SA2 CL cells per well were used in all experiments.

All further batches of conditioned medium were concurrently tested in the microtiter assay. There was always almost 100% correlation between the microtiter and colony assay

## **2.2 IN VITRO DOSE RESPONSE STUDIES OF WEHI-3B CM, L929 CM AND rGM-CSF ON LEUKAEMIC AND NORMAL BONE MARROW CELLS**

The bone marrow cells of the murine myeloid leukaemias were characterised *in vitro* by studying their growth response patterns with and without the stimulators of normal bone marrow WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay after four days in culture. Normal and leukaemic bone marrow cells from each of the leukaemias were set up with varying doses of each growth factor and the dose-response relationship for each leukaemia was determined. From these studies the concentration of growth factor which induced optimal proliferation of the leukaemic cells was determined. This optimal concentration of each growth factor was used to further determine the differences in proliferative response between normal and leukaemic cells.

### **2.2.1 Experimental Procedure**

Dose-response studies were set up in the microtitre assay. Single cell suspensions of  $7 \times 10^4$  normal and leukaemic bone marrow cells were cultured with FCS alone and with increasing concentrations of WEHI-3B CM (5 to 20% v/v), L929 CM (5 to 20% v/v) and rmGM-CSF (10 to 100 IU/ml) for two, four and six days. Cultures were set up for  $^3\text{H}$ -TdR uptake studies and for the determination of total cell number per well.

### **2.2.2 Results**

Normal and leukaemic bone marrow cells were cultured in FCS alone and with increasing doses of WEHI-3B CM, L929 CM and rGM-CSF. Normal bone marrow (Fig. 2.2) the SA7 (Fig. 2.3), SA8 (Fig. 2.4) and the SA2 HP (Fig. 2.5) leukaemias were growth factor dependent for proliferation *in vitro*. The SA2 LP (Fig's. 2.6 and 2.7) and SA2 CL (Fig. 2.8) were observed to proliferate autonomously *in vitro*.

The responses to stimulators of normal and the growth factor dependent leukaemias were dose dependent. The dose of each growth factor for maximal stimulation of both normal and leukaemic bone marrow cells was the same with only one exception. WEHI-3B and L929 CM were both found to be maximal at 15% v/v. rGM-CSF was found to be maximal from 40 to 100 IU/ml except in the case of the SA8 leukaemia where maximal stimulation was observed with 60 IU/ml.

Maximal proliferation of normal bone marrow (Fig. 2.2), the SA7 (Fig. 2.3) and the SA8 (Fig. 2.4) leukaemias was observed when the cells were stimulated with WEHI-3B CM. Maximal stimulation of the SA2 HP leukaemia (Fig. 2.5) was observed when the cells were stimulated with rGM-CSF.

Although the required concentration of each of the growth factors for optimal stimulation of normal and leukaemic cells was the same the rate of proliferation and the day to which proliferation was sustained differed. The highest proliferative responses of normal bone marrow with each of the CSFs were observed on day six. With the SA7 leukaemia maximal proliferative responses were observed on day six when stimulated with WEHI-3B CM and L929 CM and on day two and four when stimulated with rGM-CSF. Maximal proliferation was observed with the SA8 leukaemia on day six when stimulated with WEHI-3B CM, on day four when stimulated with L929 CM and on day two when stimulated with rGM-CSF. The SA2 HD leukaemia proliferated maximally on day four when stimulated with WEHI-3B CM and L929 CM and on day two when stimulated with rGM-CSF. The time study differences in proliferative response to the CSFs are investigated in detail in the next section.

Autonomous cell growth *in vitro* was observed with the SA2 LP leukaemia (Fig's. 2.6 and 2.7) and the SA2 CL (Fig 2.7) which was derived from the SA2 LP leukaemia (passage number 32). The interesting feature of the SA2 leukaemia was that in the early passages studied (passage 34 to 40) the dominant clone was

independent of growth factor for proliferation *in vitro* (Fig. 2.6) however, with continuous passaging minimal autonomous proliferation was observed at passage number 54 (Fig. 2.7) and by passage number 77 (Fig. 2.5) a subclone was obtained which was growth factor dependent for proliferation. The growth factor dependent clone was observed to be more highly proliferative than the SA2 LP clones. The SA2 transplanted cell line had been rederived from frozen cells of passage number 32 and this pattern of change, from independent to growth factor dependent cell growth *in vitro* was observed in two series of transplanted cell lines established.

A dose response relationship of the CSF's and the SA2 LP leukaemia was not observed (Fig. 2.6). The CSF's did not alter the proliferative response observed with FCS alone.

The SA2 CL was observed to be highly proliferative however, a dose response relationship of the CSF's and the SA2 CL was not observed. With the addition of L929 CM a significant decrease in the proliferative response was observed on day two (Fig 2.7) compared to FCS alone. The inhibitory effect of L929 CM appeared not to be sustained and no differences in proliferative response were observed on days four and six compared to FCS alone. Minimal decreases in the proliferative response of the SA2 CL were observed with the addition of WEHI-3B CM on day two and with the addition of rGM-CSF on day four compared to FCS alone. Conditioned medium was collected from the SA2 CL and used to stimulate normal bone marrow in microtitre and colony assay cultures and no proliferative response was observed (results not shown).

Minimal autonomous proliferation was observed with the SA8 and the SA2 HP leukaemias to day two but this was not sustained.

The results obtained for the determination of total cell number per well paralleled the  $^3\text{H}$ -TdR uptake studies.

## **2.3 TIME STUDY OF THE PROLIFERATIVE RESPONSES OF NORMAL AND LEUKAEMIC BONE MARROW CELLS TO OPTIMAL CONCENTRATIONS OF WEHI-3B CM, L929 CM AND rGM-CSF**

The main differences in the proliferation responses between normal and the growth factor dependent leukaemic bone marrow cells, as observed in section 2.2, were the proliferative rates of the cells to each of the CSF's and the day to which the cells were sustained in culture. The aim of this study was to investigate these differences by studying the proliferative responses of the leukaemic cells to the individual CSF's progressively over seven days in the microtitre culture.

### **2.3.1 Experimental Procedure**

The proliferation characteristics of leukaemic cells in FCS alone and to optimal concentrations of WEHI-3B CM, L929 CM and rGM-CSF, used singularly, were studied daily over seven days in the microtitre assay. Single cell suspensions of  $7 \times 10^4$  leukaemic and normal bone marrow cells and cells from the SA2 CL were incubated with 15% WEHI-3B CM (V/V), 15% L929 CM (V/V) and 60 U/ml rGM-CSF. Proliferation was measured utilizing  $^3\text{H}$ -TdR uptake. The results obtained were compared to the responses obtained with normal bone marrow cells.

### **2.3.2 Results**

#### **2.3.2.1 Proliferative responses in FCS**

NBM, the SA7, SA8 and the SA2 HP leukaemias were growth factor dependent for proliferation (Fig. 2.9 A). Two differences in the proliferative response between NBM and these three leukaemias were observed in FCS:-

- Proliferation of normal bone marrow was observed only during the first eight hours of culture whereas a proliferative response of the SA7 and SA8 leukaemias was observed to day two and of the SA2 HP leukaemia to day three of culture.

- After eight hours of culture the proliferative response observed with the SA7 and the SA2 HP leukaemia were double and triple the response observed with normal bone marrow respectively.

The SA2 LP leukaemia used for this study was from passage number 49. A minimal autonomous proliferative response was observed with the SA2 LP leukaemia to day seven of culture.

An autonomous and highly proliferative response was observed with the SA2 CL (Fig. 2.9). The maximal proliferative response was observed on day one. The proliferative response decreased on each consecutive day from day one to day seven.

#### **2.3.2.2 Proliferative responses to WEHI-3B CM**

WEHI-3B CM induced a maximal proliferative response compared to the other two growth factors of NBM, the SA7, SA8 and SA2 LP leukaemias but not the SA2 HP (Fig. 2.9 B). rGM-CSF induced a maximal proliferative response of the SA2 HP leukaemia.

Increasing proliferation of normal and leukaemic bone marrow cells was not sustained in culture for the same number of days. Increasing proliferation of NBM, SA8 and SA2 LP leukaemic cells was sustained to day six, of the SA7 to day five and of the SA2 HP only to day four. The maximal proliferation obtained on these days by normal bone marrow, the SA7, SA8HD and the SA2 HP leukaemic cells was similar (approx. 20,500 CPM). The maximal proliferation of the SA2 LP leukaemia as observed on day six was significantly less than that observed with normal bone marrow (14,644 CPM).

Another main difference between normal and leukaemic marrow was the proliferative rate of the cells at the earlier time points in culture (from day one to day four). The proliferative rate of the SA7 leukaemia was marginally higher

than normal bone marrow, and the proliferative rates of the SA8 and the SA2 HP leukaemias were double and triple the rate observed with normal bone marrow respectively. The proliferative rate of the SA2 LP leukaemia was significantly less than the response observed with normal bone marrow at every time point.

An inhibitory effect on the proliferation of the SA2 CL was observed with the addition of WEHI-3CM compared to FCS alone between eight hours of culture and day one (Fig 2.9). From day two to day seven there was no difference in the proliferative response compared to FCS alone.

### **2.3.2.3 Proliferative responses to L929 CM**

L929 CM was not as effective at stimulating the proliferation of normal and leukaemic cells as WEHI-3B CM or rmGM-CSF (Fig. 2.9 C). The differences in the patterns of proliferative response between normal and leukaemic marrow were similar to those observed with WEHI-3B CM.

There were differences in the number of days that increasing proliferation was sustained. Increasing proliferation of normal bone marrow and the SA2 LP leukaemia was sustained to day five, of the SA7 and the SA8 to day four and the SA2 HP leukaemia only to day three.

At the early time points, between day one and day four, the SA7, the SA8 and the SA2 HP leukaemias had a significantly higher proliferative response than normal bone marrow. The SA2 LP had a lower proliferative rate than normal bone marrow to day six of culture.

The difference between L929 and WEHI-3B CM was that L929 CM induced a higher maximal response from the SA7, SA8 and the SA2 HP leukaemia than normal bone marrow.

An inhibitory effect on the proliferation of the SA2 CL was observed with the addition of L929 CM to day two in culture compared to FCS alone. On day three

the inhibitory effect of L929 CM observed on the earlier days was overridden and an increase in the proliferative response was observed on day three. The proliferative response observed on day three and day four was higher than the response observed with FCS alone.

#### **2.3.2.4 Proliferative responses to rGM-CSF**

At the early time points in culture (eight hours to day three) an equivalent proliferative response was observed when normal bone marrow cells were cultured with rmGM-CSF or WEHI-3B CM. However, rGM-CSF induced a higher proliferative response at these time points with all the leukaemias studied than WEHI-3B CM (Fig. 2.9 D).

rGM-CSF was more effective than WEHI-3B and L929 CM for the proliferation of the SA2 HP leukaemia.

rGM-CSF did not sustain increasing proliferation of normal and leukaemic cells for as long as it was sustained with the other two growth factors. The increasing proliferation of NBM, the SA7 and SA2 HP leukaemias was sustained to day three and of the SA8 leukaemia to day two. The exception was the SA2 LP leukaemia, proliferation was sustained to day six, although this was not exponential cell growth.

As with the other two growth factors the SA7, SA8 and the SA2 HP leukaemias had higher proliferative responses when stimulated with rGM-CSF at the early time points in culture than normal bone marrow. The maximal proliferative response observed with the SA7 and the SA8 leukaemias was double the response observed with normal bone marrow and the response observed with the SA2 HP was four times greater than normal.

The proliferative response of the SA2 CL when stimulated with rGM-CSF was greater than the response observed with FCS during the first eight hours of

culture. From days one to day seven there was no difference in the response between the two treatments.

### **2.3.2. Summary of the proliferative responses of leukaemic and normal bone marrow cells to WEHI-3B CM, L929 CM and rGM-CSF**

Heterogeneous proliferative responses to the growth factors were observed with the different leukaemias (Fig 2.9). There were three main differences:-

- 1). The same growth factor did not induce maximal proliferation of each leukaemia. WEHI-3B CM induced maximal stimulation of NBM. Maximal stimulation of the different leukaemic cells was achieved with different growth factors.
- 2). Except for the SA2 LP leukaemia, a higher proliferative response was observed with the leukaemic cells with each of the growth factor at the earlier time points in culture than NBM.
- 3). The leukaemic cells were generally sustained in culture for a shorter time period than NBM
- 4) Autonomous proliferation was observed with the SA2 LP leukaemia and the SA2 CL. Autonomous proliferation was also observed with the other leukaemias for the first two to three days in culture

The SA7 leukaemia was maximally stimulated to proliferate with WEHI-3B CM. Increasing proliferation was observed to day five with WEH-3B CM, day three with L929 CM and rGM-CSF. A higher proliferative response was observed with each of the growth factors at ealier time points than NBM. rGM-CSF induced a higher maximal proliferative response of the SA7 leukaemia than NBM. Autonomous proliferation was double that observed with NBM after eight hours of culture and minimal autonomous proliferation was observed to day two of culture.

The SA8 leukaemia was maximally stimulated with WEHI-3B CM. Increasing proliferation was observed to day six with WEHI-3B CM, day four with L929 CM and day three with rGM-CSF. A higher proliferative response was observed with each of the growth factors at the earlier time points than NBM. L929 CM and rGM-CSF induced a higher maximal proliferative response of the SA8 leukaemia than NBM. The level of proliferation during the first eight hours in each of the culture conditions was similar to that of NBM and minimal autonomous proliferation was observed to day two.

The SA2 HP leukaemia was maximally stimulated with rGM-CSF. Increasing proliferation was observed only to day three with all three growth factors. A higher proliferative response was observed with each of the growth factors at earlier time points than NBM and the other leukaemias. The autonomous proliferative response observed after eight hours of culture was three times higher than NBM and autonomous proliferation was observed to day three.

The SA2 LP leukaemia differed from the other leukaemias. The overall proliferative responses observed were lower than the other leukaemias and generally lower than NBM. The SA2 LP leukaemia was maximally stimulated with WEHI-3B CM. Increasing proliferation was sustained to day six with WEHI-3B CM, day five with L929 CM and day four with rGM-CSF. The passage number of the SA2 LP leukaemia was number 49 and a minimal autonomous proliferative response was observed to day seven in culture.

The SA2 CL was highly proliferative in culture. Maximal stimulation was observed with FCS alone. The addition of growth factors induced an inhibitory effect on the proliferation of the SA2 CL at early time points in culture. The greatest inhibitory effect was observed with L929 CM.

## **2.4 TIME STUDY OF THE PROLIFERATIVE RESPONSES OF NORMAL AND LEUKAEMIC BONE MARROW CELLS TO COMBINATIONS OF WEHI-3B CM, L929 CM AND rGM-CSF**

To further characterise the various leukaemic populations we investigated the interactions of WEHI-3B CM, L929 CM and rGM-CSF in controlling myeloid leukaemic cells by using combinations of growth factors *in vitro*. The regulators of normal haemopoiesis affect overlapping stages of the haematopoietic pathways, that is, they exert stimulating effects upon the same target cells (Metcalf, 1988). They have also been shown to have synergistic proliferative effects on the same target cell when used in combination. (McNeice et al, 1988 ). The cooperative interactions between these molecules in controlling myeloid leukaemic cell growth and how they compared to normal were investigated. With the use of combinations of growth factors we have attempted to identify aberrations of synergistic responses observed with normal bone marrow cells and to possibly identify specific subsets of myeloid leukaemic cells according to these responses. The comparative variations in response between the leukaemias and compared to normal myeloid cells may provide indicators of classifying leukaemic cell populations with biologically different disease and may prove to be of prognostic value.

### **2.4.1 Experimental Procedure**

The proliferation characteristics of leukaemic cells to optimal concentrations of WEHI-3B CM, L929 CM and rGM-CSF, used in combination, were studied daily over seven days in the microtitre assay. Single cell suspensions of  $7 \times 10^4$  leukaemic and normal bone marrow cells and cells from the SA2 CL were incubated with 15% WEHI-3B CM (v/v, 15% L929 CM (v/v) and 60 U/ml rGM-CSF in double combinations. Proliferation has measured utilizing  $^3\text{H-TdR}$  uptake. The results obtained were compared to the responses obtained with normal bone marrow cells.

## **2.4.2 Results**

### **2.4.2.1 Proliferative responses of leukaemic and normal bone marrow cells to the combination of WEHI-3B CM and L929 CM**

After the first eight hours of culture the proliferative response of normal bone marrow was equivalent to the response observed with FCS. From day one to day three the response was equivalent to the response with WEHI-3B CM alone (Fig. 2.11 A). On days four and five the response was additive and on day six synergistic.

No synergistic response was observed with the SA7 leukaemia over the time period studied and the maximal proliferative response observed with the combination of growth factors was less than the maximal response observed with WEHI-3B CM alone (Fig. 2.11 B). Over the first two days of culture the response was equivalent to the response observed with WEHI-3B CM alone. An additive response was observed on day three and a less than additive response was observed on day four.

A synergistic and additive responses similar to normal bone marrow were observed with the SA8 leukaemia. A synergistic response was observed on day five and an additive response was observed on day three and four (Fig. 2.11 C). The difference in response as compared to normal was at the early time points, days one and two, where the response observed was less than the response observed with WEHI-3B CM alone; L929 CM appeared to down regulate the response to WEHI-3B CM.

A synergistic response was also observed with the SA2 LP leukaemia on day five (Fig. 2.11 D); an additive response was observed on days four and six. Similar to normal bone marrow and the SA7 leukaemia the response observed at the early time points in culture were similar to the response with WEHI-3B CM alone.

No synergistic response was observed with the SA2 HP leukaemia (Fig. 2.11 E). A less than additive response was observed on day two and an additive response on day three. On day one the response observed was less than the response observed

with WEHI-3B CM alone and as with the SA8 leukaemia L929 CM appeared to down regulate the response to WEHI-3B CM.

Summary of the results:-

- a) A synergistic response was observed only with normal bone marrow, the SA8 and the SA2 LP leukaemias
- b) Additive responses were observed with normal bone marrow and all four of the transplanted cell lines.
- c) A higher maximal proliferative response was observed with the combination of WEHI-3B plus L929 CM with normal bone marrow, the SA8, SA2 LP and SA2 HP but not the SA7 leukaemia as compared to either factor alone.
- d) L929 CM appeared to down regulate the proliferation response to WEHI-3B CM of the SA8 and SA2 HP leukaemias at the early time points.

#### **2.4.2.2 Proliferative responses of leukaemic and normal bone marrow cells to the combination of WEHI-3B CM and rGM-CSF**

A synergistic response was observed with normal bone marrow cells on days four and five of culture (Fig. 2.12 A), an additive response was observed on days three and six.

With this combination of growth factors a synergistic response was observed with the SA7 leukaemia (Fig. 2.12 B); the synergistic response was observed on day four and additive proliferative responses were observed on days three five and six. An interesting response was observed with the SA7 leukaemia after eight hours of culture and on day one. The maximal proliferative responses at these time points were observed with the addition of rGM-CSF alone, when WEHI-3B CM was added the response observed was equivalent to the response observed with WEHI-3B CM alone i.e. it was less than the response observed with

rGM-CSF. Hence, WEHI-3B CM appears to down regulate the relatively high proliferative response of these leukaemic cells to rGM-CSF.

Synergistic or additive proliferative responses were not observed with the SA8 leukaemia (Fig. 2.12 C). The responses were generally equivalent to the maximal responses observed with either factor alone, rGM-CSF alone gave the highest response on days two and three and WEHI-3B CM from day four to six. Hence, no down regulatory effect was observed.

A synergistic response was observed with the SA2 LP leukaemia on day five and additive responses were observed on days three and four.(Fig. 2.12 D).

Synergy was not observed with the SA2 HP leukaemia (Fig. 2.12 E). On days two and three less than additive responses were observed. After eight hours of culture rGM-CSF alone induced a very high proliferative response of this leukaemia. This response was down regulated with the addition of WEHI-3B CM and the response observed with the combination of growth factors was equivalent to the response observed with WEHI-3B CM alone.

In summary:-

- a) A synergistic response was observed with normal bone marrow, SA7 and the SA2 LP leukaemias but not with the SA8 or SA2 HP leukaemias.
- b) Additive responses were observed with normal bone marrow, the SA7, SA2 LP and SA2 HP leukaemias, but not the SA8 leukaemia.
- c) WEHI-3B CM down regulated the relatively high proliferative responses observed with rGM-CSF alone of the SA7 and SA2 HP leukaemias at early time points in culture.

#### **2.4.2.3 Proliferative responses of leukaemic and normal bone marrow cells to the combination of L929 CM and rGM-CSF**

No additive or synergistic responses were observed with normal bone marrow with this combination of growth factors during the first four days in culture when the cells were in exponential cell growth (Fig. 2.13 A). Additive responses were observed on days four to six.

A synergistic response was observed on day four with the SA7 leukaemia and additive responses were observed on day two and three (Fig. 2.13 B).

An increased but less than additive responses was observed with the SA8 and no down regulatory effect was observed by L929 CM on the high proliferative response observed with rmGM-CSF (Fig. 2.13 C).

A marked synergistic response was observed with the SA2 LP leukaemia from day three to day six (Fig. 2.13 D)

An increased but less than additive response was observed with the SA2 HP leukaemia on day three of culture (Fig. 2.13 E). L929 CM did appear to down regulate the high proliferative response observed with rmGM-CSF alone after eight hours of culture.

It must be noted that significantly higher ( $p < 0.05$ ) proliferative responses were observed with this factor combination than either factor alone for both normal and leukemic marrow at the later time points in culture when the cells were no longer in exponential cell growth.

In summary:-

- a) Synergy was not observed with normal bone marrow and the SA8 and SA2 HP leukaemias
- b) Synergy was observed with the SA7 and SA2 LP leukaemia.
- c) Additive responses were observed with the SA7HD, SA8HD and SA2 HP leukaemic bone marrows.

d) This factor combination sustained a higher proliferative response than either factor alone at the later time points in culture for normal marrow and all the transplanted leukaemic cell lines.

#### **2.4.2.4 Proliferative responses of the SA2 CL to the combinations of growth factors**

The combination of any of the growth factors did not significantly alter the response of the SA2 CL as compared to FCS or to either one of the growth factors alone (Appendix Table A2.10)

### **2.5 PROLIFERATIVE RESPONSES OF LEUKAEMIC AND NORMAL BONE MARROW CELLS TO OPTIMAL CONCENTRATIONS OF WEHI-3B CM, L929 CM AND rMGM-CSF, ALONE AND IN COMBINATION, IN THE COLONY ASSAY**

The responses of leukaemic cells to single and combinations of growth factors were also investigated in the colony assay. The colony assay depicts the number of progenitor cells able to form colonies in culture in response to a particular growth stimulus (Chomienne, 1990). Major differences were observed between the microtitre assay and the colony assay in terms of the growth factor inducing maximal stimulation of a particular leukaemic cell population. Differences were also observed in the synergistic responses observed with the combinations of growth factors in the two methods. The response of the leukaemic cells to growth factors in the colony assay and the differences observed between the two methods are described in this section.

#### **2.5.1 Experimental Procedure**

Single cell suspensions of normal and leukaemic bone marrow cells and cells from the SA2 CL were set up in the colony assay with FCS alone and with optimal concentrations of WEHI-3B CM (15% V/V), L929 CM (15% V/V) and rGM-CSF (60

U/ml), alone and in double combinations. Colonies were scored on day seven. The results obtained were compared to the results of the microtitre assay.

## **2.5.2 Results**

### **2.5.2.1 Responses to single growth factors**

The number of clonogenic cells stimulated to form colonies, from normal and leukaemic bone marrows, in response to WEHI-3B CM, L929 CM and rmGM-CSF, were assessed (Table 2.1). A number of major differences between the colony and microtitre assay were observed:-

- In the microtitre assay, where the proliferative responses of the total bone marrow cellular populations were determined utilizing the uptake of <sup>3</sup>H-TdR, it was observed that rmGM-CSF induced a higher proliferative response than L929 CM, especially of the leukaemic cell populations. In the colony assay, where a specific subset of the total bone marrow population is induced to form colonies in culture, L929 CM induced a higher proportion of progenitor cells to form colonies than rmGM-CSF.
- In the microtitre assay WEHI-3B CM induced a higher proliferative response of the SA7 leukaemia than the other two growth factors whereas in the colony assay L929 CM induced the greatest number of colonies.
- The SA2 LP leukaemia was growth factor dependent for colony formation whereas in the microtitre assay a minimal proliferative response was observed at each time point studied. This difference may be due to the lower number of bone marrow cell plated in the colony assay.

The main similarities between the two culture systems were:-

- The high proliferative responses observed with the SA2 HP and SA2 CL leukaemias in the microtitre assay were paralleled with the high number of colony forming cells observed in the colony assay. The number of clonogenic cells

COLONIES ± STANDARD ERROR

BONE MARROW No. CELLS PLATED	NBM 2.5 x 10 <sup>4</sup>	SA7 HD 2.5 x 10 <sup>4</sup>				SA8 HD 2.5 x 10 <sup>4</sup>				SA2 HD LP 2.5 x 10 <sup>4</sup>				SA2 HD HP 10 <sup>3</sup>			
		GROWTH FACTOR(s)															
MEDIUM	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0
WEHI-3B CM	37 4	43 1	50 5	37 3	37 3	37 3	37 3	37 3	37 3	37 3	37 3	37 3	37 3	37 3	37 3	37 3	37 3
L929 CM	26 4	55 1	37 2	35 6	35 6	35 6	35 6	35 6	35 6	35 6	35 6	35 6	35 6	35 6	35 6	35 6	35 6
rmGM-CSF	16 4	30 1	17 2	9 1	9 1	9 1	9 1	9 1	9 1	9 1	9 1	9 1	9 1	9 1	9 1	9 1	9 1
WEHI-3B + L929 CM	40 5	52 3	73 2	43 2	43 2	43 2	43 2	43 2	43 2	43 2	43 2	43 2	43 2	43 2	43 2	43 2	43 2
WEHI-3B + rmGM-CSF	39 5	57 4	68 3	30 1	30 1	30 1	30 1	30 1	30 1	30 1	30 1	30 1	30 1	30 1	30 1	30 1	30 1
L929 CM + rmGM-CSF	24 4	40 5	40 1	24 1	24 1	24 1	24 1	24 1	24 1	24 1	24 1	24 1	24 1	24 1	24 1	24 1	24 1
WEHI-3B + L929 CM + rmGM-CSF	34 3	47 3	52 3	31 1	31 1	31 1	31 1	31 1	31 1	31 1	31 1	31 1	31 1	31 1	31 1	31 1	31 1

Table 2.1 Proliferative responses of normal and leukaemic cells to WEHI-3B BM, L929 CM and rGM-CSF singularly and in combination in the colony assay.

from the SA2 HP leukaemic bone marrow and the cells from the SA2 CL were found to be relatively high, and these cells were plated at  $10^4$  and  $10^3$  cells per plate respectively. Normal bone marrow and the other leukaemias were plated at  $2.5 \times 10^4$  cells per plate.

- Normal bone marrow and the transplanted leukaemic cell lines which were growth factor dependent in the microtitre assay (the SA7, SA8 and the SA2 HP leukaemia) were also growth factor dependent in the colony assay.

#### **2.5.2.2 Responses to combinations of growth factors**

Major differences were observed with the combination of growth factors between the colony and microtitre assays. The main difference was that no synergistic or additive responses were observed with normal bone marrow with any of the growth factor combinations. The responses of the leukaemic cells to each of the combinations is described below.

##### WEHI-3B CM plus L929 CM

A synergistic or additive response was not observed with the SA7 or SA2 HP leukaemias as in the microtitre assay. Synergy was not observed with the SA2 LP leukaemia, however it was observed in the colony assay.

An additive response was observed with the SA8 leukaemia, however in the microtitre assay both additive and a synergistic responses were observed.

As noted above no synergistic response was observed with the SA2 HP leukaemia. L929 CM alone induced the greatest number of colonies from this leukaemia and the combination with WEHI-3B CM appeared to down regulate this response.

#### WEHI-3B CM plus rGM-CSF

This combination of growth factors enhanced the number of colonies formed from the SA7 and the SA2 HP leukaemic cells as compared to either factor alone, however the responses was less than additive. These result were similar with the microtitre assay.

An additive response was obtained with the SA8 leukaemia in the colony assay but not with the microtitre assay. The opposite was observed with the SA2 LP leukaemia were additive and synergistic responses were observed in the microtitre assay but not in the colony assay.

#### L929 CM plus rGM-CSF

rmGM-CSF down regulated the number of colonies observed with L929 CM alone with the SA7, SA2 LP and the SA2 HP leukaemias. This is opposite to the results obtained with the microtitre assay where L929 CM down regulated the response to rmGM-CSF at the early time points in culture. Also synergistic responses were observed with these three leukaemias in the microtitre assay but not in the colony assay.

No additive or synergistic response was observed with the SA8 leukaemia whereas an enhanced response was observed with the microtitre assay.

## **2.6 DISCUSSION**

The proliferative dose response relationships of the leukaemic cells to three growth factors of normal haemopoietic cells, WEHI-3B CM as a source of IL-3, L929 CM as a source of M-CSF and rGM-CSF were examined *in vitro*. These three growth factors and G-CSF are the only known proliferative stimuli of normal haemopoietic cells both *in vitro* and *in vivo* and hence it was relevant to consider the effects of these growth factors on myeloid leukaemic cells.

Growth factor dependent and autonomously proliferative responses were observed with the leukaemias studied. The SA7, SA8 and SA2 HP leukaemias were growth factor dependent *in vitro*. Hence, it appears that some leukaemias can be induced to proliferate by the regulators of normal haemopoiesis. The role and degree to which *in vitro* responsive leukaemic cells are sustained *in vivo* by the regulators of normal haemopoiesis cannot be inferred from these studies. Autonomous proliferation was observed with the SA2 LP leukaemia and the SA2 CL. The proliferative response of the SA2 LP leukaemia at passage number 36 was not affected by the addition of the CSF's on day four of culture. At passage number 54 where a minimal autonomous proliferative response was observed the addition of growth factors enhanced the proliferative response. A significant inhibitory effect on the proliferation of the SA2 CL was observed on day two of culture with the addition of L929 CM.

The patterns of proliferative response observed with the different leukaemias were heterogeneous. No specific "leukaemic" response could be detected. The CSF which induced maximal stimulation varied between the leukaemias. WEHI-3B CM induced a maximal proliferative response of NBM, the SA7 and SA8 leukaemia, and rGM-CSF stimulated maximal proliferation of the SA2 HP leukaemia. The *in vitro* time course for maximal-proliferative-response differed between normal and leukaemic cells and between the CSF studied. Maximal proliferation of leukaemic cells was often observed at early time points, day two or day four and of NBM on day six. These observations prompted further proliferative studies whereby normal and leukaemic cells were cultured with optimal concentrations of each growth factor and proliferative responses were measured daily over seven days in the microtitre assay and in the colony assay.

There were two main similarities between the proliferative responses observed with normal bone marrow and the growth factor dependent leukaemic bone marrow cells. All the transplanted leukaemic cell lines were induced to proliferate with each of the growth factors studied indicating that leukaemic cells may retain some of the proliferative characteristics of normal cells. In studies on human myeloid leukaemic cells it has been shown that IL-3 and GM-CSF frequently induce the proliferation of AML cells however M-CSF infrequently induces proliferation (Delwel et. al. 1988). Hence these transplanted leukaemic cell lines may provide a good model for studying M-CSF responsive leukaemic cells. Secondly, the same concentration of each growth factor required to induce optimal proliferation of normal cells was also required to induce optimal proliferation of the leukaemic cells. It has also been documented that for human bone marrow cells the same concentrations of growth factors were required for optimal proliferation for normal and leukaemic cells (Metcalf 1988).

It was evident from these studies that the growth factor dependent leukaemic cells responded differently from normal cells to these growth factors. Although the concentration of growth factor required to induce maximal proliferation of normal and leukaemic cells was the same, the level of proliferation between normal and leukaemic cell differed. The leukaemic cells proliferated *in vitro* in response to regulators of normal haemopoietic cells however, they proliferated at a higher rate than normal cells at early time points in culture and they were not sustained in culture for as long as normal cells. Further studies are required to determine whether the responses observed *in vitro* are able to be paralleled *in vivo*. The use of physiological growth factors for therapeutic regimes for leukaemia should be viewed with caution because of their proliferative effects on leukaemic cells *in vitro*. However, because of the differences in proliferative responses obtained between normal cells and leukaemic cells the physiological

growth factors may be effective in decreasing the leukaemic burden or preferentially sustaining the proliferation of suppressed normal progenitor cells. Studies with human AML cells have shown that specific growth factors arrest progenitor cells at specific points in the cell cycle and hence the administration of growth factors with therapeutic agents may be beneficial (Bhalla et al, 1991; Hassan and Maurer, 1991). However there are studies which are contradictory to this possibility. (Koistinen et al, 1991).

Autonomous proliferation was observed with the SA2 CL and the SA2 LP leukaemia was observed to undergo changes with serial transplantation from being growth factor independent to growth factor dependent. The characterization of leukaemic cells which proliferate autonomously *in vitro* has opened major discussions as to whether or not leukaemia arises because of the emergence of an autocrinally regulated cell (Metcalf, 1988). Studies on oncogenes, cellular proto-oncogenes and specific chromosomal translocations involving these genes supports the hypothesis that cancer development is due to abnormalities of growth factors or their cell membrane receptors. However, growth factor genes do not generally induce leukaemia when inserted into normal haemopoietic cells, although they do so if they are inserted into immortalized haemopoietic stem cell lines (Testa and Dexter, 1989). A discussion on leukaemogenesis and the role of the haemopoietic growth factors in this process, is beyond the scope of this study (for a review of autocrinal concepts in leukaemogenesis see Metcalf, 1988). However, the change in the proliferative response observed with the SA2 leukaemia with continued transplantation and the minimal proliferative responses observed with the growth factor dependent leukaemias to day two or three in the microtitre cultures readdresses the question as to whether autonomous cell growth is a prerequisite of leukaemogenicity. Myeloid leukaemia appears to be an "evolving" disease. With the long term progression of the disorder, as is the

case with continued transplantation, a change in the dominant clone of a highly proliferative cell population is probably likely. It appeared that leukaemic populations undergo varied genetic changes with time. Hypothetically, there are two possibilities:-

- the dominant leukaemic clone progressed from being autocrinal and sustainable in the culture system to being autocrinal and unsustainable, supporting the theory that autocrinal regulation, whatever the mechanism, is essential for the leukaemic process or
- after leukaemogenesis the emergence of an autocrinal dominant population may be part of the evolution of a particular leukaemia and with the progression of the disorder a dominant clone with different characteristics may evolve. Normal haemopoietic cells produce haemopoietic growth factors hence this explanation is possible.

With progressive improvements in culture techniques for human bone marrow cells, most clinical cases of AML have been found to be susceptible to growth factor stimulation for colony formation, DNA synthesis, enhanced survival of AML progenitors *in vitro* and for the support of the self renewal of AML precursors (Lowenberg et al, 1990). Liquid cultures (Pebusque, 1988) or a liquid phase prior to semi-solid culture (Lowenberg et al, 1980) has been shown to improve the *in vitro* responses of human leukaemic cells to growth factors. However, from this data and studies by others, the possibility that, once leukaemogenesis has occurred, the affected cells are no longer dependent upon growth factors and that proliferation of these cells is achieved by a novel cytoplasmic or nucleic mechanism cannot be excluded. The difficulty arises with the growth factor dependent leukaemias where it is difficult to assess with the methodology used for these studies whether a small proportion of the cells in culture are autonomous and whether it is these cells that constitute the leukaemic clone. These questions will be

addressed with *in vivo* clonogenicity assays in the next section. The cells from early transplantations of the SA7 or SA8 were not studied therefore it was not possible to assume that the progression from growth factor independent to growth factor dependent leukaemic cells is characteristic of these transplanted cell lines. The haemopoietic growth factors affect overlapping stages of normal haemopoietic pathways and the cell membrane receptors for these growth factors appear to interact with each other (Nicola,1987). Hence, the haemopoietic growth factors exert stimulating effects upon the same target cells. A possible reason as to why a particular progenitor is able to be induced to proliferate with a range of growth factors may be attributed to haemopoietic cellular cooperation requirements in immune responses (Nicola, 1987).

The heterogeneity of AML imposes difficult problems for the prognosis and treatment of the disease. The varied clinical responses to chemotherapy observed in AML patients within the FAB classification system, the marked variation in leukaemic cell phenotype (Griffin and Lowenberg, 1986), and the heterogeneity of response to growth factors by different leukaemias observed *in vitro* (Moore et al, 1974) implies that the acute myeloid leukaemias may need to be reclassified. Specific leukaemic progenitor cell subgroups according to growth factor response may be identifiable and this possible classification of myeloid leukaemic cells may have some prognostic value. Hence, the response of leukaemic progenitor cells to multiple growth factors and how they compared to the response of normal haemopoietic progenitors was assessed.

The leukaemic cells did not show the same proliferative patterns as normal cells to the combinations of growth factors and there were also differences in response amongst the leukaemias. As expected the combination of growth factors induced synergistic and additive responses of normal bone marrow cells indicating that

different and overlapping subsets of cells have been stimulated to proliferate. The combination of WEHI-3B CM and L929 CM induced additive responses with normal bone marrow, the SA7 and the SA2 HP leukaemias and synergistic responses from the SA8 and SA2 LP leukaemias. The combination of WEHI-3B CM plus rmGM-CSF induced synergistic responses of normal bone marrow and the SA2 LP leukaemia; additive responses with the SA7 and SA2 HP leukaemia; and no additive response of the SA8 leukaemia indicating that these two growth factors affect the same cells of the SA8 leukaemia. The combination of L929 CM plus rmGM-CSF induced an additive response of normal bone marrow cells the SA7, SA8HD and SA2 HP and a high synergistic response of the SA2 LP leukaemia. The difficulty in assessing additive and synergistic responses with the microtitre culture system is that individually, the growth factors sustain proliferation of the cells for a different number of days. Different leukaemic subgroups according to response to combinations of growth factors were observed. A comparative study of *in vitro* responses of human AML cells to growth factors prior to remission induction and the outcome of therapy would be needed to determine the prognostic value of this classification system.

Differences in responses of leukaemic cells to growth factor combinations as compared to normal were also observed when the colony assay was used. Differences in responses however, were also observed with the leukaemic cells when the two culture systems were compared. For example in some cases a different growth factor was observed to induced maximal responses of the same leukaemia in each of the assays; synergistic responses were observed with the combination of growth factors of a particular leukaemia in one assay but not the other. In a study on human AML cells by Pebusque et al (1988) it was shown that a synergistic response was observed with the combination of IL-3 and G-CSF when the cells were cultured in the microtitre assay but only three of five samples

confirmed this result when studied in the colony assay. In a study by Dewel et al, (1987) on human AML cells using colony assays no synergistic responses were observed with double combinations of IL-3, GM-CSG and G-CSF. In a study by Lemoli et al (1991) of human AML cells in the colony assay in most cases an additive response was observed compared to either factor alone by the same combinations used in the Dewel study. In studies by Buick et al (1977), Nara and McCulloch (1985) and McCulloch et al (1988) it has been shown that:-

- the colony assay principally measures terminal divisions and minimal self renewal can be detected by replating cells recovered from colonies.
- in the suspension assay, clonogenic cells increase with time, a change that reflects self renewal.

Responses of leukaemic cells to combinations of growth factors need to be investigated further with recombinant growth factors and a standardized technique for comparative studies needs to be established if clear patterns of proliferative responses are to be determined.

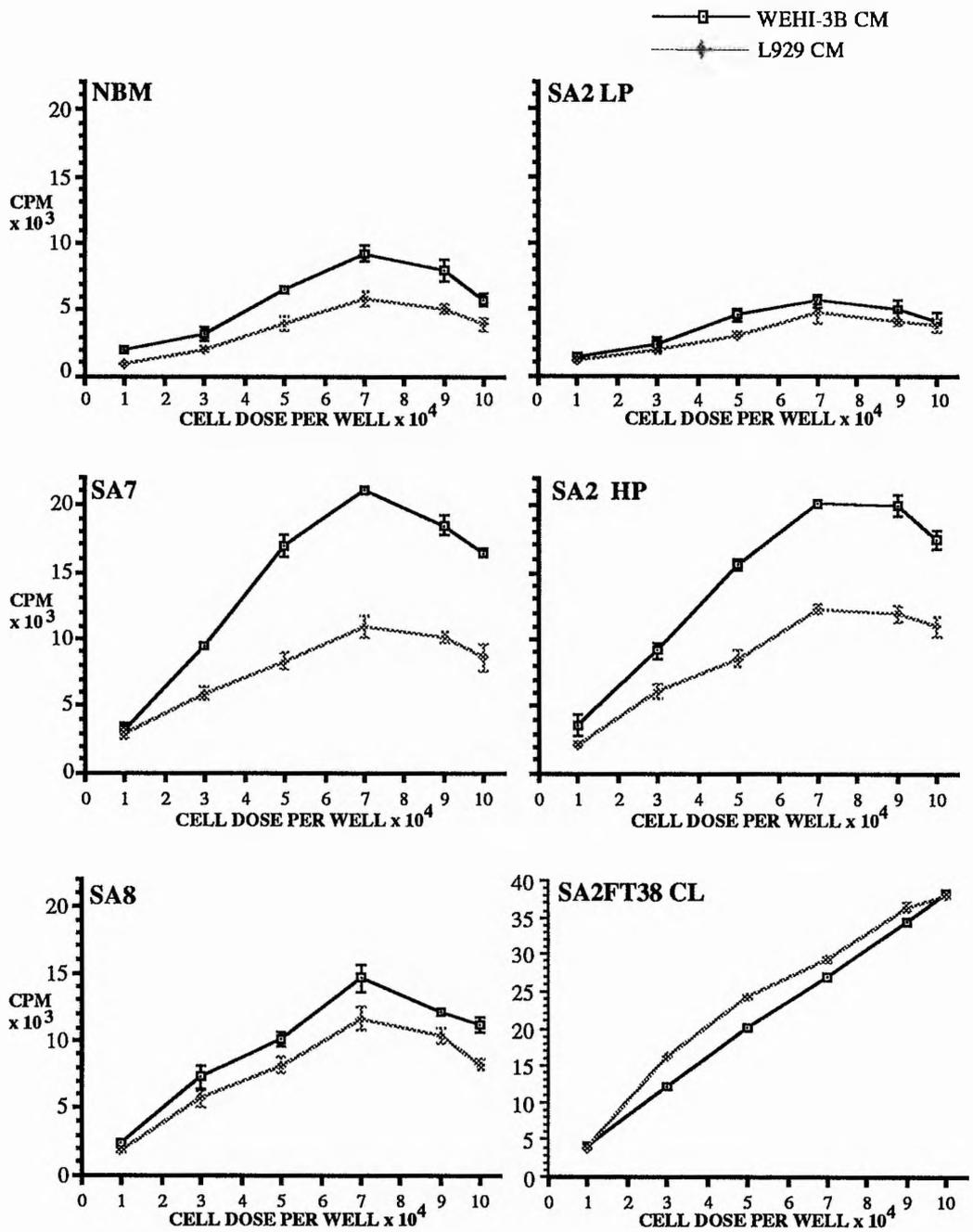
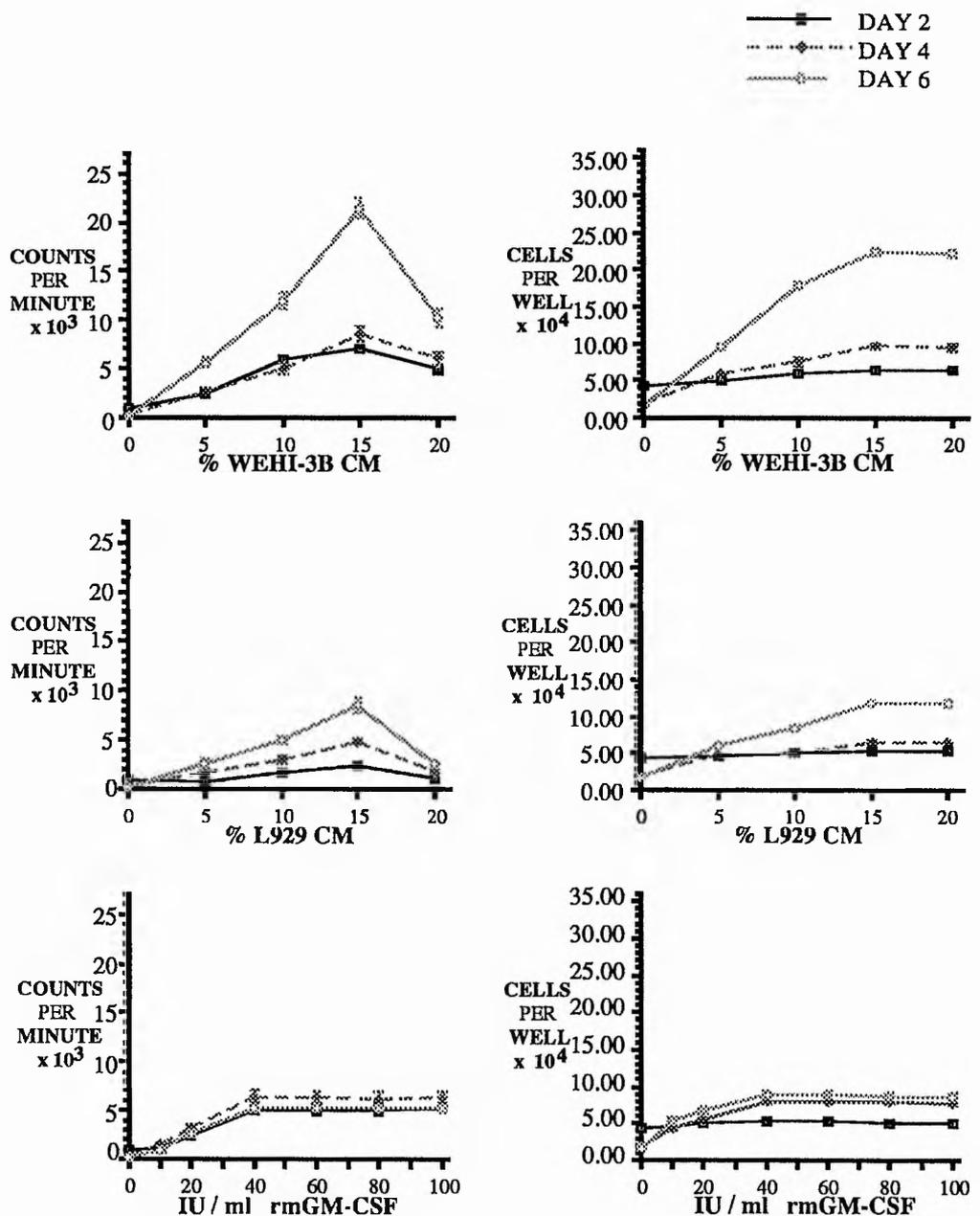
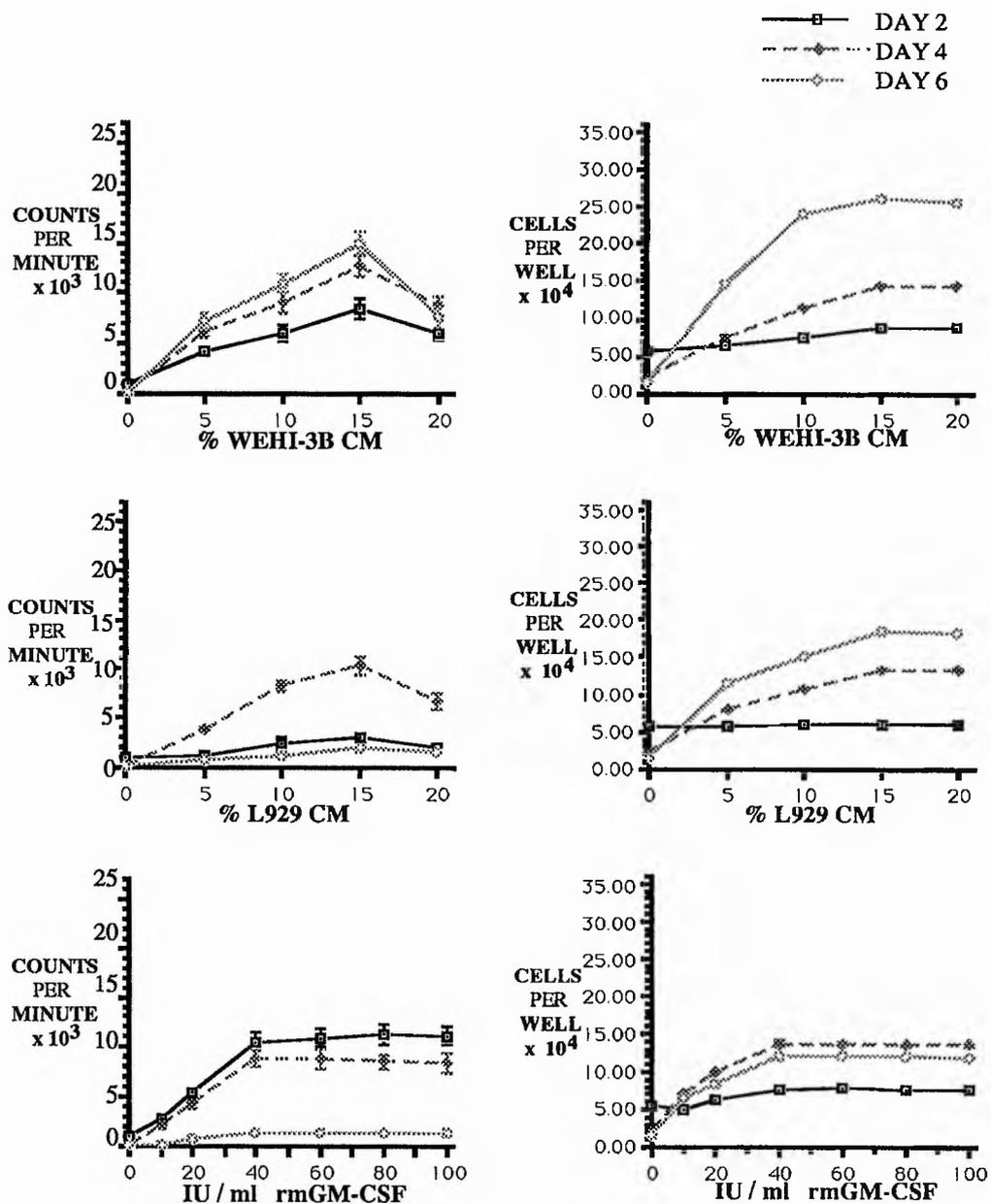


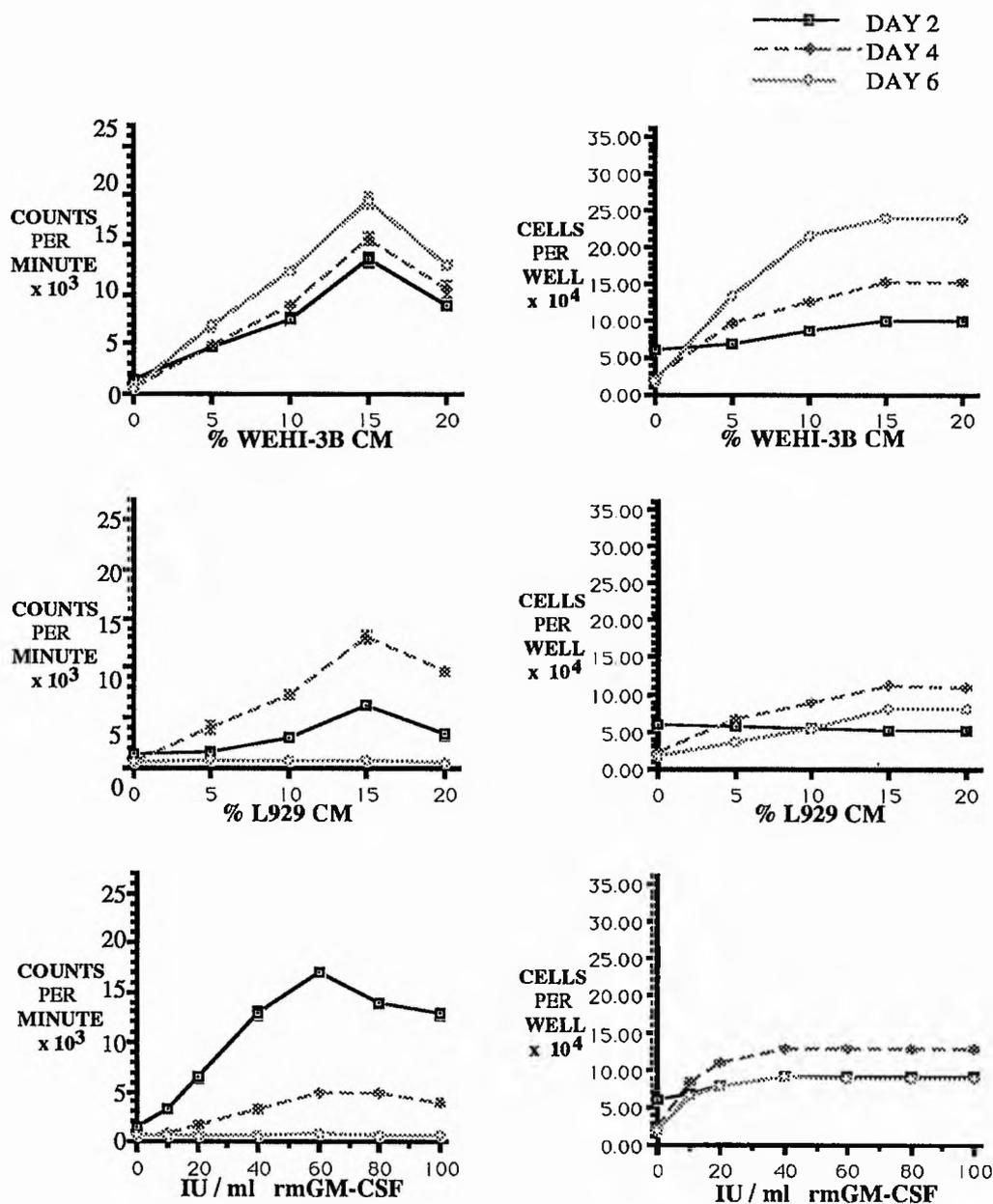
Figure 2.1 Cell Dose Study. Normal and leukaemic cells between 1 to 10 x 10<sup>4</sup> cells were cultured with optimal concentrations of WEHI-3B CM and L929 CM to determine the optimal cell number required for proliferation studies in the microtitre culture.



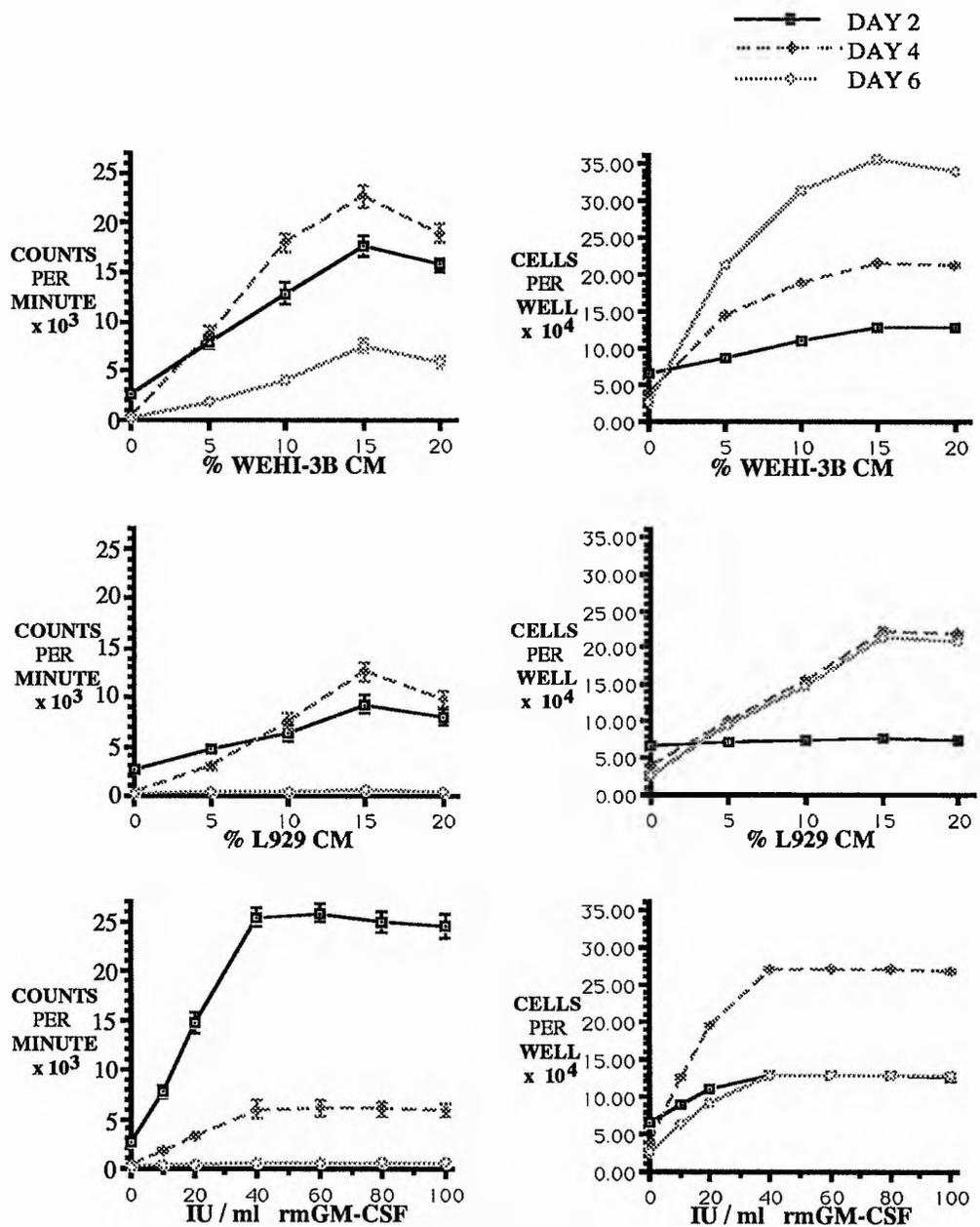
**Figure 2.2** Normal Bone Marrow. Growth Factor Dose Response Study. Normal bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with <sup>3</sup>H-TdR uptake and the results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.



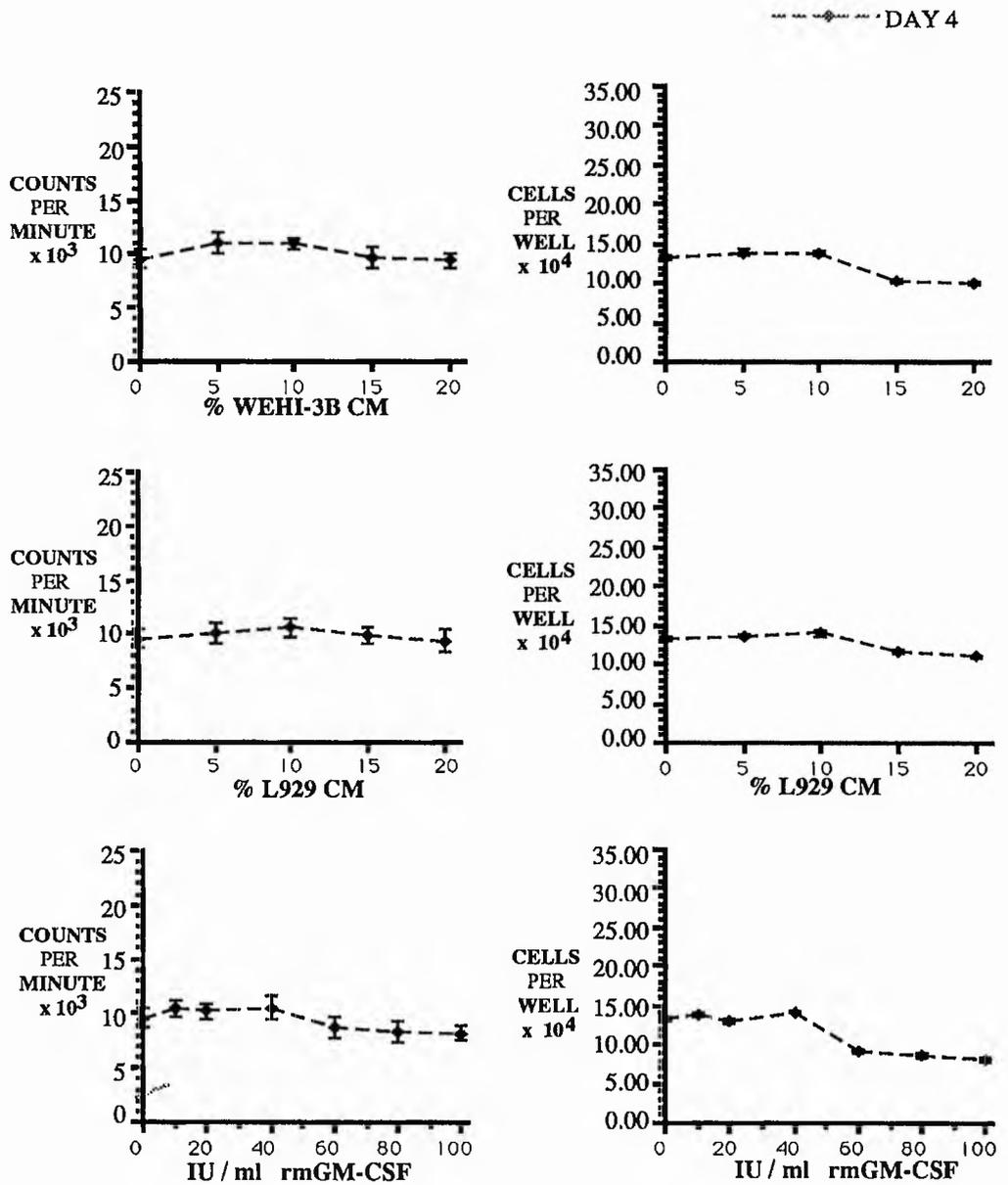
**Figure 2.3** SA7 leukaemic bone marrow. Growth Factor Dose Response Study. SA7 leukaemic bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with <sup>3</sup>H-TdR uptake and the results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.



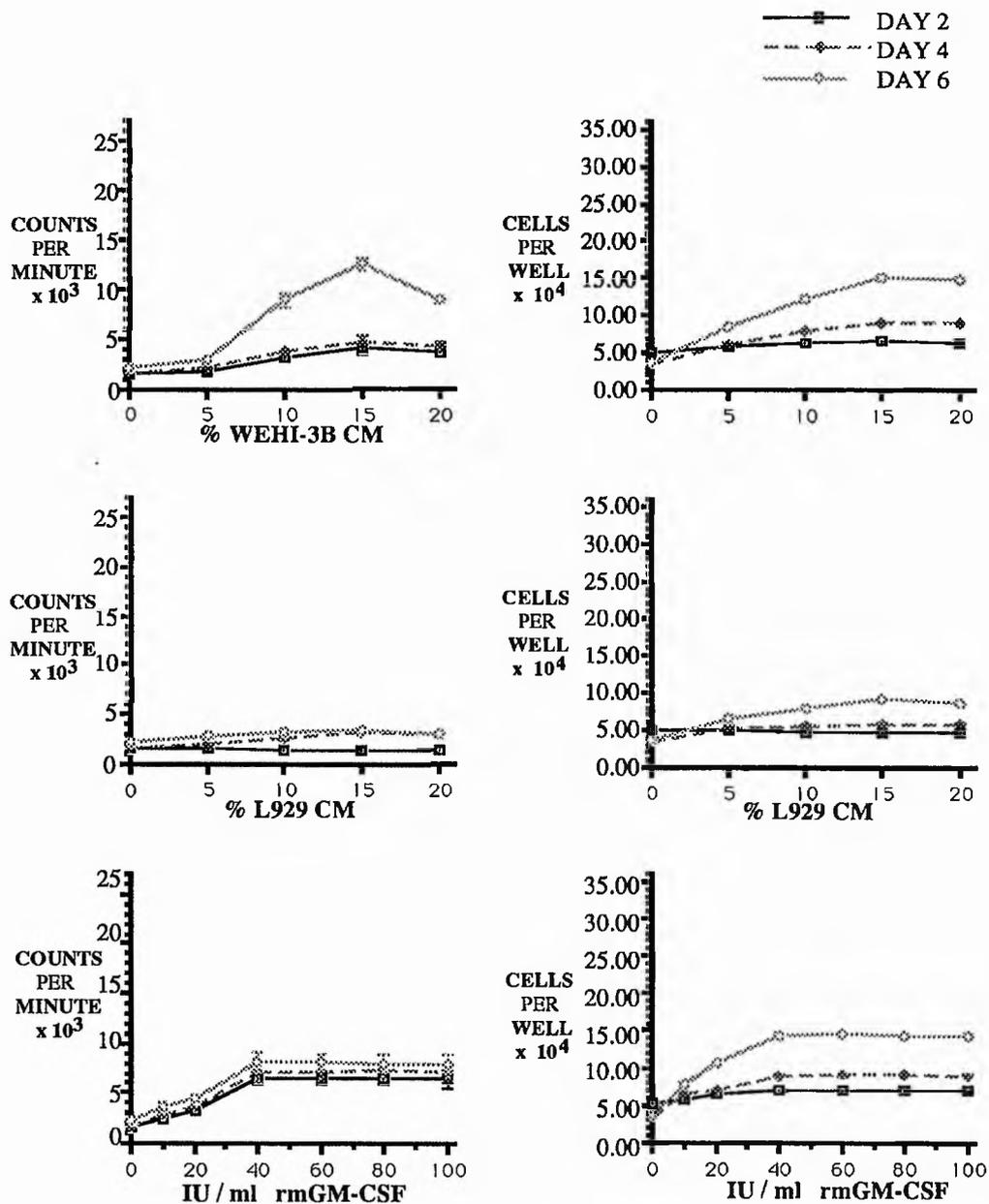
**Figure 2.4** SA8 leukaemic bone marrow . Growth Factor Dose Response Study. SA8 leukaemic bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with <sup>3</sup>H-TdR uptake and the results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.



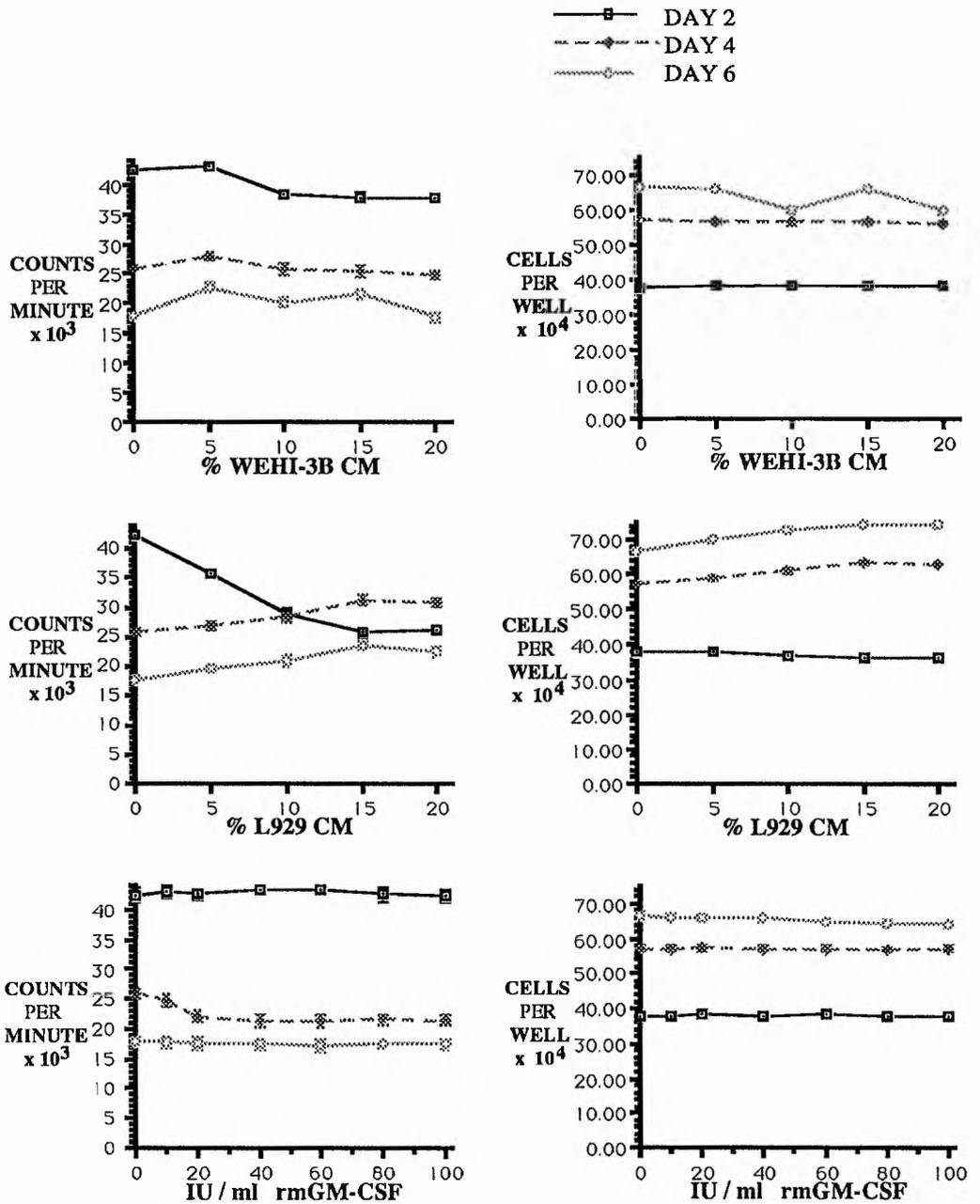
**Figure 2.5** SA2 HP (passage number 77) leukaemic bone marrow. Growth Factor Dose Response Study. SA2 leukaemic bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with <sup>3</sup>H-TdR uptake and the results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.



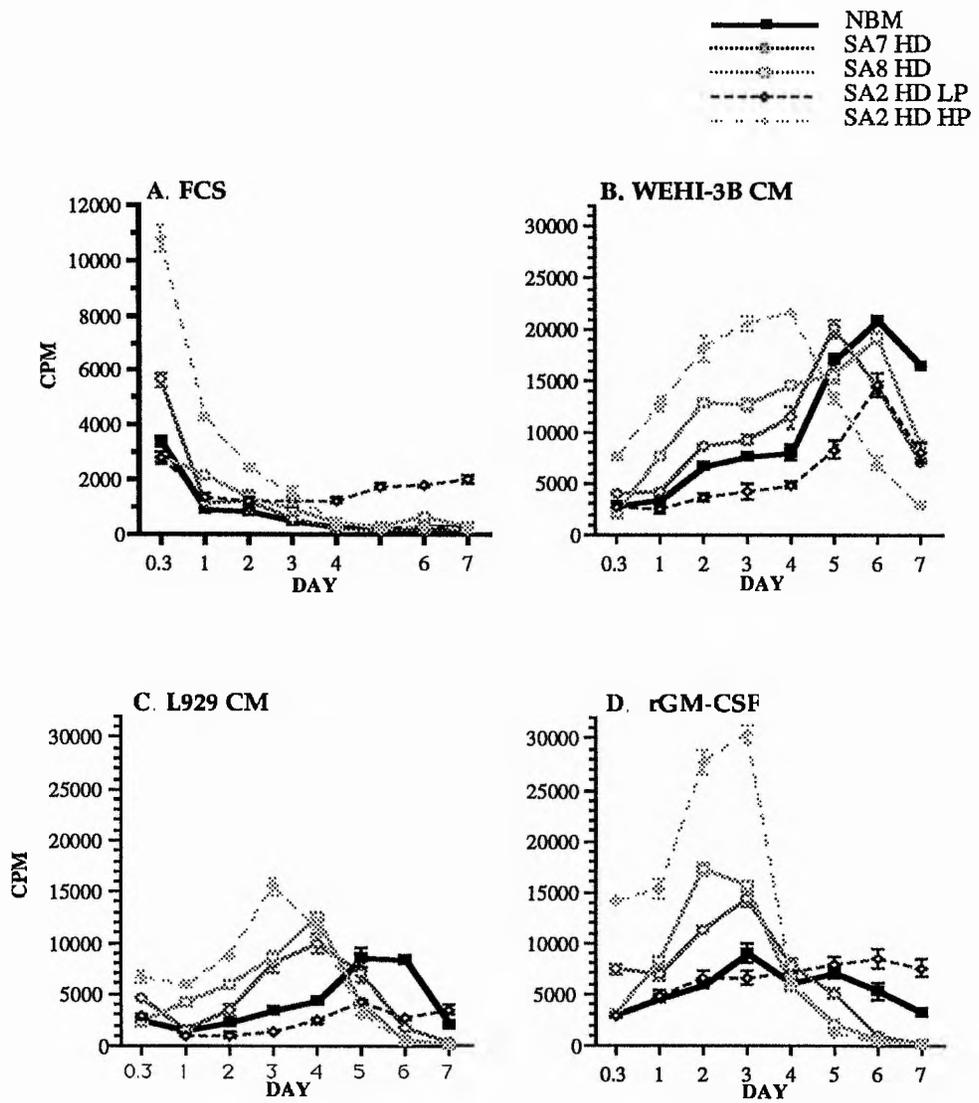
**Figure 2.6** SA2 LP (passage number 36) leukaemic bone marrow. Growth Factor Dose Response Study. SA2 leukaemic bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for four to determine the dose-response relationship. Proliferation was measured with <sup>3</sup>H-TdR uptake and the results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.



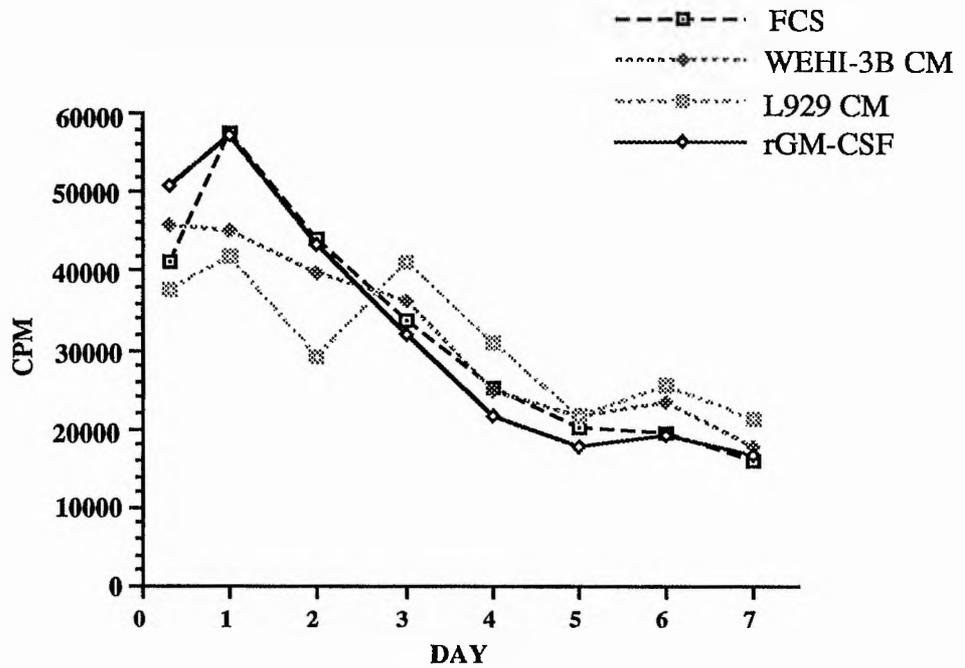
**Figure 2.7** SA2 LP (passage number 54) leukaemic bone marrow. Growth Factor Dose Response Study. SA2 leukaemic bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with <sup>3</sup>H-TdR uptake and the results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.



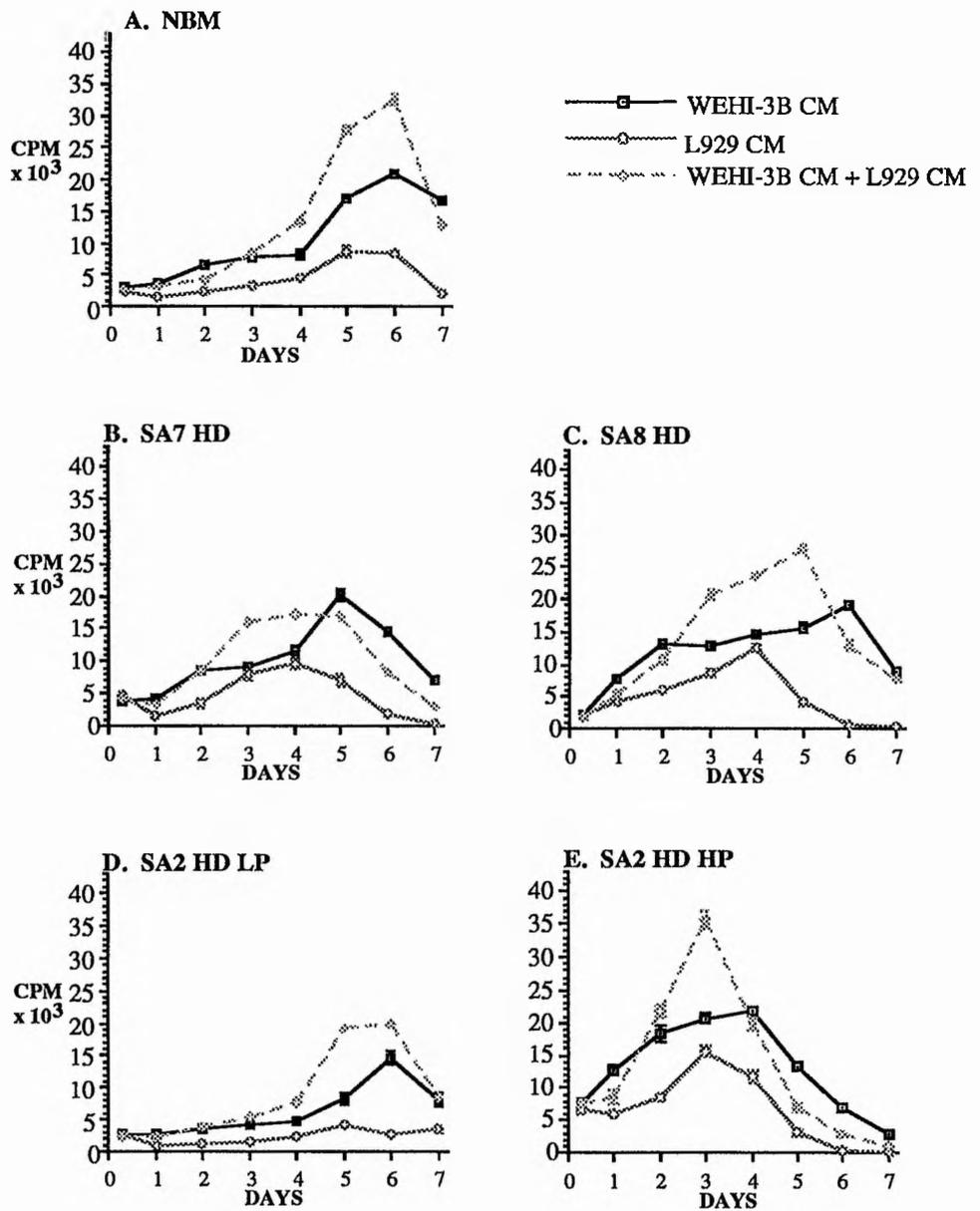
**Figure 2.8** SA2 CL leukaemic cells. Growth Factor Dose Response Study. The SA2 CL was cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with <sup>3</sup>H-TdR uptake and the results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.



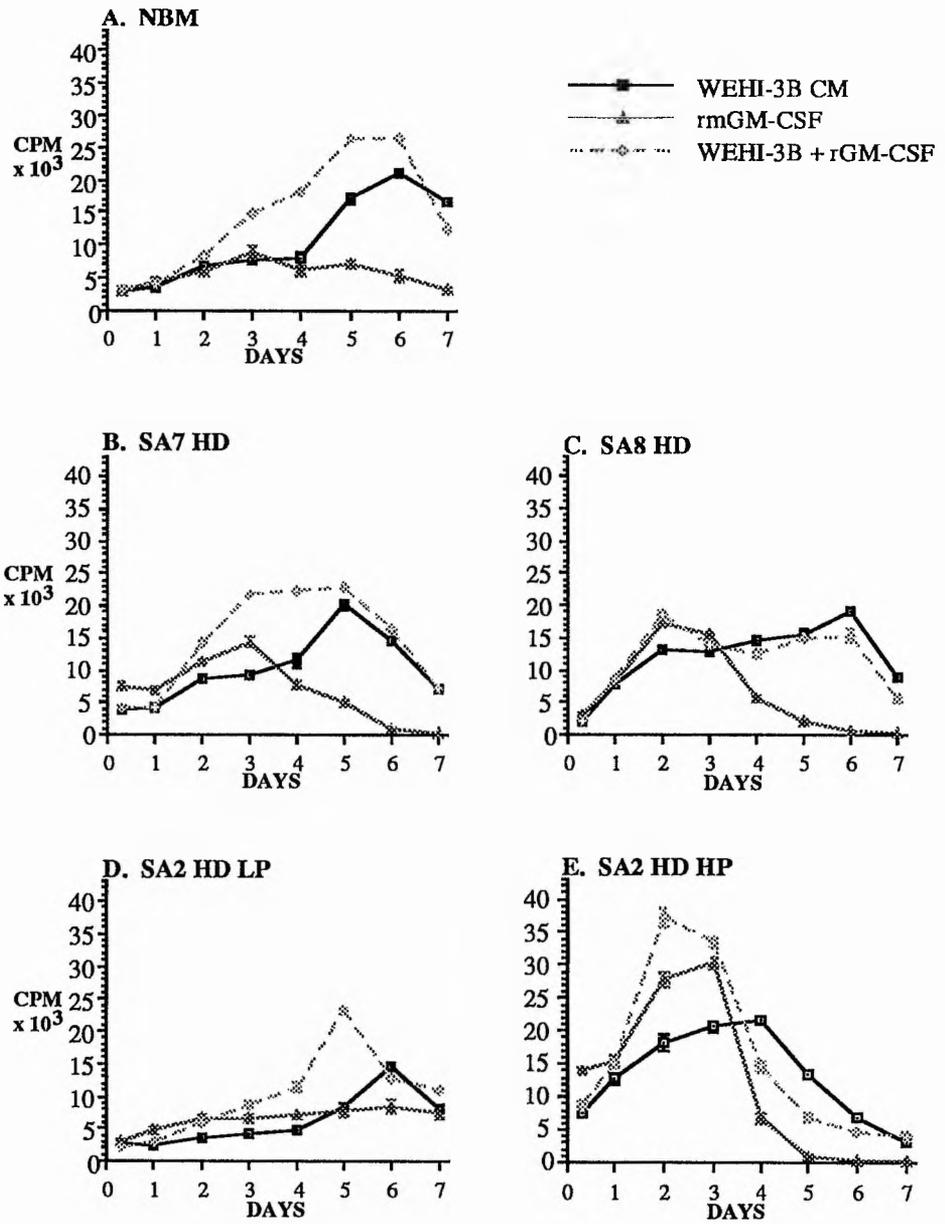
**Figure 2.9** Proliferative effects of WEHI-3B, L929 CM, and rGM-CSF on normal and leukaemic cells. Cells were cultured in the microtitre assay for eight hours to seven days. Proliferation was measured with <sup>3</sup>H-TdR uptake.



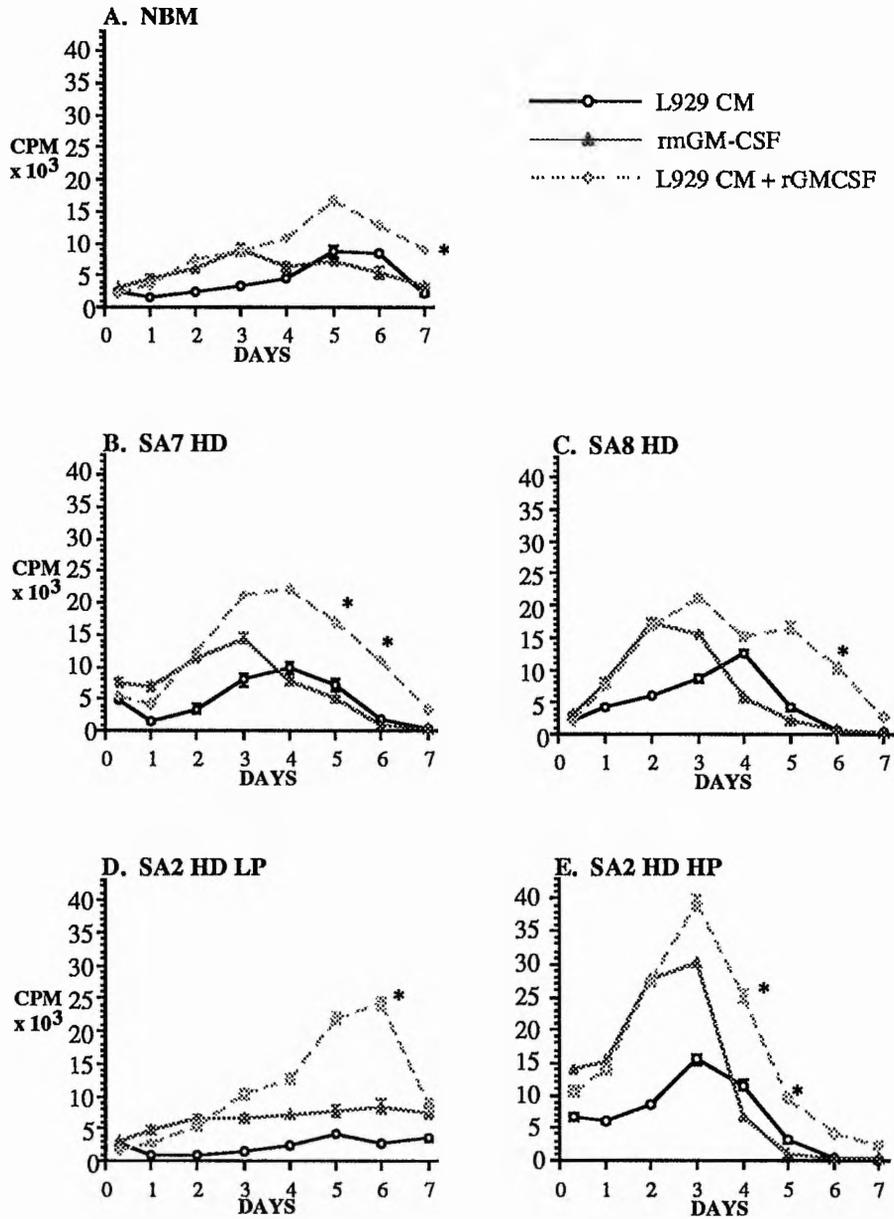
**Figure 2.10** Proliferative effects of WEHI-3B, L929 CM, and rGM-CSF on the SA2 CL. Cells were cultured in the microtitre assay for eight hours to seven days. Proliferation was measured with  $^3\text{H}$ -TdR uptake.



**Figure 2.11** Proliferation effects of combinations of growth factors, WEHI-3B CM, and L929 CM on normal and leukaemic cells. Cells were cultured in the microtitre assay for eight hours to seven days. Proliferation was measured with the uptake of <sup>3</sup>H-TdR.



**Figure 2.12** Proliferation effects of combinations of growth factors, WEHI-3B CM, and rGM-CSF on normal and leukaemic cells. Cells were cultured in the microtitre assay for eight hours to seven days. Proliferation was measured with the uptake of  $^3\text{H-TdR}$ .



**Figure 2.13** Proliferation effects of combinations of growth factors, L929 CM and rGM-CSF on normal and leukaemic cells. Cells were cultured in the microtitre assay for eight hours to seven days. Proliferation was measured with the uptake of <sup>3</sup>H-TdR. (\* p<0.05, Comparing the proliferative response between combined and single factors. A significant increase in the proliferative response was observed with the combination of growth factors compared to either factor alone for normal and leukaemic bone marrow at the later time points in culture).

## CHAPTER THREE

### ***IN VITRO* DIFFERENTIATION EFFECTS OF WEHI-3B CM, L929 CM AND rGM-CSF ON LEUKAEMIC AND NORMAL BONE MARROW CELLS**

The aim of these experiments was to determine whether the leukaemic bone marrow cells had maintained or lost their capacity for differentiation by studying their differentiation responses to physiological regulators of normal haemopoietic proliferation and differentiation. These types of studies could assist in determining the aberrant characteristics of different types of leukaemic cells and in determining whether normal physiological growth factors could possibly be used therapeutically for controlling the proliferation, via differentiation induction, of leukaemic cells *in vivo* or to be used in purging the leukaemic population from bone marrow samples for autologous transfusion or to assess the possible use of physiological regulators in conjunction with conventional therapeutic regimes. Another important aspect of the usefulness of these types of studies would be for the identification of subgroups of acute leukaemia according to their differentiability by physiological agents and may assist in the improved prognosis and treatment of the disease. The response or lack of response of certain leukaemias to normal physiological regulators and possible preferential stimulation the regulators may have on the normal suppressed haemopoietic cell population as opposed to the leukaemic cell populations, may be able to be utilized for subgrouping of leukaemias or for alternative therapeutic regimes.

The current most widely accepted model of the leukaemic cell population is that it represents a heterogeneous population of cells. These include a minority of blasts with *in vitro* proliferative potential with abnormally high levels of self renewal characteristics (clonogenic cells) and a majority of cells with little or no

proliferative capability (Buick et al, 1979; Lange et al, 1984; Griffin and Lowenberg, 1986). It is not yet clear whether differentiation inducers can force the leukaemic clonogenic cell fraction towards differentiation at the expense of self renewal or merely induce terminal differentiation of the partially differentiated cell population. Another important question which needs to be elucidated is whether induction of differentiation of the leukaemic clonogenic cells may be compensated by an increase in their proliferation rate. We have aimed to answer these questions by examining the *in vitro* effects of the WEHI-3B CM, L929 CM and rGM-CSF in an *in vivo* leukaemic clonogenic cell assay utilizing the transplanted leukaemic cell lines. An effective differentiation inducer should ultimately not enhance and preferably decrease clonogenic cell numbers. Measuring the number of clonogenic cells is easily facilitated when using transplantable leukaemic models *in vivo*. In this study the *in vivo* leukaemic clonogenic cell assay was used to determine the effectiveness of differentiation induction in reducing the number of leukaemic clonogenic cells.

### **3.1 IN VITRO DIFFERENTIATION EFFECTS OF WEHI-3B CM, L929 CM AND rGM-CSF ALONE AND IN COMBINATION ON LEUKAEMIC AND NORMAL BONE MARROW CELLS**

#### **3.1.1 Experimental procedure**

a). Cytospins were prepared and stained with Jenner Giemsa from single cell suspensions of normal and leukaemic bone marrow and with cells from the SA2 CL and differentials counted.

b). Normal and leukaemic bone marrow cells and cells from the SA2 CL were cultured in the microtitre assay with optimal concentrations of WEHI-3B CM, L929 CM and rGM-CSF, both singularly and in combination, for eight hours, two, four and six days.

c). At these time points the total number of cells per well for each treatment was counted using the Coulter Counter.

d). Three cytopspins were prepared for each treatment, stained with Jenner Giemsa and at least 500 cell differentials were counted for each slide. A full differential was counted with the cell classification being:-

Blasts, promyelocytes, immature macrophages, myelocytes, metamyelocytes, bands, segmented cells, monocytes and macrophages. Tables of the differentials of the single cell suspensions and the cultured cells are listed in the Appendix. The absolute number of each cell type per well was determined using the total number of cells per well counted *vide supra* (c).

e) From the differential data, graphs were prepared depicting the absolute number of immature cells (blasts and promyelocytes), intermediate cells (myelocytes, metamyelocytes and immature macrophages) and mature cells (bands, granulocytes, monocytes and macrophages) per well to determine whether partial or complete differentiation had occurred with each of the treatments.

f). The Maturation Index was calculated from the differential data and graphed for each treatment. The Maturation Index = The absolute number of mature cells per well / The absolute number of immature cells per well; whereby 1 is equivalent to an equal number of mature and immature cells, a value of <1 is equivalent to a predominance of immature cells and a value >1 is equivalent to a predominance of mature cells. The maturation index is a comparative measure between the number of immature cells sustained in culture and the effectiveness of differentiation induction by a particular growth treatment.

g). Induction of differentiation was also measured determining the number of phagocytic cells. Normal and leukaemic bone marrow cells and cells from the SA2 CL were cultured in the microtitre assay for eight hours, two, four and six days. Eight hours prior to the preparation of cytopspins 0.8 $\mu$  latex particles were added

to the cultures. Three cytopspins were prepared and stained as above and the percentage and absolute numbers of phagocytic cells per well were determined and graphed. At least 1000 cells were counted per slide.

### 3.1.2 Results

The bone marrow of the transplanted leukaemic cell lines contained higher percentages of immature cells (blasts and promyelocytes) and lower percentages of intermediate cells (myelocytes, metamyelocytes and immature macrophages) than normal bone marrow (Table 3.1).

CELLS	PERCENTAGE No. OF CELLS OF *SCS					
	NBM	SA7	SA8	SA2 LP	SA2 HP	SA2 CL
IMMATURE	18.5	66.6	35.7	43.6	38.0	100
INTERMEDIATE	77.8	28.5	51.5	53.3	54.4	0
MATURE	3.5	4.9	13.5	2.9	7.5	0

**Table 3.1** Percentage number of immature, intermediate and mature cells of the bone marrow single cell suspension \*(SCS) from the leukaemic transplanted cell lines and the SA2 CL and normal.

The cellular classification of "immature macrophages" was included in the differentials of the cultured leukaemic marrows only. These cells were the size of macrophages, contained dark grey blue cytoplasm and an immature blastic nucleus with numerous nucleoli. They were classified as intermediate cells.

The differentiability of the bone marrow cells from each of the transplanted leukaemic cell lines to WEHI-3B CM, L929 CM and rGM-CSF, singularly and in combination, as compared to normal bone marrow is discussed below. The effects of the growth factors on the autonomously proliferating SA2 CL will be discussed separately.

### 3.1.2.1 WEHI-3B CM

WEHI-3B CM sustained the proliferation of normal bone marrow cells to day six with a 3.4 fold increase in the absolute number of cells per well. WEHI-3B CM was effective in sustaining immature normal cells *in vitro*; there was a 13 fold increase in the number of immature cells (Fig. 3.1 A) The most significant increase was in the number of promyelocytes. The number of intermediate cells decreased 1.2 fold. Differentiation induction was observed in both the monocytic and granulocytic series with a 17 fold increase in the absolute number of mature cells per well. WEHI-3B CM was more effective in sustaining normal immature cells in culture than inducing differentiation. Immature cells constituted 59% of the normal bone marrow differential at day six when stimulated with WEHI-3B CM (Appendix Table A3.1). These results were depicted with a negative gradient in the maturation index (Fig. 3.2 A). The differential data was paralleled with the phagocytosis study where a relatively low percentage (Fig.3.3 A) and absolute number of phagocytic cells (Fig. 3.4 A) were observed in the normal bone marrow cultures.

WEHI-3B CM also sustained the proliferation of the SA7 leukaemic bone marrow cells to day six, the increase in the absolute number of cells per well was higher than that of normal, 4.2 fold (Fig. 3.1 B). WEHI-3B CM was not as effective at sustaining immature cells of the SA7 leukaemia as it was of normal bone marrow. There was only a 1.5 fold increase in the number of immature cells and immature cells only constituted 24% of the SA7 differential on day six (Appendix Table A3.4). Hence, WEHI-3B CM appeared to be a more potent stimulator of normal blast cells than the SA7 leukaemic blast cells. Differentiation induction of the SA7 leukaemic bone marrow was more effective than that observed with normal. Intermediate cells increased 3.7 by day six in culture and the number of mature cells increased 33 fold, the most significant increase being in the number of

macrophages. The proportion of mature cells of the SA7 leukaemia increased with time in culture whereas the proportion of mature cells of normal bone marrow decreased. These two major differences between normal and SA7 leukaemic bone marrow cells was depicted in the maturation index. A positive gradient was observed with the SA7 leukaemia (Fig 3.2 B) and a negative gradient was observed with normal bone marrow (Fig.3.2 A). A higher percentage and absolute number of phagocytic cells was observed with the SA7 leukaemia than normal bone marrow (Fig's. 3.3 B and 3.4 B).

The SA8 leukaemia was also sustained in culture to day six when stimulated with WEHI-3B CM (Fig. 3.1 C). The proliferative response was higher than normal on day four and equivalent to normal on day six. There was a 3.6 fold increase in the total number of cells per well by day six. Unlike the SA7 leukaemia the total number of immature cells from the SA8 leukaemia sustained to day six was high and only minimally less than the absolute number sustained with normal bone marrow. Immature cells constituted 50% of the SA8 differential on day six. Differentiation induction was not as prominent as that observed with normal bone marrow or the SA7 leukaemia. There was a 1.6 fold increase in the number of intermediate cells and only a 7.7 fold increase in the number of mature cells by day six in culture. The predominantly negative gradient of the maturation index (Fig. 3.2 C) depicts the decrease in the proportion of mature cells to immature cells over time in culture when the SA8 leukaemic bone marrow cells were stimulated with WEHI-3B CM. The number of macrophages observed in the differential of the SA8 leukaemia (Appendix Table A3.8) were higher than that of normal bone marrow (Appendix Table A3.2) and this is depicted in the phagocytosis study (Fig's 3.3 C and 3.4 C).

The SA2 LP leukaemia was sustained in culture to day six when stimulated with WEHI-3B CM however the proliferative response observed was lower than that

observed with normal bone marrow or the other leukaemias (Fig. 3.1 D). There was only a 2.2 fold increase in the total number of cells per well by day six. Immature cells increased 3.8 fold and the absolute number of immature cells was high; immature cells constituted 75% of the differential on day six (Appendix Table A3.10). Differentiation induction was limited. There was a 1.6 fold decrease in the number of intermediate cells and only a 7.4 fold increase in the number of mature cells. Depicting these results, the maturation index of the SA2 LP leukaemia decreased over time in culture (Fig. 3.2 D). The lack of a differentiation response was observed with the phagocytosis study (Fig's 3.3 D and 3.4 D).

A high proliferative response was observed with the SA2 HP leukaemia (Fig. 3.1 E). There was a 4.9 fold increase in the total number of cells per well. Immature cells increased 4.7 fold. Differentiation induction was higher than that of normal and similar to that observed with the SA7 leukaemia by day six. There was a two fold increase in the number of intermediate cells and a 26 fold increase in the number of mature cells, predominantly the number of macrophages increased. Immature cells constituted 37% of the differential by day six (Appendix Table A3.13) and a positive gradient was observed with the maturation index indicating differentiation induction (Fig. 3.2 E).

In summary:-

- WEHI-3B CM was more effective in sustaining normal blast cells in culture than the blasts from the SA7, SA8 and SA2 HP leukaemias but not the SA2 LP leukaemia.
- A progressive increase in the maturation index over time in culture was only observed with the SA7 and SA2 HP leukaemias. WEHI-3B CM was more

effective in inducing differentiation than sustaining immature cells from these two leukaemias than with normal bone marrow.

- Differentiation induction of the SA8 and SA2 LP leukaemias was minimal and less than that of normal bone marrow.
- WEHI-3B CM may be effective in preferentially stimulating normal bone marrow and reducing the leukaemic burden of the SA7 and SA2 HP leukaemias.

#### 3.1.2.2 L929 CM

L929 CM was not as effective at stimulating the proliferation of normal bone marrow cells as WEHI-3B CM. There was a 2.2 fold increase in the total number of cells per well by day six (Fig. 3.5 A). Normal immature cells were not sustained in culture. The number of blast cells decreased progressively, none were detected in the differential counts by day six. Promyelocyte number increased two fold by day 2 and then decreased. Immature cells constituted 20% and 2% of the differential on day four and six respectively. The number of intermediate cells also decreased, 2.7 fold by day six. Differentiation induction was observed; absolute numbers of mature cells increased 54 fold, the most significant increase being in the number of macrophages. These results are reflected with the steep increase observed with the maturation index between day four and day six (Fig. 3.6 A). The number of macrophages obtained with the differential of normal bone marrow cells (66.5%) (Appendix Table A3.1) is depicted in the phagocytosis study (Fig's. 3.7 A and 3.8 A).

L292 CM did not sustain the immature cells of the SA7 leukaemia in culture (Fig. 3.5 B). There was a 30 fold decrease in the number of immature cells by day six and they constituted 11% and <1% of the differential on days four and six respectively. Differentiation was evident. The number of intermediate cells increased 1.4 fold, the main increase being in the number of 'immature

macrophages'. The absolute number of mature cells per well increased 51 fold. L929 CM appeared to be an effective inducer of differentiation of the SA7 leukaemia however, the increase in the maturation index was not as high as that of normal (Fig. 3.6 B). This was mainly attributed to the partial differentiation to immature macrophages which were classified as immature cells for the determination of the maturation index. A higher number of macrophages were counted in the differential of the SA7 leukaemia (Appendix Table A3.4) than normal bone marrow and this is depicted by a higher number of phagocytic cells (Fig's. 3.7 B and 3.8 B).

Increasing cell numbers of the SA8 leukaemic bone marrow were only observed to day four in culture when stimulated with L929 CM (Fig. 3.5 C). A possible reason for this was that L929 CM appeared to have an "immediate" differentiation effect on the SA8 leukaemia. Unlike normal bone marrow and the SA7 leukaemia, proliferation was observed in the first eight hours of culture accompanied with a significant drop in the number of immature cells, an increase in intermediate cell numbers (intermediate cells of normal bone marrow and the SA7 leukaemia decreased at this time point) and a more significant increase in the number of mature cells. By day four immature cell numbers were markedly reduced. There was an eight fold decrease in the number of immature cells by day four and a 23 fold decrease by day six. Immature cells constituted 2.7% and 1.2% of the differential on days four and six respectively. On day four there was a 1.6 fold increase in the number of intermediate cells and by day six they had decreased three fold. Mature cells increased 8 fold by day four, the most significant increase being in the number of macrophages. The number of mature cells dropped minimally by day six. A positive gradient was observed with the maturation index from day two to day six and the value of the maturation index was higher than that of normal only on day four (Fig. 3.6 C). The relatively high

differentiation effect observed with the SA8 leukaemia was stimulated with L929 CM and was also observed with the phagocytosis study where the number of phagocytic cells observed with the SA8 leukaemia was higher than normal bone marrow and the other leukaemias (Fig's. 3.7 C and 3.8 C).

L929 CM was not an effective stimulator of the SA2 LP leukaemia, there was only a 1.3 fold increase in the total number of cells per well by day six (Fig. 3.5 D). Higher numbers of immature cells were sustained in culture than normal bone marrow. Immature cells constituted 32% and 6% of the differential on day four and six respectively. There was a five fold decrease in the number of intermediate cells however, differentiation induction was evident with a 36 fold increase in the number of mature cells, mainly macrophages. Differentiation induction was less than that observed with normal bone marrow. A positive gradient was observed with the maturation index between days four and six only (Fig.3.6D), however, the values are less than that of normal because more immature cells were sustained in culture and less differentiation than normal bone marrow was observed. The high percentage of macrophages in the differential (Appendix Table A3.10) were also observed with the phagocytosis study (Fig. 3.7 D). The absolute number of phagocytes was less than observed with normal bone marrow (Fig. 3.8 D).

Compared to normal bone marrow and to the other leukaemias a relatively high proliferative response was observed with the SA2 HP when stimulated with L929 CM (Fig. 3.5 E). Similar to the SA8 leukaemia, proliferation of the SA2 HP leukaemia was only sustained to day four with L929 CM. Unlike the SA8 leukaemia an "immediate" differentiation response was not observed. The opposite effect was observed whereby there was a relatively high increase in the number of immature cells in the first eight hours of culture and no differentiation induction was evident. Intermediate and mature cells number actually decreased

in the first eight hours. A progressive decrease in the number of immature cells was then observed to day six. There was a 1.2 fold decrease in the number of immature cells by day four and a 3.7 fold decrease by day six. Immature cells constituted 11% and 3% of the differential on day four and six respectively. Differentiation was evident with a 1.2 fold increase in the number of intermediate cells by day four, this number however, decreased minimally by day six. There was a 27 fold increase in the number of mature cells by day four and a 32 fold increase by day six. There was a higher increase in the number of intermediate cells and mature cells than normal bone marrow. Differentiation induction was reflected in the maturation index with a positive gradient from day two to day six (Fig. 3.6 E), however because higher numbers of immature cells were sustained in culture than normal bone marrow the maturation index was lower than that of normal at each time point. The higher numbers of macrophages observed in the differential of the SA2 HP leukaemia compared to normal bone marrow was depicted in the phagocytosis study (Fig's. 3.7 E and 3.8 E).

In summary:-

- L929 CM did induce differentiation of leukaemic cells and differentiation induction in two cases, the SA7 and SA2 HP leukaemias were higher than that of normal.
- However, L929 CM sustained more immature leukaemic cells in culture with every leukaemia studied than normal immature cells.
- The maturation index was higher for normal bone marrow than the leukaemias at each time point, except for the SA8 leukaemia on day four. In the case of the SA7 HD leukaemia this was due to the partial differentiation of this leukaemia to immature macrophages. In the case of the SA2 LP leukaemia this was because more immature cells were sustained in culture and less differentiation was

observed than normal; and in the case of the SA2 HP because higher numbers of immature cells were sustained in culture even though differentiation induction was higher than that of normal.

- L929 CM may be effective in reducing the leukaemic burden of the SA8 leukaemia.

### 3.1.2.3 rGM-CSF

rGM-CSF sustained increasing cell growth of normal bone marrow cells to day six in culture with a 1.2 fold increase in total cell number per well (Fig. 3.9 A). rGM-CSF sustained relatively high numbers of immature normal cells in culture to day six. Immature cells increased to day two and then decreased to numbers minimally lower than the SCS. Immature cells constituted 12% and 9% of the differential on day four and six respectively (Appendix Table. A3.1) Intermediate cell numbers decreased. Differentiation induction was observed with the predominant mature cells being monocytes and macrophages. A positive gradient of the maturation index was observed from day two to day six (Fig. 3.10 A). 33% macrophages were observed with the differential and this result is paralleled in the phagocytosis study (Fig's. 3.11 A and 3.12 A).

rGM-CSF only sustained increasing cell growth of the SA7 leukaemic bone marrow cells to day four in culture (Fig. 3.9 B). There was a 2 fold increase in the total number of cells per well by day four, which was higher than that of normal. No "immediate" differentiation induction was observed after eight hours of culture. rGM-CSF sustained higher numbers of immature cells from the SA7 to day four in culture than normal bone marrow. Immature cells constituted 33% and 5% of the differential on days four and six respectively. Differentiation induction was evident but not as complete as that of normal bone marrow. Partial differentiation to the intermediate cell stages was more pronounced than that of

normal bone marrow, there was a 2.2 fold increase in the number of intermediate cells. However, full maturation was significantly less ( $p < 0.01$ ) than that observed with normal bone marrow with only a 9 fold increase in mature cells: absolute numbers of mature cells were similar. An equal increase in the number of segmented cells and macrophages was observed. A positive gradient was observed with the maturation index, however the value at each time point was less than that of normal bone marrow (Fig. 3.10 B). The percentage and absolute number of macrophages observed with the differentials of the SA7 leukaemia (Appendix Table A3.4) were similar to the percentage and absolute number of phagocytic cells (Fig's. 3.11 B and 3.12 B). The absolute number of phagocytes observed with the SA7 leukaemia was higher than the number observed with normal bone marrow.

rGM-CSF sustained increasing cell growth of the SA8 leukaemia to day four in culture with a 2 fold increase in the total number of cells at this time point (Fig. 3.9 C). As with L929 CM "immediate" differentiation and a decrease in immature cell numbers was observed after the first eight hours of culture. rGM-CSF was not effective in sustaining high numbers of immature cell from the SA8 leukaemia in culture as normal bone marrow. The absolute number of immature cells decreased progressively over the six days in culture with a 25 fold decrease by day four. Immature cells constituted  $< 1\%$  of the differential at day four. On day six however, a recovery phase was observed with the absolute number of immature cells, mainly promyelocytes increasing in number. Differentiation induction was evident. Intermediate cells increased to day two and then decreased to numbers similar to that of the single cell suspension. Mature cells, predominantly granulocytic cells, increased to numbers higher than that of normal bone marrow on day four. The decreasing number of immature cells and the accompanied differentiation induction of the SA8 leukaemia was reflected in the maturation

index where there was a steep positive gradient between day two and day four (Fig. 3.10 C). The late recovery phase in the number of immature cells was reflected with a negative gradient between day four and day six. On day four of culture the percentage and absolute number of phagocytic cells observed with the SA8 leukaemia were higher than the number observed with normal bone marrow (Fig's. 3.11 C and 3.12 C). This was also the case with the number of macrophages observed in the differential studies (Appendix Tables. A3.1 and A3.7).

As with normal bone marrow, rGM-CSF sustained increasing cell growth of the SA2 LP leukaemia to day six in culture (Fig. 3.9 D). There was a two fold increase in the total number of cells per well by day six. However, unlike normal bone marrow, rGM-CSF was very effective in increasing the absolute number of immature cells of the SA2 LP leukaemia to day six. Immature cells constituted 42% and 44% of the differential on days four and six respectively. Intermediate cells initially decreased and were then sustained at the same level as that of the single cell suspension. An increase in the absolute number of mature cells was observed, however the increase in mature cells was less than that observed with normal bone marrow. Reflecting these results a negative gradient was observed with the maturation index (Fig. 3.10 D). rGM-CSF appeared not to be effective in reducing immature leukaemic cells from the SA2 LP leukaemia, or inducing enhanced differentiation. Relatively low percentage and absolute numbers of phagocytic cells were also obtained (Fig's. 3.11 D and 3.12 D).

rGM-CSF sustained increasing cellular growth of the SA2 HP leukaemia to day four in culture with a 3.7 fold increase in the total number of cells per well (Fig. 3.9 E). The proliferative response observed was greater than that of normal bone marrow. Absolute numbers but not the percentage numbers of immature cell numbers were higher than that observed with normal bone marrow on day four. By day six the number of immature cells was significantly lower ( $p < 0.05$ ) than

that of normal. Immature cells constituted 7% and 1% of the differential on day four and six respectively. As with the SA7 leukaemia there was prominent partial differentiation to the intermediate cell stages on day four with a four fold increase in the number of these cells. Unlike the SA7 leukaemia the predominant intermediate cells were the metamyelocytes. Differentiation to intermediate and mature cells was more prominent with the SA2 HP leukaemia than normal bone marrow. The predominant mature cells were the macrophages. The maturation index reflected these differentiation results with a relatively steep positive gradient between day two and day four (Fig. 3.10 E) and higher values on day four and day six than normal bone marrow. The higher numbers of mature cells as compared to normal bone marrow was also observed with the phagocytosis study (Fig's. 3.11 E and 3.12 E).

In summary:-

- rGM-CSF did not sustain immature cells, and enhanced differentiation induction of the SA8 leukaemia more effectively than that of normal bone marrow. However, a recovery phase of immature cells was observed on day six of culture.
- rGM-CSF sustained very high numbers of immature cells of the SA2 LP leukaemia to day six in culture. Differentiation was observed with this leukaemia, however a negative maturation index was observed indicating that rGM-CSF was more effective at sustaining immature cells than enhancing differentiation of this leukaemia.
- Prominent partial differentiation was observed with the SA7 leukaemia. Differentiation to fully mature cells was also observed, however this was less than that observed with normal bone marrow.
- The SA2 HP leukaemia was highly proliferative in culture with relatively high numbers of immature cells being sustained to day four. By day six however,

the number of immature cells were far fewer than that observed with normal bone marrow. Differentiation induction of this leukaemia was greater than that of normal.

- The predominant mature cells obtained with normal bone marrow and each of the leukaemias differed. The predominant mature cells observed with normal bone marrow were monocytes and macrophages, with the SA7 leukaemia there were equal numbers of granulocytes and macrophages, with the SA8 and SA2 LP leukaemia there were greater numbers of granulocytes and with the SA2 HP leukaemia a greater number of macrophages.
- It appeared that rGM-CSF preferentially sustained normal immature cells and induced a more enhanced differentiation response of the SA8 and SA2 HP leukaemias however, a recovery phase was observed with the SA8 leukaemia and a very high initial proliferative response was observed with the SA2 HP leukaemia.

#### **3.1.2.4 WEHI-3B CM + L929 CM**

The results obtained with the combination of WEHI-3B CM plus L929 CM with normal and leukaemic bone marrows were generally similar to the results obtained with WEHI-3B CM alone.

With normal bone marrow there was an enhanced, but less than additive, proliferative response with this growth factor combination (Fig. 3.13 A). There was a significant increase in the number of immature cells sustained in culture to day six compared to WEHI-3B CM alone. Differentiation was not as high as that observed with L929 CM alone however the numbers of both intermediate and mature cells were greater than the number obtained with WEHI-3B CM alone. The increase in the number of immature cells was reflected in the maturation index which had a steeper negative gradient than that of WEHI-3B CM (Fig.

3.14 A). The percentage and absolute number of phagocytic cells was also less than the numbers observed with either factor alone (Fig's. 3.15 A and 3.16 A).

An enhanced, but less than additive, proliferative response was observed with the SA7 leukaemia on day four (Fig. 3.13 B). An increase in cell number was observed in all three cell groups on day four. On day six the total number of cells obtained was similar to that of WEHI-3B CM alone. The number of immature cells sustained to day six was greater than the number sustained with WEHI-3B CM alone. The numbers of intermediate and mature cells were similar with the two treatments. The enhanced survival of immature cells with the combination of growth factors as compared to WEHI-3B CM alone was reflected in the maturation index (Fig. 3.14 B). The maturation index at day six with the combination of growth factors was lower than the maturation index of WEHI-3B CM alone. The percentage and absolute number of phagocytic cells were similar to the numbers observed with WEHI-3B alone and less than the numbers observed with L929 CM alone. Hence, the combination of these two growth factors would not be as effective in reducing the leukaemic cells as either factor alone.

No enhanced proliferative response was observed with the SA8 leukaemia when stimulated with this factor combination. The cellular proliferative response of the SA8 leukaemia was similar to that of WEHI-3B CM alone at each time point studied (Fig. 3.13 C). On day four there were fewer immature cells than WEHI-3B CM alone and there was an enhanced differentiation response with higher numbers of intermediate and mature cells than with either factor alone. On day six however, there was a sharp increase in the number of immature cells. A greater number of immature cells were sustained to day six than with WEHI-3B CM alone. There were fewer intermediate cells than with WEHI-3B CM alone and fewer mature cells than with either factor alone. A positive gradient was observed with the maturation index between day two and day four and a negative

gradient was observed between day four and day six (Fig. 3.14 C). Hence at early time points in culture, this growth factor combination reduced the rate of immature cells produced compared to WEHI-3B CM and increased differentiation induction compared to either factor alone. By day six however, a recovery of immature cell numbers was observed to levels higher than those observed with WEHI-3B CM alone and differentiation was reduced compared to either factor alone. The number of phagocytic cells was similar to the number observed with WEHI-3B alone (Fig's. 3.15 C and 3.16 C).

An additive proliferative response was observed with the SA2 LP leukaemia when stimulated with this factor combination (Fig. 3.13 D). Enhanced survival of immature cells was observed on days four and six compared to WEHI-3B CM alone. Differentiation compared to WEHI-3B CM alone was also enhanced with an increase in both the number of intermediate and mature cells. The number of mature cells on day four was similar to the number obtained with L929 CM alone and the number obtained on day six was exactly half way between the number observed with each of the factors alone. From these results it appeared that two different cell types constituted the SA2 LP leukaemia. One which was stimulated to proliferate, and differentiate only minimally with WEHI-3B CM and the other which was stimulated to proliferate and differentiate by L929 CM. A predominantly negative gradient was observed in the maturation index with this factor combination, reflecting the sustained proliferation of immature cells and limited differentiation induction (Fig. 3.14 D). The number of phagocytic cells observed was higher than the number observed with WEHI-3B CM alone and less than the number observed with L929 CM alone (Fig's. 3.15 D and 3.16 D). Hence, this growth factor combination would not be effective in reducing the number of cells from this leukaemia compared to either factor alone. Differentiation was increased compared to WEHI-3B CM alone but more

immature cells are sustained in culture. More immature cells are sustained in culture than L929 CM alone and less differentiation was observed.

No enhanced proliferative response was observed with the SA2 HP leukaemia with this growth factor combination. On day four of culture higher numbers of immature and mature cells, and fewer intermediate cells were observed than with WEHI-3B CM alone (Fig. 3.13 E). The number of mature cells on day four was similar to the number observed with L929 CM alone. On day six only half the number of blasts sustained with WEHI-3B CM alone was sustained with the growth factor combination. The numbers of intermediate and mature cells were equivalent to the numbers obtained with WEHI-3B CM alone. The number of mature cells observed on day six was less than the number observed with L929 CM alone. The maturation index over time with the growth factor combination was similar to that of WEHI-3B CM alone except with the value at day six which was higher with the growth factor combination (Fig. 3.14 E). This reflects the fewer immature cells sustained to day six compared to WEHI-3B CM alone. The number of phagocytic cells was less than the number observed with L929 CM alone and greater than the number observed with WEHI-3B CM (Figs 3.15 E and 3.16 E). The differences in the phagocytic cell numbers were similar to the differences in the macrophage numbers observed in the differentials. These results suggest that the SA2 HP leukaemia consists of a cell population which is responsive for proliferation and differentiation to both WEHI-3B CM and L929 CM.

In summary:-

- The combination of WEHI-3B CM plus L929 CM sustained higher numbers of immature cells in culture than either growth factor alone from normal bone marrow, the SA7, SA8 and the SA2 LP leukaemias.

- With the SA2 HP leukaemia the number of immature cells sustained in culture was less than the number observed with WEHI-3B CM alone.
- The combination of WEHI-3B CM plus L929 CM induced the enhanced proliferation of normal bone marrow and the SA7 leukaemia. For both these bone marrows, differentiation was greater than that observed with WEHI-3B alone but less than the differentiation observed with L929 CM alone.
- Enhanced differentiation induction with the combination of WEHI-3B CM plus L929 CM was only observed with the SA8 leukaemia on day four in culture. However this effect was overridden by day six as an increase in immature cells was observed.
- An additive proliferative response was observed with the SA2 LP leukaemia. Differentiation was greater than that observed with WEHI-3B CM alone but less than with L929 CM alone.

#### **3.1.2.5 WEHI-3B CM + rGM-CSF**

A synergistic proliferative response was observed with normal bone marrow at day six when stimulated with this factor combination. Enhanced numbers of immature cells and enhanced differentiation were observed on day four and six (Fig. 3.17 A). This factor combination was more effective in sustaining immature cells than inducing differentiation and thus the maturation index at each of the time points studied was less than that of either factor alone (Fig. 3.18 A). The percentage and absolute number of phagocytic cells were less than the numbers observed with either factor alone (Fig's 3.19 A and 3.20 A).

An enhanced but less than additive proliferative response was observed on day four with the SA7 leukaemia. Enhanced numbers of immature cells were observed on days four and six (Fig. 3.17 B). Differentiation, compared to either factor alone was reduced. This growth factor combination was more effective at sustaining

immature cells and less effective at inducing differentiation of the SA7 leukaemias than either factor alone. As with normal bone marrow the maturation index was less at each time than with either factor alone (Fig. 3.18 B). The percentage and absolute number of phagocytic cells were less than the number observed with either factor alone (Fig's. 3.19 B and 3.20 B).

An enhanced but less than additive proliferative response was observed with the SA8 leukaemia on day four and six. The number of immature cells observed on day four was markedly lower than the number observed with WEHI-3B CM alone, but higher than the number observed with rGM-CSF alone (Fig. 3.17 C). By day six the number of immature cells was only minimally less than the number of observed with WEHI-3B CM alone. Differentiation induction was significantly enhanced compared to either factor alone on days four and six. The maturation index was higher at each time point than with WEHI-3B CM alone but less than that of rGM-CSF (Fig. 3.18 C). The number of phagocytic cells was higher than with either factor alone (Fig's 3.19 C and 3.20 C).

An enhanced but less than additive response was observed with the SA2 LP leukaemia on days four and six. The number of immature cells sustained in culture was less than the number sustained with WEHI-3B CM alone and greater than the number sustained with rGM-CSF alone (Fig 3.17 D). Differentiation was minimally enhanced on day four and markedly enhanced on day six compared to either factor alone. Hence this factor combination enhances the differentiation of the SA2 LP leukaemia without increasing the number of immature cells sustained in culture above the number observed with WEHI-3B CM alone. This response was reflected in the maturation index with a positive gradient from day two to day six compared to a negative gradient observed with either growth factor alone (Fig. 3.18 D) The number of phagocytic cells were also higher than with either factor alone (Fig's 3.19 D and 3.20 D).

An enhanced but less than additive response was observed with the SA2 HP leukaemia with this growth factor combination on day four. On day six the proliferative response was less than with WEHI-3B CM alone and greater than with rGM-CSF (Fig. 3.17 E). Enhanced numbers of immature cells were observed on day four however, by day six the numbers were significantly less than that observed with WEHI-3B CM alone. Differentiation induction was enhanced on day four but not on day six compared to either factor alone. The maturation indices were generally lower than with either factor alone (Fig. 3.18 E). The number of phagocytic cells were less than the number observed with WEHI-3B CM alone and similar to the number observed with rGM-CSF alone.

In summary:-

- The combination of WEHI-3B CM plus rGM-CSF enhanced the differentiation induction of the SA8 and the SA2 LP leukaemias compared to either factor alone. The number of immature cells sustained in culture was not enhanced; the numbers were similar to those observed with WEHI-3B CM alone..
- Enhanced numbers of immature cells and reduced differentiation were observed with the SA7 leukaemia compared to either factor alone.
- With the SA2 HP leukaemia immature cell numbers sustained were similar to the number sustained with WEHI-3B CM alone and enhanced differentiation was only observed on day four.

#### **3.1.2.6 L929 CM + rGM-CSF**

An additive proliferative response was observed with normal bone marrow with the combination of L929 CM plus rGM-CSF on day six. Enhanced numbers of immature cells were observed on days four and six compared to either factor alone (Fig. 3.21 A). Differentiation was enhanced on day four; there were higher

numbers of intermediate cells and mature cells. By day six higher numbers of intermediate cell were observed but the number of mature cells was equivalent to the number observed with L929 CM alone. The maturation index was lower at each time than with either factor alone reflecting the increased number of immature cells (Fig 3.22 A). The number of phagocytic cells was minimally higher than the number observed with rGM-CSF and less than the number observed with L929 CM alone (Fig's. 3.23 A and 3.24 A).

An enhanced but less than additive proliferative response was observed with the SA7 leukaemia with this growth factor combination. On day six the proliferative response was similar to that observed with L929 CM alone and greater than that observed with rGM-CSF (Fig. 3.21 B). The number of immature cells was greater than the number observed with either either factor alone. Differentiation induction was less than that observed with L929 CM alone and greater than rGM-CSF alone. The enhanced numbers of immature cells were reflected in the maturation index which was lower than with either factor alone between day 2 and day six (Fig. 3.22 B). The number of phagocytic cells was less than the number observed with L929 CM alone and greater than the number observed with rGM-CSF alone.

The combination of L929 CM plus rGM-CSF sustained increasing cell proliferation of the SA8 leukaemia longer than either factor alone (Fig. 3.21 C). An enhanced but less than additive proliferative response was observed on day four and a synergistic response was observed on day six. The number of immature cells sustained in culture was higher than with either factor alone on days four and six. The number of intermediate cells were higher on day four and on day six than with either factor alone. The number of mature cells was similar to the number observed with L929 and greater than the number observed with rGM-CSF alone on day four. On day six enhanced differentiation was observed with higher numbers

of both intermediate and mature cells than the number observed with either factor alone. The increase in the number of immature cells sustained in culture with this growth factor combination was reflected in the maturation index which was lower than with either factor alone on day six (Fig. 3.22 C). The enhanced differentiation and increase in immature cells numbers is reflected in the phagocytosis study. The percentage number of phagocytic cells observed with the growth factor combination was less than the percentage observed with L929 CM and similar to rGM-CSF alone (Fig. 3.23 C). The absolute number of phagocytic cells with the growth factor combination was similar to the number observed with L929 CM and greater than the number observed with rGM-CSF (Fig. 3.34 C).

An additive proliferative response was observed with the SA2 LP leukaemia. The number of immature cells was greater than the number observed with L929 CM alone and less than the number observed with rGM-CSF alone (Fig. 3.21 D). Differentiation was markedly enhanced with increased numbers of intermediate and mature cells compared to either factor alone by day six. The maturation index at each time point was greater than that of rGM-CSF alone and less than that of L929 CM alone (Fig. 3.22 D). Two cell populations appear to constitute the SA2 LP leukaemia. The percentage and absolute number of phagocytic cells were greater than the numbers observed with rGM-CSF: percentage numbers were less but absolute numbers greater than with L929 CM alone (Fig's. 3.23 D and 3.24 D).

An enhanced but less than additive proliferative response was observed with the SA2 HP with this growth factor combination on day four and day six. Increasing cell proliferation was only sustained to day four as with each growth factor alone. Enhanced numbers of immature cells were sustained to day six in culture compared to either growth factor alone (Fig. 3.21 E). Partial differentiation induction was increased with higher numbers of intermediate cells being observed on day six. Differentiation induction to mature cells was not enhanced with

numbers being equivalent to those observed with L929 CM alone on day six. The increase in immature cells was reflected in the maturation index which was lower than with either growth factor alone on days four and six (Fig. 3.22 E). The number of phagocytic cells was similar to the number observed with L929 CM alone (Figs 3.23 E and 3.24 E).

In summary:-

- Enhanced numbers of immature cells without enhanced differentiation were observed with the SA7 leukaemia.
- Enhanced differentiation was observed with the SA8 leukaemia, however this was accompanied by enhanced numbers of immature cells and the maturation index was lower than with either factor alone on day six.
- Enhanced differentiation was observed with the SA2 LP leukaemia. The number of immature cells of the SA2 LP leukaemia sustained in culture was less than the number sustained with rGM-CSF alone and greater than the number observed with L929 CM. The maturation index was greater than with rGM-CSF alone but less than with L929 CM alone
- Enhanced numbers of immature cells and intermediate cells but not mature cells were observed with the SA2 HP leukaemia.

### 3.1.2. The SA2 CL

The autonomously, high proliferative SA2 CL could not be induced to terminally differentiate in culture (Fig 3.25) with and without the addition of growth factor. In the initial differential ninety-nine percent of the cells were blasts and one percent were blastic cells with azurophilic granules and were classified as promyelocytes. In FCS alone partial differentiation was observed to 'immature macrophages'. This partial differentiation was reduced with the addition of

single growth factors and no difference compared to either factor alone was observed with the combination of WEHI-3B CM plus L929 CM or rGM-CSF. An enhanced differentiation effect was observed with the combination of L929 CM plus rGM-CSF compared to FCS and either growth factor alone. The lack of differentiation to terminal end cells of the SA2 CL was also observed with the phagocytosis study where less than one percent blasts were observed with every treatment.

### **3.2 THE *IN VITRO* EFFECTS OF WEHI-3B CM AND L929 CM ON THE LEUKAEMIC CLONOGENIC CELLS FRACTION AS MEASURED IN THE *IN VIVO* CLONOGENIC CELL ASSAY**

*In vivo* leukaemic clonogenic cell assays were carried out to determine the effectiveness of the physiological regulators to decrease the clonogenic fraction of the leukaemic cell population. Because of limited resources only the SA7 leukaemia and two of the growth factors were used for this study. WEHI-3B CM was used as it sustained a high percentage of immature cells in culture, and L929 CM as it did not sustain high numbers of immature cells and was an effective differentiation inducer.

#### **3.2.1 Experimental procedures**

A single cell suspension of SA7 leukaemic bone marrow cells was prepared. A proportion of the cells were injected into syngeneic recipients at varying cell concentrations ( $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$  and 10 total cells). The remaining cells were cultured in the microtitre assay for four days with either FCS, or optimal concentrations of WEHI-3B CM or L929 CM. On day four the cells were harvested, washed in non-supplemented RPMI, counted and injected into syngeneic recipients at the same varying concentrations as above. At least five mice were used per

treatment and percentage survival was monitored over 90 days. The results are expressed as the means of three experiments.

### 3.2.2 Results

The results obtained with the uncultured SA7 leukaemic bone marrow cells are well established in this laboratory and form the criteria of maintaining the transplanted leukaemic cell lines for study (Riches, unpublished data). Thirty percent take was observed with as few as 10 cells being injected and 100% take was observed with at least  $10^3$  cells being injected (Table 3.2). A significant percentage of the leukaemic clonogenic cell fraction of the SA7 leukaemia was maintained in FCS alone to day four in the microtitre assay. Proliferation was not observed, however differentiation to the macrophage lineage was observed in FCS alone in the previous study (Appendix Table A3.5). Only a minimal decrease in the clonogenic fraction was observed, and it was not possible to predict whether this was due to spontaneous differentiation or to the lack of growth factor for enhanced cell survival in vitro. At least  $10^4$  cells were required for a 100% take, the injection of  $10^3$  cells resulted in a 70% take and 100% survival to day 90 was observed with the injection of 10 cells. The addition of WEHI-3B CM or L929 CM to the cultures resulted in a one log reduction of the leukaemic clonogenic cell fraction (Fig. 3.27). The addition of L929 CM to cultures resulted in a higher percentage survival than with WEHI-3B CM. With both treatments  $10^5$  cells were required for 100% take of the leukaemia. 90% take was observed when  $10^4$  WEHI-3B CM treated cells were injected and 80% take was observed with L929 CM. With the injection of  $10^3$  cells 30% take and 10% take were observed with WEHI-3B CM and L929 CM respectively. The injection of  $10^2$  cells treated with either CM resulted in 100% survival to 90 days.

It was difficult from this study to assess whether the conditioned mediums used were unable to sustain the leukaemic clonogenic cells in culture or whether the clonogenic cells had been induced to differentiate and to consequently lose their self-renewal capacity. From the differential data however the SA7 leukaemia was shown to have maintained its proliferative and differentiative responses to physiological regulators. Both absolute immature and mature cell numbers were shown to increase with stimulation with WEHI-3B CM and mature cells increased with stimulation with L929 CM. It would appear therefore that at least part of the decrease in leukaemic clonogenic cell numbers observed with the addition of growth factors may be due to differentiation induction of these cells.

No. Cells Injected	PERCENTAGE TAKE OF LEUKAEMIA			
	UNCULTURED	FCS	WEHI-3B CM	L929 CM
10 <sup>5</sup>	100 by day 7	100 by day 13	100 by day 16	100 by day 13
10 <sup>4</sup>	100 by day 11	100 by day 18	90 by day 23	80 by day 16
10 <sup>3</sup>	100 by day 13	70 by day 28	30 by day 17	10 by day 18
10 <sup>2</sup>	70 by day 14	20 by day 23	0 to day 30	0 to day 30
10	30 by day 16	0 to day 30	ND	ND

**Table 3.2** *In vivo* Clonogenic Cell Assay. Single cell suspension of the SA7 leukaemic bone marrow at varied cell concentrations (10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> and 10 total cells) was injected into syngeneic recipients. Cells from the same single cell suspension were cultured for 4 days in the microtitre assay with FCS, and optimal concentrations of WEHI-3B CM or L929 CM. The cells were harvested washed in non-supplemented RPMI and injected into syngeneic recipients as above. Percentage survival was monitored over 90 days. Results were the means of three experiments.

### 3.3 DISCUSSION

The leukaemias studied had very heterogeneous responses to the different growth factors. The leukaemias had not completely lost their capacity for differentiation caused by the physiological regulators of normal haemopoiesis studied. The most effective response for the control of leukaemic cell growth

would be one where differentiation induction was obtained without immature leukaemic cells being sustained and consequently allowing the reestablishment of a normal dominant haemopoietic clone. If this type of response was possible, physiological growth factors may have a therapeutic role in the treatment of the disease. This type of response was observed with at least one of the leukaemias with each of the growth factors studied singly. WEHI-3B CM was more effective in sustaining normal immature cells than SA7 leukaemic immature cells and the differentiation induction of the SA7 leukaemia was greater than that of normal. L929 CM was more effective in sustaining immature cells of normal bone marrow than the SA8 leukaemia and the differentiation induction of this leukaemia was greater than that of normal bone. rGM-CSF was more effective in sustaining immature cells of normal bone marrow than the SA8 and the SA2 HP leukaemias and differentiation induction of these leukaemias was greater than that of normal. However, in the case of rGM-CSF a recovery of immature cells was observed on day six with the SA8 leukaemia and the SA2 HP had a very high proliferative response to day four in culture compared to normal. If this data can be projected to *in vivo* responses, growth factors which induced differentiation of leukaemic cells and preferentially sustained normal immature cells may be useful therapeutically for each particular leukaemia.

Relatively high numbers of mature cells were observed with the SA2 HP leukaemia with each of the growth factors. The differentiation responsiveness of this leukaemia may account for it not being sustained in culture for as long as normal bone marrow or the other leukaemias.

The SA2 LP was the least proliferative leukaemia in culture with each of the growth factors. It was also the leukaemia which did not differentiate in culture in response to WEHI-3B CM and rGM-CSF. Differentiation was observed with L929 CM however this was less than the differentiation observed with normal

bone marrow and higher numbers of immature cells were sustained in culture than with normal bone marrow. It appears therefore, that growth factors would not be able to be used as differentiation inducers therapeutically in all cases of leukaemia.

The combination of growth factors generally did not produce more effective responses than when they were used singly. In some cases, where differentiation induction was enhanced the number of immature cells sustained in culture also increased making the maturation index lower than both or one of the growth factors used. In the case of the SA7 leukaemia the combination of rGM-CSF with WEHI-3B CM or L929 CM sustained higher numbers of immature cells and reduced differentiation compared to either factor alone. In the case of the SA2 LP leukaemia differentiation was enhanced with the growth factor combinations however the number of immature cells sustained in culture was also increased. This data contradicts some studies on human AML cells in which improved differentiation responses were observed with combinations of growth factors (Lowenberg et al, 1988; Salem et al, 1989).

The SA2 CL was not induced to differentiate in the growth factors studies. The growth factors had adverse effects on the spontaneous differentiation observed with this leukaemia when grown in FCS alone. WEHI-3B CM decreased the partial differentiation, L929 CM decreased the partial differentiation and increased the proliferative response and rGM-CSF appeared to have no effect. The combination of growth factors did not enhance differentiation.

The maturation index was an effective way to represent the relationship between the number of immature cells sustained in culture and differentiation induction. The determination of the maturation index to determine the effectiveness of both physiological and non-physiological growth factors on leukaemic cell growth and

differentiation could be easily implemented in conjunction with automated total cell and differential cell counting.

The phagocytosis assay was used to help confirm the differential data. The use of this assay alone and the reporting of only the percentage of phagocytic cells however, would have given misleading results.

No specific pattern for subgrouping the leukaemias became apparent with this study, however only a small sample was used. From the heterogeneity of the responses obtained it appears that the effects of growth factors on leukaemias will need to be evaluated on an individual basis.

From the clonogenic cell assay it was evident that the treatment of leukaemic cells with physiological growth factors reduced the number of leukaemic clonogenic cells. This may have bearing on the possibility of direct use of physiological growth factors as part of a therapeutic regime or to purge the leukaemic cells from long term bone marrow cultures for autologous transfusion.

There has been a lot of controversy from numerous studies on the suitability of physiological growth factors as therapeutic agents for myeloid leukaemia as the haemopoietic growth factors may potentially activate residual leukaemic cells expressing CSF receptors and messenger RNA. In numerous studies it was shown that GM-CSF stimulated the growth of blast progenitors suggesting that it may be contraindicated in the treatment of any condition related to AML or capable of progression to that disease (Hoang et al, 1986; Nara and McCulloch, 1985). However, in Hoang's study partial differentiation of some of the blasts studied was obtained. What became evident was that both normal and leukaemic cells are stimulated by the normal physiological growth factors, the response by leukaemic cells being varied between patients. However, what had not been established was whether in some leukaemic conditions the normal cell population might be more responsive to growth factor than the leukaemic population and

therefore the suppressed normal clones may be restored as dominant. This may become important as an effective maintenance therapeutic regime, keeping minimal residual disease in check.

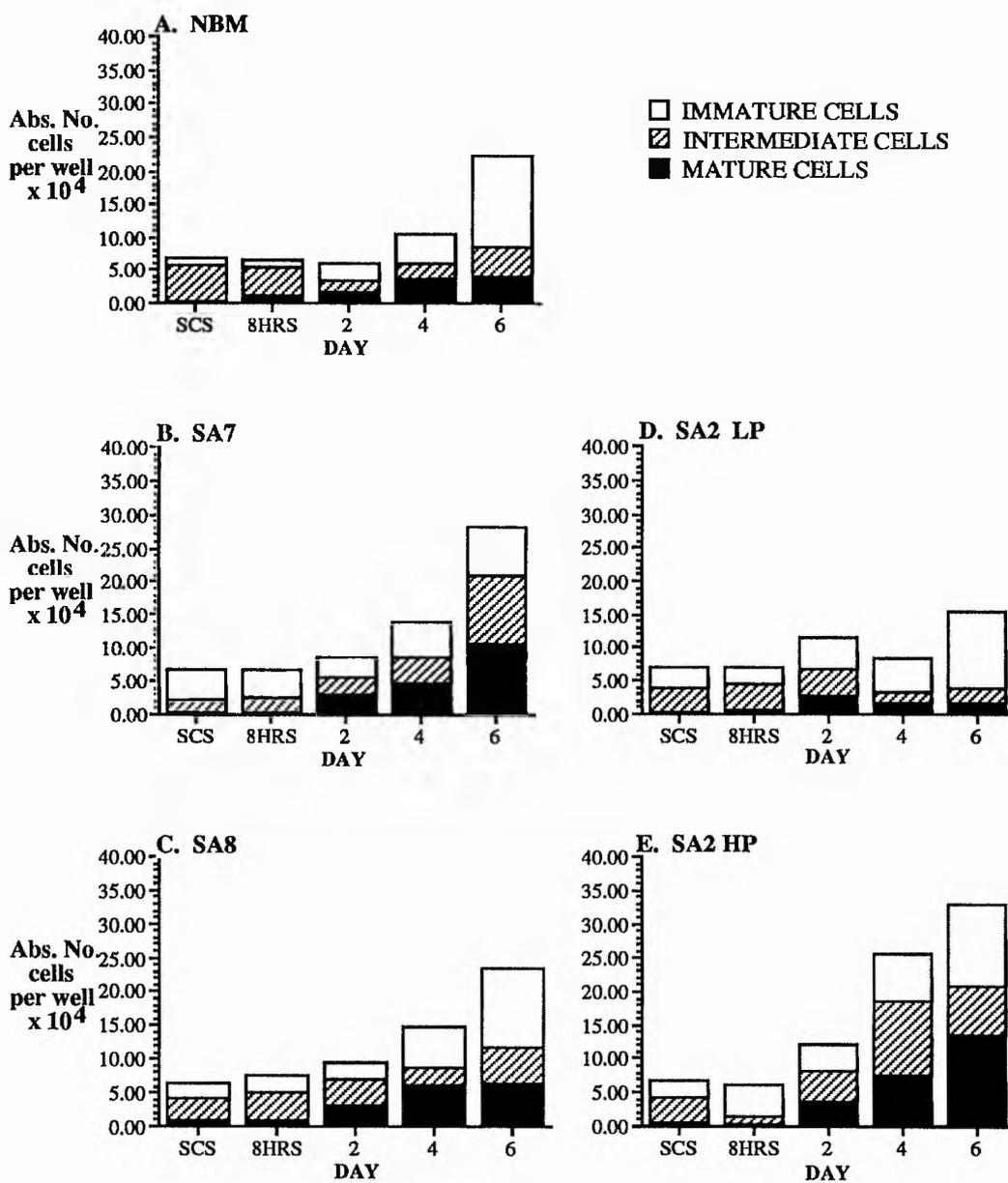
In this study, as far as we were aware, we were the first to identify murine leukaemic clones which were induced to differentiate with M-CSF. The existence of such clones has been previously postulated as M-CSF has been shown to increase the functional activities of mature myeloid cells (Ziboh et al, 1982; Tushinski and Stanley, 1983; Vadas et al, 1983; Chen and Lin, 1984; Gasson et al, 1984) and there are myeloid leukaemic cell lines which have M-CSF receptors (Guilbert and Stanley, 1980). Therefore it had been predicted that it may also act as a physiological differentiation inducing agent. In the study of human AML M-CSF stimulated little or no proliferation of AML blasts (Miyauchi et. al, 1988; Suzuki et. al, 1988) and its major effect seemed to be the induction of differentiation (Lowenberg et al, 1988; Salem et al, 1989). In Miyauchi's study an increase in the number of adherent macrophages was observed in suspension cultures of AML blasts with the addition of M-CSF. It was proposed that the adherent cells were progeny of leukaemic and not normal blasts. Miyauchi et al and Suzuki et al showed that M-CSF, in contrast to IL-3, GM-CSF and G-CSF did not stimulate the self-renewal of AML blasts and that its major effect on such cells was the stimulation of terminal cell divisions. Our differential data was in keeping with these two observations. Mayani in 1991 studied the effects of exogenously added M-CSF on human AML blasts in long term bone marrow cultures. They did not have direct evidence on the differentiative effects of M-CSF on leukaemic blasts, however they showed that the addition of M-CSF resulted in the stimulation of putative normal haemopoiesis and inhibition of its putative leukaemic counterpart. A significant decrease in the number of blasts in the cultures were observed. In a very recent study by Ferrero et al, (1992) it was shown that M-CSF

reduced the number of leukaemic colony forming units (L-CFU) in some cases of myelomonocytic leukaemia.

The action of GM-CSF on human myeloid leukaemic cells has been found to be primarily proliferative with uncoupling of differentiation (Moore, 1987; Lowenberg et al, 1988) however differentiation of the HL-60 cell line with GM-CSF has been reported (Moore, 1987).

We show that the radiation induced murine leukaemic models varied in their response to differentiation induction with the growth factors used. From our study and studies by others it appears to be indicated that most and possibly all the physiological regulatory proteins of the normal myeloid haemopoietic system have a potential use in differentiation induction therapy in myeloid leukaemia and that the leukaemic clonogenic cell fraction can be reduced with the use of physiological regulators of normal haemopoiesis.

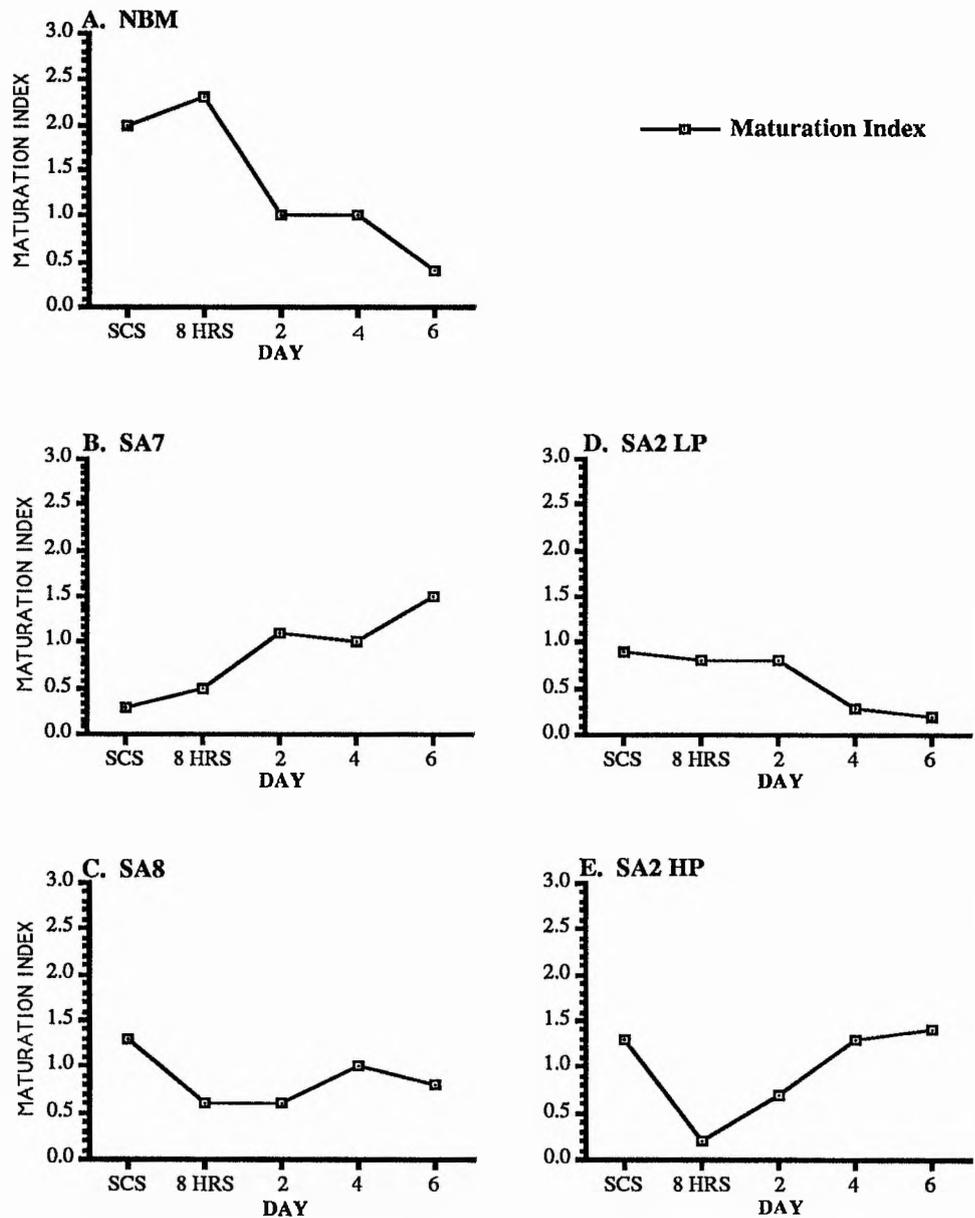
Differentiation study:  
WEHI-3B CM



**Figure 3.1** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of WEHI-3B CM in the microtitre assay. Cytospins were prepared and differentials were counted.

Immature cells = blasts, promyelocytes  
 Intermediate cells = myelocytes, immature monocytic cells and metamyelocytes  
 Mature cells = bands, segmented, monocytes and macrophages

Differentiation study:  
Maturation Index - WEHI-3B CM



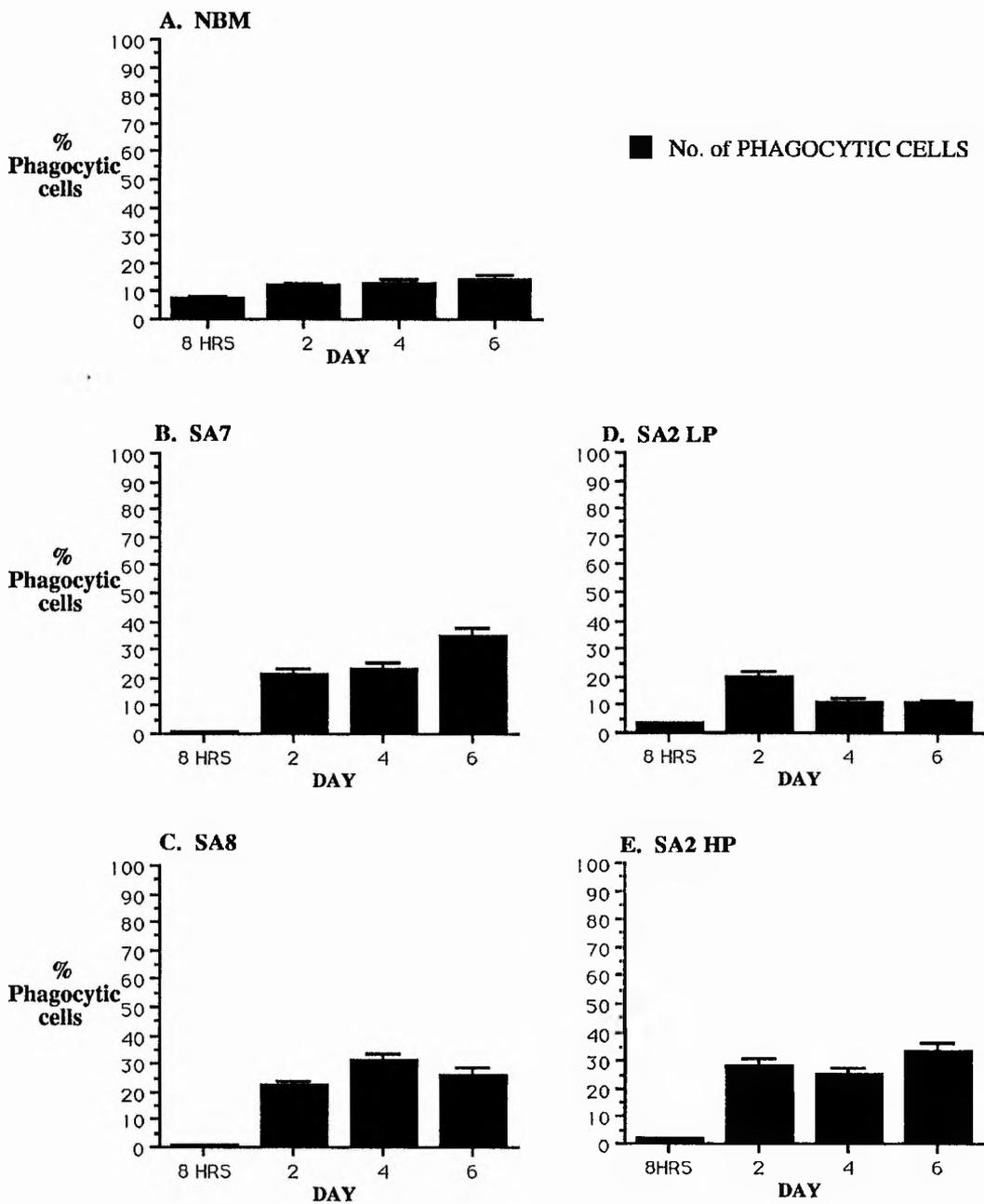
**Figure 3.2** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of WEHI-3B CM in the microtitre assay. Cytospins were prepared, differentials were counted and the maturation index determined.

Maturation Index = mature cells/immature cells

Mature cells = metamyelocytes, bands, segmented, monocytes, macrophages

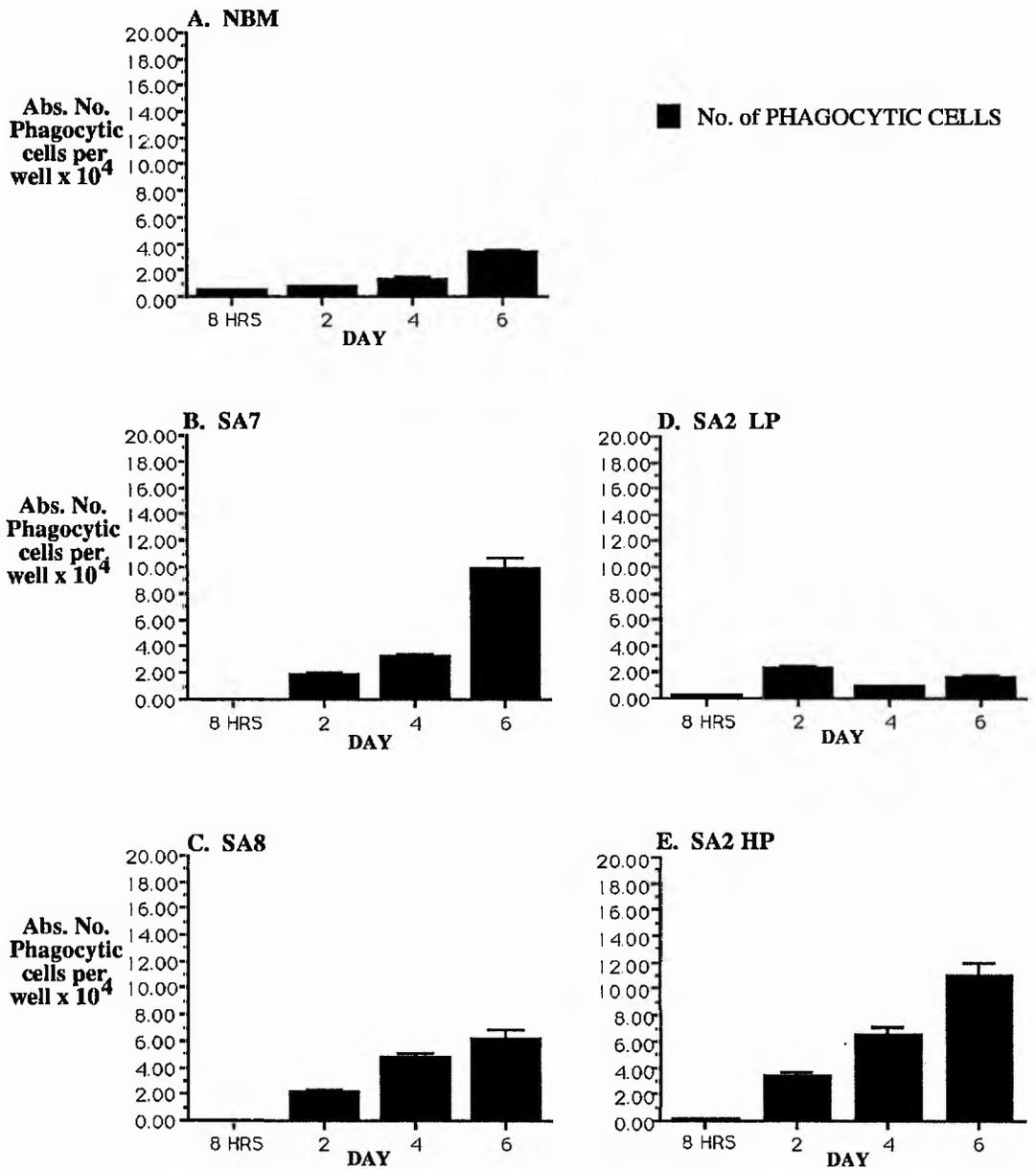
Immature cells = blasts, promyelocytes, myelocytes, immature monocytic cells

Differentiation study:  
WEHI-3B CM



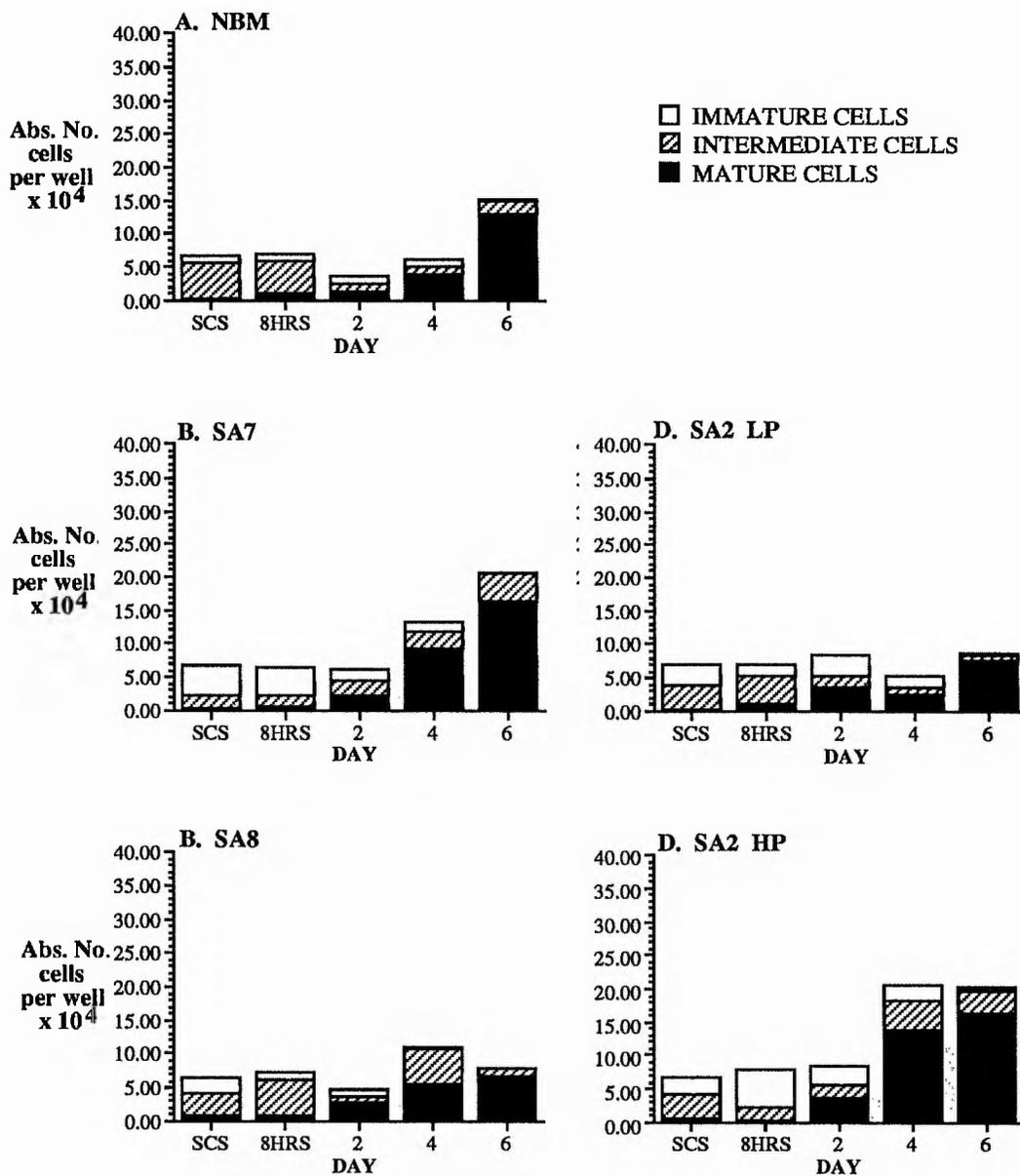
**Figure 3.3** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentration of WEHI-3B CM. Cells were incubated for eight hours with 0.8 $\mu$  latex particles prior to cytopins being prepared. The percentage number of phagocytic cells was determined.

Differentiation study:  
WEHI-3B CM



**Figure 3.4** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentration of WEHI-3B CM. Cells were incubated for eight hours with 0.8 $\mu$  latex particles prior to cytopins being prepared. The absolute number of phagocytic cells was determined.

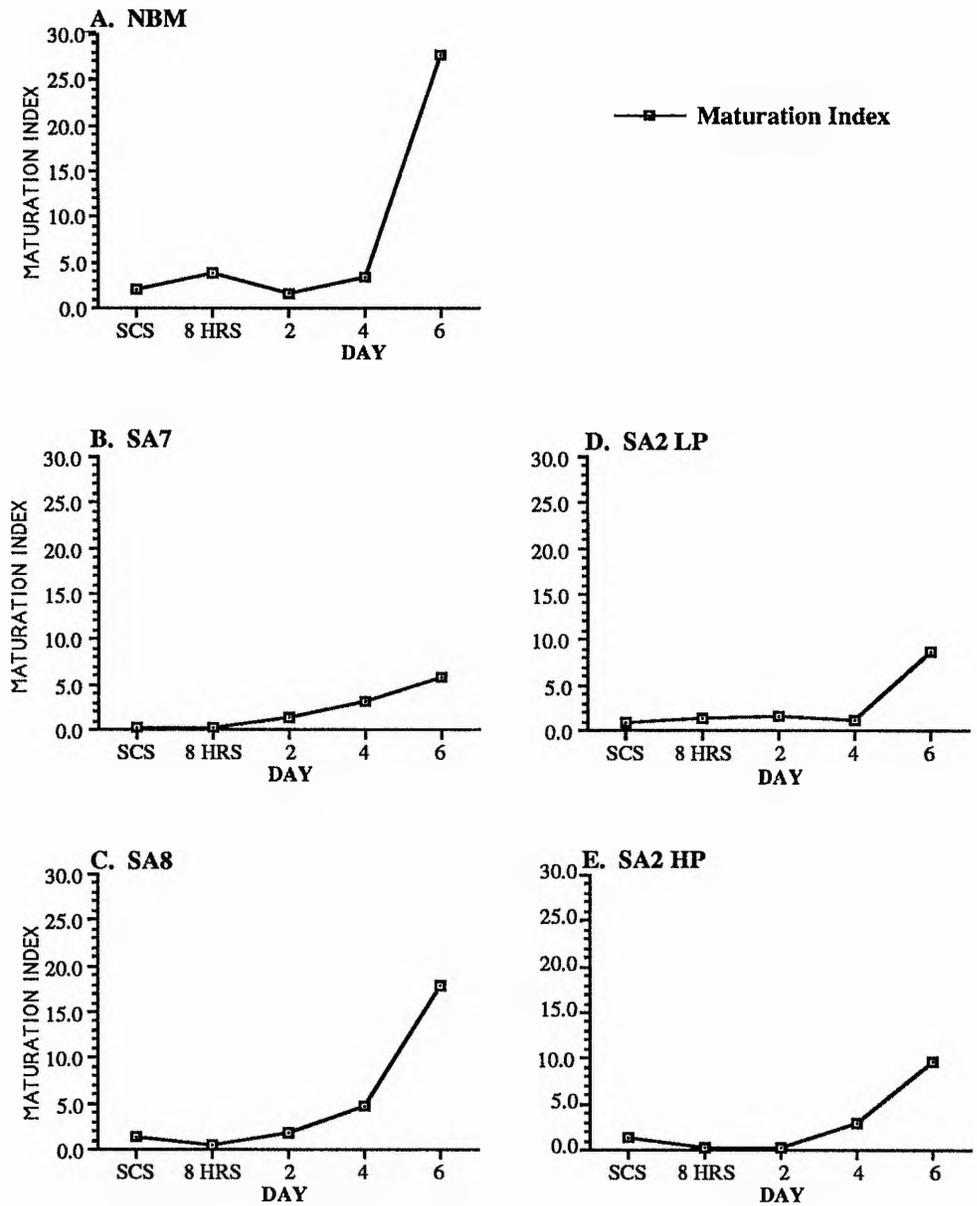
Differentiation study:  
L929 CM



**Figure 3.5** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of L929 CM in the microtitre assay. Cytospins were prepared and differentials were counted.

Immature cells = blasts, promyelocytes  
 Intermediate cells = myelocytes, immature monocytic cells and metamyelocytes  
 Mature cells = bands, segmented, monocytes and macrophages

Differentiation study:  
Maturation Index - L929 CM



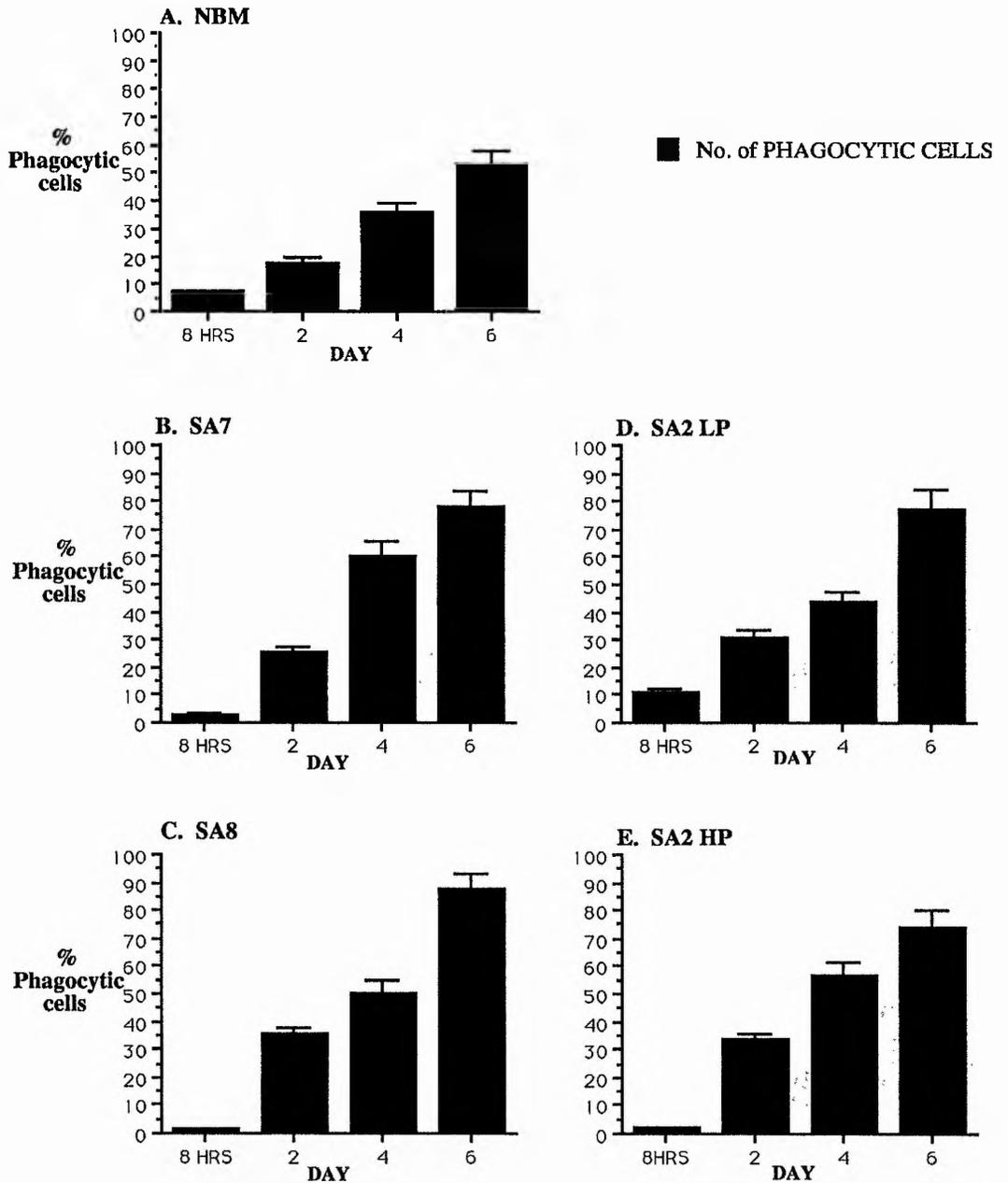
**Figure 3.6** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of L929 CM in the microtitre assay. Cytospins were prepared, differentials were counted and the maturation index determined.

Maturation Index = mature cells/immature cells

Mature cells = metamyelocytes, bands, segmented, monocytes, macrophages

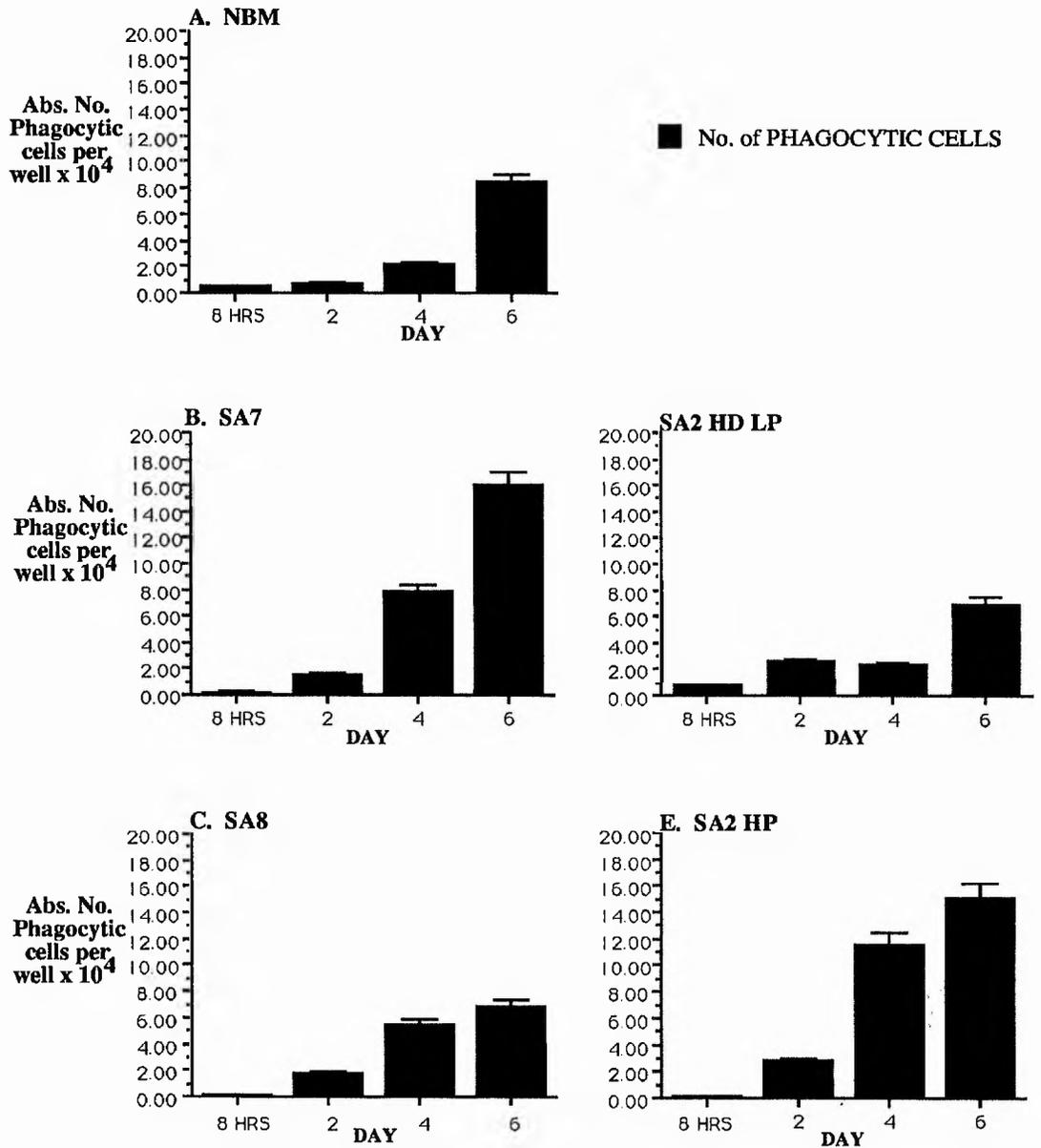
Immature cells = blasts, promyelocytes, myelocytes, immature monocytic cells

Differentiation study:  
L929 CM



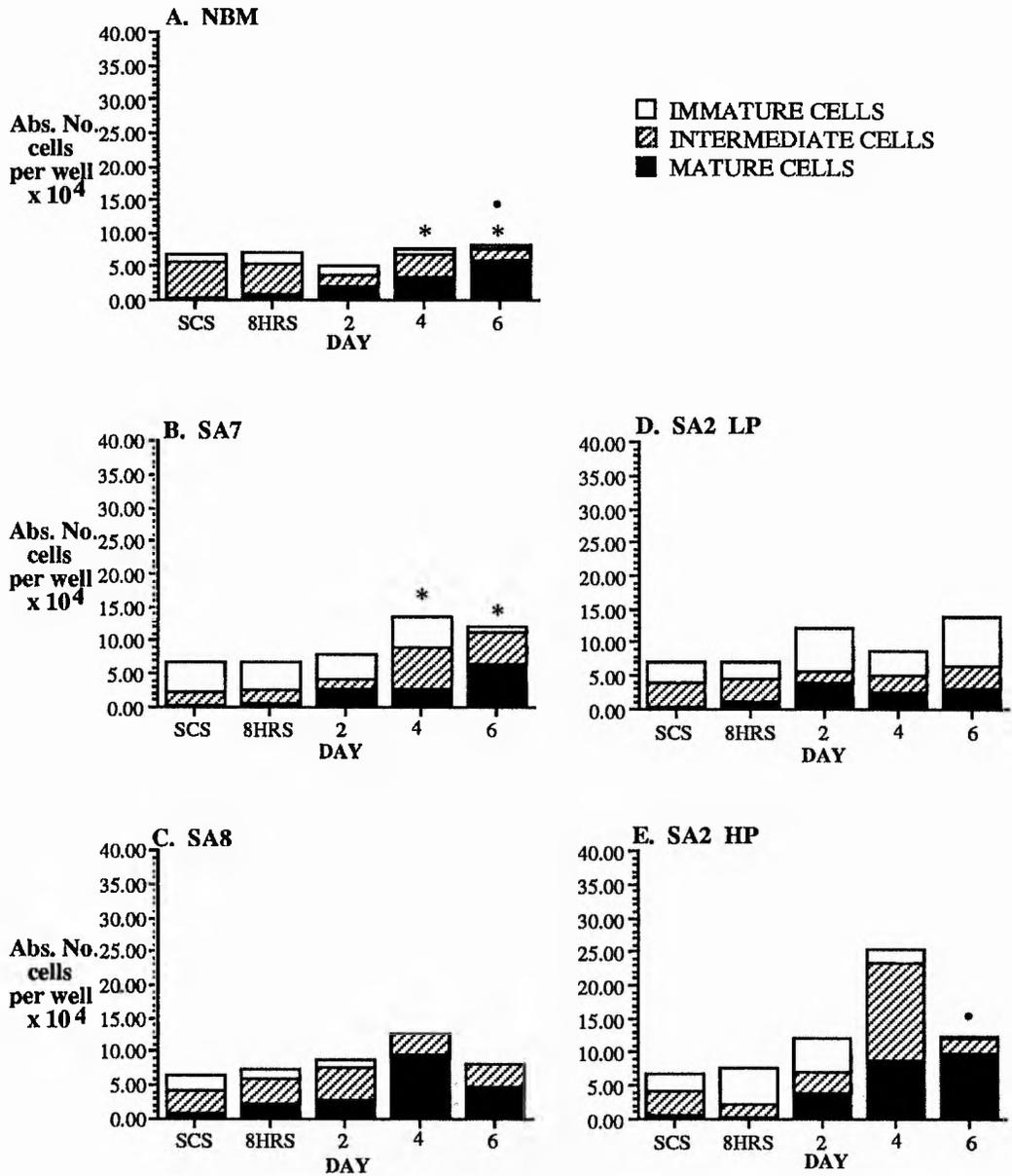
**Figure 3.7** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentration of L929 CM. Cells were incubated for eight hours with  $0.8\mu$  latex particles prior to cytopins being prepared. The percentage number of phagocytic cells was determined.

Differentiation study:  
L929 CM



**Figure 3.8** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentration of L929 CM. Cells were incubated for eight hours with 0.8 $\mu$  latex particles prior to cytopins being prepared. The absolute number of phagocytic cells was determined.

Differentiation study:  
rGMCSF



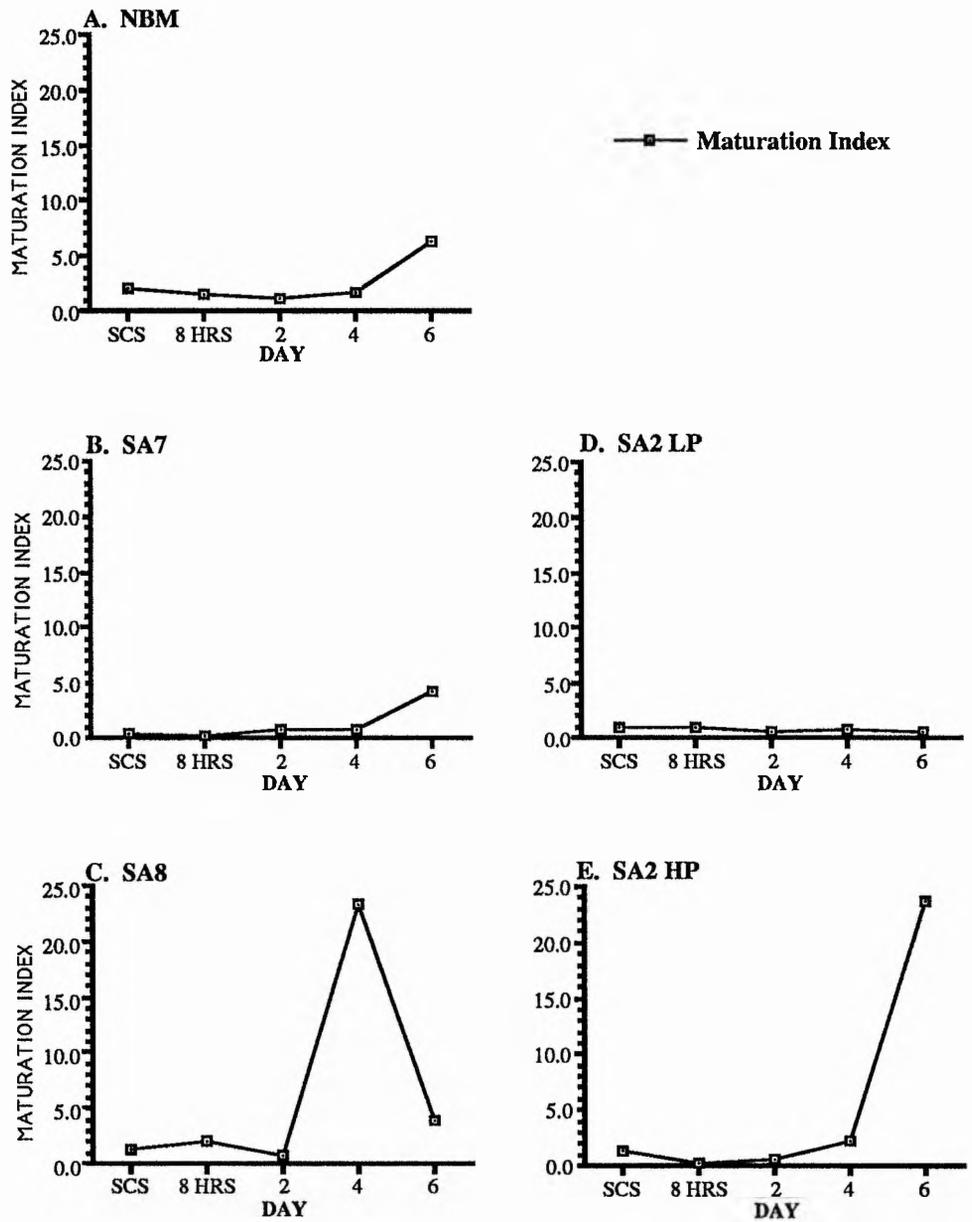
**Figure 3.9** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of rGM-CSF in the microtitre assay. Cytospins were prepared and differentials were counted. (\* $p < 0.01$  A significantly higher percentage number of mature cell were obtained with NBM compared to the SA7 leukaemia. • $p < 0.05$  A significantly lower number of immature cells were observed with the SA2 HP leukaemia than NBM).

Immature cells = blasts, promyelocytes

Intermediate cells = myelocytes, immature monocytic cells and metamyelocytes

Mature cells = bands, segmented, monocytes and macrophages

Differentiation study:  
Maturation Index - rGM-CSF



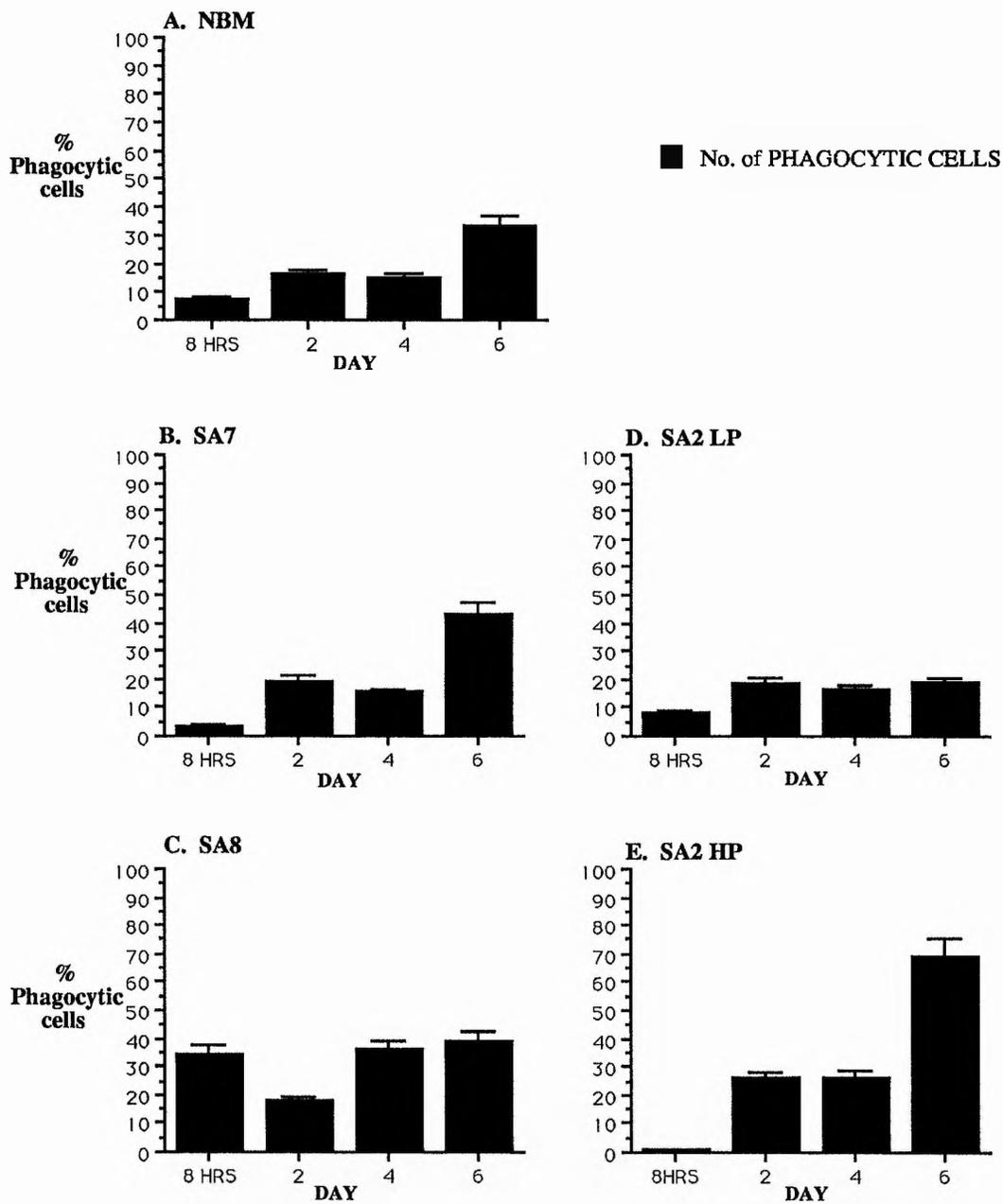
**Figure 3.10** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of rGM-CSF in the microtitre assay. Cytospins were prepared, differentials were counted and the maturation index determined.

Maturation Index = mature cells/immature cells

Mature cells = metamyelocytes, bands, segmented, monocytes, macrophages

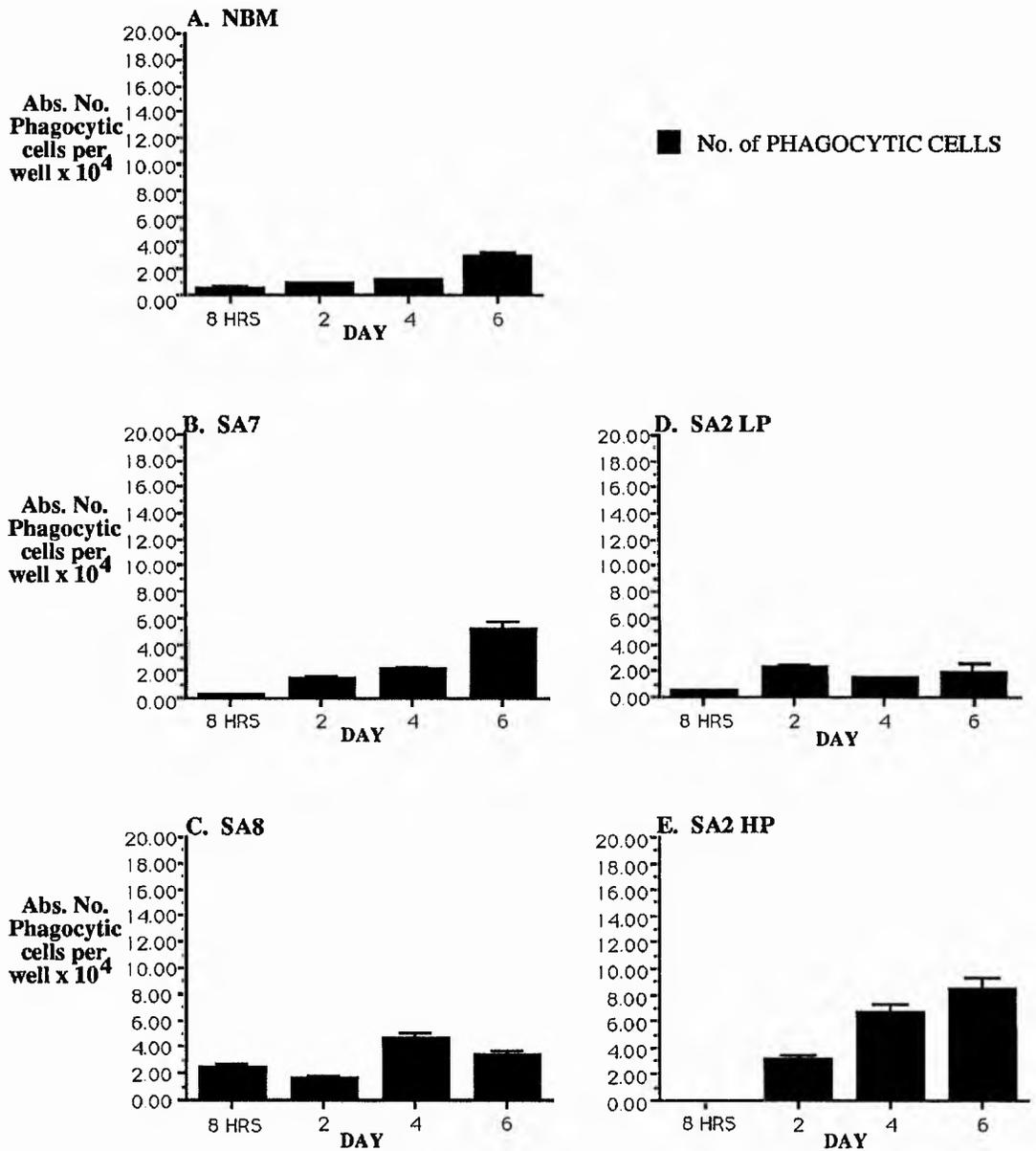
Immature cells = blasts, promyelocytes, myelocytes, immature monocytic cells

Differentiation study:  
rGM-CSF



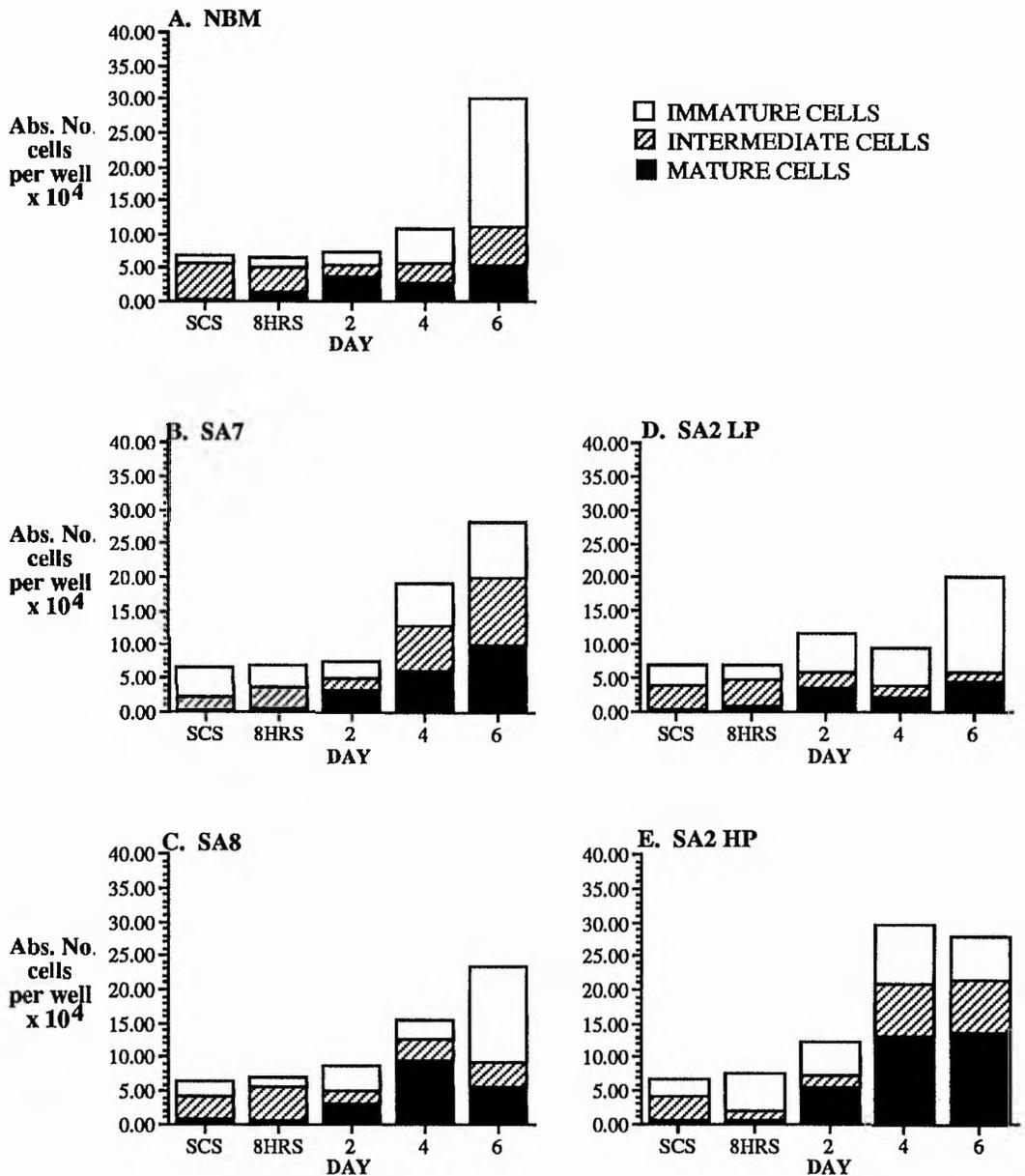
**Figure 3.11** Normal and leukaemic cells were cultured for eight hours, two four and six days in the microtitre assay with optimal concentration of rGM-CSF. Cells were incubated for eight hours with 0.8 $\mu$  latex particles prior to cytopins being prepared. The percentage number of phagocytic cells was determined.

Differentiation study:  
rGM-CSF



**Figure 3.12** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentration of rGM-CSF. Cells were incubated for eight hours with 0.8 $\mu$  latex particles prior to cytopins being prepared. The absolute number of phagocytic cells was determined.

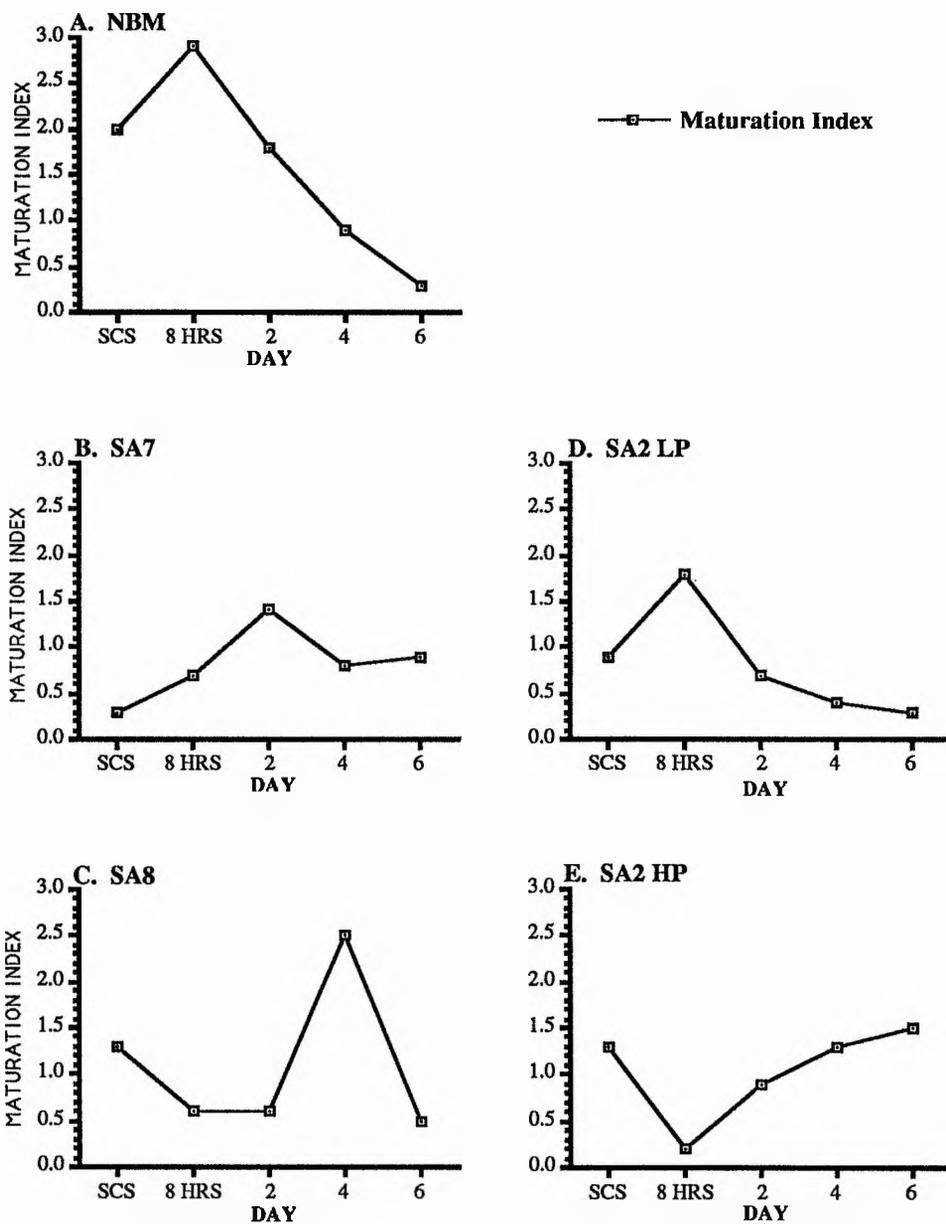
Differentiation study:  
WEHI-3B CM + L929 CM



**Figure 3.13** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM plus L929 CM in the microtitre assay. Cytospins were prepared and differentials were counted.

Immature cells = blasts, promyelocytes  
 Intermediate cells = myelocytes, immature monocytic cells and metamyelocytes  
 Mature cells = bands, segmented, monocytes and macrophages

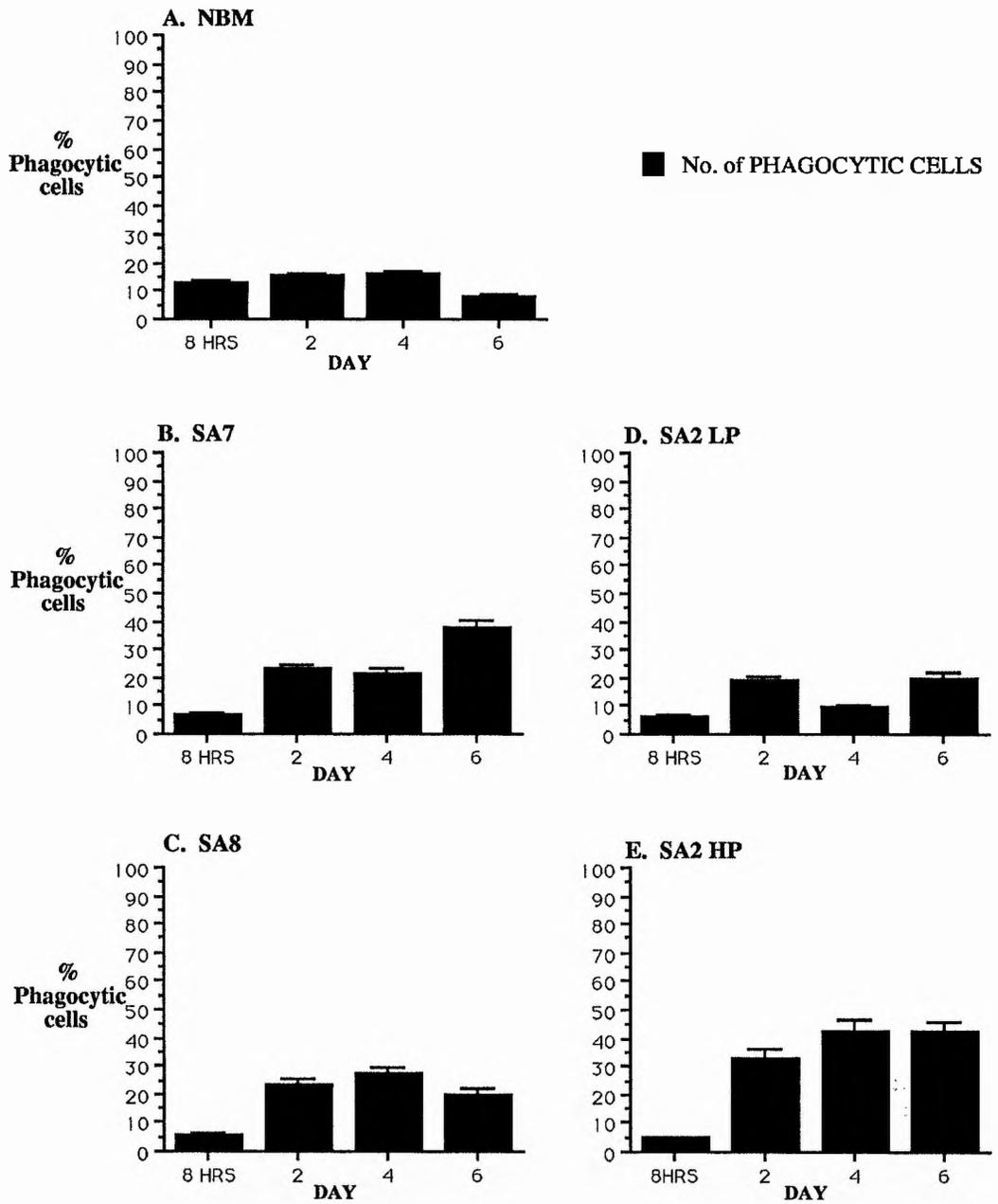
Differentiation study:  
Maturation Index - WEHI-3B CM + L929 CM



**Figure 3.14** Differentiation study. Cells were cultured for eight hours, two, four and days with optimal concentration of WEHI-3B plus L929 CM in the microtitre assay. Cytospins were prepared, differentials were counted and the maturation index determined.

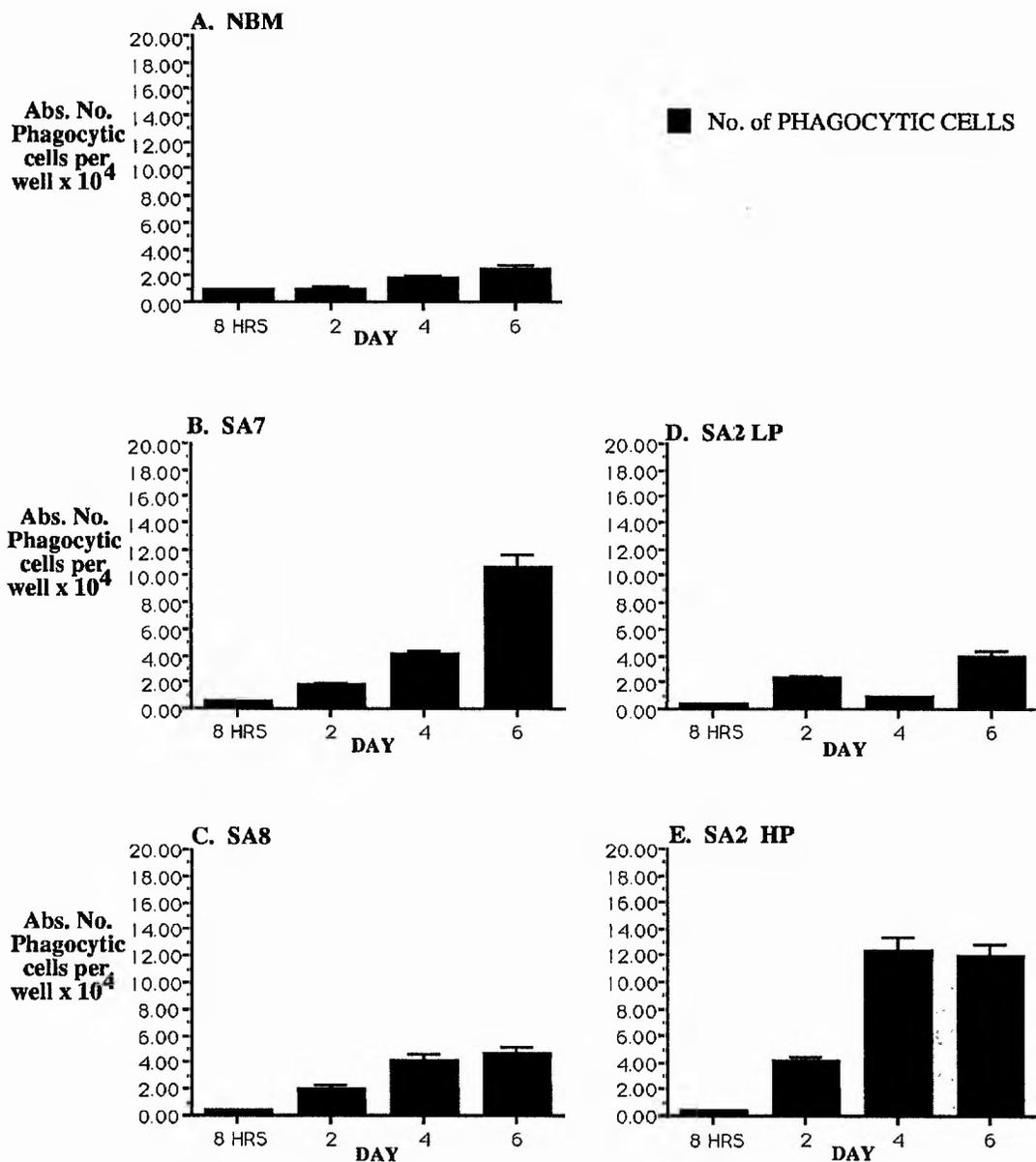
Maturation Index = mature cells/immature cells  
 Mature cells = metamyelocytes, bands, segmented, monocytes, macrophages  
 Immature cells = blasts, promyelocytes, myelocytes, immature monocytic cells

Differentiation study:  
WEHI-3B CM + L929 CM



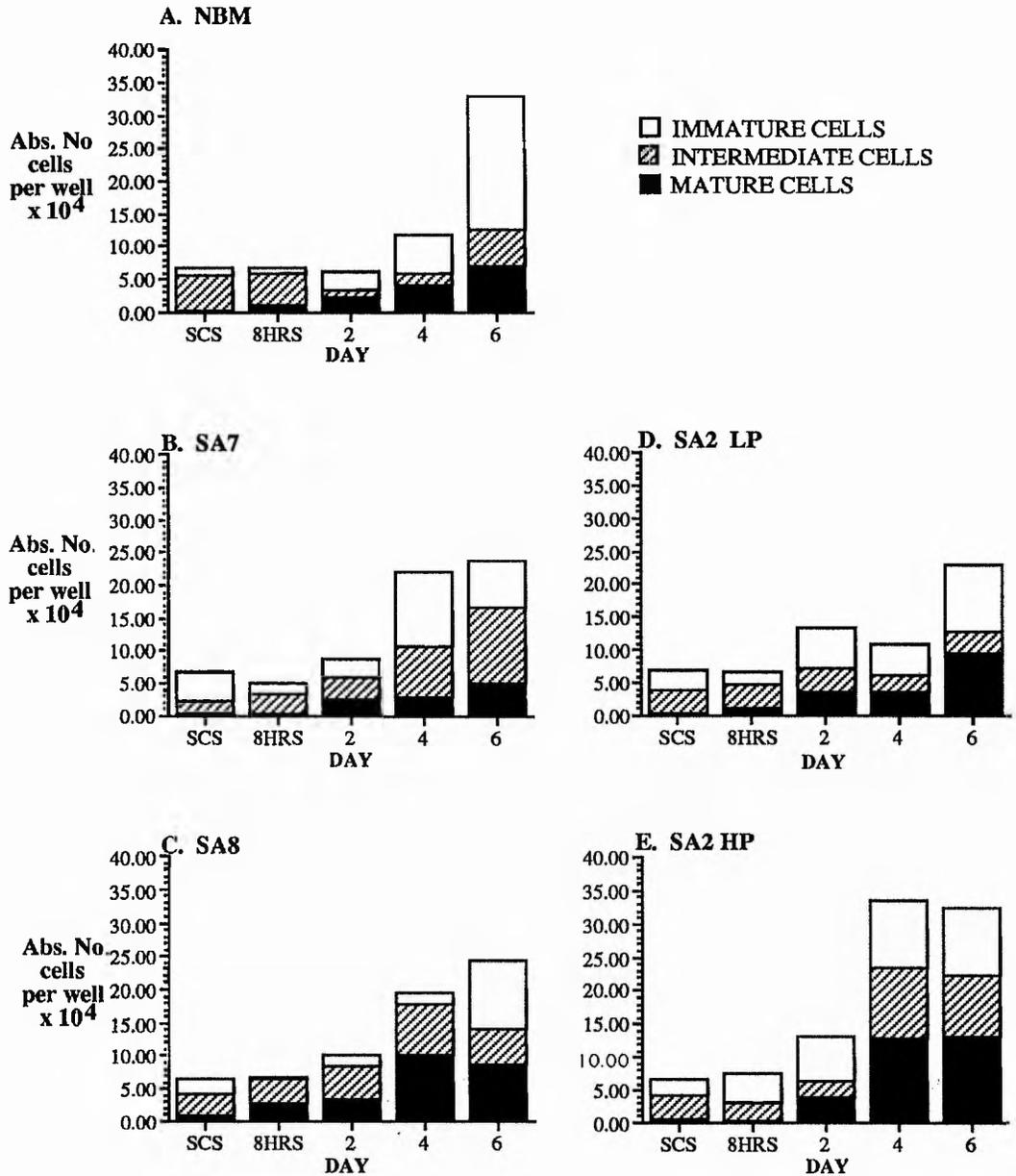
**Figure 3.15** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentration of WEHI-3B CM plus L929 CM. Cells were incubated for eight hours with  $0.8\mu$  latex particles prior to cytopspins being prepared. The percentage number of phagocytic cells was determined.

Differentiation study:  
WEHI-3B CM + L929 CM



**Figure 3.16** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentrations of WEHI-3B CM plus L929 CM. Cells were incubated for eight hours with 0.8 $\mu$  latex particles prior to cytopins being prepared. The absolute number of phagocytic cells was determined.

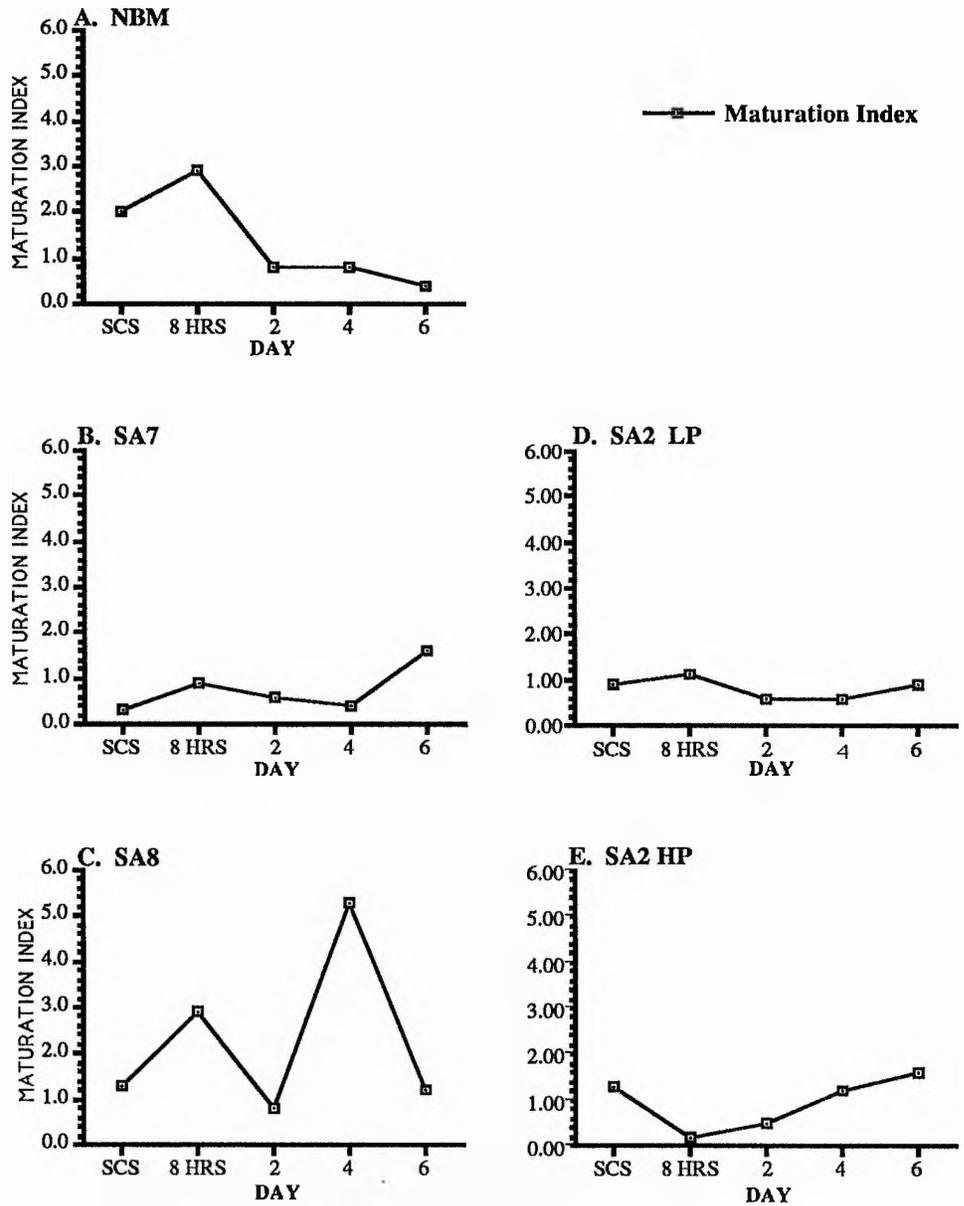
Differentiation study:  
WEHI-3B CM + rGMCSF



**Figure 3.17** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of WEHI-3B CM plus rGM-CSF in the microtitre assay. Cytospins were prepared and differentials were counted.

Immature cells = blasts, promyelocytes  
 Intermediate cells = myelocytes, immature monocytic cells and metamyelocytes  
 Mature cells = bands, segmented, monocytes and macrophages

Differentiation study:  
Maturation Index - WEHI-3B CM + rGM-CSF



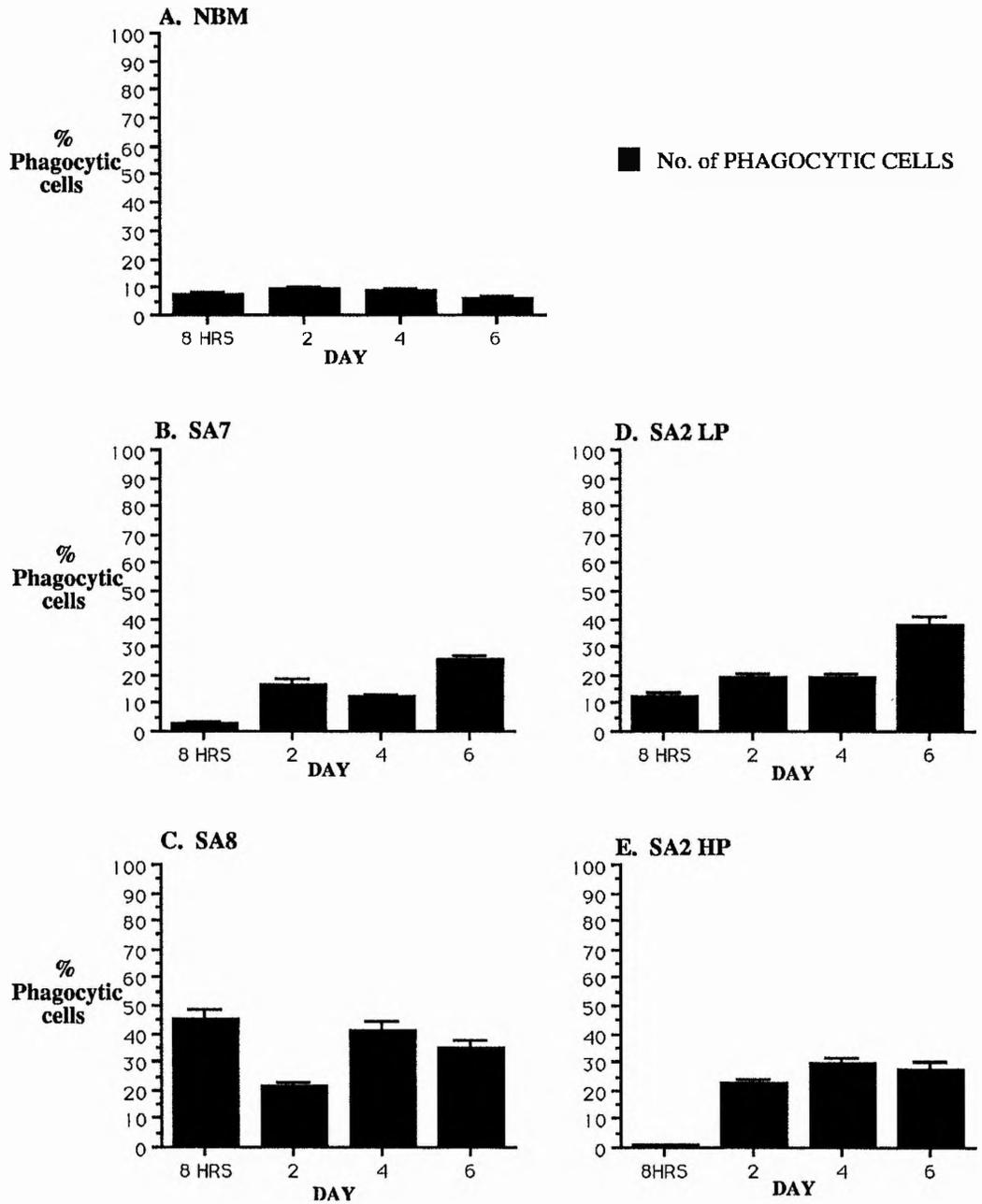
**Figure 3.18** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of WEHI-3B CM plus rGM-CSF in the microtitre assay. Cytospins were prepared, differentials were counted and the maturation index determined.

Maturation Index = mature cells/immature cells

Mature cells = metamyelocytes, bands, segmented, monocytes, macrophages

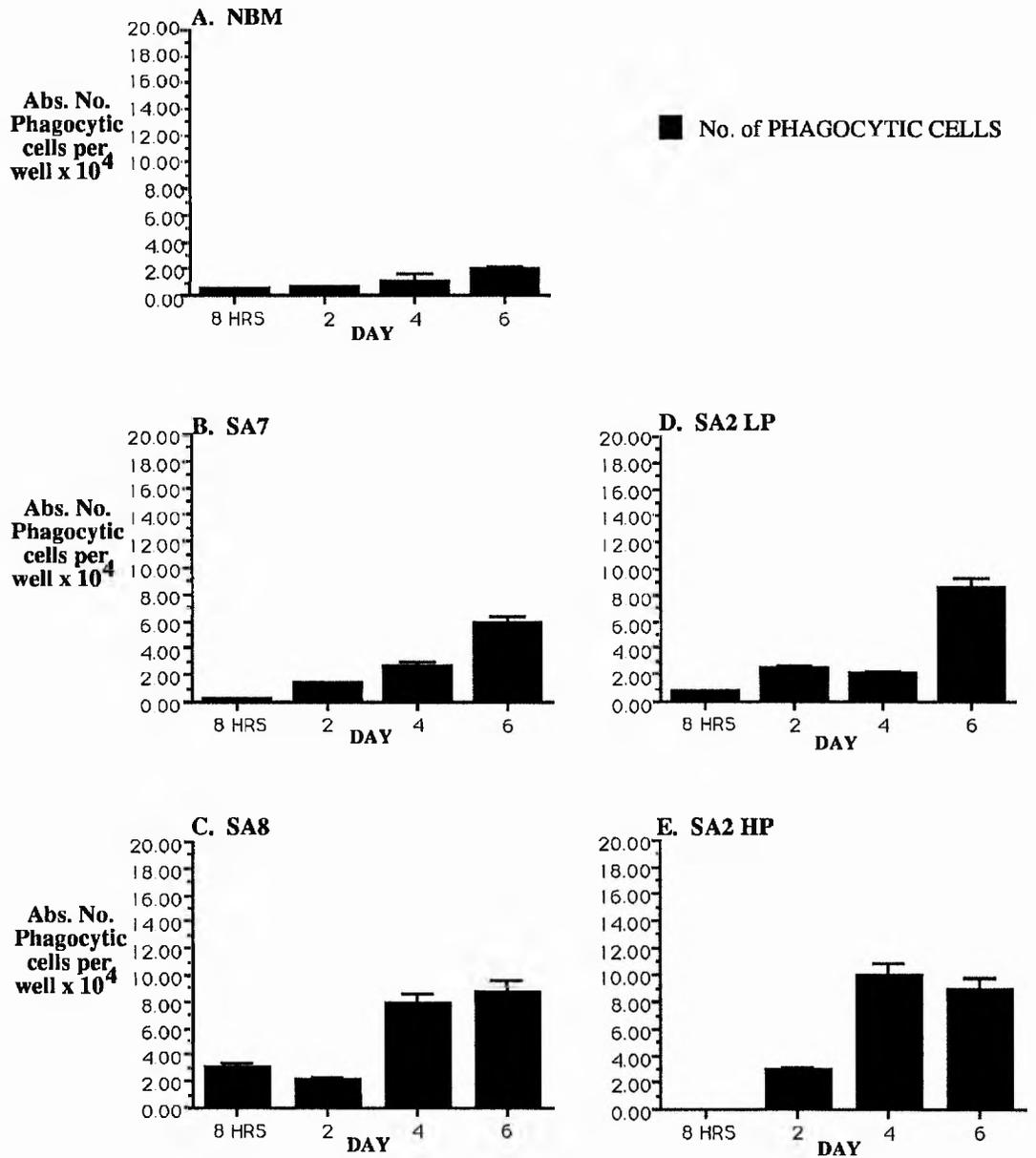
Immature cells = blasts, promyelocytes, myelocytes, immature monocytic cells

Differentiation study:  
WEHI-3B CM + rGM-CSF



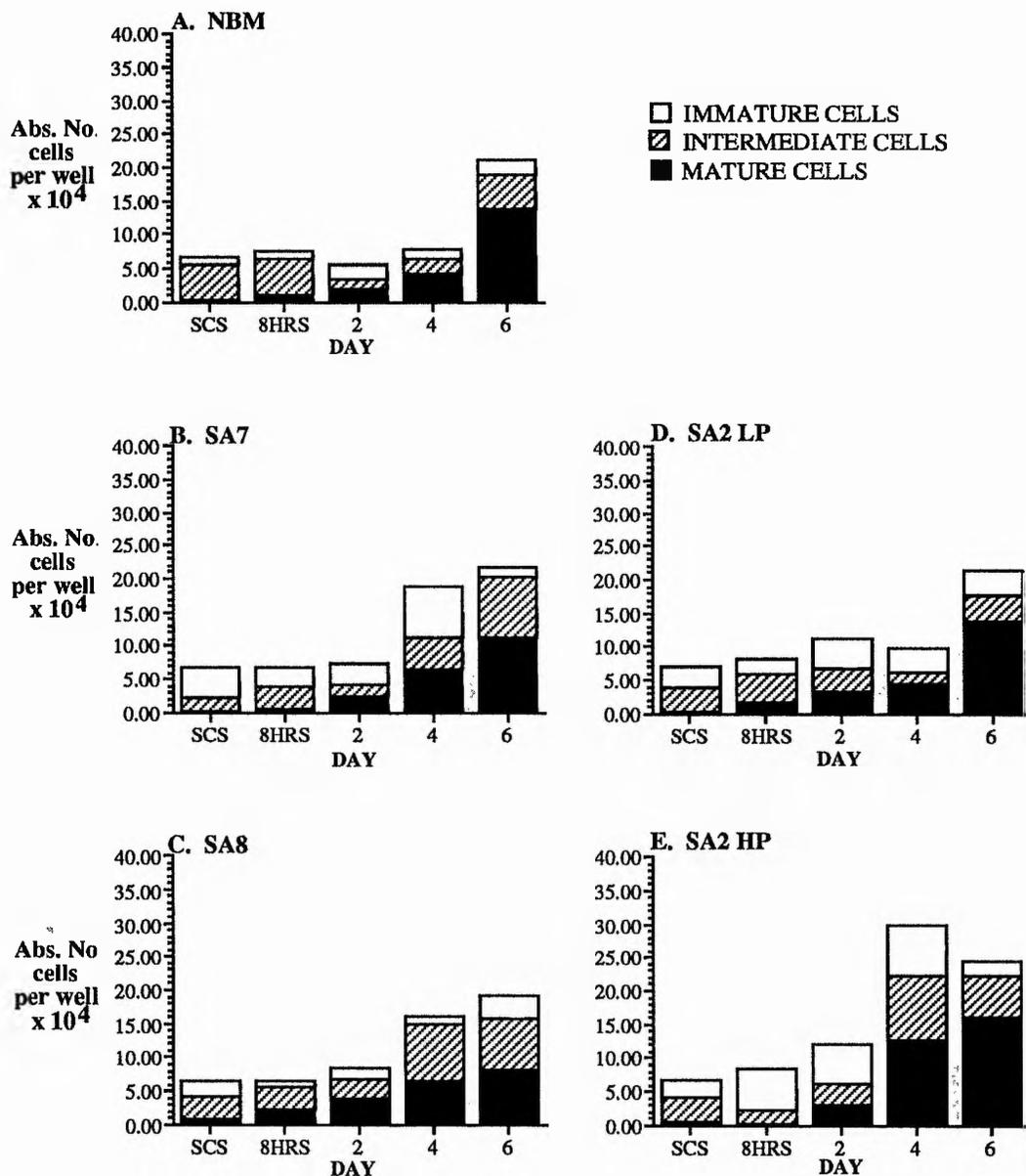
**Figure 3.19** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentration of WEHI-3B CM plus rGM-CSF. Cells were incubated for eight hours with 0.8 $\mu$  latex particles prior to cytopins being prepared. The percentage number of phagocytic cells was determined.

Differentiation study:  
WEHI-3B CM + rGM-CSF



**Figure 3.20** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentrations of WEHI-3B CM plus rGM-CSF. Cells were incubated for eight hours with 0.8 $\mu$  latex particles prior to cytopspins being prepared. The absolute number of phagocytic cells was determined.

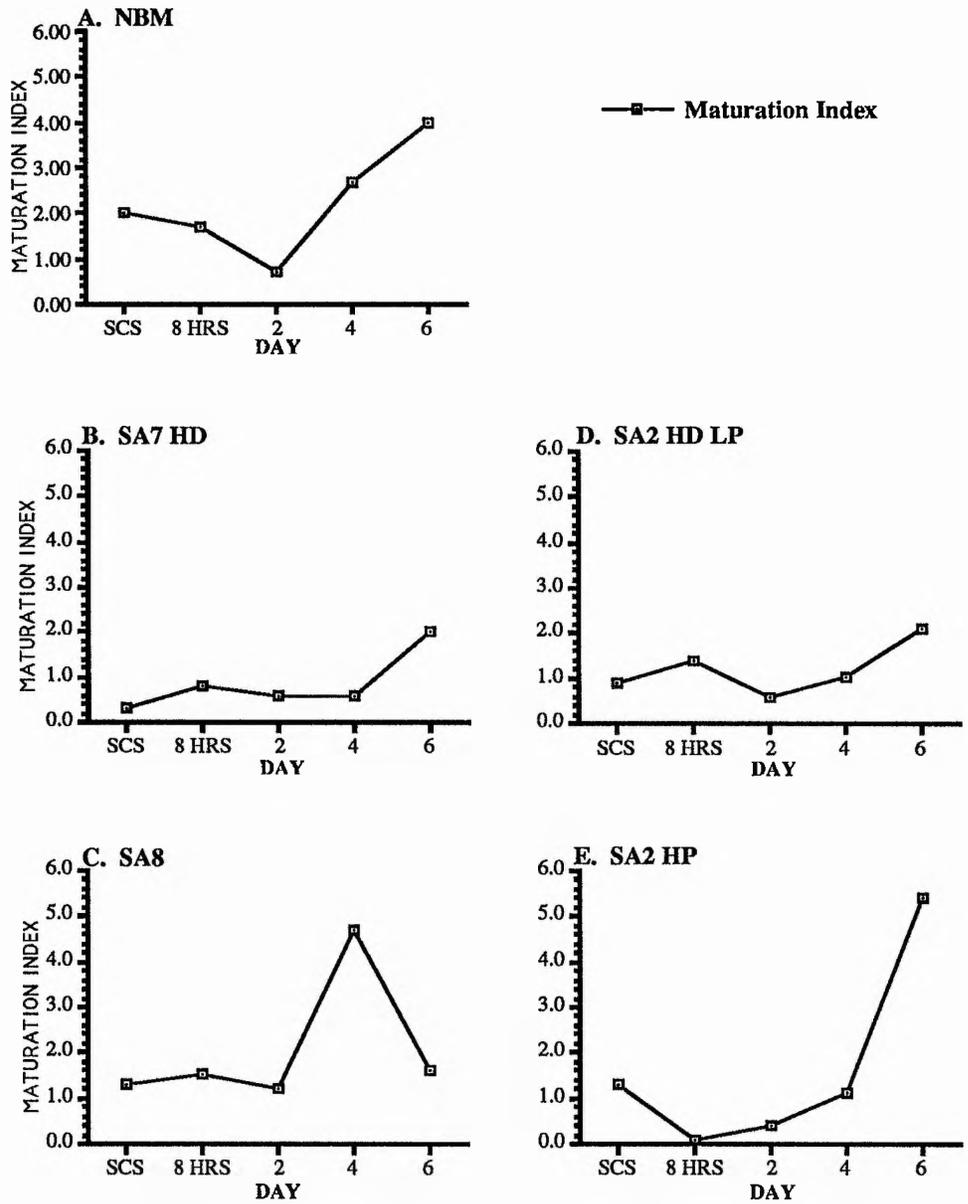
Differentiation study:  
L929 CM + rGMCSF



**Figure 3.21** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of L929 CM plus rGM-CSF in the microtitre assay. Cytospins were prepared and differentials were counted.

Immature cells = blasts, promyelocytes  
 Intermediate cells = myelocytes, immature monocytic cells and metamyelocytes  
 Mature cells = bands, segmented, monocytes and macrophages

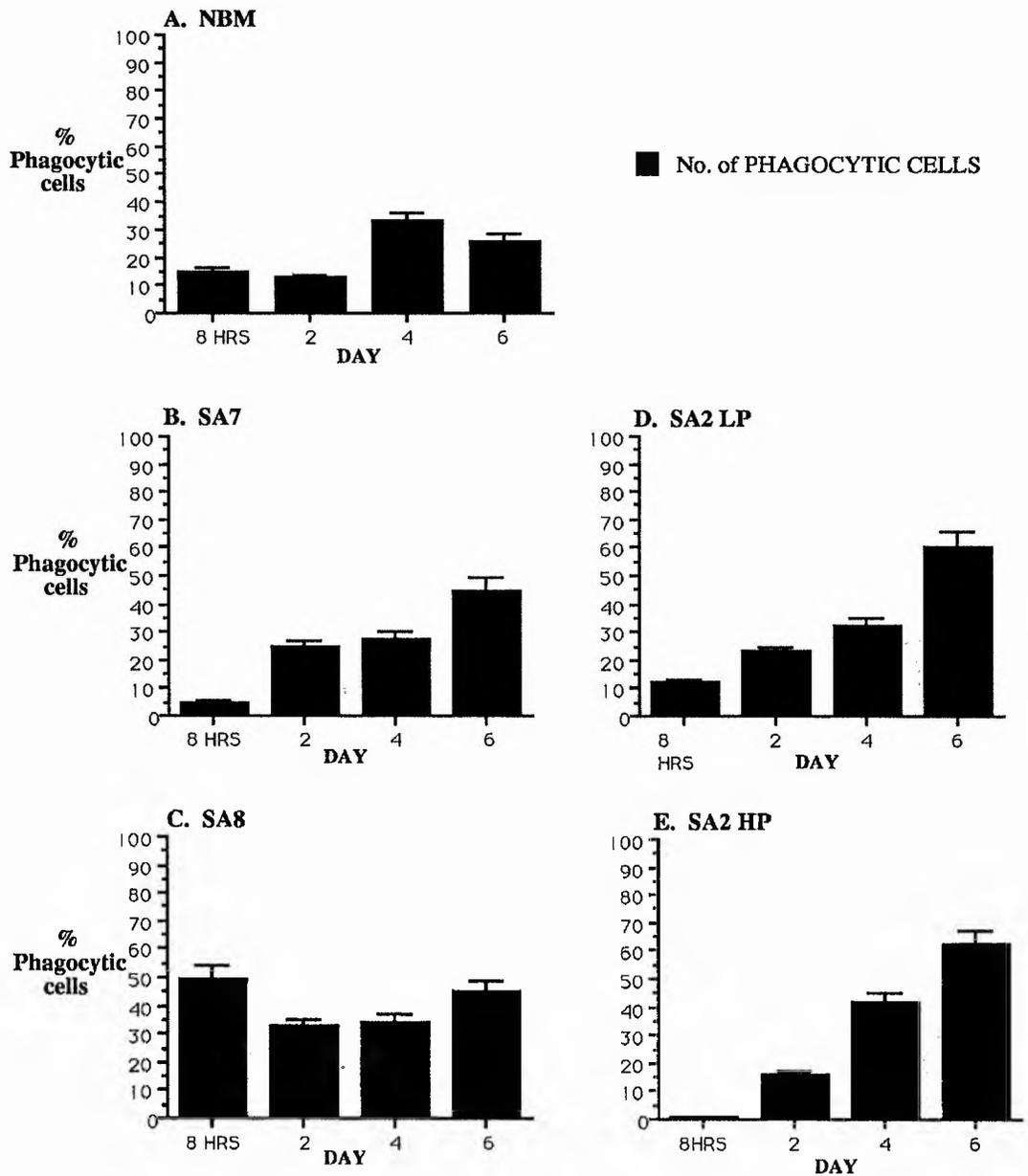
Differentiation study:  
Maturation Index - L929 CM + rGM-CSF



**Figure 3.22** Differentiation study. Cells were cultured for eight hours, two, four and six days with optimal concentration of L929 CM plus rGM-CSF in the microtitre assay. Cytospins were prepared, differentials were counted and the maturation index determined.

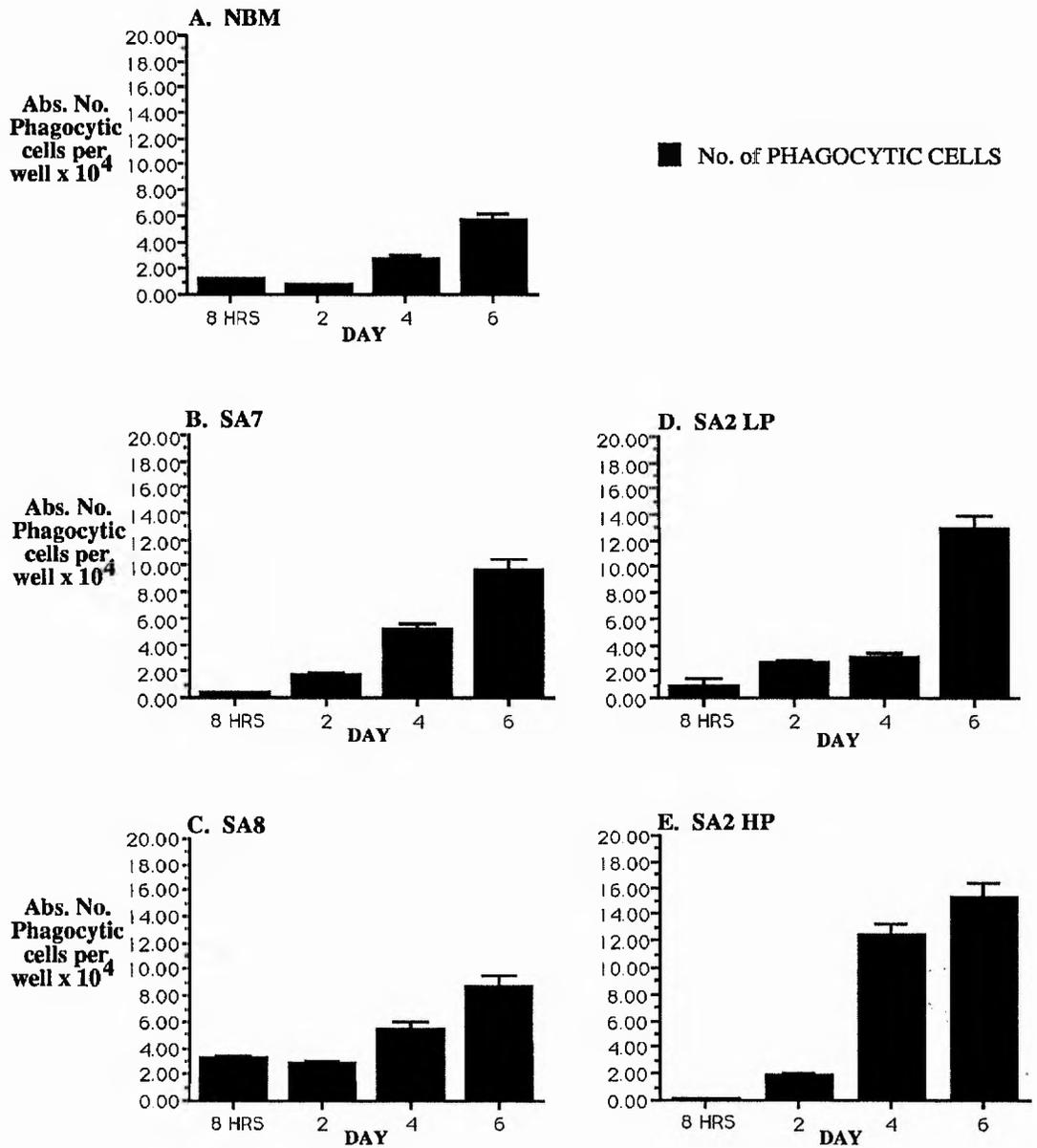
Maturation Index = mature cells/immature cells  
 Mature cells = metamyelocytes, bands, segmented, monocytes, macrophages  
 Immature cells = blasts, promyelocytes, myelocytes, immature monocytic cells

Differentiation study:  
L929 CM + rGM-CSF



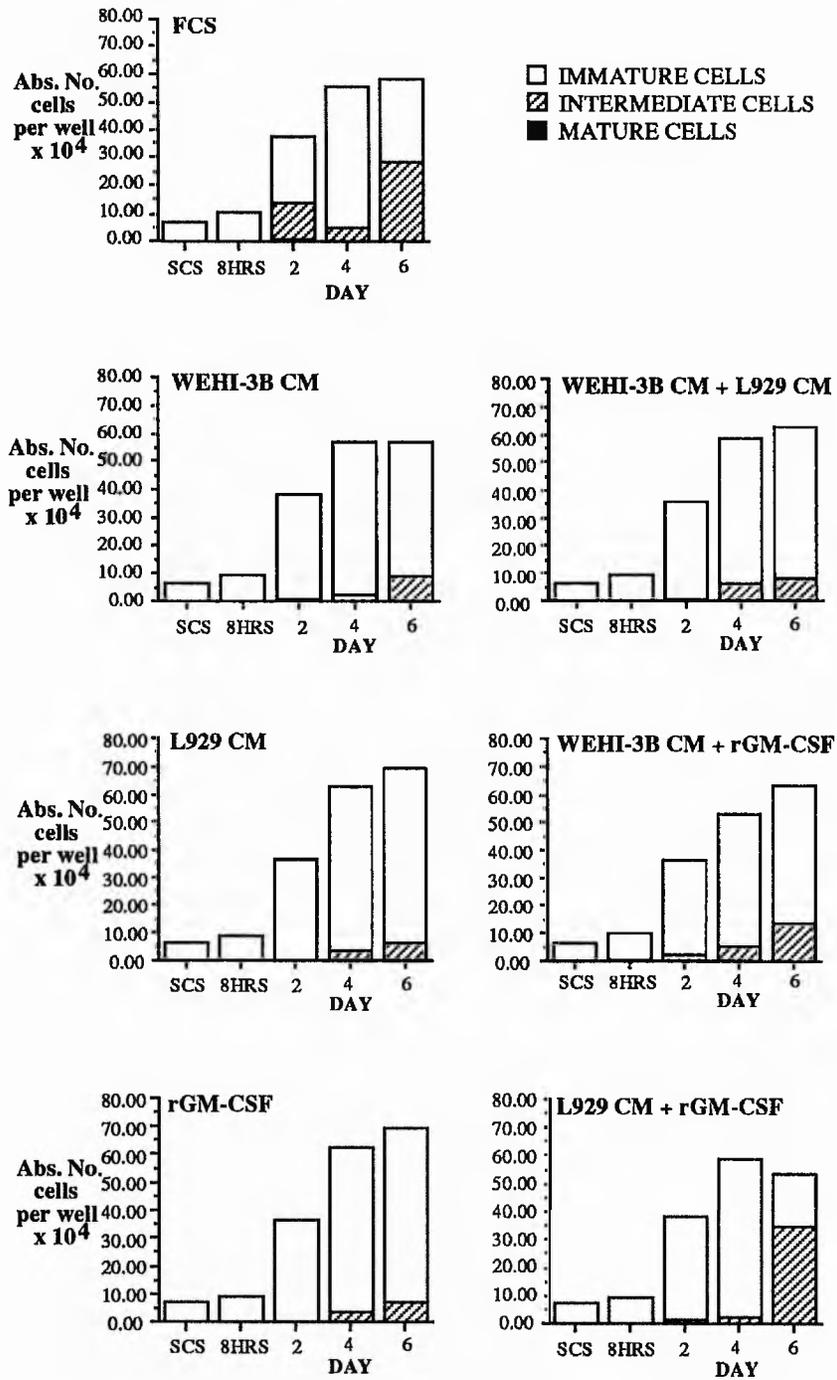
**Figure 3.23** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentration of L929 CM plus rGM-CSF. Cells were incubated for eight hours with  $0.8\mu$  latex particles prior to cytopins being prepared. The percentage number of phagocytic cells was determined.

Differentiation study:  
L929 CM + rGM-CSF



**Figure 3.24** Normal and leukaemic cells were cultured for eight hours, two, four and six days in the microtitre assay with optimal concentration of L929 CM plus rGM-CSF. Cells were incubated for eight hours with 0.8 $\mu$  latex particles prior to cytopins being prepared. The absolute number of phagocytic cells was determined.

Differentiation study:  
The SA2 CL



**Figure 3.25** Differentiation study. The SA2 CL was cultured for eight hours, two, four and six days with optimal concentration of growth factor(s) in the microtitre assay. Cytospins were prepared and differentials were counted.

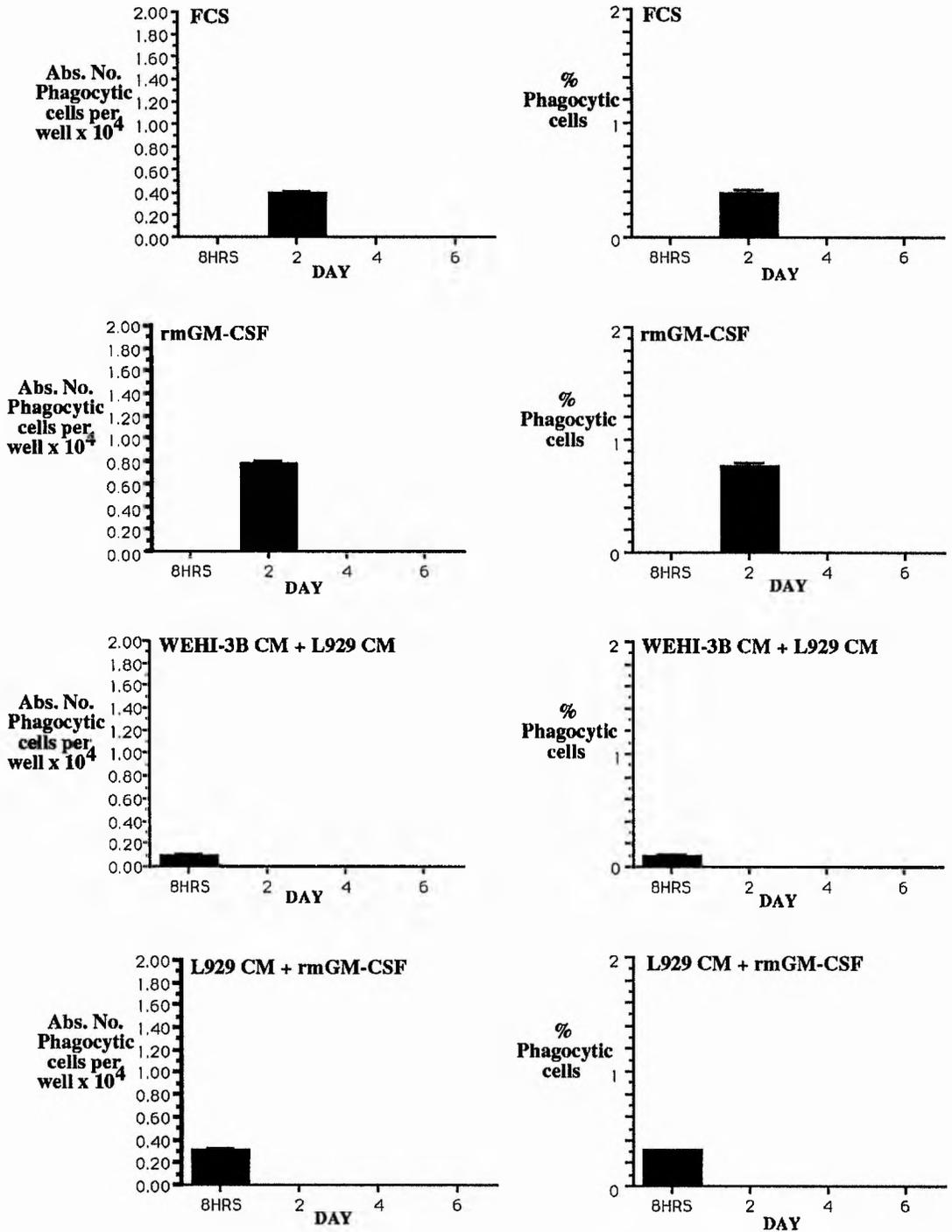
Immature cells =blasts, promyelocytes

Intermediate cells=myelocytes, immature monocytic cells, metamyelocytes

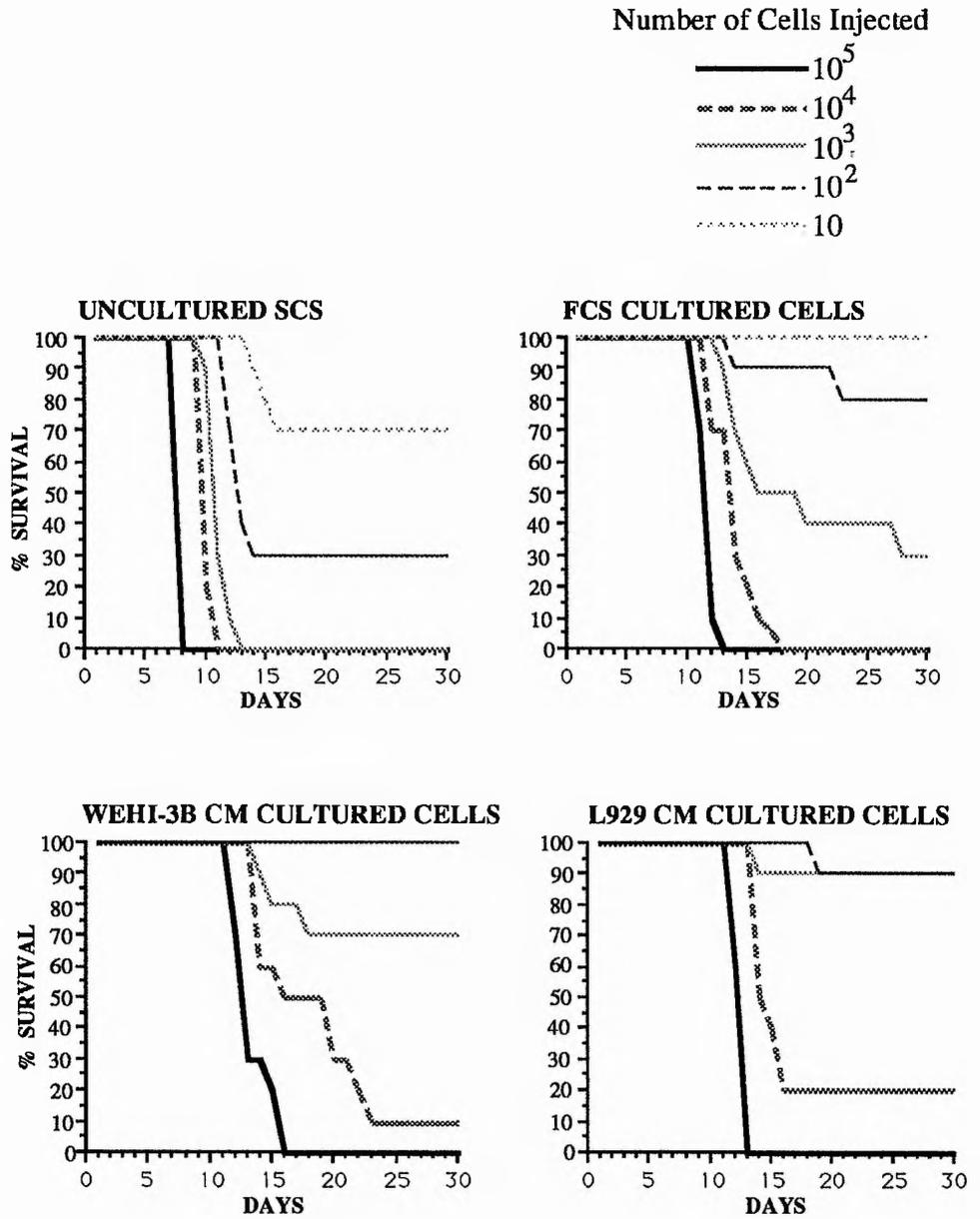
Mature cells =bands, segmented, monocytes and macrophages

Differentiation study:  
The SA2 CL

■ No. of PHAGOCYtic CELLS



**Figure 3.26** Differentiation study. Cells from the SA2 CL were cultured for eight hours, two, four and six days with optimal concentration of growth factor(s) in the microtitre assay. Cytopins were prepared and differentials were counted.



**Figure 3.27.** *In vivo* Clonogenic Cell Assay. Uncultured single cell suspension and cells cultured for four days in the microtitre assay with FCS, and optimal concentrations of WEHI-3B CM or L929 CM from the SA7 leukaemic bone marrow were injected into syngeneic recipients at varied cell concentrations ( $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ , 10 total cells). Percentage leukaemia free survival was monitored for 90 days.

## CHAPTER FOUR

### DIFFERENTIATION EFFECTS OF $\beta$ -ALL TRANS RETINOIC ACID ON LEUKAEMIC AND NORMAL BONE MARROW CELLS

Current therapeutic regimes for the treatment of acute myeloid leukaemia (AML) rely upon non-specific cytotoxicity and although a number of advances have been made with various combinations and scheduling of chemotherapeutic agents a significantly high percentage of patients relapse. Often the leukaemic clones present in relapse are aggressive and refractory to treatment (Chomienne, 1991). Differentiation induction regimes may be able to provide a maintenance rather than cure strategy approach for the treatment of AML. What remains to be elucidated is whether long term use of differentiation inducing agents would prevent the emergence of refractory leukaemic clones and maintain the patient in long term complete remission.

It has been postulated that induction of differentiation of leukaemic cell populations may be compensated by an increase in the proliferation of leukaemic clonogenic cell fraction (Francis et al 1985). Studies however, have not been extended to determine whether differentiation induction with  $\beta$ atRA increases or decreases the leukaemic clonogenic cell fraction. The role of  $\beta$ atRA in the maintenance of remission has not been elucidated. These studies are important in determining whether complete long term remission is obtainable in  $\beta$ atRA responsive patients. The transplanted murine leukaemic cell lines provide a useful tool for *in vivo* studies on leukaemic clonogenic cells.

The aim of this study was three fold:-

1. To study the differentiation effect of  $\beta$ atRA on bone marrow cells from the myeloid leukaemic models *in vitro*.

2. To determine any differences in response to  $\beta$ tRA when growth factors with different modes of action on normal and leukaemic cells were used in the culture system. The two growth factors studied were WEHI-3B CM, which was shown to sustain relatively high numbers of immature leukaemic cells in culture, and L929 CM which was more effective as a differentiation inducer of leukaemic cells than WEHI-3B CM and sustained fewer immature cells.

3. To determine whether differentiation induction by  $\beta$ tRA decreases the clonogenic leukaemic cell fraction *in vitro*. An *in vivo* Clonogenic Cell Assay was used for this study.

Studies *in vitro* have shown that RA was a potent inducer of myeloid differentiation both in the promyelocytic cell line HL-60 and fresh promyelocytes from patients with acute promyelocytic leukaemia (APL) (Lippman et al, 1987; Breitman et al, 1981; Douer and Koeffler, 1982a; Fabian et al, 1986; Imaizumi et al, 1987; Lawrence et al, 1987). RA induced differentiation at concentrations which were pharmacologically tolerable to humans and differentiation induction has been achieved *in vivo* in patients with APL (Flynn et al., 1983; Nilsson, 1984; Daenen et al, 1986; Fontana et al, 1986). Previous studies have employed 13-*cis* RA, our aim was to evaluate the efficacy of  $\beta$ tRA.

#### **4.1 IN VITRO PROLIFERATIVE EFFECTS OF $\beta$ -ALL TRANS RETINOIC ACID ON LEUKAEMIC AND NORMAL CELLS.**

Preliminary *in vitro* studies were carried out to determine the concentration of  $\beta$ tRA necessary to reduce the proliferation of normal and leukaemic bone marrow cells and cells from the SA2 CL by utilizing the uptake of  $^3\text{H}$ -TdR in the microtitre assay.

#### 4.1.1 Experimental Procedure

Single cell suspensions of normal and leukaemic bone marrow cells and cells from the SA2 CL were cultured in the microtitre assay for four days with decreasing concentrations of  $\beta$ atRA ( $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$  M total concentration per well). For the growth factor dependent cells, WEHI-3B CM was used in culture as the growth factor source as it was observed to induce the highest proliferative and least differentiative effects of the growth factors used in this study (see Chapter 4). On day four the cells were pulsed with  $^3\text{H-TdR}$  for eight hours, harvested and the  $^3\text{H-TdR}$  uptake measured on the scintillation counter. The CPM results were expressed as 'Percentage of Untreated Cells' and plotted.

#### 5.1.2 Results

$10^{-5}\text{M}$  all-trans  $\beta$ atRA had a toxic effect on normal and leukaemic bone marrow cells and the proliferative response of the SA2 CL as measured by the uptake of  $^3\text{H-TdR}$  was 14% of the untreated control. Concentrations of  $10^{-6}\text{M}$  and/or  $10^{-7}\text{M}$   $\beta$ atRA were effective in reducing the proliferative response of normal and leukaemic bone marrow cells and cells from the SA2 CL.(Fig. 4.1). The SA2 leukaemias were more susceptible to these two concentrations of  $\beta$ atRA than normal bone marrow, the SA7 and SA8 leukaemias.  $10^{-6}\text{M}$   $\beta$ atRA reduced the proliferation of normal bone marrow, SA7 and SA8 leukaemic cells to approximately 55%, the SA2 LP leukaemia to 8%, the SA2 HP leukaemia to 40% and the SA2 CL to 25%.  $10^{-7}\text{M}$   $\beta$ atRA reduced the proliferation of normal and the SA7, SA8 and SA2 HP leukaemias approximately 84%, the SA2 LP to 33% and the SA2 CL to 64%. Minimal to negligible decreases in proliferation were observed with  $10^{-8}\text{M}$  and  $10^{-9}\text{M}$   $\beta$ atRA with normal bone marrow and all the leukaemias except the SA2 LP which was reduced to 74% with  $10^{-8}\text{M}$  and 83% with  $10^{-9}\text{M}$ .

For the study of the differentiation effects of  $\beta$ atRA on the leukaemic cells,  $10^{-6}$ M and  $10^{-7}$ M total concentration per well were used.

#### **4.2 IN VITRO DIFFERENTIATION EFFECTS OF $\beta$ -ALL TRANS RETINOIC ACID ON LEUKAEMIC AND NORMAL CELLS**

The differentiation effects of  $\beta$ atRA on normal and leukaemic bone marrow cells and cells from the SA2 CL were investigated utilizing the microtitre assay, preparation of cytopins and counting differentials. The differences in differentiation responses between the combination of  $\beta$ atRA and WEHI-3B CM (which induced a high proliferative response and sustained a relatively high proportion of immature cells in culture) and L929 CM (which induced a lower proliferative response, sustained a relatively low proportion of immature cells and enhanced differentiation) were investigated. The addition of growth factors was necessary to maintain the growth factor dependent bone marrow cells in culture. The growth factors have been shown to have differentiative effects on the leukaemic and normal bone marrow cells used in these studies and these may augment the responses observed with the addition of  $\beta$ atRA. Consequently, the responses observed with the  $\beta$ atRA treated cells were compared to the responses observed with growth factor alone.

##### **4.2.1 Experimental Procedure**

a) Single cell suspensions of normal and leukaemic bone marrow cells and cells from the SA2 CL were cultured for four days in the microtitre assay with two concentrations of  $\beta$ atRA shown to have an effect on the proliferation on normal and leukaemic cells as investigated in section 5.1 ( $10^{-6}$  M and  $10^{-7}$  M  $\beta$ atRA total concentration per well). WEHI-3B CM and L929 CM were used as sources of growth factor and the differences in differentiation response between each of the

two factors and  $\beta$ atRA were compared. The cells were cultured in FCS alone as a control.

b) On day four the total cell count per well was determined, cytopins were prepared, stained and full differentials counted. At least 500 cells were counted per slide. Differentials were determined as both percentage and absolute number per well. All experiments were carried out in quadruplicate.

c) The differentials were plotted as 'stack graphs' showing the absolute number of immature cells (blast and promyelocytes), intermediate cells (myelocytes, metamyelocytes and 'immature macrophages') and mature cells (segmenteds, bands, monocytes and macrophages).

d) From the differential data the maturation index was determined.

e) The number of phagocytic cells for each test group was determined. The cells were cultured as described above and on day four they were incubated with 0.8 $\mu$  latex particles for eight hours. Cytopins were prepared, stained and the percentage and absolute number of cells per well containing greater than 10 particles was determined. At least 1000 cells were counted per slide and the results were reported as both percentages and absolute number per well.

## **4.2.2 Results**

### **4.2.2.1 FCS + $\beta$ -all *trans* Retinoic Acid**

Decreases in the total cell number per well were observed in cultures containing FCS alone with the growth factor dependent leukaemic bone marrow cells and normal bone marrow. Cell counts and the percentage of viable cells were low by day four and consequently these cultures were not used for comparative studies (Table 4.1).

**TOTAL CELL COUNT PER WELL x 10<sup>4</sup> ± STANDARD ERROR**

TREATMENT	NBM	SA7	SA8	SA2 LP	SA2 HP	SA2 CL
SCS	7.02 0.05	6.98 0.03	7.00 0.08	7.00 0.03	6.97 0.05	7.00 0.01
FCS	2.10 0.15	2.65 0.15	2.14 0.18	3.12 0.22	3.73 0.25	21.90 2.50
+ 10 <sup>-6</sup> M RA	1.73 0.10	2.12 0.14	1.77 0.14	1.85 0.20	3.50 0.40	10.83 0.95
+ 10 <sup>-7</sup> M RA	1.81 1.81	2.01 0.16	1.80 0.15	2.90 0.15	3.90 0.35	11.80 0.85
WEHI-3B CM	10.08 0.88	14.67 1.32	15.05 1.35	8.95 0.65	19.44 1.76	24.92 2.15
+ 10 <sup>-6</sup> M RA	9.65 0.85	12.33 1.14	14.50 1.55	8.83 0.55	10.31 0.97	10.98 1.25
+ 10 <sup>-7</sup> M RA	10.76 1.10	14.19 1.12	17.00 1.63	8.32 0.91	19.06 1.53	12.85 1.35
L929 CM	5.94 0.43	14.93 1.33	10.93 0.98	5.42 0.62	15.45 1.33	17.63 1.55
+ 10 <sup>-6</sup> M RA	5.97 0.61	15.49 1.28	12.06 1.31	8.16 0.85	6.32 0.53	10.85 0.90
+ 10 <sup>-7</sup> M RA	9.49 1.02	22.07 1.84	12.17 1.15	8.08 0.76	8.85 0.93	12.33 1.32

Table 4.1 Single cell suspensions (SCS) of leukaemic and normal bone marrow and the SA2 CL were cultured for four days in the microtitre assay in FCS alone, WEHI-3B CM and L929 CM ± 10<sup>-6</sup>M or 10<sup>-7</sup> M βATRA. The results show the total cell number per well.

An increase in the total cell number per well of the SA2 CL was observed in FCS cultures (Table.4.1). With the addition of  $\beta$ atRA an inhibitory response was observed without evidence of differentiation from the morphological studies (Fig. 4.2 F). The absolute number of blast cells decreased to half the number observed with FCS alone with the addition of both concentrations of  $\beta$ atRA. The addition of WEHI-3B CM or L929 CM did not alter the response observed with the FCS cultures.

#### 4.2.2.2 WEHI-3B CM + $\beta$ -all *trans* Retinoic Acid

$10^{-6}$ M  $\beta$ atRA enhanced the differentiation of normal bone marrow cells (Fig. 4.2 A). The proportion of immature:intermediate:mature cells of normal bone marrow when cultured with WEHI-3B CM alone were 45:25:30 (Appendix Table A4.2). With the addition of  $10^{-6}$ M  $\beta$ atRA the proportion of immature cells decreased 13%, the decrease being in the number of promyelocytes; intermediate cell numbers were unchanged and an increase in the number of mature cells, both in the granulocytic and monocytic series, was observed. The proportions of the three cell groups were 32:22:46. No effect on the proportion of cells was observed with  $10^{-7}$  M  $\beta$ atRA. The total cell count per well of normal bone marrow decreased minimally with the addition of  $10^{-6}$ M  $\beta$ atRA and increased mildly with  $10^{-7}$ M  $\beta$ atRA as compared to WEHI-3B CM alone. The differential results were reflected in the maturation index; the maturation index for WEHI-3B CM alone and with  $10^{-7}$ M  $\beta$ atRA was 0.9 and with  $10^{-6}$ M  $\beta$ atRA 1.5 (Table 4.2). The percentage and absolute number of phagocytic cells were increased compared to WEHI-3B CM alone with the addition of both concentrations of  $\beta$ atRA,  $10^{-6}$ M  $\beta$ atRA being more effective (Fig's.4.3 A and 4.4 A).

A more enhanced differentiation effect was observed with the SA7 HD leukaemia with the addition of  $10^{-6}$ M  $\beta$ atRA than with normal bone marrow

(Fig.4.2 B). The proportion of immature:intermediate:mature cells with WEHI-3B CM alone was 34:31:34 whereas with the addition of  $10^{-6}$ M  $\beta$ atRA the proportion was 16:23:61 (Appendix Table A4.4). The immature cells decreased 18% the intermediate cells decreased 8% and the proportion of mature cells almost doubled. Unlike normal bone marrow, with the SA7 HD leukaemia an enhanced differentiation effect was also observed with the addition of  $10^{-7}$ M  $\beta$ atRA although, this concentration was not as effective as the higher concentration. The proportion of the three cell types with  $10^{-7}$  M  $\beta$ atRA was 20:35:44. The total cell number per well was lower with the addition of  $\beta$ atRA than with WEHI-3B CM alone (Table 4.1). The results were reflected in the maturation index whereby the value for WEHI-3B CM alone was 1.0, with  $10^{-6}$  M  $\beta$ atRA 2.2 and with  $10^{-7}$  M  $\beta$ atRA 1.3. The percentage and absolute number of phagocytic cells were also higher with the addition of  $\beta$ atRA than with WEHI-3B CM alone (Fig's. 4.3 B and 4.4 B).

TREATMENT	NBM	MATURATION INDEX			
		SA7	SA8	SA2 LP	SA2 HP
FCS	25.3	2.3	25.8	0.6	5.5
+ $10^{-6}$ M RA	34.4	4.4	4.4	1.4	3.7
+ $10^{-7}$ M RA	17.1	2.4	10.3	0.9	6.4
WEHI-3B CM	0.9	1.0	2.0	0.3	1.3
+ $10^{-6}$ M RA	1.5	2.2	0.8	1.1	1.6
+ $10^{-7}$ M RA	0.8	1.3	2.5	0.8	1.2
L929 CM	3.1	3.1	8.1	1.2	3.0
+ $10^{-6}$ M RA	148.3	10.1	69.9	5.2	9.1
+ $10^{-7}$ M R	58.4	7.2	151.1	10.2	5.3

**Table 4.2** Differentiation study. Cells were cultured for 4 days in the microtitre assay with FCS, WEHI-3B CM and L929 CM with and without  $\beta$ -all *trans* RA. Cytospins were prepared, stained and full differentials counted. The maturation index was determined from the differential cell counts.

Maturation Index = The number of mature cells / The number of immature cells

Immature cells = Blasts, promyelocytes, myelocytes and immature macrophages.

Mature cells = Metamyelocytes, bands, segmented, monocytes and macrophages

An enhanced differentiation effect was observed with the SA8 HD leukaemia with the addition of  $10^{-7}$ M  $\beta$ atRA but not with  $10^{-6}$ M (Fig. 4.2 C). With the addition of  $10^{-6}$ M  $\beta$ atRA partial differentiation was observed as blast cell numbers were less than with WEHI-3B CM alone and promyelocyte number was greater. Intermediate cell numbers were similar to the number observed with WEHI-3B CM alone. Mature cell numbers were less than WEHI-3B CM alone. The total cell number per well was less than the number observed with WEHI-3B CM alone as was the proliferative response observed in section 4.1. Maturation in the granulocytic series was reduced compared to WEHI-3B CM alone. With the addition of  $10^{-7}$ M  $\beta$ atRA there was an increase in the total cell number per well, however immature cell numbers were half the number observed with WEHI-3B CM alone, and intermediate and mature cell numbers were greater. Maturation in the granulocytic series and in the monocytic series was observed with this concentration of  $\beta$ atRA. The proportions of the three cell types were 28:27:45, 37:28:36, 14:36:49 with WEHI-3B CM alone, plus  $10^{-6}$ M  $\beta$ atRA and plus  $10^{-7}$ M  $\beta$ atRA respectively (Appendix Table A4.6). These results were reflected in the maturation index (Table 4.2) with the index being lower with the addition of  $10^{-6}$ M  $\beta$ atRA and higher with the addition of  $10^{-7}$ M  $\beta$ atRA than with WEHI-3B CM alone. The results observed with the phagocytosis study also paralleled the differential data with fewer phagocytic cells being observed with the addition of  $10^{-6}$ M  $\beta$ atRA and greater numbers of phagocytic cells being observed with the addition of  $10^{-7}$ M  $\beta$ atRA compared to WEHI-3B CM alone (Fig's. 4.3 C and 4.4 C). The addition of  $\beta$ atRA at both concentrations studied enhanced the differentiation of the SA2 LP leukaemia, with  $10^{-6}$ M  $\beta$ atRA being the more effective (Fig. 4.2 D). The proportions of the three cell types were 62:20:19 with WEHI-3B CM alone, 32:21:46 with  $10^{-6}$ M  $\beta$ atRA and 41:22:37 with  $10^{-7}$ M  $\beta$ atRA (Appendix Table 4.8). The greatest fold increase in mature cell numbers was

observed with the SA2 LP leukaemia. These data were supported by the results in section 4.1 where the greatest reduction in proliferation was observed with the SA2 LP leukaemia. Enhanced differentiation was observed to macrophages but not in the granulocytic series. The total cell number per well with the addition of  $10^{-6}\text{M}$   $\beta\text{atRA}$  was similar to the number observed with WEHI-3B CM alone, and minimally less with the addition of  $10^{-7}\text{M}$   $\beta\text{atRA}$  (Table 4.1). The maturation index was 0.3, 1.1 and 0.8 for WEHI-3B CM alone, plus  $10^{-6}\text{M}$   $\beta\text{atRA}$  and  $10^{-7}\text{M}$   $\beta\text{atRA}$  respectively. The percentage and absolute numbers of phagocytic cells were also observed to be significantly higher with the addition of both concentrations of  $\beta\text{atRA}$  than with WEHI-3B CM alone (Fig's. 4.3 D and 4.4 D).

The bone marrow cells of the SA2 HP leukaemia have been shown to be highly proliferative in culture when stimulated with growth factors (Chapter Three). With the addition of  $10^{-6}\text{M}$   $\beta\text{atRA}$  a significant decrease in the proliferation of the leukaemia was observed with the total cell number per well being decreased to almost half the number observed with WEHI-3B CM alone (Table 4.1). A decrease was observed predominantly in the absolute number of intermediate cells (Fig.4.2 E). The absolute number of immature cells was half the number observed with WEHI-3B CM alone. The absolute number of mature cells was similar to the number observed with WEHI-3B CM alone, however because of the decrease in total cell number with the addition of  $10^{-6}\text{M}$   $\beta\text{atRA}$ , mature cells constituted a higher percentage of total cells compared to WEHI-3B CM alone. The addition of  $10^{-7}\text{M}$   $\beta\text{atRA}$  only minimally decreased the total cell number of the SA2 HP leukaemia compared to WEHI-3B CM alone (Table 4.1). The percentage of cells in each group was similar to the number observed with WEHI-3B CM. There were differences in the differentiation pattern between WEHI-3B CM alone and the addition of  $10^{-7}\text{M}$   $\beta\text{atRA}$ . Fewer blasts and more promyelocytes, and fewer metamyelocytes and more bands were observed with the addition of  $10^{-7}\text{M}$   $\beta\text{atRA}$

(Appendix Table A4.11). The absolute number of macrophages observed with the addition of both concentrations of  $\beta$ atRA was less than with WEHI-3B CM alone and the number of granulocytic cells was higher. The proportions of each of the cell groups were 28:43:30, 25:23:52, and 30:38:32 for WEHI-3B CM alone, plus  $10^{-6}$ M  $\beta$ atRA and plus  $10^{-7}$ M  $\beta$ atRA respectively (Appendix Table A4.10). The maturation index obtained with the addition of  $10^{-6}$ M  $\beta$ atRA was minimally higher than with either WEHI-3B CM alone or with the addition of  $10^{-7}$ M  $\beta$ atRA (Table 4.2). In this case, where a decrease in total cell number per well was observed, the difference in the maturation index between WEHI-3B CM alone and the addition of  $10^{-6}$ M  $\beta$ atRA was not large. The maturation index needs to be assessed in conjunction with the proliferation results. As described above the absolute number of macrophages observed with the addition of  $\beta$ atRA was less than the number observed with WEHI-3B CM alone; this was reflected in the phagocytosis study where the absolute number of phagocytic cells was less with the addition of  $\beta$ atRA than with WEHI-3B CM alone (Fig. 4.4 E), however the percentage of phagocytic cells with the addition of  $10^{-6}$ M  $\beta$ atRA was minimally higher than the number observed with WEHI-3B CM alone (Fig. 4.3 E).

#### 4.2.2.3 L929 CM + $\beta$ -all *trans* Retinoic Acid

L929 CM was shown to induce a lower proliferative response than WEHI-3B BM of normal and leukaemic bone marrow. Generally it did not effectively sustain immature cells in culture and induced differentiation mainly to macrophages. The addition of both concentrations of  $\beta$ atRA to cultures of normal cells grown in L929 CM enhanced differentiation markedly (Fig. 4.2 A). The proportions of immature:intermediate:mature cells were 21:19:60, 0:4:96 and 1:6:93 with L929 CM alone, plus  $10^{-6}$ M  $\beta$ atRA and  $10^{-7}$ M  $\beta$ atRA respectively (Appendix Table A4.2). The total number of cells per well with the addition of  $10^{-6}$ M  $\beta$ atRA was similar

to the number observed with L929 CM alone. The total cell number observed with the addition of  $10^{-7}$ M  $\beta$ atRA was higher than L929 CM alone however the main increases were in the number of myelocytes and macrophages (Table 4.1). The enhanced differentiation was reflected in the maturation index. There was a marked increase in the maturation index with the addition of  $10^{-6}$ M  $\beta$ atRA. The maturation indices were 3.1, 148.3 and 58.4 for L929 CM alone, plus  $10^{-6}$ M  $\beta$ atRA and  $10^{-7}$ M  $\beta$ atRA respectively. The differential results were paralleled with the phagocytosis study. The percentage and absolute numbers of phagocytic cells were higher with the addition of  $\beta$ atRA than with L929 CM alone (Fig's 4.3 A and 4.4 A). The combination of L929 CM plus  $\beta$ atRA was more effective in inducing the differentiation of normal bone marrow cells than WEHI-3B CM in combination with  $\beta$ atRA.

L929 CM had a higher proliferative effect on the SA7 leukaemia than normal bone marrow. The addition of  $\beta$ atRA enhanced differentiation but not to the same degree as that of normal marrow (Fig. 4.2 B). The proportions of the three cell groups were 11:18:71, 4:6:89 and 4:12:84 for L929 CM alone, plus  $10^{-6}$ M  $\beta$ atRA and  $10^{-7}$ M  $\beta$ atRA respectively (Appendix Table A4.4). The addition of  $\beta$ atRA increased the total cell number per well compared to L929 CM alone however, the increases observed were in the number of mature macrophages (Appendix Table A4.5). The total cell numbers per well observed were  $14.93$ ,  $15.49$  and  $22.07 \times 10^4$  with L929 CM alone, plus  $10^{-6}$ M  $\beta$ atRA and  $10^{-7}$ M  $\beta$ atRA respectively. The maturation index with the addition of  $\beta$ atRA was higher than with L929 CM. The values were 3.1 for L929 CM alone, 10.1 with the addition of  $10^{-6}$ M  $\beta$ atRA and 7.2 with the addition of  $10^{-7}$ M  $\beta$ atRA. The increase in the number of macrophages observed with the differential data was paralleled with the phagocytosis study with the highest absolute number of phagocytic cells being observed with  $10^{-7}$ M  $\beta$ atRA (Fig. 4.4 B). The combination of L929 CM plus  $\beta$ atRA

was more effective in inducing the differentiation of the SA7 leukaemic bone marrow cells than WEHI-3B CM in combination with  $\beta$ atRA.

The addition of  $10^{-7}$ M  $\beta$ atRA to L929 stimulated bone marrow cells of the SA8 leukaemia markedly enhanced differentiation (Fig. 4.2 C). The differentiation observed was similar to the level observed with normal bone marrow cells cultured with L929 CM plus  $10^{-6}$ M  $\beta$ atRA. The proportions of the three cell groups were 3:32:65 with L929 CM alone, 0:9:91 with  $10^{-6}$ M  $\beta$ atRA and 0:4:96 with  $10^{-7}$ M  $\beta$ atRA (Appendix Table A4.6). The total numbers of cells per well obtained with the two concentrations of  $\beta$ atRA were higher than the number observed with L929 CM alone, the difference being in the number of macrophages (Appendix Table A4.7). The maturation indices with the addition of  $\beta$ atRA were markedly higher than that of L929 CM alone. The values for the maturation indices were 8.1 for L929 CM alone, 69.9 with  $10^{-6}$ M  $\beta$ atRA and 151.1 with  $10^{-7}$ M  $\beta$ atRA. The numbers of phagocytic cells were higher with the addition of  $\beta$ atRA than with L929 CM alone (Fig's. 4.3 C and 4.4 C). The combination of L929 CM plus  $\beta$ atRA was more effective in inducing the differentiation of the SA8 leukaemic bone marrow cells than WEHI-3B CM in combination with  $\beta$ atRA.

Both concentrations of  $\beta$ atRA had an enhanced and similar effect on the differentiation of the SA2 LP leukaemia compared to L929 CM alone (Fig. 4.2 D). The proportion of the three cell groups were 32:22:46, 2:16:82, and 2:16:82 with L929 CM alone,  $10^{-6}$ M  $\beta$ atRA and  $10^{-7}$ M  $\beta$ atRA respectively (Appendix Table A4.8). The total cell count per well was higher with the addition of  $\beta$ atRA than with L929 CM alone, the increase was in the number of macrophages (Appendix Table A4.9). The differential results were reflected in the maturation index and the phagocytosis studies. The maturation indices were 1.2, 5.2 and 10.2 for L929 CM, plus  $10^{-6}$ M  $\beta$ atRA and  $10^{-7}$ M  $\beta$ atRA respectively. The absolute numbers of phagocytic cells were higher with the addition of  $\beta$ atRA than with L929 CM

alone (Fig. 4.4 D). The combination of L929 CM plus  $\beta$ atRA was more effective in inducing the differentiation of the SA2 LP leukaemic bone marrow cells than WEHI-3B CM in combination with  $\beta$ atRA.

There was a major difference in the response of the SA2HP leukaemia when cultured with L929 CM plus  $\beta$ atRA compared to NBM and the other transplanted leukaemic cell lines. With the SA2 HP leukaemia a decrease in the total cell number per well was observed with the addition of  $\beta$ atRA compared to L929 CM alone (Table 4.1). With the other leukaemic cells and normal bone marrow an enhanced differentiation effect was accompanied with an increase in the total cell number per well compared to L929 CM alone, the difference being in the greater absolute number of mature cells. With the SA2 HP leukaemia, as with the other cells, a decrease was observed in the absolute number of immature cells, however compared to L929 CM alone a decrease was also observed in the absolute number of mature cells. The percentage number of mature cells however, was greater than with L929 CM alone (Fig. 4.2 E). The proportion of each of the cell groups were 11:22:67 with L929 CM alone, 1:20:79 with  $10^{-6}$ M  $\beta$ atRA and 0:21:79 with  $10^{-7}$ M  $\beta$ atRA (Appendix Table A4.10). The maturation indices with the addition of  $\beta$ atRA were higher than with L929 CM alone (Table 4.2). The absolute number of macrophages was reduced with the addition of  $\beta$ atRA however the percentage number of macrophages was higher because of the decrease in the total cell count and these results are reflected in the phagocytosis study (Fig's. 4.3 E and 4.4 E). With these results it was difficult to assess whether the addition of  $\beta$ atRA to L929 CM enhanced the differentiation response or whether  $\beta$ atRA at these concentrations had a cytotoxic effect on bone marrow cells from the SA2 HP leukaemia.

**4.2.2.4 Summary of the *in vitro* all-trans Retinoic Acid Induced Differentiation of Normal and Leukaemic Cells**

In summary, the addition of  $\beta$ atRA was effective in enhancing the differentiation of normal and leukaemic bone marrow cells from the transplanted leukaemic cell lines. The results are summarised in Table 4.3. The combination of WEHI-3B CM plus  $\beta$ atRA was more effective in enhancing the proliferation of leukaemic bone marrow cells from the transplanted leukaemic cell lines than normal bone marrow, the most enhanced differentiation being observed with the SA2 LP leukaemia. Only partial differentiation with a minimal decrease in total cell number per well was observed with the SA8 leukaemia with  $10^{-6}$ M  $\beta$ atRA, however differentiation induction was observed with  $10^{-7}$ M  $\beta$ atRA. The lower concentration of  $10^{-7}$ M  $\beta$ atRA also enhanced the differentiation of the SA7 and SA2 LP leukaemia but not normal bone marrow cells or the SA2 HP leukaemia. No differentiation effect was observed with the SA2 CL.

**COMPARATIVE DEGREE OF ENHANCED DIFFERENTIATION**

BM	WEHI-3B CM		L929 CM	
	+ $10^{-6}$ M RA	+ $10^{-7}$ M RA	+ $10^{-6}$ M RA	+ $10^{-7}$ M RA
NBM	+	-	++++	+++
SA7	++	+	+++	++
SA8	*Partial Diff $\square$	++	+++	++++
SA2 LP	+++	+++	++++	++++
SA2 HP	+/? Cytotoxic	-	? Diff $\square$ /Cytotoxic	? Diff $\square$ /Cytotoxic
SA2 CL	-	-	-	-

**Table 4.3** Summary of the degree of enhanced differentiation with the addition of all-trans retinoic acid to cultures of normal and leukaemic bone marrow cells from the transplanted leukaemic cell lines and the SA2 CL compared to growth factor alone.

\*Diff $\square$  = Differentiation

The combination of L929 CM plus  $\beta$ atRA was effective in enhancing differentiation of normal bone marrow cells and leukaemic bone marrow cells from

the SA7, SA8 and SA2 LP leukaemias. The most enhanced differentiation effect was observed in normal bone marrow with  $10^{-6}$ M  $\beta$ atRA and the SA2 LP leukaemia with both concentrations of  $\beta$ atRA. Where differentiation induction was observed it was more enhanced than with the combination of WEHI-3B CM plus  $\beta$ atRA.  $10^{-6}$ M as well as  $10^{-7}$  M  $\beta$ atRA enhanced the differentiation of the SA8 leukaemia when cultured with L929 CM. The effect of this combination on the SA2 HP leukaemia was difficult to assess as a decrease in the total cell number per well was observed with an increase in the percentage but not the absolute number of mature cells. No enhanced differentiation was observed with the SA2 CL although the proliferative response was decreased.

#### **4.3 THE *IN VIVO* CLONOGENIC CELL ASSAY FOR THE DETERMINATION OF THE NUMBER OF CLONOGENIC LEUKAEMIC CELLS REMAINING IN CULTURE AFTER TREATMENT WITH $\beta$ -ALL TRANS RETINOIC ACID**

For this study the number of clonogenic leukaemic cells remaining in culture following differentiation induction with  $10^{-6}$ M  $\beta$ atRA was measured by injecting known numbers of treated and untreated cells into syngeneic recipients and recording the difference in percentage survival over time between the two groups. Only the SA7 leukaemia was used for this study. Single cell suspension of leukaemic cells were cultured for four days with FCS  $\pm$   $10^{-6}$ M  $\beta$ atRA as control, WEHI-3B CM  $\pm$   $10^{-6}$ M  $\beta$ atRA and L929 CM  $\pm$   $10^{-6}$ M  $\beta$ atRA. In the previous study  $10^{-6}$ M  $\beta$ atRA was found to enhance the differentiation of SA7 leukaemic cells compared to growth factor alone. Differentiation induction was more enhanced with the combination of  $10^{-6}$ M  $\beta$ atRA plus L929 CM than with WEHI-3B CM.

#### 4.3.1 Experimental Procedure

Single cell suspension of SA7 leukaemic bone marrow cells were cultured in the microtitre assay for four days with either FCS, or optimal concentrations of WEHI-3B CM or L929 CM  $\pm 10^{-6}$  M  $\beta$ atRA. On day four the cells were harvested, washed in non-supplemented RPMI, counted and injected into syngeneic recipients at varying cell concentrations ( $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$  and 10 total cells in 0.5ml non supplemented RPMI). At least five mice were used per treatment and percentage survival was monitored over 90 days. The results were the means of three experiments.

For controls:-

- the initial single cell suspension was injected into syngeneic recipients at the same concentrations listed above.
- differentials were counted for each treatment of the cultured cells and compared to the experiments in section 5.2.

#### 4.3.2 Results

The results obtained of the single cell suspension of uncultured SA7 leukaemic bone marrow cells are well established in this laboratory and form the criteria of maintaining the transplanted leukaemic cell lines for study (Riches, unpublished data). The results of the survival studies using the uncultured single cell suspension were as expected (see section 3.2.2). Leukaemic clonogenic cells were sustained in FCS only cultures to day four. The addition of  $10^{-6}$ M  $\beta$ atRA did not significantly decrease the leukaemic clonogenic cell number. With  $10^5$  FCS cultured cells being injected into syngeneic recipients no leukaemia free survival was observed by day 13; with the addition of  $\beta$ atRA 20% survival was observed to day 90 (Fig. 4.5 A). No difference in percentage survival was observed when  $10^4$  and  $10^3$  FCS  $\pm \beta$ atRA cultured cells were injected (Fig's 4.5 B and 4.5 C). When  $10^2$

cells were injected 100% survival was observed with  $\beta$ atRA treated cells and 80% survival was observed with FCS only treated cells.

WEHI-3B CM was shown to sustain immature leukaemic cells to day four in culture as described in Chapter Three. Enhanced differentiation induction was observed when  $\beta$ atRA was added to WEHI-3B CM stimulated cells as observed in the morphological studies described in section 5.2. However, no difference in the number of clonogenic leukaemic cells being sustained in culture was observed with the Clonogenic Cell Assay. There was no difference in percentage survival between groups being injected with WEHI-3B CM treated cells and WEHI-3B CM plus  $\beta$ atRA treated cells (Fig. 4.6).

L929 CM was more effective in inducing differentiation of leukaemic cells and sustained fewer immature cells in culture than WEHI-3B CM. The combination of L929 CM plus  $\beta$ atRA was more effective in inducing differentiation than the combination of WEHI-3B CM plus  $\beta$ atRA (Section 5.2). With the Clonogenic Cell Assay it was found that there was an improvement in percentage survival when  $10^4$  L929 CM plus  $\beta$ atRA treated cells were injected compared to L929 CM treated cells. The percentage of leukaemia free survival with the former was 60% and 20% by day 90 (Fig. 4.7).

#### 4.4 DISCUSSION

From the proliferation studies utilizing the uptake of 3H-TdR it was noted that  $10^{-5}$  M  $\beta$ atRA had a toxic effect on normal and leukaemic bone marrow cells *in vitro*. At concentrations of  $10^{-6}$ M and  $10^{-7}$  M  $\beta$ atRA a reduction in proliferation was observed in all the cell groups. Minimal or no effects on proliferation were observed with concentrations of  $10^{-8}$ M or less. Consequently concentrations of  $10^{-6}$ M and  $10^{-7}$ M  $\beta$ atRA were used for the differentiation studies. Retinoids have

been shown to enhance clonal proliferation of normal human erythroid (CFU-E) and normal myeloid (CFU-GM) precursors in the colony assay (Douer and Koeffler, 1982b), however in our study of normal murine progenitors in the microtitre assay proliferation of myeloid progenitors decreased with the addition of  $10^{-6}$ M and  $10^{-7}$ M  $\beta$ tRA.

*In vitro* studies of fresh human bone marrow cells from patients with acute promyelocytic leukaemia (APL) have been carried out in suspension cultures without the addition of growth factor (Flynn et al, 1983; Huang Meng-er et al, 1988; Chomienne et al, 1990). In our study, suspension cultures in the microtitre assay were used with the addition of growth factor as it was found that cell numbers and viability in cultures with FCS alone were too low to provide acceptable and statistically significant comparative results. Good correlation however, was found between our studies and both *in vitro* and *in vivo* studies on human APL and is discussed below.

$\beta$ tRA was found to enhance the differentiation of both normal and leukaemic bone marrow cells but not the SA2 CL. The SA2 LP, which has been shown to be a slow proliferating leukaemia in Chapter Two, was more responsive to differentiation induction by  $\beta$ tRA than the other leukaemias. Unlike human acute myeloid leukaemias where only the partially differentiated APL cells have been induced to differentiate with  $\beta$ tRA (Breitman et al, 1981) no relationship was found between the bone marrow and blood differentials of the transplanted leukaemic cell lines and their response to  $\beta$ tRA. Cytogenetic studies of the transplanted myeloid leukaemic cell lines would be useful in identifying any similarity of genetic abnormalities to human myeloid leukaemias.

Differentiation induction of the transplanted leukaemic cell lines depended on the concentration of  $\beta$ tRA, and on the growth factor used in the culture system.

The concentration of  $\beta$ atRA required for a maximal differentiation response varied with the leukaemia studied and the growth factor used in the culture system. When WEHI-3B CM was used as the growth factor source the addition of  $\beta$ atRA was more effective at inducing the differentiation of the leukaemic bone cells than normal bone marrow cells.  $10^{-6}$ M but not  $10^{-7}$ M  $\beta$ atRA induced the differentiation of bone marrow cells and the SA2 HP leukaemia;  $10^{-6}$ M  $\beta$ atRA was more effective than  $10^{-7}$ M with the SA7 leukaemia;  $10^{-7}$ M  $\beta$ atRA induced the differentiation of the SA8 leukaemia,  $10^{-6}$ M  $\beta$ atRA only induced partial differentiation; and equivalent differentiation induction of the SA2 LP leukaemia was observed with both concentrations. When L929 CM was used as the growth factor source the addition of  $\beta$ atRA was most effective at inducing the differentiation of NBM and the SA2 LP leukaemia. Again  $10^{-6}$ M  $\beta$ atRA was more effective at inducing the differentiation of the SA7 leukaemia than  $10^{-7}$ M. Equivalent differentiation induction was observed with both concentrations with the SA8 and the SA2 LP leukaemia. These results were consistent with results obtained by *in vitro* studies on human leukaemic cells from patients with acute promyelocytic leukaemia whereby maximal differentiation induction was observed with both  $10^{-6}$ M and  $10^{-7}$ M  $\beta$ atRA (Chomienne et al, 1990).

Increases in total cell count with the addition of  $\beta$ atRA compared to untreated controls were observed in conjunction with differentiation induction: these increases were in the absolute number of mature cells. The percentages of mature cells in  $\beta$ atRA treated samples were also higher than controls. In the study by Chomienne et al (1990), using suspension cultures without the addition of growth factor a 'trend' in the increase of cell concentration in  $\beta$ atRA-treated samples was noted and the total cell number in their study was also higher in  $\beta$ atRA treated samples than in the controls. They did not specify in which cell population the increases were observed; however, they reported that there was no decrease in

the percentage of differentiated cells. From this evidence they suggested that  $\beta$ atRA may play a role in the proliferation and survival of some APL clones. In clinical trials of patients with APL treated with  $\beta$ atRA increases in the peripheral white blood cell count were observed with maturation of the leukaemic promyelocytes (Huang Meng-er et al, 1988; Chen et al, 1991). It may be argued that the addition of growth factor may mask or enhance the specific effect of  $\beta$ atRA; however, suspension cultures with growth factor reflected the results obtained in the *in vivo* studies.

The second aspect of this study was to determine the differences in response of leukaemic cells to combinations of  $\beta$ atRA with growth factors having different modes of action on normal and leukaemic cells. Except in the case of the SA2 HP leukaemia, it was found that the combination of  $\beta$ atRA with L929 CM was more effective for inducing differentiation than with WEHI-3B CM. L929 CM has been shown to be a more effective inducer of differentiation and sustains fewer immature cells in cultures of the leukaemic cells than WEHI-3B CM (Chapter Three). The bone marrow cells from the transplanted leukaemic cell lines used in this study were growth factor dependent therefore, comparison of the results could not be made to cultures containing  $\beta$ atRA without growth factor. It may be concluded however, that a synergistic differentiation response was observed with the combination of the two differentiation inducers,  $\beta$ atRA and L929 CM. The differentiation inducing factor, G-CSF, which is involved in the terminal differentiation of myeloid cells to granulocytes has been shown to co-operatively induce granulocyte differentiation of APL cells *in vitro* with the addition of  $\beta$ atRA (Nakamaki et al, 1989). In another study G-CSF but not GM-CSF co-operated with  $\beta$ atRA on the induction of differentiation of HL60 cells as measured by the induction of functional N-formyl-methionyl-phenylalanine receptors (Sakashita et al, 1991). The results obtained for the highly

proliferative SA2 HP leukaemia differed from those of the other leukaemias and were difficult to assess. Differentiation induction was observed with the combination of  $10^{-6}$ M but not  $10^{-7}$ M  $\beta$ atRA plus WEHI-3B CM and a decrease in proliferation without direct evidence of differentiation was observed with the combination of both concentrations of  $\beta$ atRA and L929 CM. With the addition of  $\beta$ atRA the percentage numbers of mature cells were greater than the percentage observed with L929 CM alone however, the absolute numbers of mature cells were less. With the methodology used and the data obtained it was not possible to determine whether the reduction in proliferation with the addition of  $\beta$ atRA was due to differentiation induction, cytotoxicity alone or a combination of both. In a study on human AML cells it was found that 13-*cis* RA inhibited the *in vitro* colony growth of 17/35 non APL bone marrow samples and that there was no evidence of differentiation induction in these samples (Lawrence et al, 1987). In the light of these current studies, by us and others, the re-evaluation of  $\beta$ atRA in combination with physiological regulators of proliferation and differentiation of normal haemopoiesis in the treatment of AML is warranted.

The third part of this study was to determine the effects of  $\beta$ atRA on the leukaemic clonogenic cell fraction. The SA7 leukaemia which was induced to differentiate with  $\beta$ atRA in combination with both growth factors was used for this study. Differentiation induction by  $\beta$ atRA did not appear to significantly decrease the leukaemic clonogenic cell fraction compared to untreated controls as assessed by the *in vivo* Clonogenic Cell Assay. Although  $\beta$ atRA enhanced the differentiation induction as observed by the morphological studies, it did not significantly reduce the proportion of cells required to induce leukaemia in syngeneic recipients. The difference in percentage survival at  $10^4$  cells injected between treated and untreated cells was minimally better with the combination of  $\beta$ atRA and L929 CM than with  $\beta$ atRA and WEHI-3B CM, which supported the

*in vitro* morphological data. However, no increase and a minimal decrease in the leukaemic clonogenic cell fraction was observed with differentiation induction. This evidence, although not conclusive as the cell culture technique used may not have adequately supported the leukaemic clonogenic cells, suggests that the differentiation induction of the leukaemic clone is not necessarily compensated by an increase in the leukaemic 'stem' or clonogenic cell compartment.

In clinical trials of RA relapse was a common occurrence with patients treated with  $\beta$ atRA and 13-*cis* RA for APL and other tumours, (Lippman et al, 1987; Daenan et al, 1986; Fontana et al, 1986; Huang Meng-er et al, 1988; Castaigne et al, 1990; Chen et al, 1991). Two cases of APL treated with 13-*cis* RA reported by Daenan et al (1986) and Fontana et al (1986) relapsed in six and twelve months respectively. The high percentage of APL patients who successfully achieved complete remission (CR) in the clinical trials of  $\beta$ atRA all relapsed between two to ten months even with continuous use of  $\beta$ atRA. (Huang Meng-er et al, 1988; Castaigne et al, 1990; Chen et al, 1991). Huang Meng-er et al, (1988) observed the persistence of abnormal clones with cytogenetic studies and suggested that intensive chemotherapy following CR induction with  $\beta$ atRA would be beneficial. In the study by Huang Meng-er et al it was shown using bone marrow samples from patients with APL in the *in vitro* colony assay that normal haemopoiesis was restored during  $\beta$ atRA induced CR. Prior to treatment leukaemic-colony forming unit (L-CFU) growth was predominant and normal GM-CFU growth was suppressed (<1 colony). During CR GM-CFU reached normal levels and little or no growth of L-CFU was observed. On bone marrow samples from patients with APL in the clinical trial by Castaigne et al (1990) proliferation studies were carried out on  $\beta$ atRA treated cells and controls (Chomienne et al, 1990). Bone marrow cells were grown without growth factor in suspension cultures for five days. After one hour, on day three and on day five, treated and untreated cells were removed,

plated in microtitre wells and pulsed with 3H-TdR for one hour. A marked reduction in the uptake of 3H-TdR was observed with  $\beta$ atRA treated cells as compared to controls from day three and day five samples (78% reduction). It was concluded from these studies that  $\beta$ atRA treated cells showed a decrease or absence of self-renewal as compared to controls. In our study we attempted to quantify the effect of  $\beta$ atRA on clonogenic leukaemic cells and found that they persisted, could be minimally reduced and were not increased with differentiation induction. Restoration of normal haemopoiesis with the use of  $\beta$ atRA has been observed in APL patients (Huang Meng-er et al, 1989); however relapse was a common occurrence in patients achieving CR. The reason for this may have been provided by a pharmacological study by Muindi et al, (1992). It has been shown in these studies that oral administration of  $\beta$ atRA was associated with a significant decrease in plasma peak levels and a significant decrease in the area under the concentration time curve when measured two to six weeks after treatment. These decreases highly correlated with clinical relapse and were attributed to the induction of accelerated catabolism of  $\beta$ atRA with continuous administration by an unspecified cytochrome enzyme. It was suggested in this study that treatment may be improved by discontinuous dosing schedules. With further understanding of the pharmacology of  $\beta$ atRA, a maintenance rather than a cell kill strategy therapy for APL, using this differentiation inducing agent still appears as a possibility.

The methods used in this study of cell culture of whole leukaemic bone marrow samples stimulated with growth factor and the reliance on changes in differential cell counts to quantitate differentiation do not provide direct evidence on the differentiation effect of  $\beta$ atRA. Similar methodology in studies using growth factor independent human leukaemic cell lines such as HL60, K-562, U-937 and THP-1 which are relatively homogeneous provide more conclusive

evidence on differentiation induction. Indirect evidence of the differentiation properties of  $\beta$ atRA *in vitro* was also reported in conjunction with the *in vivo* clinical trials of  $\beta$ atRA in patients with APL (Huang Meng-er et al, 1988; Chomienne et al, 1990). In the study by Huang Meng-er et al, (1988) decreases in the number of promyelocytes and increases in the number of mature cells were observed and the rate of nitroblue tetrazoleum (NBT) reduction was higher with  $\beta$ atRA treated samples as compared to controls. They observed the progression of cellular differentiation (from promyelocytes to myelocytes to mature granulocytes) with morphological studies on the cultured cells over consecutive days from days one to eight. Cytochemical analysis and transmission electronmicroscopic examination showed evidence of differentiation in  $\beta$ atRA treated cells. Chomienne et al, (1990) also employed morphological and functional criteria including NBT reduction and granulocyte respiratory burst function. With the morphological studies they observed asynchrony of maturation between the nucleus and cytoplasm with the persistence of Auer Rods in differentiated cells. With  $\beta$ atRA treated cells, as compared to control, they also reported an increase in the CD15 differentiation cell surface antigen and a decrease in cellular proliferation. Direct evidence of the differentiation induction of cells can be provided only when chromosomal abnormalities characteristic of the leukaemic cell population can be detected in mature cells. However, the preparation of karyotypes of non dividing mature cells is very difficult to achieve. Recently, Lo Coco et al (1991) were able to definitively show the differentiation effects of  $\beta$ atRA on human APL cell. They presented a molecular evaluation of response to  $\beta$ atRA in patients with APL by analysing the RA receptor  $\alpha$  (RAR $\alpha$ ) locus in the patients' cells during treatment. The gene for the RAR $\alpha$  has been shown to be rearranged in all cases of APL (de The et al, 1990). Le Coco et al showed that the RAR $\alpha$  rearrangement persisted in the APL samples

containing maturing myeloid cells two to three weeks after the start of treatment but disappeared after five to eight weeks when patients achieved CR.

The differentiation induction properties of RA have been studied on a variety of tumours including myeloid leukaemias. A variety of tumour types have been induced to differentiate, including teratocarcinomas (Pierce and Wallace, 1971), neuroblastomas (Schubert et al, 1971), squamous cell carcinoma (Pierce and Wallace, 1971), adenocarcinomas of the breast (Decosse et al, 1973) leukaemias (Metcalf et al, 1969, Paran et al, 1970, Gootwine et al, 1982). Several *in vitro* studies have shown that retinoids, especially those with a free carboxyl group such as 13-*cis* RA and  $\beta$ atRA have potent haematopoietic activity (Lippman et al, 1987). These reports showed that RA induced terminal differentiation and cessation of growth of several human leukaemic cell lines and marrow cultures from certain patients with MDS and AML (Lippman et al, 1987; Breitman et al, 1981; Douer and Koeffler, 1982a; Fabian et al, 1986; Imaizumi et al, 1987; Lawrence et al, 1987). Breitman et al (1981) were the first to study the effects of  $\beta$ atRA on human AML cells *in vitro*. Bone marrow cells from 21 patients with AML were cultured with  $10^{-6}$ M  $\beta$ atRA in suspensions cultures not stimulated with growth factor. Differentiation induction was monitored morphologically and with reduction of NBT. Only cells from patients with acute promyelocytic leukaemia were shown to differentiate *in vitro*. During the early eighties attention focused on 13-*cis* RA. Flynn et al (1983) described the first case of APL treated with 13-*cis* RA. This patient was refractory to conventional chemotherapy. Bone marrow cells from this patient were shown to differentiate with the addition of 13-*cis* RA *in vitro* and following oral administration a significant increase in peripheral blood maturing myeloid cells was observed after two weeks of treatment. Favourable results were also obtained with other patients with APL treated with 13-*cis* RA (Nilsson, 1984; Daenen et al, 1986;

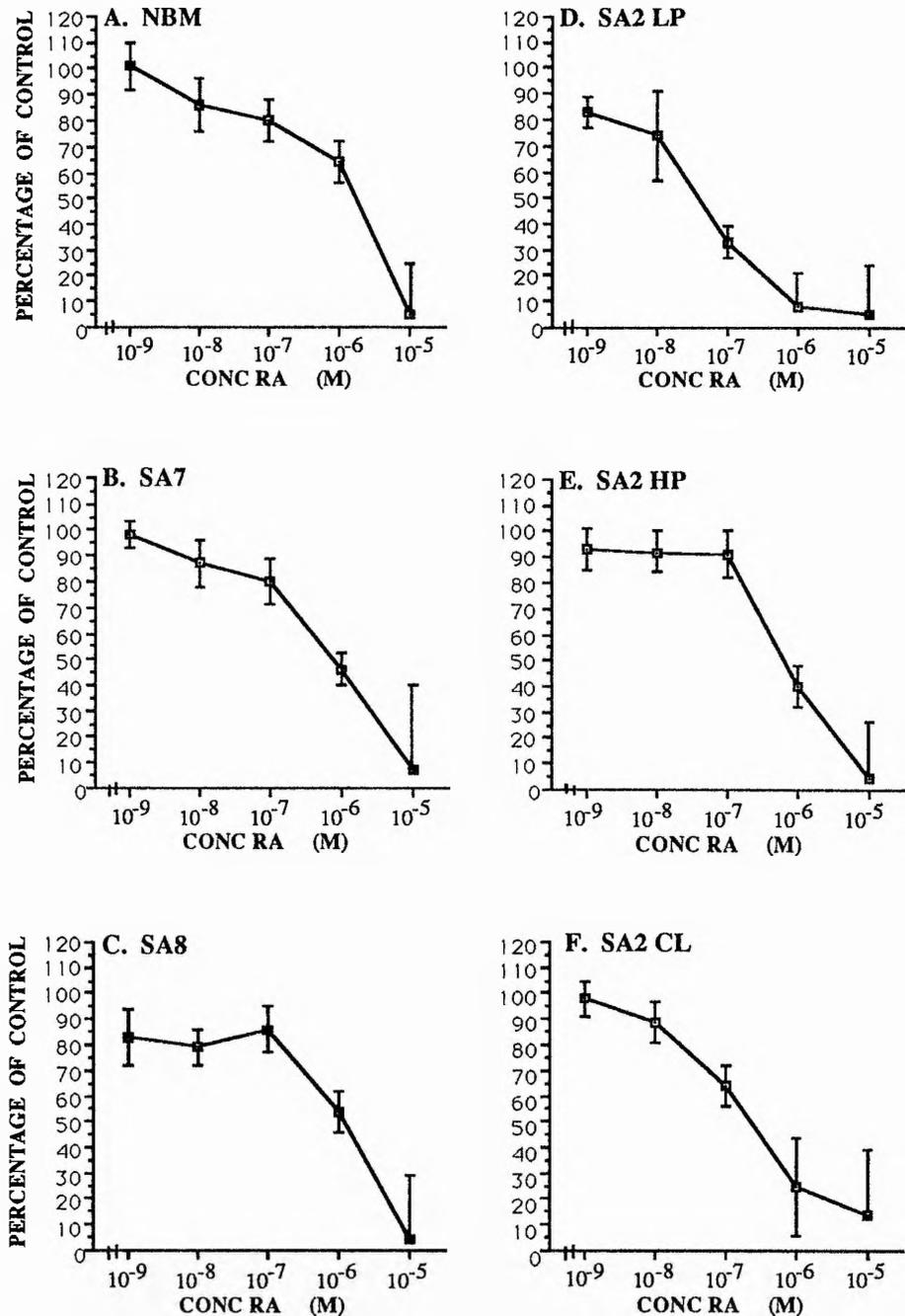
Fontana et al, 1986). In a study by Lawrence et al (1987) on bone marrow cells from 35 patients with AML in the colony assay, and stimulated by growth factor, it was shown that only cells from the two patients with acute promyelocytic leukaemia were induced to differentiate with 13-*cis* RA. The six FAB subgroups of AML were represented in the study. In 17/35 cases inhibition of leukaemic cell growth was observed however, in 10/35 cases an enhanced stimulatory response was observed.

It was not till the late eighties that the first clinical trial of  $\beta$ atRA was conducted on 24 patients with APL (Huang Meng-er et al, 1988). The results were exceptionally promising. 23/24 patients attained CR with  $\beta$ atRA treatment alone. Bone marrow samples from these patients were initially treated with  $\beta$ atRA *in vitro* (suspension cultures without the addition of growth factor) and these studies were found to be good predictors of the *in vivo* response. In studies by Chomienne et al (1989, 1990) it was shown that  $\beta$ atRA was more effective for differentiation induction of APL cells both *in vitro* and *in vivo* than 13-*cis* RA. It was also shown that both isomers induced maximal differentiation at  $10^{-6}$ M however, maximal differentiation was maintained at  $10^{-7}$ M with  $\beta$ atRA but not 13-*cis* RA. A second clinical trial of  $\beta$ atRA on patients with APL was conducted by Castaigne et al (1990). The high percentage of complete remissions obtained with the first study were also obtained with the second. The induction of CR with  $\beta$ atRA on patients with APL had a dual impact. Firstly, the theoretical notion that differentiation inducers may play a role in the treatment of myeloid leukaemia (Sachs, 1978a, 1978b) was realised. Secondly,  $\beta$ atRA induced CR in a high percentage of APL patients and avoided the aggravation of disseminated intravascular coagulation, which was often the cause of death, than conventional induction chemotherapy (Avvisati et al, 1992).

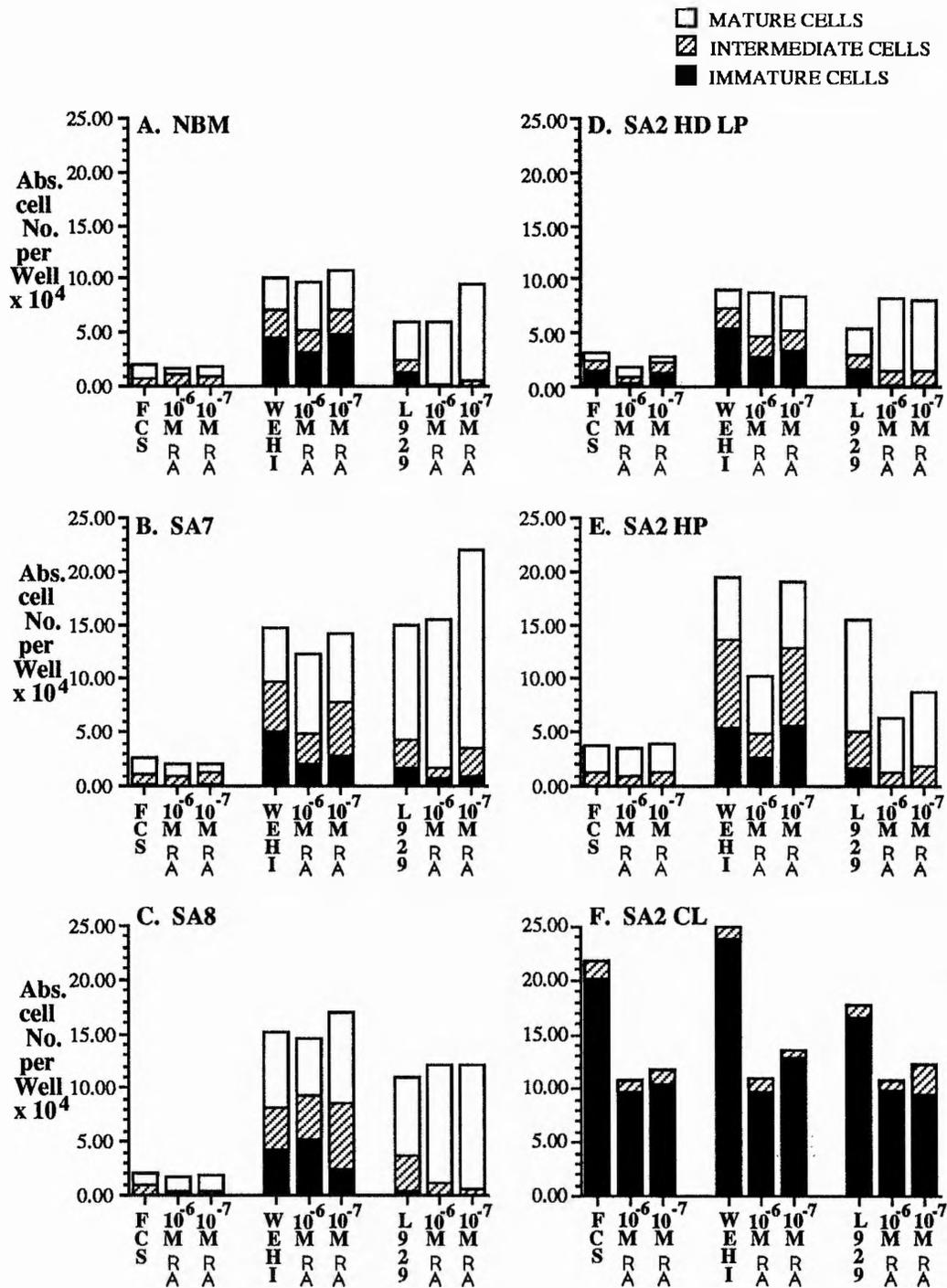
The mechanism through which RA induces leukaemic cell differentiation has not been elucidated although many effects subsequent to RA action have been observed at different levels of the cell and activity has been reported to be mediated by binding to specific receptors (Chomienne et al, 1990). The action of retinoid and RA on cellular differentiation and development might be attributed to the induction of protein kinase activity which in turn triggers multiple cellular mechanisms to achieve differentiation. (Sporn and Roberts, 1983; Plet and Evain, 1982)

The characteristic chromosomal abnormality of APL is the t(15;17) translocation. (Golomb et al, 1976). Recently, the breakpoint on chromosome 17 has been localized within the RAR $\alpha$  locus while the breakpoint on the chromosome 15 has been localized within a new gene named MYL (de The et al, 1990). As a consequence of the translocation a new chimeric gene with residual RAR $\alpha$ /MYL sequences is produced and actively transcribed in APL (de The et al, 1990; Borrow et al, 1990; Longo et al, 1990 Alcalay et al 1991). It has been suggested that the MYL gene may represent a novel transcription factor and the perturbation by the aberrant RAR $\alpha$ -MYL fusion product of the MYL transcriptional pathway may contribute to APL (Kakizuka et al, 1991). Another explanation put forward is that the RAR $\alpha$ -MYL fusion product may generate an RAR $\alpha$  mutant that contributes to acute promyelocytic leukaemogenesis through interference with promyelocytic differentiation. (de The et al, 1991). However, it has also been shown that the biological effect of RA on APL cells was not related to the level of expression of RAR $\alpha$  nor to its modulation by RA (Gallagher et al, 1989). It would be of interest to determine whether the genetic abnormalities of the transplanted myeloid leukaemias which were induced to differentiate with  $\beta$ atRA, eg the SA2 LP leukaemia, shared any of the genetic abnormalities observed in APL.

The results obtained with the *in vitro* studies of RA on bone marrow cells from radiation induced murine myeloid leukaemia models have paralleled both the *in vitro* and *in vivo* studies of human myeloid leukaemia. Our studies have shown that  $\beta$ atRA could induce the differentiation of myeloid leukaemic cells but could not significantly decrease the leukaemic clonogenic cell fraction. Clinical trials have shown  $\beta$ atRA to induce but not sustain CR. This may however, be due to the pharmacology of RA. The myeloid leukaemic models provided a useful and sensitive tool for the determination of the effects of proliferation and differentiation inducing agents on leukaemic cells.

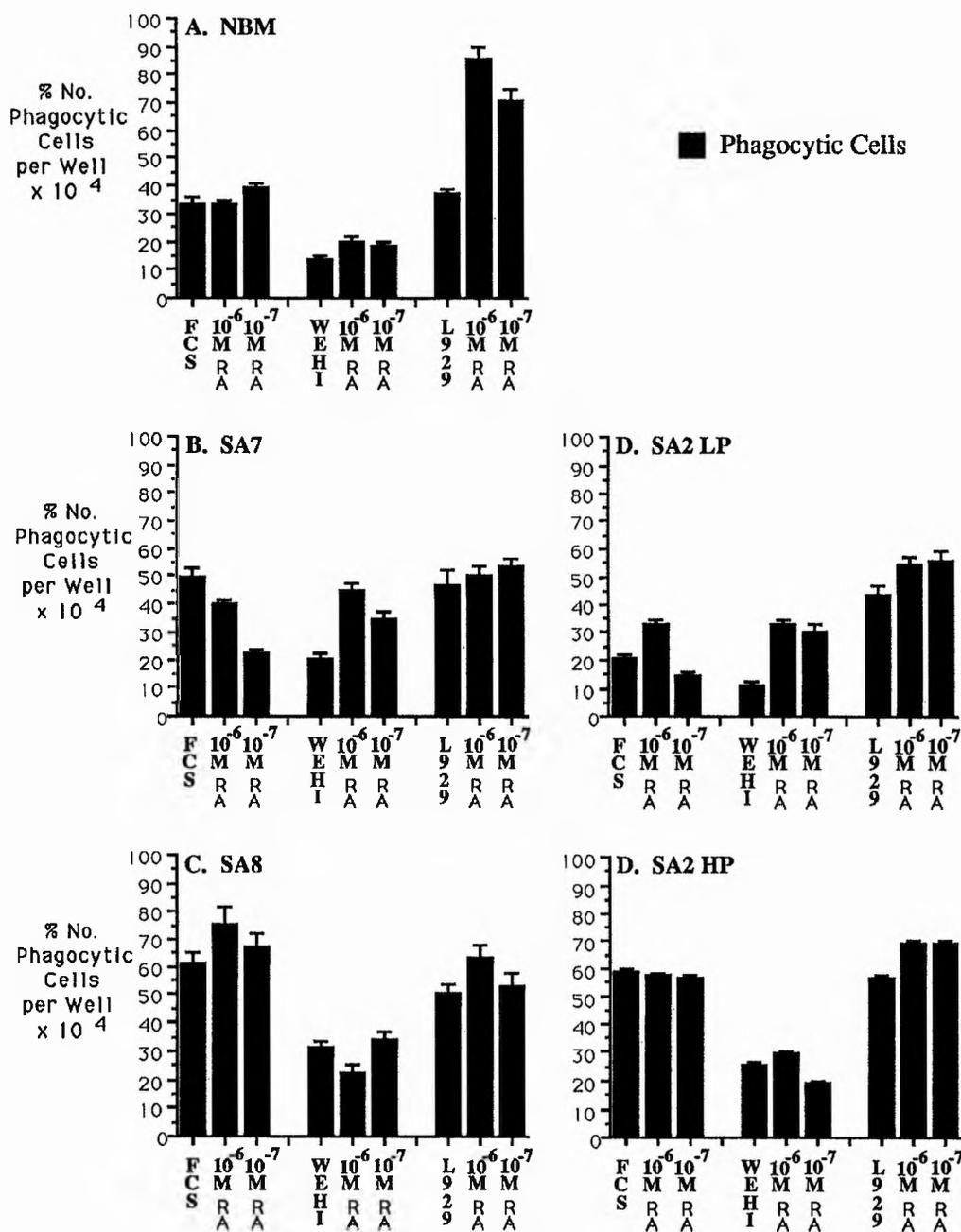


**Figure 4.1** Preliminary study on the effect of  $\beta$ -all *trans* retinoic acid on the proliferation of normal and leukaemic bone marrow cells and leukaemic cells from the SA2 CL. Single cell suspensions were cultured in the microtitre assay for four days with varying concentrations of  $\beta$ atRA. Proliferation was measured utilising the uptake of  $^3\text{H}$ -TdR. For growth factor dependent cells WEHI-3B CM was used to stimulate proliferation and growth factor independent cells were cultured in FCS alone. The scintillation CPM were converted to "Percentage of Control". Control cells were not treated with RA.  $10^{-6}\text{M}$  and  $10^{-7}\text{M}$  RA were consequently used for the differentiation studies.

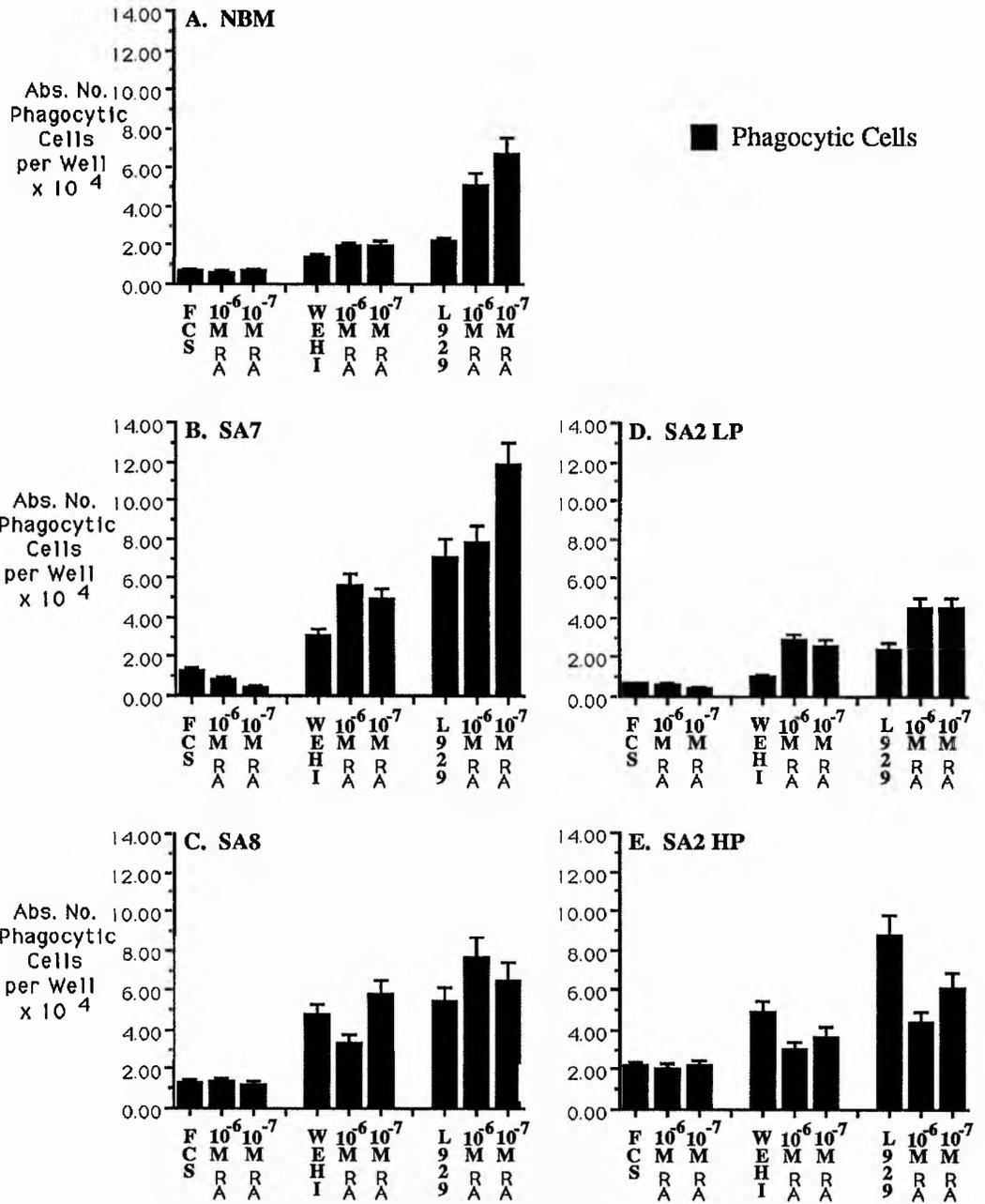


**Figure 4.2** Differentiation Study: Cells were cultured for four days in the microtitre assay with FCS, WEHI-3B CM or L929 CM with and without the addition of  $\beta$ TRA. Cytospins were prepared stained and differentials counted. At least 500 cells were counted per slide.

Mature cells = bands, segmented, monocytes and macrophages  
 Intermediate cells = myelocytes, immature monocytic cells and metamyelocytes  
 Immature cells = blasts, promyelocytes

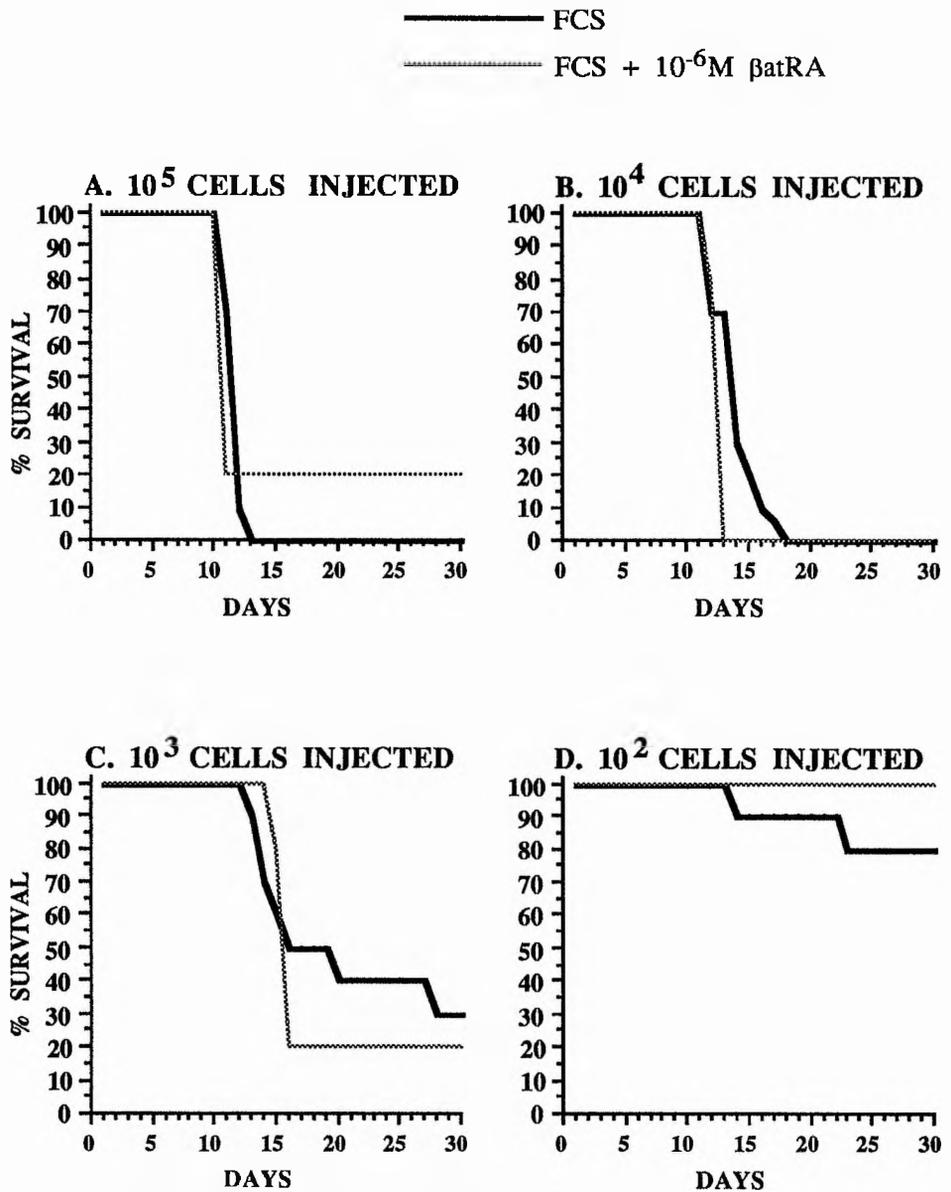


**Figure 4.3** Differentiation study. Cells were cultured for four days in the microtitre assay with FCS, WEHI-3B CM and L929 CM with and without  $\beta$ taraxerol. Two concentrations of  $\beta$ taraxerol were used,  $10^{-6}$  M and  $10^{-7}$  M. On day four cells were incubated with  $8\mu$  latex particles for eight hours. Cytospins were prepared, stained and the number of cells containing greater than 10 particles was determined. The results depict the percentage number of phagocytic cells per well.



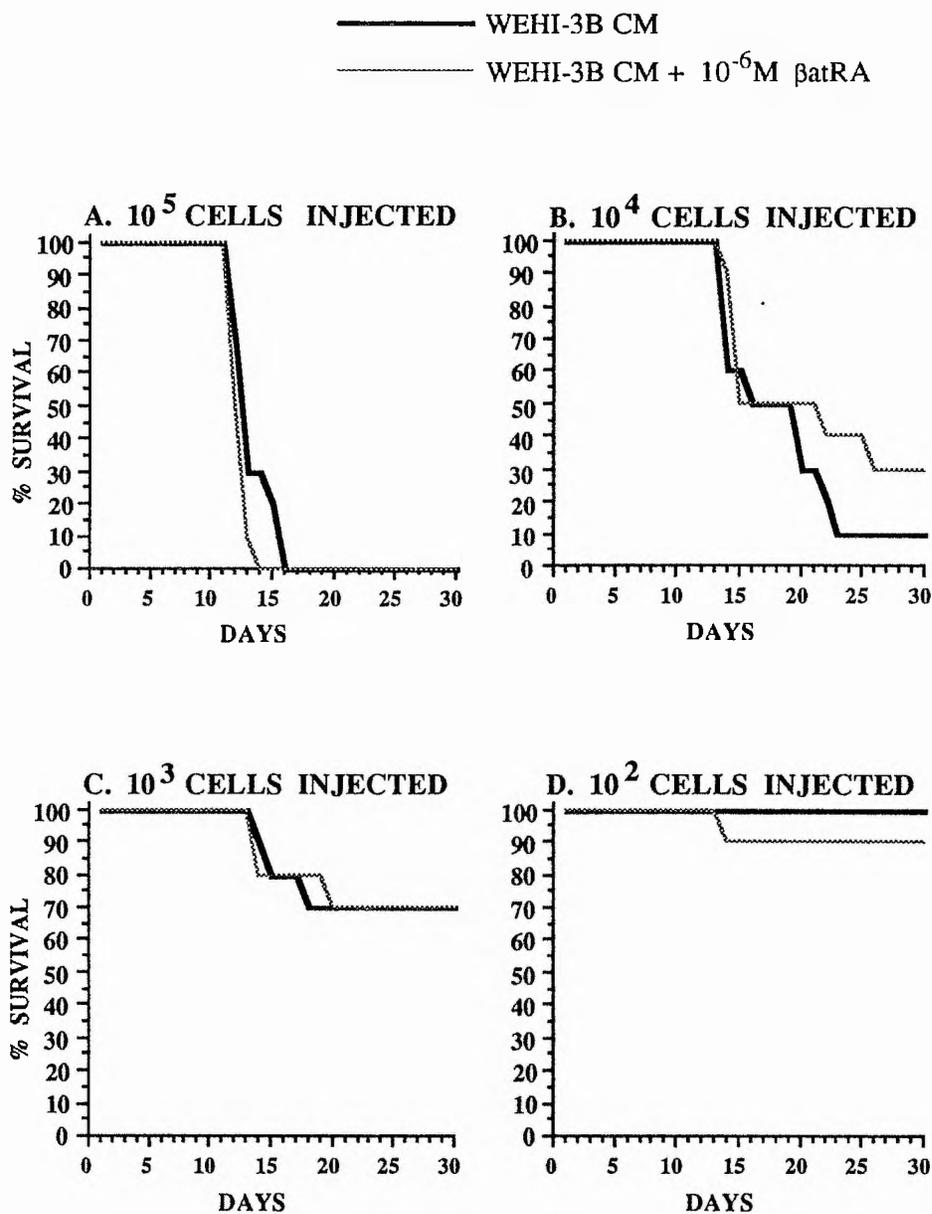
**Figure 4.4** Differentiation study. Cells were cultured for four days in the microtitre assay with FCS, WEHI-3B CM and L929 CM with and without  $\beta$ TRA. Two concentrations of  $\beta$ TRA were used,  $10^{-6}$  M and  $10^{-7}$  M.

On day four cells were incubated with  $8\mu$  latex particles for eight hours. Cytospins were prepared, stained and the number of cells containing greater than 10 particles was determined. The results depict the absolute number of phagocytic cells per well.



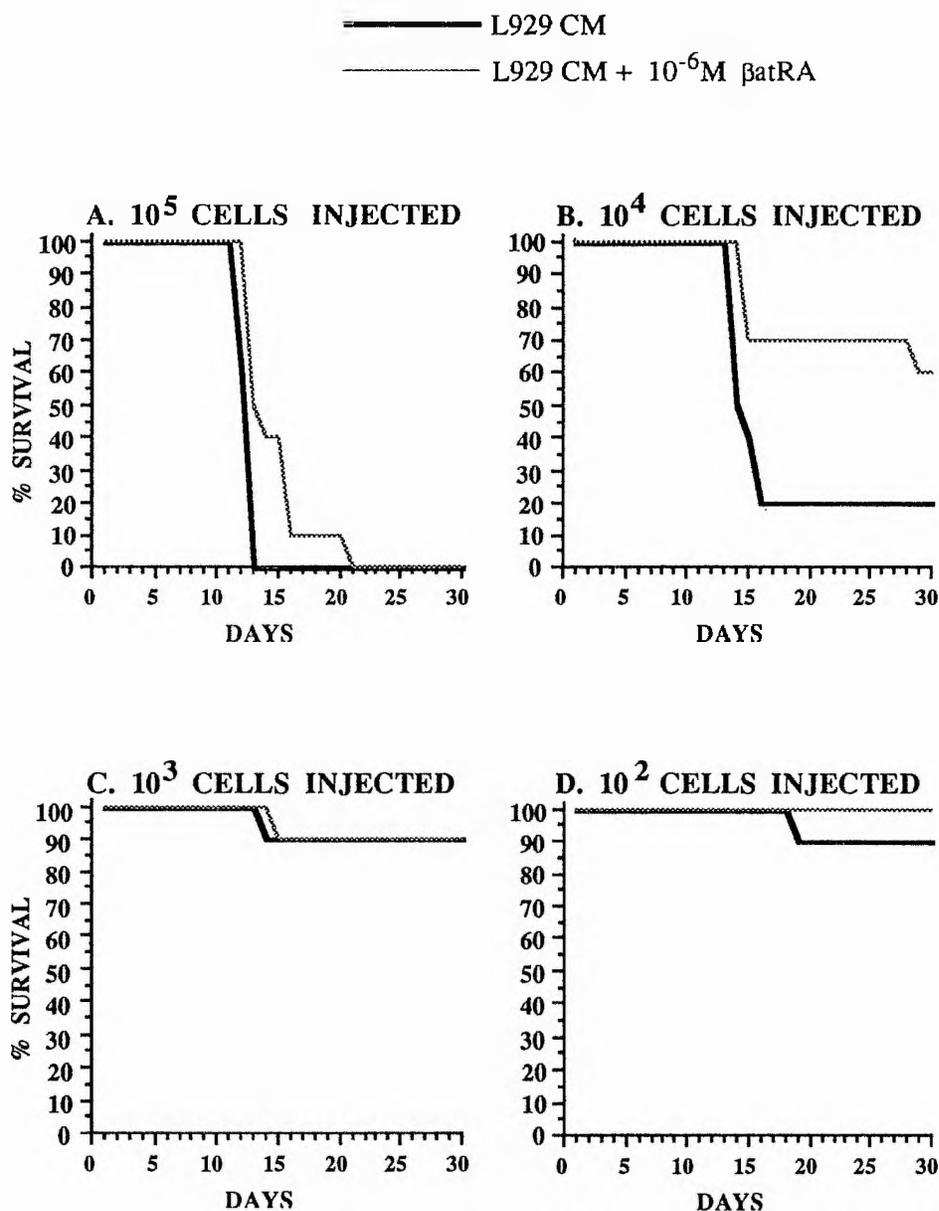
**Figure 4.5** *In Vivo* Clonogenic Cell Assay.

Single cell suspension of SA7 leukaemic bone marrow cells were cultured in the microtitre assay with FCS  $\pm 10^{-6}$  M  $\beta$ atRA. On day four of culture the cells were harvested with a pasteur pipette, the number of viable cells counted and varied concentrations of cells were injected into syngeneic recipients to determine the number of clonogenic leukaemic cells remaining in culture after each treatment. *in vitro*. Five mice were injected per test group and the percentage survival was monitored for 90 days. The results were the means of three experiments and the standard error was not greater than 5% for each treatment.



**Figure 4.6** *In Vivo* Clonogenic Cell Assay.

Single cell suspension of SA7 leukaemic bone marrow cells were cultured in the microtitre assay with WEHI-3B CM  $\pm$   $10^{-6}$  M  $\beta$ atRA. On day four of culture the cells were harvested with a pasteur pipette, the number of viable cells counted and varied concentrations of cells were injected into syngeneic recipients to determine the number of clonogenic leukaemic cells remaining in culture after each treatment. *in vitro*. Five mice were injected per test group and the percentage survival was monitored for 90 days. The results were the means of three experiments and the standard error was not greater than 5% for each treatment.



**Figure 4.7** *In Vivo* Clonogenic Cell Assay.

Single cell suspension of SA7 leukaemic bone marrow cells were cultured in the microtitre assay with L929 CM  $\pm$   $10^{-6}$  M  $\beta$ atRA. On day four of culture the cells were harvested with a pasteur pipette, the number of viable cells counted and varied concentrations of cells were injected into syngeneic recipients to determine the number of clonogenic leukaemic cells remaining in culture after each treatment. *in vitro*. Five mice were injected per test group and the percentage survival was monitored for 90 days. The results were the means of three experiments and the standard error was not greater than 5% for each treatment.

## CHAPTER FIVE

### **AN *IN VITRO* STUDY OF THE COMBINATION OF A DIFFERENTIATION INDUCER, $\beta$ -ALL TRANS RETINOIC ACID, AND A DNA SYNTHESIS INHIBITOR, ARA-C, ON MURINE MYELOID LEUKAEMIAS.**

Combinations of differentiation inducing agents and DNA synthesis inhibitors have been shown to have synergistic differentiation effects on bone marrow cells from patients with acute myeloid leukaemia (AML) and myelodysplastic syndromes (MDS) (Francis et al, 1985, 1987; Ho et al, 1987; Hoffman et al, 1985; Hassan and Rees, 1988; Hellstrom et al, 1987). The resultant responses observed *in vitro* suggested that, with the combination of agents which used different modes of action, inhibition of proliferation and induction of differentiation, lower, less toxic doses of each agent could be used to achieve decreases in the proliferation of the malignant clones comparable to high doses of single agents (Francis et al, 1985). Such therapeutic regimes have been suggested for patients with AML who are refractory to conventional chemotherapy, in elderly patients with AML who are unfit for aggressive chemotherapeutic protocols and with MDS patients whose cytopenias are exacerbated resulting in increased mortality rates with the use of conventional chemotherapy. Studies on the combination of these agents have been mainly limited to bone marrow cells from patients with MDS (Francis et al, 1985; Francis et al, 1987; Ho et al, 1987). Studies on bone marrow cells from patients with AML have been limited (Hoffman et al, 1985; Hassan and Rees, 1988; Hellstrom et al, 1987) and have focused on the use of 13-*cis* RA or have not specified which RA was employed. Most *in vitro* studies on the combination of differentiation inducing agents and DNA synthesis inhibitors have been primarily concerned with enhanced or synergistic increases in the differentiation response. In Chapter Four we have shown that all the transplanted leukaemic cell lines were induced to differentiate with  $\beta$ atRA in WEHI-3B CM stimulated

cultures. In this study we were primarily concerned with the enhanced or synergistic decreases in the proliferative response of leukaemic cells with the use of the combination of agents. We aimed to evaluate the combination of  $\beta$ atRA with cytosine arabinoside (ara-C) for the treatment of myeloid leukaemia by monitoring for synergistic decreases in the proliferative response. Normal bone marrow, bone marrow cells from the transplanted murine myeloid leukaemias and the SA2 CL were studied *in vitro*. The SA2 transplanted leukaemia was studied progressively on consecutive transplantations. The dominant leukaemic clone of the SA2 leukaemia has been shown to alter from growth factor independent to growth factor dependent *in vitro* (Chapter Two) and we aimed to monitor changes in the response to the combination of agents following serial transplantation.

## 5.1 EXPERIMENTAL PROCEDURE

a). Single cell suspensions of normal and leukaemic bone marrow cells and cells from the SA2 CL were cultured in the microtitre assay with varying concentrations of  $\beta$ atRA and ara-C alone and in combination ( $\beta$ atRA -  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$ M; ara-C -  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$  and  $10^{-10}$ M total concentration per well). WEHI-3B CM was used as the growth factor source for the growth factor dependent bone marrow cells. It must be noted here that growth factor sources provide an intrinsic differentiation stimulus in the culture system and this will be reflected in the results. WEHI-3B CM was used as it induced a lower differentiation response and maintained relatively higher numbers of immature leukaemic cells in culture than L929 CM or rGM-CSF, as reported in Chapter Three. Growth factor independent cells were cultured in FCS alone.

b) The SA2 transplanted leukaemic cell line which has been shown to undergo a change from being growth factor independent to growth factor dependent with serial transplantation was rederived using frozen cells from passage number 32.

The effects of the combination of  $\beta$ atRA and ara-C on the SA2 transplanted leukaemic cell line were investigated progressively on transplantation numbers 36 to 59 to monitor the differences in response as the leukaemic population altered. Four representative studies were documented, transplantation numbers 36 and 39 which were growth factor independent and transplantation numbers 55 and 59 which were growth factor dependent.

## 5.2 RESULTS

### 5.2.1 The *in vitro* proliferative effects of the combination of $\beta$ atRA and ara-C on leukaemic and normal bone marrow cells

$10^{-5}$ M  $\beta$ atRA and  $10^{-6}$ M ara-C were found to be cytotoxic on normal and leukaemic cells. Increases but not decreases in the proliferative response of the normal bone marrow cells were observed with the combination of  $\beta$ atRA and ara-C compared to either agent alone. With the addition of  $10^{-6}$ M  $\beta$ atRA to each of the concentrations of ara-C studied the proliferative responses observed were equal to the responses observed with ara-C alone (Fig. 5.1 A). With the combination of  $10^{-7}$ M  $\beta$ atRA and  $10^{-9}$ M ara-C an increase in the proliferative response was observed compared to either factor alone (Fig. 5.1 B). With the addition of  $10^{-7}$ M  $\beta$ atRA to  $10^{-8}$ M or  $10^{-7}$ M ara-C the proliferative responses observed were equivalent to the responses observed with ara-C alone. With the addition of  $10^{-8}$ M  $\beta$ atRA no differences in response were observed compared to ara-C alone (Fig. 5.1 C).

### **5.2.2 The in vitro proliferative effects of the combination of $\beta$ atRA and ara-C on the SA7 leukaemia**

The pattern of response observed with the SA7 leukaemia was similar to that of normal bone marrow. Increases but not decreases in the proliferative response of the SA7 leukaemia were observed with the combination of  $\beta$ atRA and ara-C compared to either agent alone. With the addition of  $10^{-6}$ M  $\beta$ atRA to each concentration of ara-C studied no differences in the proliferative response were observed compared to ara-C alone (Fig. 5.2 A). With the combination of  $10^{-7}$ M  $\beta$ atRA and  $10^{-9}$ M ara-C an increase in the proliferative response was observed compared to either agent alone (Fig. 5.2 B). With the addition of  $10^{-7}$ M  $\beta$ atRA to  $10^{-8}$ M or  $10^{-7}$ M ara-C the proliferative responses observed were equivalent to the responses observed with ara-C alone. With the addition of  $10^{-8}$ M  $\beta$ atRA no differences in response were observed compared to ara-C alone (Fig. 5.2 C).

### **5.2.3 The in vitro proliferative effects of the combination of $\beta$ atRA and ara-C on the SA8 leukaemia**

The combination of  $\beta$ atRA and ara-C to bone marrow cultures of the SA8 leukaemia resulted in 1) decreases in the proliferative response compared to ara-C alone and 2) synergistic decreases in proliferation compared to either agent alone. Decreases in the proliferative response compared to ara-C alone were observed with the combination of  $10^{-6}$ M  $\beta$ atRA plus  $10^{-9}$ M or  $10^{-8}$ M ara-C; the responses observed with these combinations were equivalent to the response observed with  $10^{-6}$ M  $\beta$ atRA alone (Fig. 5.3 A). The proliferative responses observed with  $10^{-6}$ M  $\beta$ atRA,  $10^{-7}$ M ara-C and their combination were equivalent. Synergistic decreases in proliferative response were observed with the combinations of either  $10^{-7}$ M or  $10^{-8}$ M  $\beta$ atRA with  $10^{-8}$ M or  $10^{-9}$ M ara-C (Figs. 5.3 B and C). The resultant proliferative responses obtained with these lower concentrations of ara-C ( $10^{-8}$  and  $10^{-9}$ M) in combination with lower concentrations

of  $\beta$ atRA ( $10^{-7}$  and  $10^{-8}$ M) were equivalent to the proliferative response obtained with the higher concentration of ara-C ( $10^{-7}$ M) alone and equivalent to the higher concentration of  $\beta$ atRA ( $10^{-7}$ M). Hence, with the combination of agents a comparable decrease in proliferation was obtained with a 10 fold decrease in the concentration of  $\beta$ atRA and a 10 and 100 fold decrease in the concentration of the more toxic agent ara-C.

#### **5.2.4 The *in vitro* proliferative effects of the combination of $\beta$ atRA and ara-C on the SA2 leukaemia**

##### **5.2.4.1 Transplantation number 36**

The SA2 LP passage number 36 (SA2 p36) leukaemic bone marrow cells were growth factor independent *in vitro* and the cells from this study were cultured in FCS alone. The proliferative response of the SA2 p36 leukaemia was inhibited by both  $\beta$ atRA and ara-C to a greater extent than the other leukaemias described (Fig's. 5.4 and 5.5).  $10^{-6}$ M  $\beta$ atRA and concentrations as low as  $10^{-8}$ M ara-C had a toxic effects on this leukaemia. An inhibitory response ( $43\pm 7\%$  of control) was observed with  $10^{-10}$ M ara-C the lowest concentration tested whereas no inhibitory response was observed with this concentration on normal bone marrow or the SA7 or SA8 leukaemias. A synergistic decrease in the proliferative response was observed with the combination of  $10^{-8}$ M  $\beta$ atRA plus  $10^{-9}$ M ara-C (Fig's. 5.6 C). With the combination of  $10^{-7}$ M  $\beta$ atRA plus  $10^{-9}$ M ara-C a marked decrease in the proliferative response was observed compared to ara-C alone; the response was also less but comparable to the response observed with  $\beta$ atRA alone (Fig. 5.6 B).

##### **5.2.4.2 Transplantation number 39**

The SA2 p39 leukaemic bone marrow cells were growth factor independent *in vitro* and the cells from this study were cultured in FCS alone. The proliferative

responses of the leukaemic bone marrow cells from the SA2 p39 to decreasing concentrations of ara-C alone and in combination with  $\beta$ tRA differed from passage number 36. The response to ara-C alone was similar to that of the SA8 leukaemia.  $10^{-6}$ M ara-C was toxic and the proliferative responses observed with  $10^{-7}$ M and  $10^{-8}$ M ara-C were  $26\pm 13\%$  and  $88\pm 9\%$  of the control respectively (Fig 5.4). The responses observed with decreasing concentrations of  $\beta$ tRA alone however, were similar to the responses observed with the bone marrow cells from passage number 36 (Fig 5.5). This was consistent with the study in Chapter 4 where the SA2 LP leukaemia was observed to be more responsive to differentiation induction with  $\beta$ tRA than the SA7 or SA8 leukaemias. Unlike the SA2 p36 leukaemia no statistically significant synergistic decrease ( $p>0.2$ ) in the proliferative response was observed with the combination of agents; however, with the combination of  $10^{-7}$ M or  $10^{-8}$ M  $\beta$ tRA and  $10^{-7}$ M ara-C the trend observed was a decrease in the proliferative response compared to either factor alone (Figs. 5.7 B and C). The responses observed with the combinations of agents were similar to the responses observed with  $\beta$ tRA alone and less than the responses observed with ara-C alone (Fig 5.7).

#### 5.2.4.3 Transplantation number 55

Leukaemic bone marrow cells from the SA2 p55 leukaemia were growth factor dependent *in vitro* and cells were cultured with WEHI-3B CM which has been shown to have a more limited effect on differentiation induction of the SA2 HP leukaemias than L929 CM. The SA2 p55 leukaemia was more sensitive to ara-C than normal bone marrow, the SA7, SA8 and SA2 p39 leukaemia. A 50% inhibitory response was observed with  $10^{-9}$ M ara-C (Fig. 5.4). The response observed with  $\beta$ tRA was similar to that observed with the SA7 and SA8 leukaemia (Fig. 5.5). Synergistic decreases in the proliferative response with the

combination of  $\beta$ tRA and ara-C were observed. The synergistic decreases in the proliferative response were observed with  $10^{-7}$ M  $\beta$ tRA plus  $10^{-9}$ M ara-C (Fig. 5.8 B), and  $10^{-8}$ M  $\beta$ tRA plus  $10^{-9}$ M or  $10^{-8}$ M ara-C.(Fig. 5.8 C). These responses were similar to the response observed with  $10^{-7}$ M ara-C alone, a 10 and 100 fold decrease in the concentration of ara-C

#### **5.2.4.4 Transplantation number 59**

Leukaemic bone marrow cells from the SA2 p59 leukaemia were growth factor dependent *in vitro* and cells were grown in WEHI-3B CM which has been shown to have a more limited effect on differentiation induction of the SA2 HP leukaemias than L929 CM. The SA2 p59 leukaemia was more sensitive to ara-C than the SA7 and SA8 leukaemias (Fig 5.4). The proliferative response with  $10^{-9}$ M ara-C was  $61\pm 8\%$  of the control. The response observed with  $\beta$ tRA was similar to the SA7, SA8, and SA2 p55 leukaemias (Fig. 5.5). A synergistic decrease in the proliferative response was observed with the combination of  $10^{-8}$ M  $\beta$ tRA and  $10^{-9}$ M ara-C (Fig 5.9 C). This response was similar to the response observed with  $10^{-8}$ M ara-C alone.

#### **5.2.5 The *in vitro* proliferative effects of the combination of $\beta$ tRA and ara-C on the SA2 CL**

The SA2 CL was growth factor independent *in vitro* and the cells for this study were cultured in FCS alone. The SA2 CL was the least sensitive of the leukaemias studied to ara-C. The proliferative response observed with  $10^{-7}$ M ara-C was  $37\pm 9\%$  of control and no inhibition of growth was observed with  $10^{-8}$ M ara-C (Fig. 5.4). In the differentiation study in Chapter Four it was shown that  $\beta$ tRA induced an inhibitory response of the SA2 CL without evidence of differentiation. The inhibitory response observed with  $10^{-6}$ M  $\beta$ tRA was  $30\pm 13\%$  of control, with

$10^{-7}$ M  $64 \pm 8\%$  and no inhibitory response was observed with  $10^{-8}$ M  $\beta$ atRA (Fig. 5.5). With the combination of  $\beta$ atRA and ara-C only decreases in proliferative response were observed compared to ara-C alone (Fig. 5.10). The proliferative responses observed with  $10^{-6}$ M  $\beta$ atRA in combination with decreasing concentrations of ara-C were equivalent to the responses observed with  $\beta$ atRA alone and less than the response observed with ara-C alone (Fig. 5.10 A). This was also the case with  $10^{-7}$ M  $\beta$ atRA and  $10^{-9}$ M ara-C (Fig. 5.10 B). The proliferative responses observed with all other combinations of  $10^{-7}$ M and  $10^{-8}$ M  $\beta$ atRA with decreasing concentrations of ara-C were equivalent to the responses observed with ara-C alone (Figs 5.10 B and C).

#### **5.2.6 Summary**

In summary (Table 5.1), of the leukaemias tested an adverse stimulatory response with the combination of agents was only observed with the SA7 leukaemia. Favourable responses were observed with the other leukaemias. An enhanced inhibitory effect compared with ara-C alone was observed with the SA8, SA2 p39, SA2 p55, SA2 p59 and the SA2 CL. Synergistic decreases in the proliferative response with the combination of agents was observed with the SA8, SA2 p36, SA2 p55 and the SA2 p59 leukaemias. The low dose of  $10^{-9}$ M ara-C in combination with  $10^{-7}$ M or  $10^{-8}$ M  $\beta$ atRA produced the synergistic responses observed and these responses were similar to the responses observed with a 10 or 100 times greater concentration of ara-C alone.. From the progressive study on the SA2 leukaemia it was observed that the leukaemic bone marrow cells became less sensitive to  $\beta$ atRA and ara-C when used alone with serial transplantation. However, the enhanced inhibitory responses and synergistic decreases in proliferation were observed at each transplantation.

**PROLIFERATIVE RESPONSE WITH COMBINATION OF  $\beta$ atPA & ARA-C**  
**STIMULATORY                      < ARA-C ALONE                      SYNERGISTIC DECREASE**

<b>NBM</b>	+	-	-
<b>SA 7</b>	+	-	-
<b>SA 8</b>	-	+	+
<b>SA2 p36</b>	-	-	+
<b>SA2 p39</b>	-	+	?
<b>SA2 p55</b>	-	+	+
<b>SA2 p59</b>	-	+	+
<b>SA2 CL</b>	-	+	-

**Table 5.1** The proliferative responses of leukaemic and normal cells to the combination of  $\beta$ atRA and ara-C. Stimulatory responses were observed with the combination of agents compared to either agent alone, or decreases in the proliferative responses were observed compared to ara-C alone and/or decreases in the proliferative response were observed compared to both agents alone (synergistic decreases).

### 5.3 DISCUSSION

With the combination of  $\beta$ atRA and ara-C synergistic decreases and enhanced decreases in the proliferative response compared to ara-C alone, were observed with two out of the four leukaemias studied. The responses observed were dependent upon the concentration of agents used. Different concentration combinations of the two agents induced synergistic decreases in the different leukaemias. An adverse stimulatory effect was observed with one of the leukaemias. A stimulatory response was observed with normal bone marrow indicating that, comparatively, the combination of agents preferentially inhibits some leukaemic cells rather than normal bone marrow cells. It appears therefore, that this combination of agents may be effective for the treatment of some leukaemias. Screening of patients would be essential for both susceptibility and dosage.

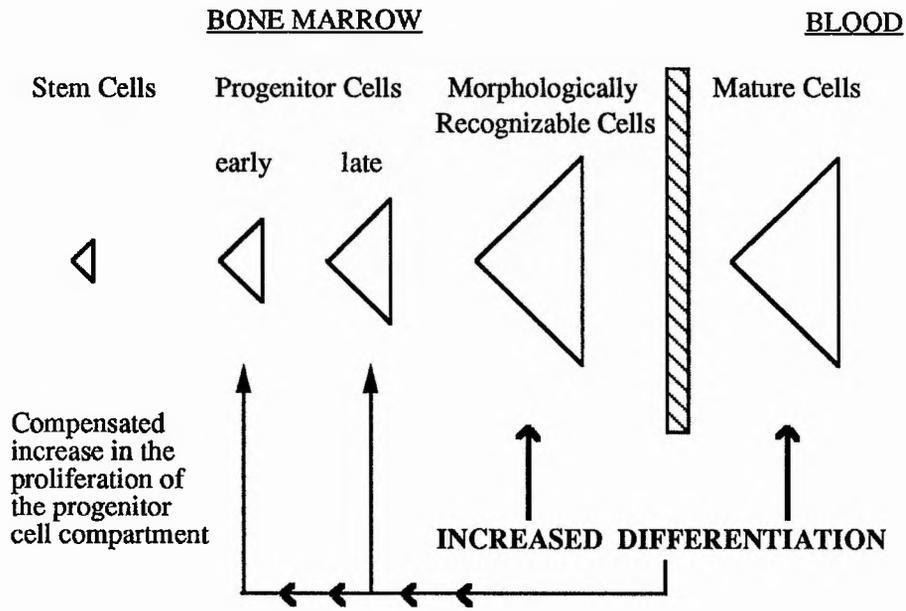
The reasoning behind using the combinations of differentiation inducing agents and DNA synthesis inhibitors for the treatment of AML stems from the concept that by using a differentiation inducing agent the resultant reduction in the

amplification of the system might be compensated by an increase in the leukaemic stem cell (clonogenic cell) proliferation rate. This possible increase in the proliferation of the stem cell pool would then be curbed by the use of a DNA synthesis inhibitor (Francis et al, 1985). As far as we are aware however, there is no experimental evidence suggesting that an increase in the proliferative rate of the leukaemic stem cell pool would result from the differentiation induction of the leukaemic clone (Fig. 5.11). From studies utilizing the leukaemic Clonogenic Cell Assay in Chapters Three and Four it was observed that there was no increase in the leukaemic clonogenic cell fraction when leukaemic cells were induced to differentiate *in vitro* with growth factors or  $\beta$ atRA. When L929 CM was used with  $\beta$ atRA, which were both shown to be effective inducers of differentiation of the leukaemia studied *in vitro*, a decrease in the leukaemic clonogenic cell fraction was observed.

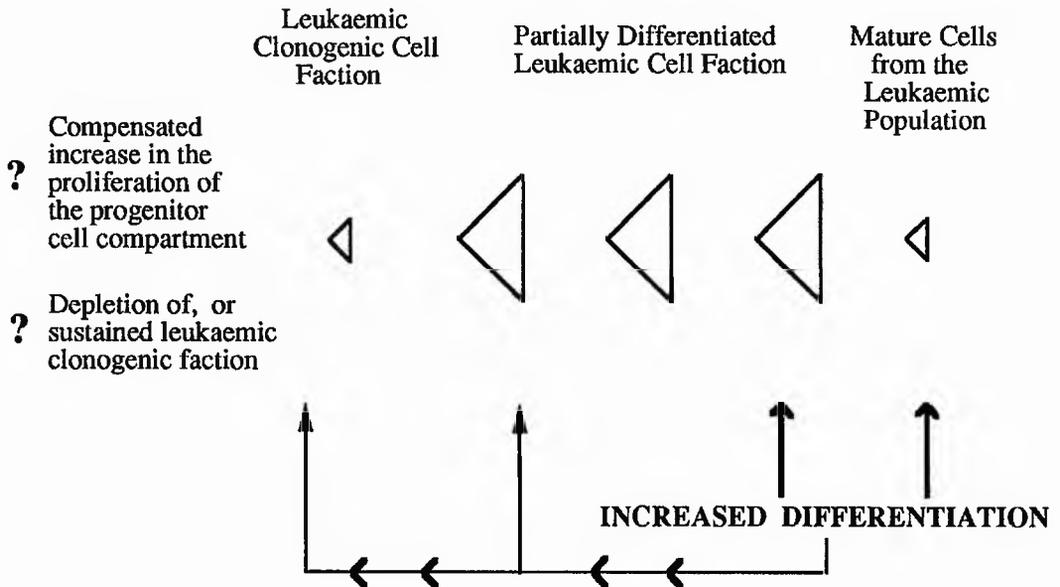
It has also been argued that the leukaemic state is not simply due to a maturation arrest or blockage but rather that there is a profound shift in the differentiation probability (Guimaraes et al, 1984). Consequently, two modes of intervention should be possible. One, with the use of agents which slow proliferation and two, with the use of agents that speed up differentiation.

There was disagreement in the literature as to whether low doses of chemotherapeutic agents acted principally as cytotoxic agents or differentiation inducers. The most widely accepted theory was that they primarily slow down DNA synthesis. Studies on differentiation commitment of leukaemic cells using chemical inducers of differentiation such as butyric acid and phorbol esters suggested that the decision point in the cell cycle regarding commitment may be at the late stage of the cell cycle or near the S phase (Boyd and Metcalf 1984). This has been referred to as the differentiation responsive "window" (Francis et al, 1985). By slowing DNA synthesis this phase of the cell cycle may be lengthened

**NORMAL HAEMOPOIESIS**



**ACUTE LEUKAEMIA**



**Figure 5.11** The possible effects of differentiation induction on the leukaemic clonogenic cell fraction

and/or the cell may be rendered more responsive to differentiation induction. The effect of low concentrations of chemotherapeutic agents have been shown to be more potent when arrest of proliferation is immediate and if the leukaemic clone is arrested in a late stage of differentiation (eg HL60, acute promyelocytic cells) (Chomienne, 1991). It has therefore, also been suggested that the inhibitory effect on leukaemic cell proliferation is an initiating factor for differentiation induction: by synchronizing the cells in a cell-cycle phase responsive to endogenous or exogenous differentiation agents, by controlling the transcription of oncogenes implicated in cell proliferation (*c-myc*, *c-fos* or with a tyrosine kinase action) or by directly modulating the activation of differentiation target genes (for review see Chomienne et al, 1991).

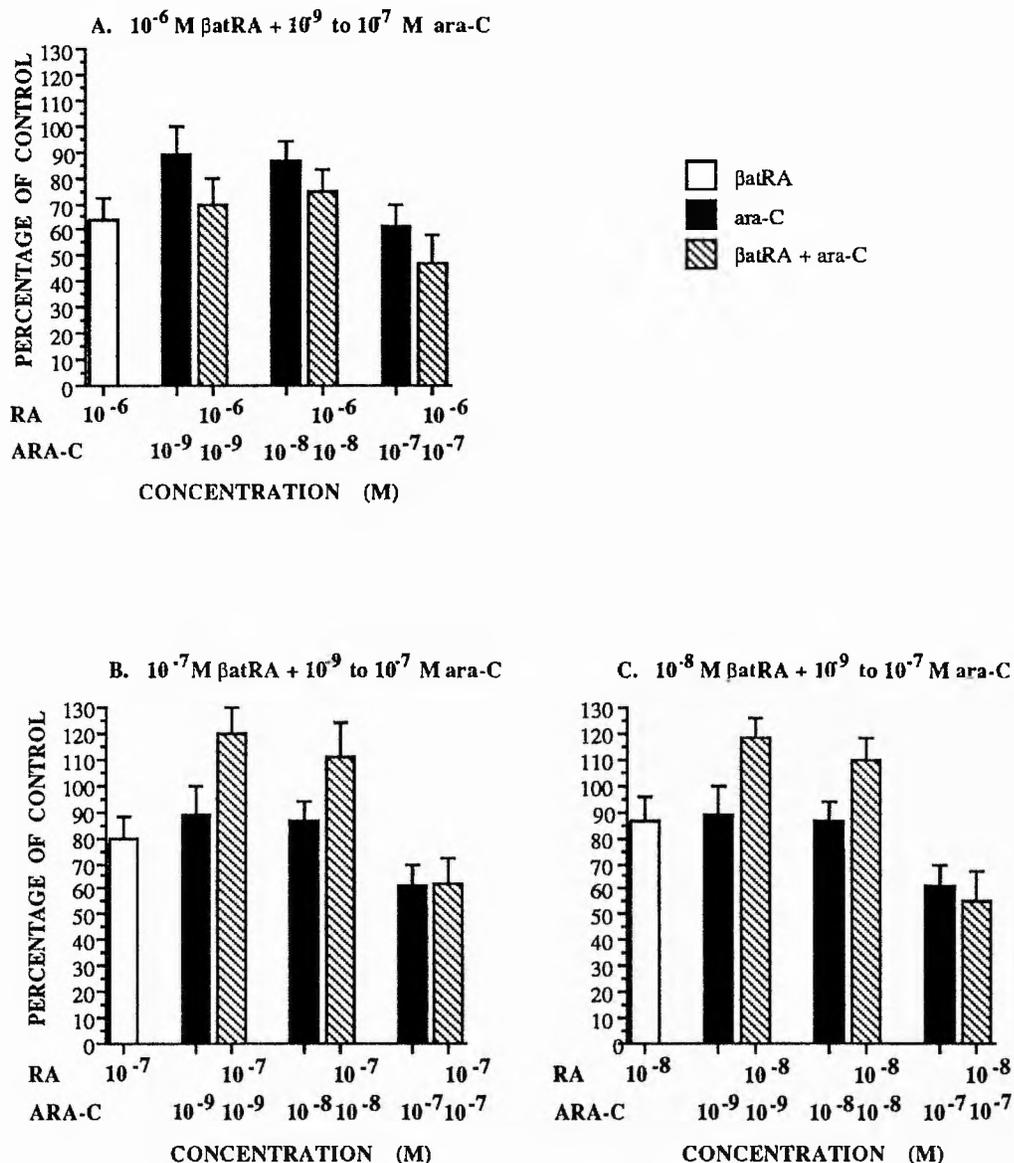
Most but not all of the initial *in vitro* data on the effectiveness of combinations of differentiation inducers and DNA synthesis were generally promising (Francis et al 1985; Clark et al, 1987; Ho et al, 1987; Hellstrom et al, 1988; Ganser et al, 1989). In a study by Waxman and Schreiber (1983) a low proportion of AML and MDS samples responded (4/28). However, in that study three patients with MDS were successfully treated with the combination of RA and hydroxyurea; one patient being relapse free for over two years. Very few combination therapy trials have been reported, most of these were on patients with MDS and unfortunately the results were not as promising as the *in vitro* data would suggest (Chomienne, 1991). Previous *in vitro* data on human AML cells has focussed on the use of 13-*cis* RA (Hoffman et al, 1985; Francis et al, 1987), and in another study the RA in combination with ara-C used was not specified (Hassan and Rees, 1988), and results have been variable. With evidence of the higher efficacy of  $\beta$ atRA compared to 13-*cis* RA (Chomienne et al 1986, 1990) further trials on fresh human AML cells with  $\beta$ atRA and ara-C were warranted. In a recent study by Hassan et al (1991) the double and triple combinations of  $\beta$ atRA, ara-C and hexamethylene

bisacetamide (HMBA) were evaluated in bone marrow cells from AML patients. A novel statistical analysis was used to assess the significance of synergy and antagonism in the studies of drug combinations. They studied AML bone marrow cells from each of the FAB classification subtypes (M1-M5) and they reported, but not specifically, successfully induced differentiation of bone marrow cells from AML patients with either single agents or with a combination of agents. Synergistic differentiation responses were observed with the combination of agents,  $\beta$ atRA plus ara-C being the most effective of the double combinations tested.

Further studies are also needed to elucidate whether the use of differentiation agents exacerbates the emergence of resistant leukaemic clones and whether mild long term maintenance of AML is possible and/or preferential over cytotoxic regimes. In a study of the long term treatment of M1 cells with the differentiation inducer Vit D3 Kasukabe et al (1987) concluded that the combination of Vit D3 with either daunomyocin or ara-C was more effective in reducing cell numbers of the M1 leukaemic cell line *in vitro* than Vit D3 alone. They also found that after 20 days of continuous treatment with Vit D3 alone a significant decrease in the proliferation of M1 cells was observed, however after 25 days proliferative resistant clones emerged in culture. It was not clear however if the treatment was ceased between day 20 and 25. They found that resistant cells did not appear with the combination therapy. They argued that the continuous treatment of myeloid cells with low doses of differentiation agents and low doses of cytotoxic agents was effective in inducing terminal differentiation of leukaemic cells and that the appearance of differentiation-resistant cells may be suppressed or retarded. In the clinical trial on patients with APL by Chen et al (1991) however, when  $\beta$ atRA was used in combination with chemotherapeutic agents as maintenance therapy after CR induction with  $\beta$ atRA, there was a high incidence

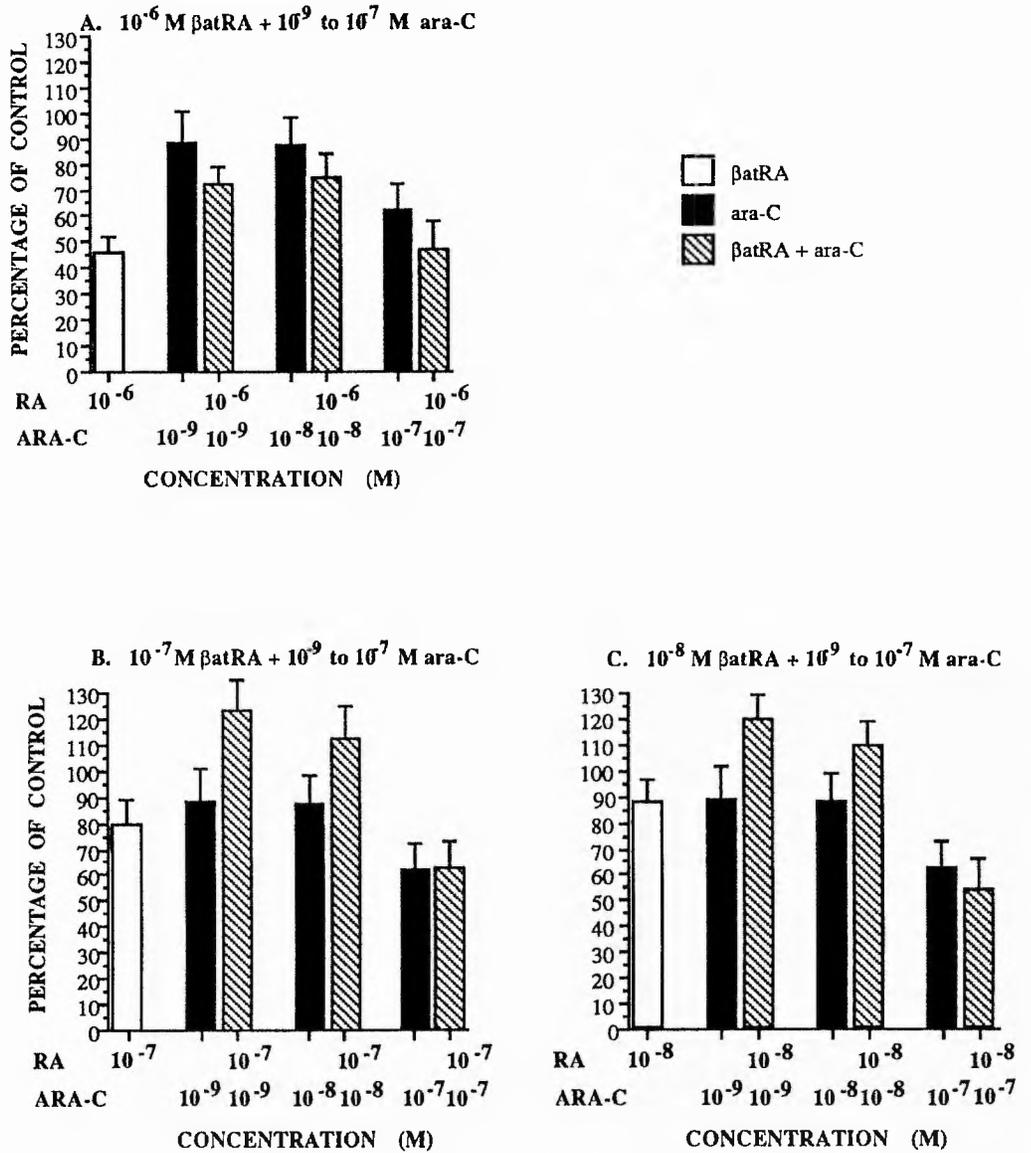
of relapse and emergence of leukaemic clones resistant to differentiation induction by  $\beta$ atRA. When chemotherapeutic agents were used alone for maintenance therapy the emergence of resistant leukaemic clones were not observed at least at first relapse.

## NBM



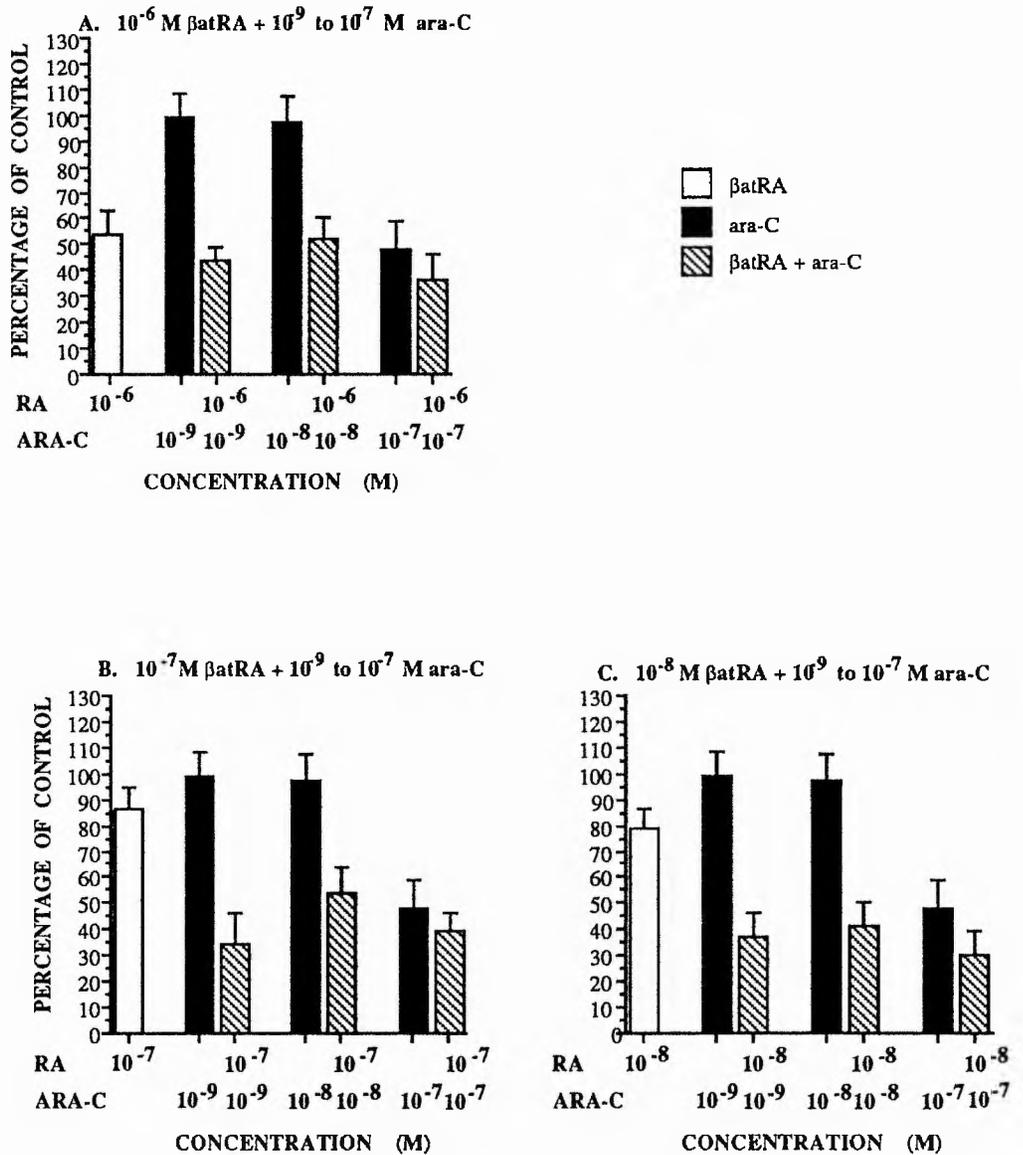
**Figure 5.1** Normal bone marrow single cell suspension was cultured in the microtitre assay with varying concentrations of  $\beta$ atRA and ara-C both alone and in combination. WEHI-3B CM was used as the growth factor source. On day four of culture the cells were pulsed with  $^3\text{H-TdR}$  for eight hours to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'.

SA7

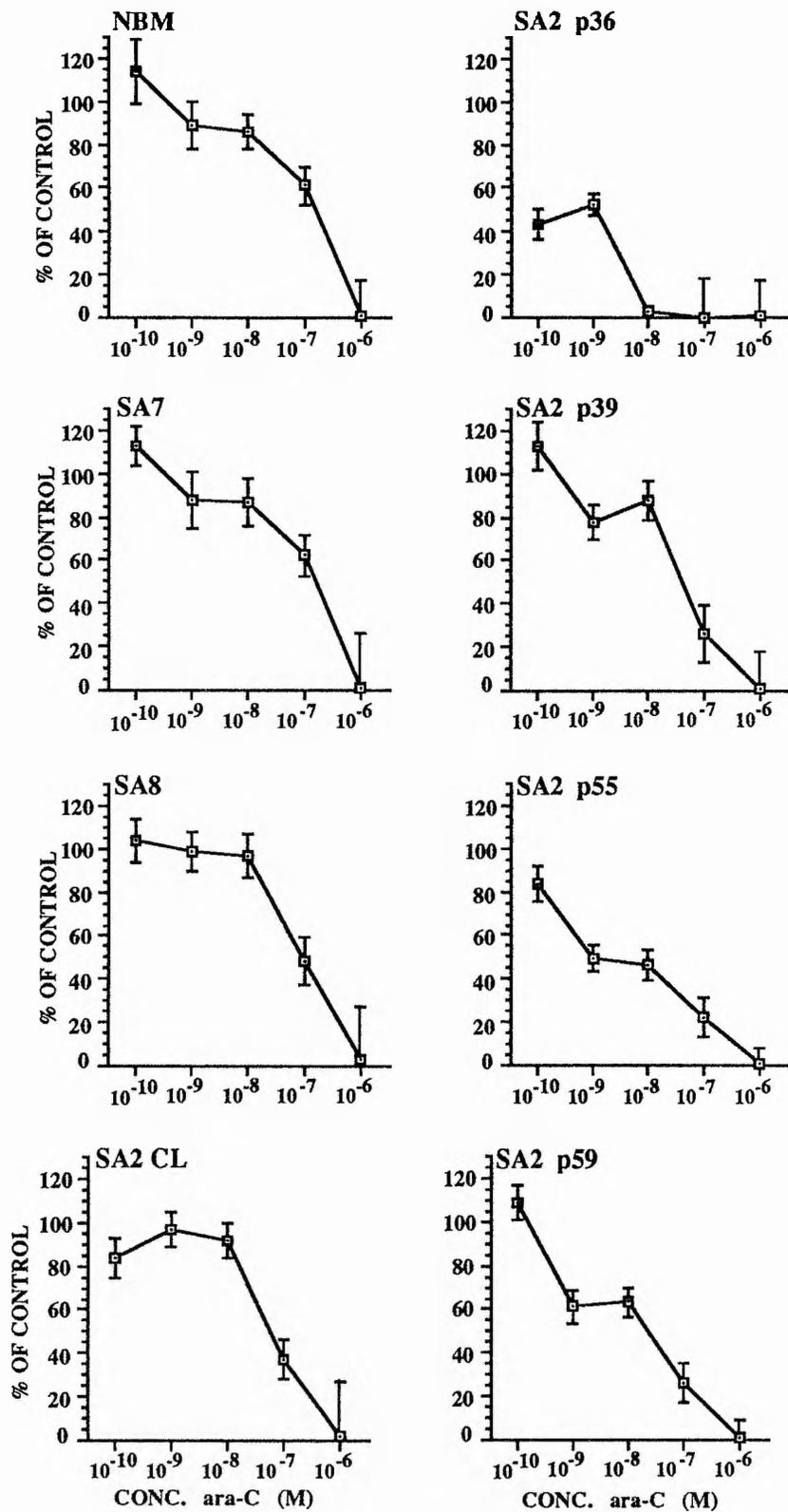


**Figure 5.2** SA7 leukaemic bone marrow single cell suspension was cultured in the microtitre assay with varying concentrations of  $\beta$ atRA and ara-C both alone and in combination. WEHI-3B CM was used as the growth factor source. On day four of culture the cells were pulsed with  $^3$ H-TdR for eight hours to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'.

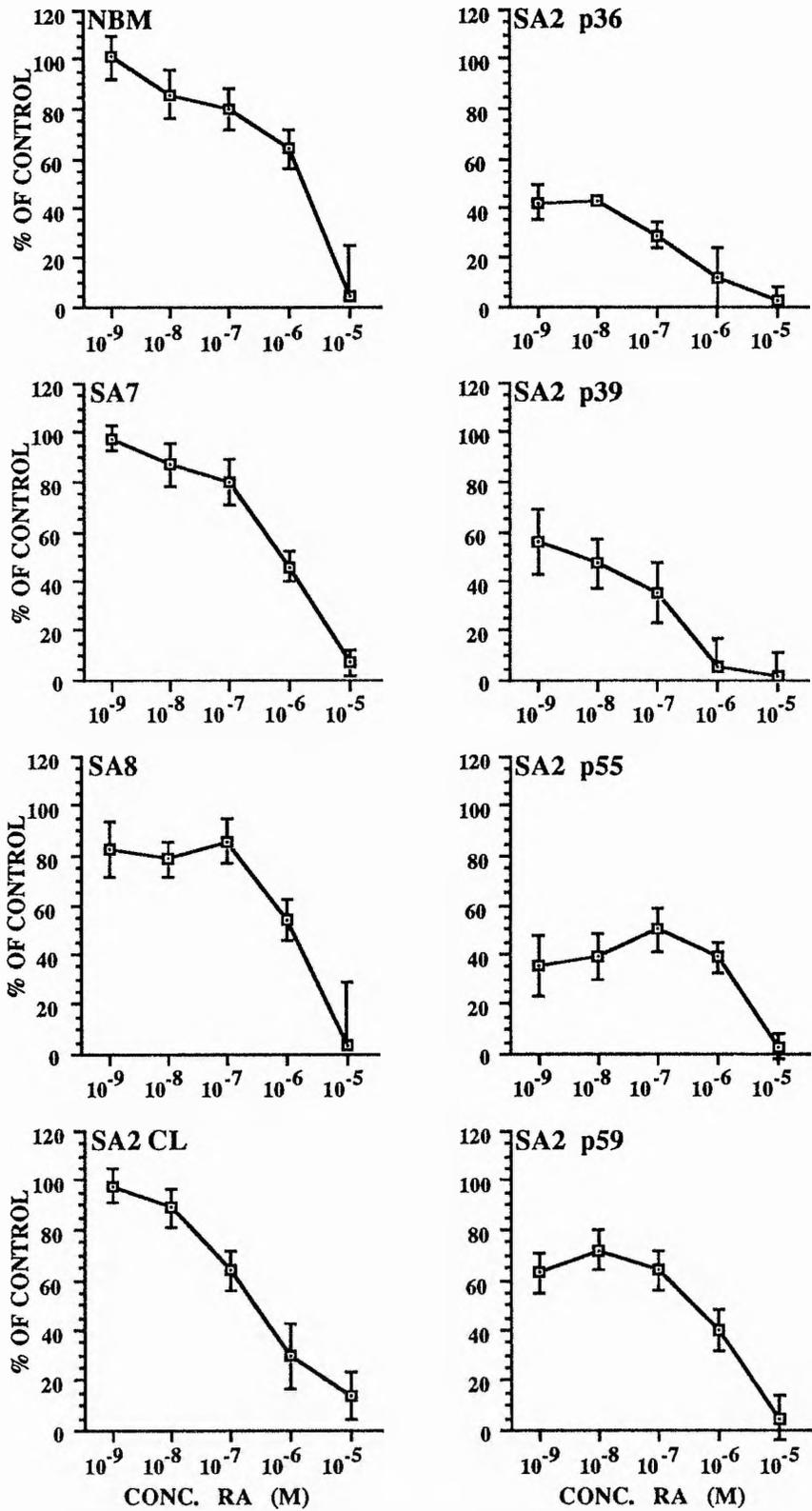
### SA8



**Figure 5.3** SA8 leukaemic bone marrow single cell suspension was cultured in the microtitre assay with varying concentrations of batRA and ara-C both alone and in combination. WEHI-3B CM was used as the growth factor source. On day four of culture the cells were pulsed with 3H-TdR for eight hours to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'.

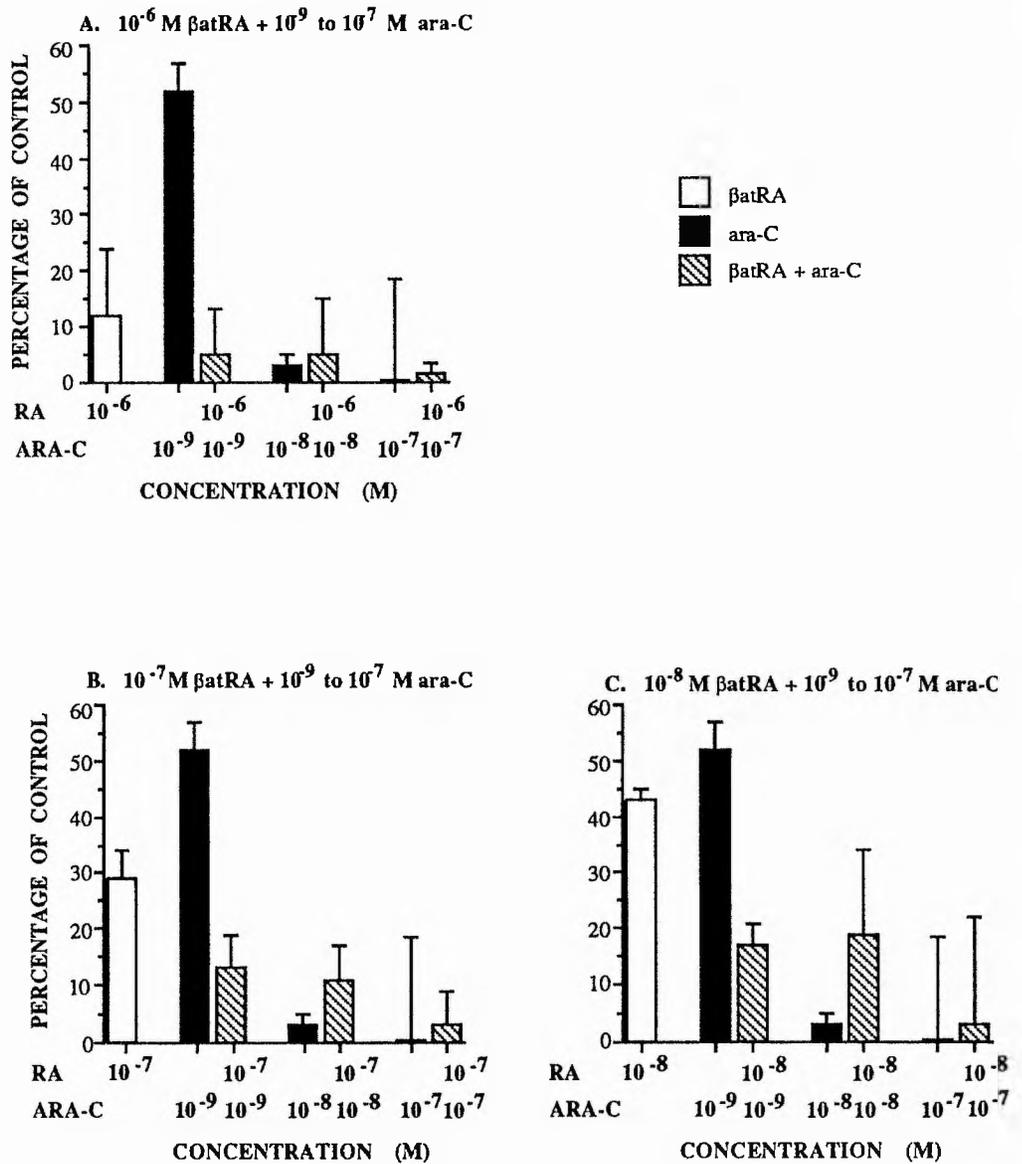


**Figure 5.4** The proliferative responses of leukaemic and normal cells to varying concentrations of ara-C. The cells were cultured in the microtitre assay for four days and pulsed with  $^3\text{H}$ -TdR to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'.



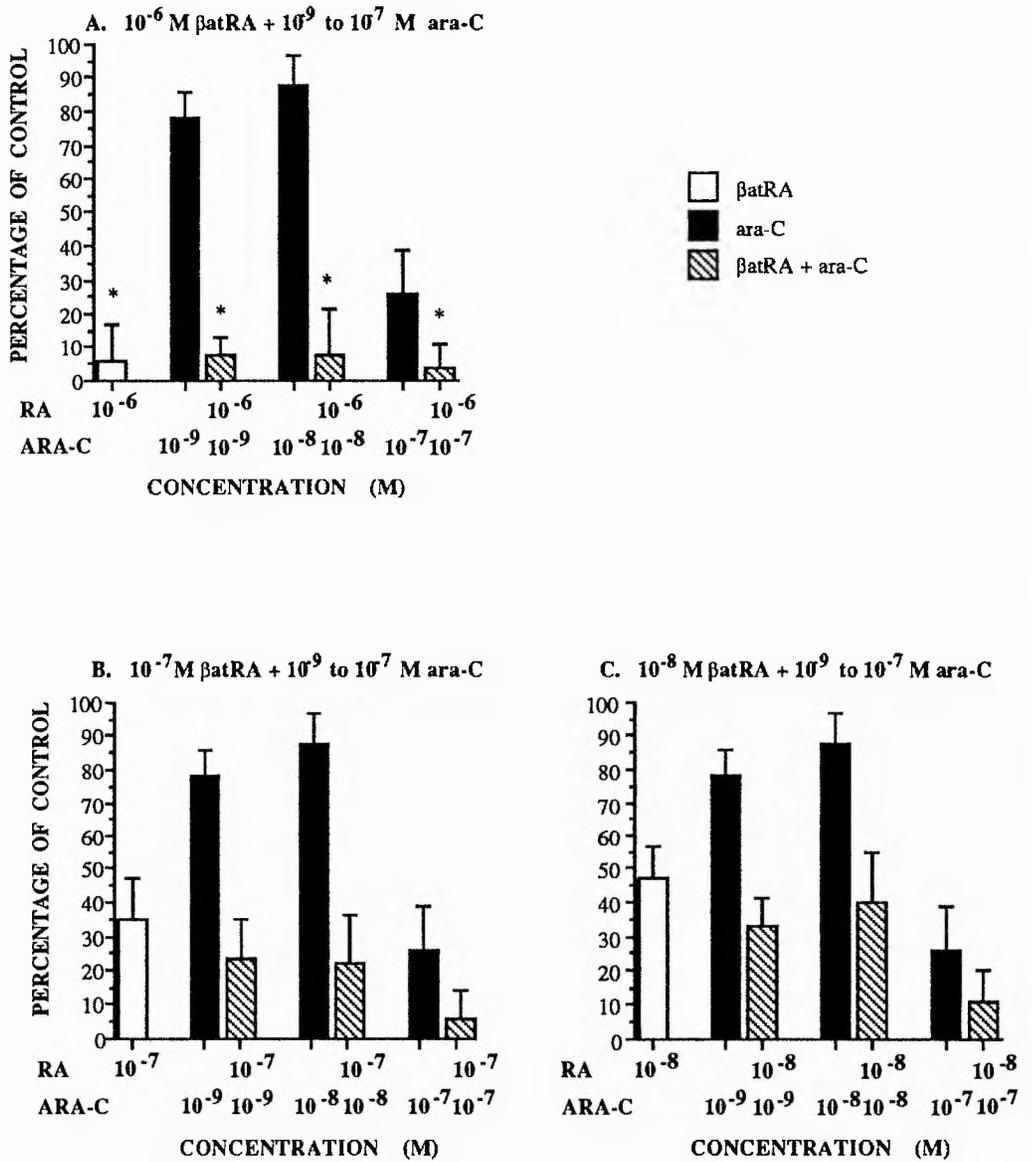
**Figure 5.5** The proliferative responses of leukaemic and normal cells to varying concentrations of batRA. The cells were cultured in the microtitre assay for four days and pulsed with  $^3\text{H}$ -TdR to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'.

## SA2 p36



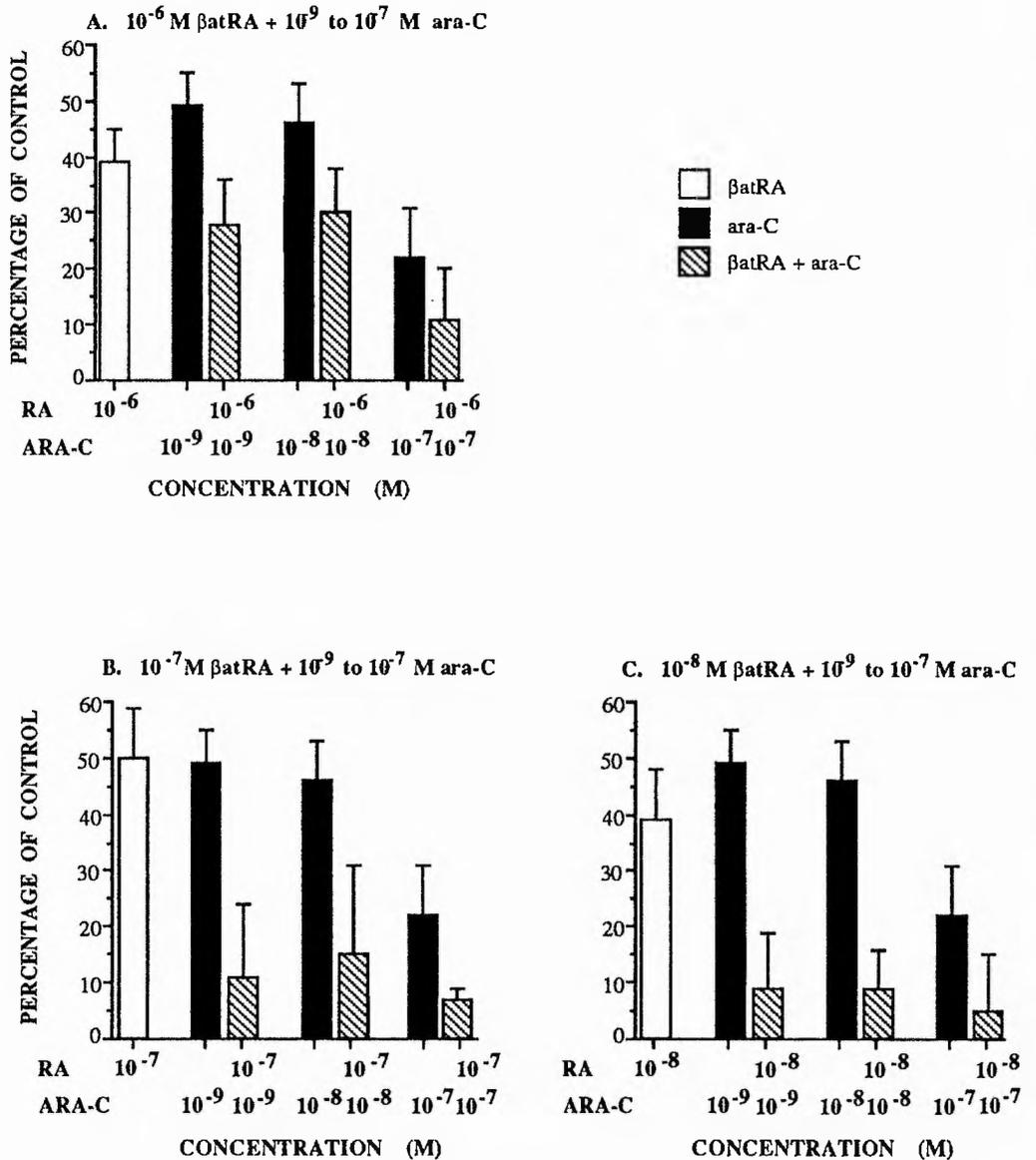
**Figure 5.6** SA2 p36 leukaemic bone marrow single cell suspension was cultured in the microtitre assay with varying concentrations of  $\beta$ atRA and ara-C both alone and in combination. The cells were cultured in FCS alone. On day four of culture the cells were pulsed with  $^3\text{H-TdR}$  for eight hours to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'.

SA2 p39



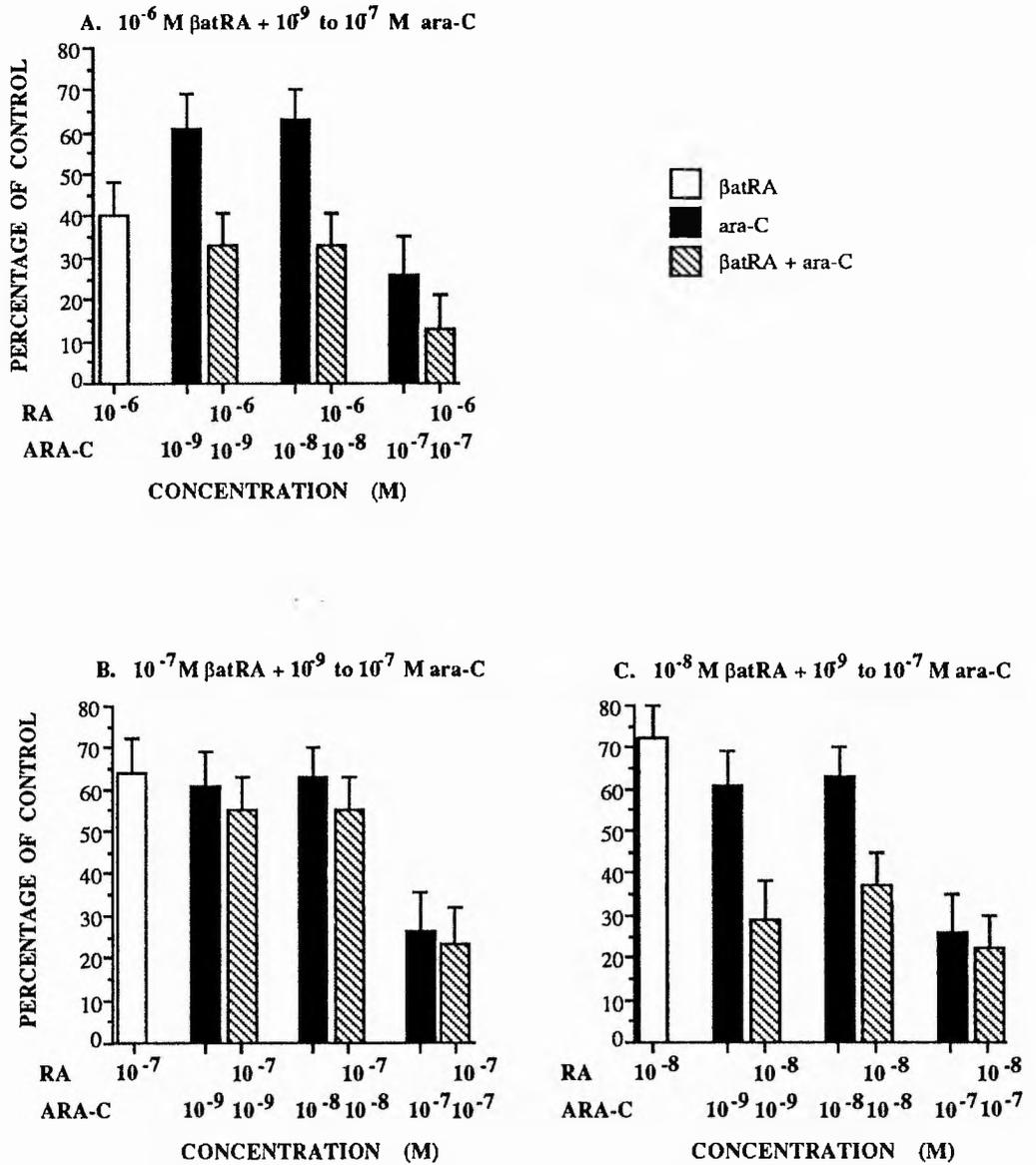
**Figure 5.7** SA2 p39 leukaemic bone marrow single cell suspension was cultured in the microtitre assay with varying concentrations of batRA and ara-C both alone and in combination. The cells were cultured in FCS alone. On day four of culture the cells were pulsed with  $^3\text{H-TdR}$  for eight hours to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'. (\* $p > 0.2$  No synergistic decrease was observed with the combination of  $10^6$  M batRA plus ara-C compared to batRA alone).

SA2 p55



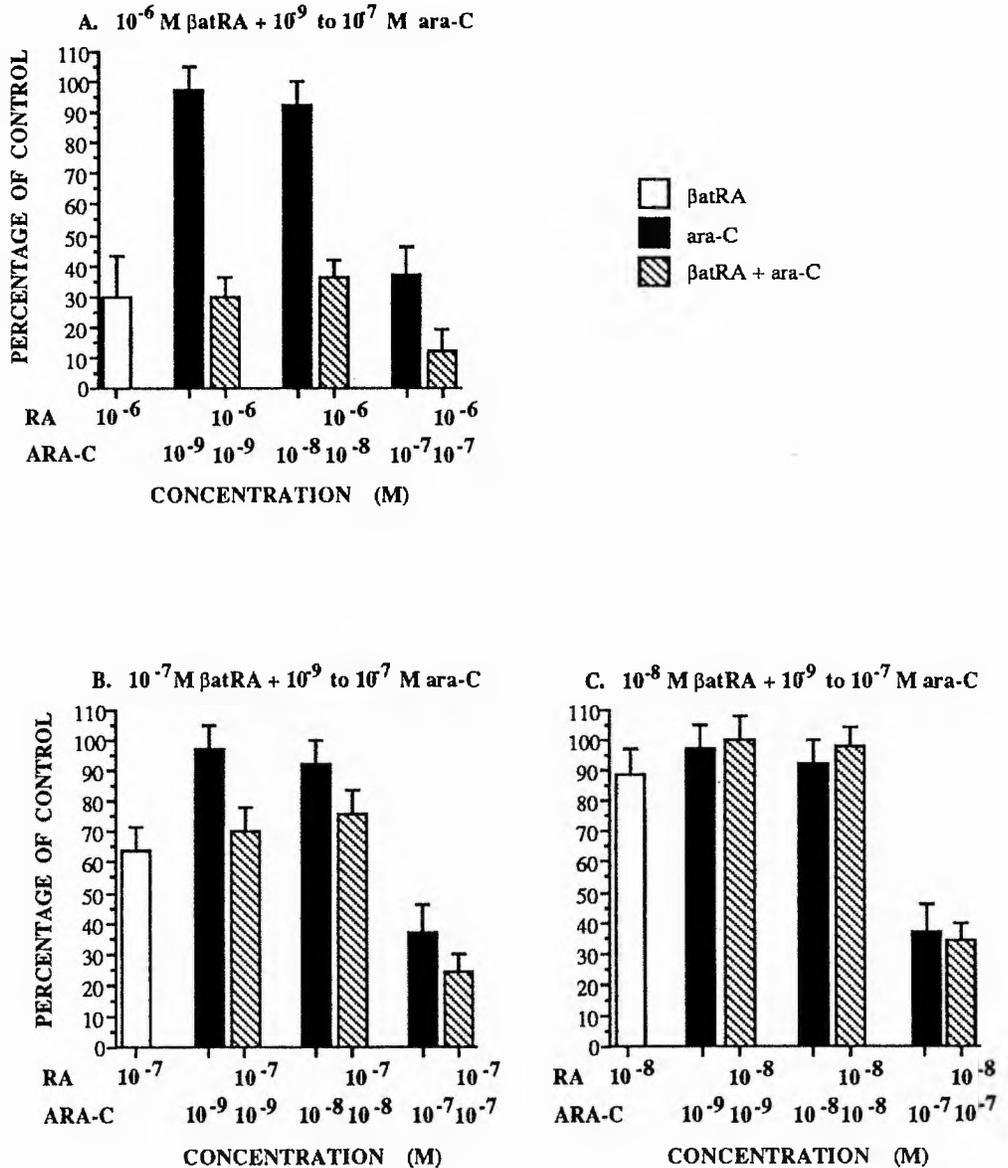
**Figure 5.8** SA2 p55 leukaemic bone marrow single cell suspension was cultured in the microtitre assay with varying concentrations of batRA and ara-C both alone and in combination. WEHI-3B CM was used as the growth factor source. On day four of culture the cells were pulsed with <sup>3</sup>H-TdR for eight hours to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'.

SA2 p59



**Figure 5.9** SA2 p59 leukaemic bone marrow single cell suspension was cultured in the microtitre assay with varying concentrations of  $\beta$ atRA and ara-C both alone and in combination. WEHI-3B CM was used as the growth factor source. On day four of culture the cells were pulsed with  $^3$ H-TdR for eight hours to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'.

## SA2 CL



**Figure 5.10** The SA2 CL was cultured in the microtitre assay with varying concentrations of  $\beta$ atRA and ara-C both alone and in combination. The cells were cultured in FCS alone. On day four of culture the cells were pulsed with  $^3\text{H}$ -TdR for eight hours to measure the effects on proliferation. The results as scintillation CPM were converted to 'Percentage of Untreated Control'.

**APPENDIX TABLES  
TO  
CHAPTER TWO**

CELL DOSE X 10 <sup>4</sup>		COUNTS PER MINUTE ± STANDARD ERROR								
BM CELLS	GROWTH FACTOR	1	3	5	7	9	10			
NBM	WEHI-3B CM	1950 256	3210 511	6558 298	9250 632	7980 812	5794 411			
	L929 CM	982 105	2022 210	4012 487	5826 522	5123 366	3954 444			
SA7	WEHI-3B CM	3145 562	9496 287	16932 863	21013 257	18451 693	16432 355			
	L929 CM	2914 422	5914 451	8321 638	10917 799	10143 424	8614 972			
SA8	WEHI-3B CM	2396 323	7314 874	10163 547	14621 962	12143 244	11158 548			
	L929 CM	1893 118	5692 586	8194 633	11614 874	10456 541	8241 364			
SA2 HP	WEHI-3B CM	3571 814	9143 568	15614 433	20142 288	20032 798	17451 622			
	L929 CM	2194 188	6114 567	8571 621	12314 335	11944 651	11011 714			
SA2 LP	WEHI-3B CM	1428 352	2445 542	4624 478	5693 433	5025 688	4092 701			
	L929 CM	1222 155	1982 187	3122 233	4821 788	4127 255	3824 511			
SA2 CL	WEHI-3B CM	4183 211	12129 845	20142 754	26841 787	34311 522	38261 178			
	L929 CM	3904 257	16341 189	24314 895	29314 688	36453 711	38143 227			

Appendix Table A2.1 Cell Dose Study. Normal and leukaemic bone marrow cells between 1 to 10 x 10<sup>4</sup> cells were cultured with optimal concentration of WEHI-3B CM and L929 CM to determine the optimal cell number required for proliferation studies in the microtitre culture.

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	COUNTS PER MINUTE $\pm$ SE					
MEDIUM	0	850	101	333	87	255	59
WEHI-3B CM	5%	2463	378	2569	337	5648	489
	10	5894	356	4998	566	11954	869
	15	7046	230	8600	741	21488	999
	20	4988	426	6111	657	10256	898
L929 CM	5%	750	79	1689	165	2689	451
	10	1659	225	2946	335	5002	358
	15	2502	365	4897	227	8569	784
	20	1203	114	1783	268	2600	247
rGM-CSF	10 IU/ml	1187	198	1488	154	1024	115
	20	2470	256	3222	226	2648	358
	40	4991	361	6400	678	5187	266
	60	5029	605	6332	564	5216	200
	80	5111	666	6236	711	5289	289
	100	5214	311	6274	523	5218	458

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	CELL COUNT PER WELL $\times 10^4 \pm$ SE					
MEDIUM	0	4.35	0.32	2.00	0.15	1.80	0.05
WEHI-3B CM	5%	4.99	0.28	5.89	0.23	9.69	0.36
	10	6.01	0.44	7.59	0.56	18.09	0.29
	15	6.54	0.51	9.80	0.33	22.60	0.41
	20	6.41	0.22	9.65	0.46	22.4	0.50
L929 CM	5%	4.69	0.16	4.89	0.25	6.00	0.19
	10	5.01	0.18	4.99	0.29	8.56	0.18
	15	5.33	0.14	6.52	0.31	11.87	0.24
	20	5.30	0.14	6.45	0.14	11.76	0.3
rGM-CSF	10 IU/ml	4.63	0.24	4.40	0.20	5.20	0.20
	20	4.98	0.29	5.60	0.31	6.80	0.17
	40	5.21	0.15	7.90	0.18	8.91	0.35
	60	5.22	0.17	7.98	0.18	8.88	0.19
	80	5.18	0.08	7.91	0.24	8.80	0.26
	100	5.14	0.16	7.85	0.27	8.76	0.34

**Appendix Table A2.2** Normal Bone Marrow. Growth Factor Dose Response Study. Normal bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with  $^3\text{H-TdR}$  uptake and results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	COUNTS PER MINUTE $\pm$ SE					
MEDIUM	0	1105	150	203	15	159	24
WEHI-3B CM	5%	4199	332	6214	654	7216	751
	10	6029	796	9052	1012	10900	896
	15	8426	987	12674	978	15006	1024
	20	6013	537	8777	954	7562	874
L929 CM	5%	1257	159	3900	223	781	169
	10	2401	553	8201	556	1255	259
	15	3010	112	10237	873	2005	333
	20	1999	226	6664	751	1553	227
rGM-CSF	10 IU/ml	2789	266	2198	457	299	66
	20	5474	449	4369	541	710	201
	40	10570	954	8829	786	1464	221
	60	10963	897	8811	963	1498	158
	80	11236	1032	8619	741	1445	165
	100	11147	852	8514	962	1389	412

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	CELL COUNT PER WELL $\times 10^4 \pm$ SE					
MEDIUM	0	5.63	0.16	2.44	0.09	1.62	0.25
WEHI-3B CM	5%	6.52	0.15	7.56	0.21	14.68	0.16
	10	7.66	0.12	11.52	0.16	23.97	0.29
	15	8.90	0.09	14.41	0.18	26.20	0.21
	20	8.82	0.14	14.42	0.14	25.68	0.18
L929 CM	5%	5.77	0.11	7.96	0.16	11.52	0.09
	10	5.89	0.09	10.77	0.18	15.04	0.21
	15	6.00	0.07	13.42	0.16	18.40	0.24
	20	6.02	0.25	13.40	0.27	18.24	0.21
rGM-CSF	10 IU/ml	5.00	0.15	7.10	0.12	6.54	0.14
	20	6.30	0.12	9.94	0.04	8.52	0.18
	40	7.70	0.21	13.72	0.40	12.00	0.11
	60	7.78	0.2	13.71	0.14	12.11	0.16
	80	7.71	0.17	13.65	0.12	12.00	0.25
	100	7.67	0.26	13.54	0.17	11.78	0.19

Appendix Table A2.3 SA7 leukaemic bone marrow. Growth Factor Dose Response Study. SA7 leukaemic bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with  $^3\text{H-TdR}$  uptake and results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	COUNTS PER MINUTE ± SE					
MEDIUM	0	1458	165	512	79	666	52
WEHI-3B CM	5%	4569	260	4896	247	6837	456
	10	7543	569	8964	169	12359	160
	15	13502	699	15696	578	19521	782
	20	8963	332	10598	789	12966	334
L929 CM	5%	1596	66	3999	568	789	60
	10	2999	158	7254	349	803	101
	15	6180	299	13126	561	880	189
	20	3325	410	9633	288	598	57
rGM-CSF	10 IU/ml	3179	336	888	116	561	154
	20	6459	459	1727	241	698	59
	40	12889	579	3226	369	712	70
	60	16879	224	5002	188	723	26
	80	13986	116	4875	268	699	112
	100	12789	472	3926	297	685	106

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	CELL COUNT PER WELL x 10 <sup>4</sup> ± SE					
MEDIUM	0	5.97	0.14	2.26	0.14	1.94	0.06
WEHI-3B CM	5%	6.90	0.18	9.60	0.15	13.50	0.28
	10	8.76	0.11	12.56	0.24	21.50	0.17
	15	10.05	0.23	15.35	0.27	24.00	0.16
	20	10.10	0.29	15.21	0.20	23.84	0.25
L929 CM	5%	5.71	0.20	6.81	0.30	3.56	3.56
	10	5.41	0.08	8.88	0.14	5.48	5.48
	15	5.31	0.14	11.20	0.07	8.15	8.15
	20	5.24	0.06	11.03	0.25	8.00	8.00
rGM-CSF	10 IU/ml	6.84	0.13	8.53	0.30	6.45	0.22
	20	7.76	0.11	11.02	0.24	7.80	0.23
	40	9.18	0.18	12.98	0.15	9.12	0.06
	60	9.22	0.24	12.97	0.07	9.00	0.19
	80	9.20	0.27	12.84	0.14	8.94	0.14
	100	9.09	0.17	12.80	0.24	8.84	0.21

Appendix Table A2.4 SA8 leukaemic bone marrow. Growth Factor Dose Response Study. SA8 leukaemic bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with <sup>3</sup>H-TdR uptake and results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	COUNTS PER MINUTE $\pm$ SE					
MEDIUM	0	2589	188	370	55	300	59
WEHI-3B CM	5%	7897	547	8746	854	1876	98
	10	12889	1024	17984	879	4003	363
	15	17590	987	22689	1134	7598	678
	20	15786	693	18965	985	5896	645
L929 CM	5%	4789	366	2996	288	355	69
	10	6355	784	7570	821	499	30
	15	9258	875	12590	985	545	101
	20	7985	742	9845	864	366	47
rGM-CSF	10 IU/ml	7832	652	1786	197	355	59
	20	14736	1035	3358	359	478	89
	40	25456	972	6007	902	566	55
	60	25873	853	6147	853	590	108
	80	24987	956	6045	784	600	68
	100	24541	1215	5982	658	532	71

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	CELL COUNT PER WELL $\times 10^4 \pm$ SE					
MEDIUM	0	6.62	0.13	3.94	0.26	2.70	0.15
WEHI-3B CM	5%	8.73	0.16	14.56	0.15	21.36	0.12
	10	10.97	0.22	18.79	0.18	31.40	0.17
	15	12.79	0.18	21.50	0.24	35.60	0.27
	20	12.82	0.15	21.20	0.30	34.02	0.25
L929 CM	5%	7.05	0.15	10.05	0.17	9.60	0.15
	10	7.32	0.17	15.62	0.19	14.82	0.14
	15	7.69	0.16	22.30	0.34	21.50	0.19
	20	7.52	0.22	21.96	0.26	21.00	0.21
rGM-CSF	10 IU/ml	8.91	0.18	12.56	0.21	6.23	0.15
	20	10.96	0.13	19.55	0.14	9.12	0.16
	40	12.88	0.24	27.15	0.18	12.90	0.18
	60	12.99	0.30	27.10	0.11	12.98	0.29
	80	12.80	0.22	27.00	0.28	12.90	0.28
	100	12.62	0.32	26.82	0.27	12.78	0.25

Appendix Table A2.5 SA2 HP leukaemic bone marrow. Growth Factor Dose Response Study. SA2 HP leukaemic bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with  $^3\text{H-TdR}$  uptake and results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.

DAYS CELLS CULTURED		DAY 4		DAY 4	
GF	GF CONC	CPM $\pm$ SE		CC/WELL x 104 $\pm$ SE	
MEDIUM	0	9552	875	13.32	0.19
WEHI-3B CM	5%	11112	952	13.95	0.36
	10	10951	568	13.85	0.28
	15	9623	963	10.42	0.27
	20	9424	644	10.16	0.26
L929 CM	5%	10056	912	13.65	0.15
	10	10587	863	13.98	0.30
	15	9835	759	11.76	0.24
	20	9372	1043	11.06	0.15
rGM-CSF	10 IU/ml	10432	782	13.86	0.31
	20	10241	654	13.00	0.16
	40	10562	1132	14.20	0.18
	60	8692	982	9.20	0.17
	80	8341	964	8.64	0.19
	100	8222	754	8.10	0.32

**Appendix Table A2.6** SA2 LP (passage number 36) leukaemic bone marrow. Growth Factor Dose Response Study. SA2 LP bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for four days to determine the dose-response relationship. Proliferation was measured with <sup>3</sup>H-TdR uptake and results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	COUNTS PER MINUTE ± SE					
MEDIUM	0	1579	60	1700	68	2241	167
WEHI-3B CM	5%	1790	101	2256	58	2988	204
	10	3158	290	3783	206	8973	687
	15	4121	533	4898	554	12564	651
	20	3876	412	4479	321	9013	455
L929 CM	5%	1566	25	2000	147	2746	344
	10	1463	154	2659	168	3192	247
	15	1490	97	3258	126	3411	289
	20	1400	88	2987	188	2987	262
rGM-CSF	10 IU/ml	2471	158	2874	301	3556	458
	20	3328	457	3752	387	4365	547
	40	6478	587	7005	852	8148	987
	60	6587	748	7146	845	8145	784
	80	6523	589	7256	678	8006	984
	100	6421	874	7122	874	7985	854

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	CELL COUNT PER WELL x 10 <sup>4</sup> ± SE					
MEDIUM	0	5.12	0.26	3.45	0.12	3.67	0.22
WEHI-3B CM	5%	5.86	0.25	6.15	0.16	8.52	0.18
	10	6.34	0.12	7.85	0.18	12.22	0.26
	15	6.51	0.08	9.00	0.07	15.01	0.17
	20	6.45	0.07	8.87	0.26	14.90	0.30
L929 CM	5%	5.03	0.26	5.24	0.15	6.47	0.14
	10	4.86	0.15	5.50	0.18	7.99	0.16
	15	4.74	0.16	5.73	0.22	9.10	0.09
	20	4.65	0.18	5.68	0.24	8.79	0.21
rGM-CSF	10 IU/ml	5.78	0.26	6.43	0.13	7.86	0.30
	20	6.41	0.18	7.01	0.16	10.60	0.14
	40	7.05	0.14	8.99	0.24	14.39	0.18
	60	7.10	0.16	9.05	0.19	14.5	0.26
	80	7.11	0.22	9.04	0.24	14.43	0.18
	100	7.02	0.25	9.00	0.24	14.32	0.21

Appendix Table A2.7 SA2 LP (passage number 54) leukaemic bone marrow. Growth Factor Dose Response Study. SA2 LP bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with 3H-TdR uptake and results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	COUNTS PER MINUTE $\pm$ SE					
MEDIUM	0	42398	787	25896	548	17889	735
WEHI-3B CM	5%	43025	589	27894	654	22758	894
	10	38257	412	25879	942	20225	745
	15	37859	658	25478	875	21548	785
	20	37655	514	24895	547	17758	854
L929 CM	5%	35897	625	26894	741	19785	583
	10	28800	941	28489	829	20999	852
	15	25894	825	31258	845	23569	748
	20	26297	569	30959	647	22569	896
rGM-CSF	10 IU/ml	42979	936	24763	947	17805	821
	20	42479	964	22148	786	17698	997
	40	43457	893	21458	1048	17489	896
	60	43457	759	21237	964	17223	1054
	80	42478	1024	21523	863	17589	518
	100	42356	978	21402	872	17454	748

DAYS CELLS CULTURED		DAY 2		DAY 4		DAY 6	
GF	GF CONC	CELL COUNT PER WELL $\times 10^4 \pm$ SE					
MEDIUM	0	38.02	0.34	57.11	0.71	66.59	0.84
WEHI-3B CM	5%	38.18	0.91	56.68	0.82	66.00	0.67
	10	38.27	0.68	56.55	0.84	59.98	0.69
	15	38.26	0.48	56.60	0.54	66.11	0.58
	20	38.09	0.56	56.00	0.61	59.88	0.84
L929 CM	5%	37.88	0.82	58.90	0.67	69.80	0.75
	10	36.97	0.57	61.00	0.81	72.80	0.92
	15	36.52	0.68	63.20	0.58	74.50	0.65
	20	36.48	0.64	62.99	0.66	74.45	0.87
rmGM-CSF	10 IU/ml	37.98	0.86	57.21	0.72	66.10	0.82
	20	38.10	0.77	57.25	0.83	65.78	0.97
	40	38.04	0.68	57.17	0.88	65.78	0.75
	60	38.12	0.75	57.20	0.85	65.00	0.58
	80	37.85	0.98	57.01	0.79	64.45	0.96
	100	37.56	0.47	57.11	0.87	64.10	0.87

Appendix Table A2.8 SA2 CL leukaemic cells. Growth Factor Dose Response Study. SA2 LP bone marrow cells were cultured in FCS alone and with varied concentrations of WEHI-3B CM, L929 CM and rGM-CSF in the microtitre assay for two, four and six days to determine the dose-response relationship. Proliferation was measured with  $^3\text{H-TdR}$  uptake and results recorded as scintillation counts per minute (CPM) and with the measurement of total cell count per well.

		<b>COUNTS PER MINUTE ± STANDARD ERROR</b>							
<b>BM</b>	<b>DAY</b>	<b>MEDIUM</b>		<b>WEHI-3B CM</b>		<b>L929 CM</b>		<b>rmGM-CSF</b>	
NBM	0.3	3383	181	2935	133	2530	54	2982	122
	1	962	60	3492	104	1533	66	4550	383
	2	880	77	6696	219	2369	138	5892	204
	3	534	29	7786	73	3367	216	8938	982
	4	313	21	8150	735	4411	291	6181	778
	5	212	36	17081	641	8607	909	7062	547
	6	279	71	20933	204	8349	422	5319	831
	7	270	115	16585	214	2029	247	3203	253
SA7 HD	0.3	5662	240	3956	81	4654	110	7418	510
	1	1170	10	4214	168	1444	149	6952	615
	2	1205	390	8625	236	3412	641	11362	298
	3	591	10	9332	474	7918	935	14369	719
	4	208	14	11504	1016	9887	900	7898	543
	5	178	21	20172	818	7097	954	5011	453
	6	113	17	14508	586	1680	231	1037	172
	7	146	13	7163	266	299	97	264	16
SA8 HD	0.3	2843	156	2028	281	2201	182	3084	209
	1	2175	128	7706	306	4180	263	8175	494
	2	1365	115	12995	134	5887	124	17261	600
	3	907	49	12772	535	8597	552	15641	239
	4	427	49	14562	349	12600	391	5768	204
	5	281	20	15652	793	4072	492	2147	166
	6	634	54	19230	623	617	71	657	23
	7	317	69	8922	100	269	64	269	18
SA2 HD LP	0.3	2783	224	2751	118	2781	285	2884	241
	1	1386	127	2535	381	1002	107	4754	557
	2	1193	139	3645	445	1042	264	6448	798
	3	1196	168	4252	800	1405	133	6566	656
	4	1237	122	4864	314	2408	328	7056	235
	5	1755	126	8365	941	4218	108	7771	836
	6	1787	38	14644	1116	2685	164	8437	1000
	7	1991	140	8050	996	3512	449	7535	854
SA2 HD HP	0.3	10768	511	7675	413	6648	589	14149	164
	1	4283	146	12802	738	5964	289	15385	978
	2	2436	168	18223	1223	8673	264	27830	1124
	3	1411	343	20626	770	15548	843	30398	751
	4	431	47	21700	165	11619	931	6754	785
	5	222	24	13429	502	3103	428	1078	164
	6	318	27	7027	658	390	46	407	33
	7	220	97	2983	247	128	29	197	54
SA2 CL	0.3	41132	103	45920	476	37538	506	50792	417
	1	57467	116	45065	341	41923	111	57278	151
	2	44118	115	39593	209	28958	185	43275	157
	3	33837	454	36132	862	41083	140	32010	380
	4	25153	876	24973	451	30901	102	21725	945
	5	20109	993	21781	475	21540	234	17910	789
	6	19669	391	23405	276	25666	856	19253	100
	7	16043	211	17650	156	21433	855	16689	112

**Appendix Table 2.9** Proliferative effects of WEHI-3B CM, L929 CM and rGM-CSF on normal and leukaemic cells. Cells were cultured in the microtitre assay for 8 hours to seven days. Proliferation was measured with uptake of <sup>3</sup>H-TdR.

**COUNTS PER MINUTE ± STANDARD ERROR**

GROWTH FACTORS		WEHI-3B		WEHI-3B CM		L929 CM	
BM	DAY	+ L929 CM		+ rmGM-CSF		+ rmGM-CSF	
NBM	0.3	2705	70	3136	71	2225	91
	1	3171	94	4241	376	3678	106
	2	4035	225	8323	712	7373	133
	3	8228	209	14781	500	8786	233
	4	13299	168	18038	212	10686	502
	5	27847	594	26354	345	16712	419
	6	32568	855	26368	100	12747	172
	7	12960	209	12594	142	8903	135
SA7 HD	0.3	4012	479	4123	399	5455	653
	1	3356	278	4079	460	4211	561
	2	8514	983	14478	964	12267	893
	3	15978	1028	21687	1171	21189	936
	4	17143	893	22257	943	22003	1045
	5	17002	981	23079	1041	16999	879
	6	8245	639	16571	921	11089	999
	7	3071	276	7075	832	3248	412
SA8 HD	0.3	1646	21	2119	78	1850	104
	1	5370	178	8560	327	7384	191
	2	10613	326	18506	621	16858	334
	3	20753	703	13899	353	21060	342
	4	23595	187	12544	512	15196	605
	5	27741	645	14889	173	16451	740
	6	12936	738	15349	1000	10312	683
	7	7737	319	5588	657	2806	216
SA2 HD LP	0.3	2243	134	2114	102	1810	114
	1	2493	143	2911	192	2611	268
	2	3725	272	6063	61	5363	577
	3	5242	90	8803	434	10345	719
	4	7711	358	11455	799	12650	835
	5	19175	151	23385	242	21871	701
	6	19782	152	12856	152	24140	945
	7	8486	196	11035	161	8673	772
SA2 HD HP	0.3	7706	422	8667	605	10658	850
	1	8671	980	15098	627	14116	580
	2	21788	858	37291	1441	27319	524
	3	35669	1229	33462	771	39333	1091
	4	19978	857	14666	717	25157	917
	5	6947	513	6890	696	9545	392
	6	2820	178	4699	293	4200	250
	7	1020	193	4024	659	2344	404
SA2 CL	0.3	40898	834	51236	587	37147	1053
	1	53865	512	60005	953	57154	693
	2	35091	714	46076	1003	49677	876
	3	37786	1143	34985	843	37246	592
	4	26247	767	23587	752	28976	927
	5	20476	689	20074	819	19174	840
	6	25543	924	22265	691	22975	421
	7	24327	853	21869	514	23875	746

**Appendix Table A2.10** Proliferative effects of combinations of growth factors, WEHI-3B CM, L929 CM and rGM-CSF on normal and leukaemic cells. Cells were cultured in the microtitre assay for 8 hours to seven days. Proliferation was measured with uptake of <sup>3</sup>H-TdR.

**APPENDIX TABLES  
TO  
CHAPTER THREE**

GF	CELL	PERCENTAGE OF TOTAL CELLS ± STANDARD ERROR									
		SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
FCS	Bl	9.5	0.8	4.4	0.3	0	0.0	0	0	0	0
	P	9.0	0.7	16.3	1.4	2.8	0.1	0	0	17.9	2.0
	M	14.2	1.5	11.7	1.4	11.5	0.2	4.0	0.4	2.6	0.1
	MM	63.6	5.1	58.1	5.0	62.9	5.2	31.2	3.3	11.5	0.8
	B	2.1	0.2	2.9	0.2	1.3	0.1	13.9	1.5	0.6	0.1
	S	0.7	0.1	4.4	0.3	11.5	0.9	16.8	1.3	0	0
	Mo	0.7	0.1	0	0	0	0	0	0	4.5	3.9
Mf	0.0	0.0	2.3	0.1	10.0	0.9	34.2	2.9	62.8	6.6	
15% WEHI-3B CM	Bl			3.8	0.3	2.2	0.2	1.3	0.1	4.0	0.3
	P			14.5	1.1	39.7	4.1	40.8	3.5	55.1	5.1
	M			6.1	0.7	7.1	0.8	8.5	0.7	10.7	0.9
	MM			12.2	1.4	21.2	1.9	14.8	1.1	8.0	0.7
	B			51.8	4.5	2.9	0.3	6.7	0.5	0	0
	S			3.8	0.2	9.9	0.7	11.6	0.8	5.3	0.4
	Mo			2.3	0.2	6.4	0.4	6.5	0.6	6.7	0.8
Mf			5.4	0.6	10.8	0.8	9.7	0.9	6.0	0.7	
15% L929 CM	Bl			4.2	0.3	0	0	3.3	0.2	0	0
	P			11.4	0.9	34.6	3.0	16.2	1.3	2.1	0.2
	M			5.0	0.6	5.1	0.4	3.3	0.4	1.4	0.1
	MM			62.2	6.5	24.7	2.6	14.2	1.6	11.8	0.9
	B			2.2	0.2	2.7	0.2	6.9	0.8	1.4	0.1
	S			5.6	0.6	15.0	1.2	8.5	0.8	5.6	0.6
	Mo			4.2	0.3	2.1	0.2	14.2	1.2	11.2	1.2
Mf			5.3	0.4	15.8	1.4	33.6	2.9	66.5	6.3	
60 U/ml rGM-CSF	Bl			5.4	0.6	10.1	0.8	0.7	0.1	2.2	0.1
	P			16.9	2.0	20.2	1.7	11.6	0.8	7.2	0.6
	M			16.9	1.5	15.9	1.2	24.7	1.9	4.3	0.5
	MM			47.6	4.0	17.3	1.3	19.8	0.9	16.1	1.4
	B			1.5	0.1	4.2	0.3	1.3	0.1	0	0
	S			7.5	0.4	14.4	1.6	19.0	0.8	20.7	1.8
	Mo			0	0	8.8	1.0	8.9	0.6	16.4	1.4
Mf			3.9	0.2	8.7	0.7	13.7	0.9	33.0	2.9	
WEHI-3B CM + L929 CM	Bl			5.3	0.4	4.0	0.3	5.4	0.4	4.1	0.4
	P			14.2	0.9	20.6	1.9	40.8	3.7	59.3	6.0
	M			6.1	0.5	11.4	1.3	6.8	0.7	11.1	0.8
	MM			54.3	5.2	14.2	1.3	19.1	2.0	7.5	0.5
	B			0.8	0.1	4.6	0.4	3.4	0.2	2.1	0.2
	S			8.3	0.8	20.6	1.8	8.1	0.7	8.3	0.6
	Mo			3.0	0.3	0	0	2.1	0.2	4.1	0.3
Mf			8.1	0.7	24.7	2.2	14.2	1.3	3.5	0.3	
WEHI-3B CM + rGM-CSF	Bl			2.2	0.2	2.2	0.2	6.9	0.6	4.2	0.3
	P			11.0	0.8	46.3	4.4	42.4	4.0	57.4	5.8
	M			12.4	1.0	8.0	0.6	5.5	0.6	8.4	0.8
	MM			58.3	5.1	9.6	0.8	8.3	0.8	8.5	0.8
	B			2.9	0.3	7.1	0.8	0.8	0.8	1.4	0.1
	S			9.6	1.0	15.3	1.4	14.6	1.5	11.1	0.9
	Mo			2.9	0.3	5.7	0.5	13.2	1.3	6.3	0.5
Mf			0.7	0.1	5.8	0.5	8.3	0.7	2.8	0.2	
L929 CM + rGM-CSF	Bl			2.9	0.2	10.8	0.9	4.2	0.4	0	0
	P			10.0	0.8	30.9	2.9	13.2	0.9	10.3	0.9
	M			24.5	2.1	15.8	1.4	9.8	0.9	9.7	0.9
	MM			48.1	5.0	8.3	0.7	18.0	1.5	14.5	1.6
	B			0.8	0.8	3.4	0.2	2.0	0.2	0	0
	S			8.6	0.8	19.4	1.5	10.4	1.1	26.2	2.8
	Mo			0.8	0.8	7.2	0.7	11.1	0.9	16.5	1.7
Mf			4.3	0.3	4.3	0.3	31.2	2.9	22.7	2.0	

Appendix Table A3.1 Normal bone marrow differential cell counts. Normal bone marrow cells were cultured in FCS and optimal concentrations of WEHI-3B CM, L929 CM and rGM-CSF alone and in combination in the microtitre assay for eight hours, two, four and six days. Cytospins were prepared stained and the differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
FCS	Bl	0.65	0.07	0.29	0.04	0	0	0	0	0	0
	P	0.61	0.07	1.08	0.15	0.11	0.01	0	0	0.28	0.05
	M	0.98	0.13	0.78	0.13	0.45	0.03	0.08	0.01	0.04	0.01
	MM	4.33	0.48	3.86	0.54	2.46	0.24	0.63	0.09	0.18	0.03
	B	0.14	0.02	0.19	0.02	0.05	0	0.28	0.04	0.01	0
	S	0.05	0.01	0.29	0.04	0.45	0.04	0.34	0.04	0	0
	Mo	0.05	0.01	0	0	0	0	0	0	0.07	0.06
	Mf	0	0	0.15	0.02	0.39	0.04	0.69	0.08	0.98	0.16
Total	cells	6.81	0.52	6.64	0.74	3.91	0.21	2.02	0.18	1.56	0.20
WEHI 3B CM	Bl			0.25	0.03	0.13	0.02	0.14	0.02	0.92	0.08
	P			0.95	0.13	2.36	0.35	4.23	0.54	12.70	1.29
	M			0.80	0.13	0.42	0.06	0.88	0.11	2.45	0.23
	MM			3.39	0.54	1.26	0.17	1.54	0.19	1.83	0.18
	B			0.25	0.03	0.17	0.03	0.70	0.08	0	0
	S			0.15	0.02	0.59	0.08	1.20	0.14	1.21	0.10
	Mo			0.40	0.06	0.38	0.05	0.67	0.09	1.53	0.19
	MF			0.35	0.05	0.64	0.08	1.01	0.13	1.37	0.17
Total	cells			6.54	0.71	5.95	0.63	10.4	0.98	22.01	0.98
15% L929 CM	Bl			0.29	0.03	0	0	0.20	0.02	0	0
	P			0.79	0.09	1.29	0.18	0.99	0.12	0.32	0.04
	M			0.35	0.05	0.19	0.03	0.20	0.03	0.21	0.02
	MM			4.33	0.56	0.92	0.14	0.87	0.13	1.79	0.18
	B			0.15	0.02	0.10	0.01	0.42	0.06	0.22	0.02
	S			0.39	0.05	0.56	0.08	0.52	0.07	0.86	0.11
	Mo			0.29	0.03	0.08	0.01	0.87	0.11	1.70	0.21
	Mf			0.37	0.04	0.59	0.08	2.06	0.26	10.10	1.17
Total	cells			6.96	0.54	3.73	0.41	6.13	0.58	15.20	1.22
60 U/ml rGM- CSF	Bl			0.38	0.05	0.52	0.08	0.06	0.01	0.18	0.02
	P			1.19	0.18	1.04	0.15	0.89	0.08	0.6	0.07
	M			1.19	0.15	0.82	0.12	1.89	0.18	0.36	0.05
	MM			3.35	0.42	0.89	0.13	1.52	0.11	1.34	0.16
	B			0.11	0.01	0.22	0.03	0.10	0.01	0	0
	S			0.53	0.06	0.74	0.12	1.46	0.10	1.72	0.21
	Mo			0	0	0.45	0.08	0.68	0.06	1.36	0.17
	Mf			0.28	0.03	0.45	0.07	1.05	0.09	2.74	0.34
Total	cells			7.03	0.65	5.13	0.63	7.65	0.43	8.30	0.72

Appendix Table A3.2 Normal bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in the microtitre assay. The total cell number per well was determined and cytopspins were prepared, stained and differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
WEHI-3B CM +	BI	0.65	0.07	0.34	0.05	0.29	0.04	0.58	0.08	1.24	0.18
	P	0.61	0.07	0.91	0.12	1.49	0.21	4.36	0.64	17.80	2.58
	M	0.98	0.13	0.39	0.06	0.83	0.13	0.73	0.11	3.32	0.42
	MM	4.33	0.48	3.48	0.53	1.03	0.15	2.04	0.32	2.26	0.28
	B	0.14	0.02	0.05	0.01	0.33	0.05	0.36	0.05	0.62	0.09
	S	0.05	0.01	0.53	0.08	1.49	0.21	0.87	0.13	2.48	0.31
	Mo	0.05	0.01	0.19	0.03	0	0	0.22	0.03	1.24	0.16
	Mf	0	0	0.52	0.08	1.79	0.25	1.52	0.22	1.05	0.14
Total cells		6.81	0.52	6.41	0.75	7.25	0.79	10.68	1.23	30.01	3.12
WEHI-3B CM +	BI			0.15	0.02	0.14	0.02	0.82	0.10	1.38	0.18
	P			0.76	0.08	2.94	0.37	5.01	0.63	18.90	2.84
	M			0.86	0.10	0.51	0.06	0.65	0.09	2.77	0.41
	MM			4.03	0.48	0.61	0.07	0.98	0.12	2.80	0.41
	B			0.20	0.03	0.45	0.06	0.09	0.09	0.45	0.06
	S			0.66	0.09	0.97	0.12	1.72	0.23	3.67	0.50
	Mo			0.20	0.03	0.36	0.04	1.56	0.20	2.07	0.28
	Mf			0.05	0.01	0.37	0.04	0.98	0.12	0.93	0.12
Total cells				6.91	0.56	6.35	0.54	11.81	0.98	32.99	3.66
L929 CM +	BI			0.22	0.03	0.60	0.08	0.33	0.04	0	0
	P			0.75	0.09	1.72	0.23	1.03	0.11	2.17	0.28
	M			1.84	0.24	0.88	0.12	0.77	0.10	2.04	0.27
	MM			3.62	0.51	0.46	0.06	1.41	0.17	3.04	0.44
	B			0.06	0.06	0.19	0.02	0.16	0.02	0	0
	S			0.65	0.09	1.08	0.14	0.81	0.11	5.51	0.79
	Mo			0.06	0.06	0.40	0.06	0.87	0.10	3.47	0.49
	Mf			0.32	0.04	0.24	0.03	2.44	0.31	4.77	0.62
Total cells				7.52	0.72	7.57	0.75	7.82	0.69	21.00	2.00

**Appendix Table A3.3** Normal bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in combination in the microtitre assay. The total cell number per well was determined and cytopspins were prepared, stained and differentials counted.

GF	CELL	PERCENTAGE OF TOTAL CELLS ± STANDARD ERROR									
		SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
FCS	Bl	48.9	5.0	34.7	3.1	4.3	0.4	2.1	0.2	0	0
	P	17.7	1.6	23.1	2.0	11.9	1.0	4.7	0.4	0.7	0.1
	Imf	0.0	0.0	4.7	0.4	5.0	0.4	8.6	0.7	10.7	0.9
	M	9.4	0.8	10.2	0.9	19.6	2.0	14.2	1.2	0.7	0.1
	MM	19.1	1.7	23.9	2.0	25.1	2.3	14.2	1.2	5.7	0.6
	B	0.0	0.0	1.3	0.1	4.3	0.4	4.7	0.4	0.7	0.1
	S	1.4	0.1	0.7	0.1	3.4	0.3	2.1	0.2	0.7	0.1
	Mo	2.1	0.2	0	0	0	0	0	0	0	0
	Mf	1.4	0.1	1.3	0.1	26.5	2.7	48.9	4.7	80.7	8.2
15% WEHI-3B CM	Bl			47.5	5.0	6.8	0.5	5.0	0.5	7.8	0.7
	P			10.0	0.9	27.3	2.4	29.5	3.0	16.6	1.5
	Imf			3.6	0.3	2.7	0.2	3.4	0.3	1.0	0.1
	M			4.4	0.4	9.6	0.8	12.3	1.0	14.9	1.3
	MM			28.8	3.0	20.0	1.8	15.5	1.4	21.8	2.0
	B			2.9	0.2	2.7	0.3	1.8	0.1	1.7	0.1
	Mf			2.1	0.2	6.2	0.6	3.4	0.3	6.1	0.6
15% L929 CM	Bl			37.1	3.8	13.0	1.2	2.3	0.2	0.7	0.1
	P			28.4	2.5	13.7	1.2	8.6	0.7	0	0
	Imf			5.8	0.5	11.0	0.9	10.6	0.9	12.7	1.3
	M			8.9	0.8	4.1	0.4	3.1	0.3	0.7	0.1
	MM			12.4	1.1	23.2	2.4	4.5	0.5	6.0	0.5
	B			0.8	0.7	5.6	0.5	0.3	0.1	0.7	0.1
	Mf			2.9	0.2	15.0	1.4	1.7	0.1	0.7	0.1
60 U/ml rGM-CSF	Bl			42.0	4.0	8.2	0.7	2.1	0.2	0.7	0.1
	P			20.3	2.0	37.8	3.5	30.8	2.9	4.2	0.4
	Imf			6.3	0.6	2.1	0.2	13.6	1.4	4.8	0.4
	M			12.6	1.0	7.5	0.8	13.1	1.4	9.0	0.8
	MM			9.2	0.9	9.5	1.0	19.9	1.8	26.1	2.5
	B			3.5	0.4	2.1	0.2	0	0	5.6	0.6
	Mf			2.1	0.2	6.8	0.7	10.3	1.1	15.8	1.4
WEHI-3B CM + L929 CM	Bl			34.0	3.5	7.8	0.6	3.0	0.3	4.8	0.5
	P			13.3	1.2	24.2	2.5	30.2	3.0	24.6	2.3
	Imf			4.2	0.4	4.3	0.4	4.0	0.4	15.7	1.4
	M			7.8	0.6	5.6	0.4	17.1	1.5	7.5	0.7
	MM			32.6	3.0	12.8	1.0	13.1	1.4	11.6	0.9
	B			1.3	0.1	2.1	0.2	3.5	0.4	2.8	0.2
	Mf			2.7	0.2	19.2	1.8	7.0	0.6	11.7	0.9
WEHI-3B CM + rGM-CSF	Bl			34.6	3.3	10.4	0.9	6.1	0.6	4.8	0.5
	P			17.3	1.5	22.8	2.0	45.1	4.4	25.2	2.4
	Imf			11.6	1.0	15.3	1.4	8.9	0.8	3.4	0.3
	M			17.3	1.5	13.2	1.4	8.9	0.8	4.8	0.5
	MM			14.3	1.5	10.4	0.8	17.8	1.6	40.0	3.8
	B			2.1	0.2	4.9	0.4	0.7	0.6	0.7	0.1
	Mf			1.4	0.1	9.1	0.8	2.1	0.2	10.2	0.8
L929 CM + rGM-CSF	Bl			28.6	2.6	15.1	1.4	6.1	0.6	3.4	0.3
	P			12.6	1.1	28.2	2.9	33.7	3.0	2.1	0.2
	Imf			1.4	1.3	7.5	0.8	13.5	1.4	17.3	1.6
	M			12.6	1.3	10.9	0.8	7.5	0.8	10.4	0.8
	MM			33.0	2.9	5.4	0.5	5.4	0.6	14.5	1.2
	B			4.9	0.4	1.5	0.1	0	0	4.1	0.4
	Mf			2.1	0.2	10.2	0.9	6.8	0.7	17.9	1.8

Appendix Table A3.4 SA7 leukaemic differential cell counts. SA7 bone marrow cells were cultured in FCS and optimal concentrations of WEHI-3B CM, L929 CM and rGM-CSF alone and in combination in the microtitre assay for eight hours, two, four and six days. Cytospins were prepared stained and the differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
FCS	Bl	3.26	0.42	2.34	0.31	0.24	0.03	0.05	0.01	0.00	0.00
	P	1.18	0.14	1.56	0.20	0.67	0.09	0.11	0.02	0.01	0.00
	Imf	0.00	0.00	0.32	0.04	0.28	0.04	0.20	0.03	0.15	0.02
	M	0.63	0.07	0.69	0.09	1.10	0.17	0.33	0.05	0.01	0.00
	MM	1.27	0.15	1.61	0.21	1.41	0.20	0.33	0.05	0.08	0.01
	B	0.00	0.00	0.09	0.01	0.24	0.03	0.11	0.02	0.01	0.00
	S	0.09	0.01	0.05	0.01	0.19	0.03	0.05	0.01	0.01	0.00
	Mo	0.14	0.02	0	0	0	0	0	0	0	0
	Mf	0.09	0.01	0.09	0.01	1.49	0.22	1.13	0.17	1.13	0.18
Total cells		6.66	0.53	6.75	0.65	5.42	0.60	2.31	0.28	1.40	0.17
15% WEHI 3B CM	Bl			3.15	0.47	0.60	0.07	0.69	0.10	2.20	0.29
	P			0.66	0.09	2.39	0.29	4.11	0.60	4.66	0.61
	Imf			0.24	0.03	0.24	0.03	0.48	0.07	0.29	0.04
	M			0.29	0.04	0.84	0.10	1.72	0.23	4.20	0.54
	MM			1.91	0.29	1.75	0.22	2.16	0.30	6.15	0.81
	B			0.19	0.02	0.24	0.03	0.25	0.03	0.49	0.05
	S			0.14	0.02	0.54	0.07	0.48	0.07	1.72	0.23
	Mf			0.05	0.01	2.16	0.29	4.06	0.60	8.44	1.20
Total cells				6.63	0.71	8.76	0.75	13.95	1.45	28.15	2.63
15% L929 CM	Bl			2.42	0.36	0.79	0.11	0.31	0.04	0.15	0.03
	P			1.85	0.26	0.83	0.11	1.13	0.16	0	0
	Imf			0.38	0.05	0.67	0.09	1.40	0.20	2.63	0.40
	M			0.58	0.08	0.25	0.04	0.41	0.06	0.15	0.03
	MM			0.81	0.11	1.41	0.21	0.59	0.09	1.24	0.17
	B			0.05	0.04	0.34	0.05	0.04	0.01	0.15	0.03
	S			0.19	0.02	0.91	0.13	0.23	0.03	0.15	0.03
	Mf			0.24	0.04	0.87	0.13	9.09	1.36	16.16	1.89
Total cells				6.52	0.70	6.07	0.64	13.20	1.51	20.63	2.31
60.00 U/ml rGM-CSF	Bl			2.79	0.38	0.64	0.09	0.28	0.04	0.09	0.02
	P			1.35	0.19	2.94	0.42	4.20	0.62	0.50	0.07
	Imf			0.42	0.06	0.16	0.02	1.86	0.29	0.58	0.08
	M			0.84	0.11	0.58	0.09	1.78	0.28	1.08	0.15
	MM			0.61	0.08	0.74	0.11	2.71	0.40	3.13	0.46
	B			0.23	0.03	0.16	0.02	0	0	0.67	0.10
	S			0.14	0.02	0.53	0.08	1.40	0.22	1.90	0.27
	Mf			0.28	0.04	2.02	0.29	1.40	0.19	4.04	0.62
Total cells				6.66	0.75	7.77	0.84	13.63	1.56	11.99	1.35

**Appendix Table A3.5** SA7 leukaemic bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in the microtitre assay. The total cell number per well was determined and cytopins were prepared, stained and differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS	8 HOUR		DAY 2		DAY 4		DAY 6	
WEHI-3B CM + L929 CM	Bl	3.26 0.42	2.36 0.34	0.60 0.07	0.58 0.07	1.37 0.21				
	P	1.18 0.14	0.92 0.13	1.85 0.26	5.79 0.74	6.97 1.00				
	Imf	0.00 0.00	0.29 0.04	0.33 0.04	0.77 0.10	4.45 0.63				
	M	0.63 0.07	0.54 0.07	0.43 0.05	3.28 0.39	2.12 0.31				
	MM	1.27 0.15	2.26 0.31	0.98 0.12	2.51 0.34	3.28 0.44				
	B	0 0	0.09 0.01	0.16 0.02	0.68 0.09	0.78 0.10				
	S	0.09 0.01	0.19 0.02	1.47 0.19	1.35 0.16	3.31 0.44				
	Mo	0.14 0.02	0 0	0 0	0 0	0 0				
Mf	0.09 0.01	0.29 0.04	1.83 0.23	4.24 0.51	6.02 0.85					
Total	cell	6.66 0.53	6.94 0.71	7.60 0.71	19.20 1.54	28.30 3.10				
WEHI-3B CM + rGM-CSF	Bl		0.44 0.06	0.91 0.11	1.34 0.19	1.14 0.16				
	P		1.22 0.15	1.99 0.24	9.86 1.39	5.97 0.80				
	Imf		0.82 0.10	1.33 0.17	1.95 0.26	0.80 0.10				
	M		1.22 0.15	1.15 0.16	1.95 0.26	1.14 0.16				
	MM		1.01 0.14	0.91 0.10	3.90 0.53	9.50 1.27				
	B		0.15 0.02	0.43 0.05	0.16 0.14	0.17 0.03				
	S		0.10 0.01	0.79 0.10	0.45 0.06	2.42 0.30				
	Mf		0.10 0.01	1.20 0.15	2.24 0.29	2.59 0.31				
Total	cells		7.06 0.62	8.71 0.74	21.85 2.22	23.73 2.24				
L929 CM + rGM-CSF	Bl		2.04 0.28	1.11 0.15	1.15 0.17	0.74 0.10				
	P		0.90 0.12	2.07 0.30	6.36 0.89	0.45 0.06				
	Imf		0.10 0.09	0.55 0.08	2.54 0.38	3.73 0.52				
	M		0.90 0.13	0.80 0.10	1.41 0.21	2.24 0.29				
	MM		2.35 0.31	0.40 0.06	1.01 0.16	3.13 0.41				
	B		0.35 0.05	0.11 0.01	0 0	0.89 0.13				
	S		0.15 0.02	0.75 0.10	1.28 0.19	3.87 0.56				
	Mf		0.34 0.05	1.56 0.22	5.10 0.74	6.55 0.95				
Total	cells		7.13 0.72	7.35 0.75	18.85 2.04	21.60 2.23				

Appendix Table A3.6 SA7 leukaemic bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in combination in the microtitre assay. the total cell number per well was determined and cytopspins were prepared, stained and differentials counted.

GF	CELL	PERCENTAGE OF TOTAL CELLS ± STANDARD ERROR									
		SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
FCS	Bl	17.2	1.6	13.8	1.4	0	0	3.5	0.4	0	0
	P	18.5	1.6	17.1	1.8	11.7	0.9	7.0	0.7	2.9	0.3
	Imf	0	0	7.2	0.7	7.5	0.8	3.5	0.3	0.0	0.0
	M	8.6	0.9	21.3	2.0	7.5	0.8	10.1	0.8	8.2	0.8
	MM	42.9	4.4	34.8	3.3	37.1	3.6	15.6	1.5	13.5	1.4
	B	2.8	0.3	5.1	0.5	8.3	0.8	1.0	0.1	0	0
	S	0.8	0.1	0.7	0.1	2.9	0.3	2.0	0.2	8.2	0.9
	Mo	8.6	0.9	0	0	0	0	0	0	0	0
	Mf	0.8	0.1	0	0	25.0	2.6	57.3	5.6	67.3	6.8
15% WEHI-3B CM	Bl			13.1	1.4	5.4	0.5	4.0	0.4	2.7	0.3
	P			20.8	1.9	20.5	1.9	38.5	3.5	47.0	4.5
	Imf			23.6	2.4	25.4	2.4	3.4	0.4	0	0
	M			4.9	0.4	9.5	0.8	3.4	0.4	4.7	0.5
	MM			27.7	2.5	6.2	0.6	10.2	0.8	18.0	1.9
	B			4.9	0.5	2.8	0.3	0.7	0.1	2.0	0.2
	S			2.1	0.2	5.4	0.4	7.4	0.7	6.0	0.5
	Mf			2.8	0.2	24.7	2.3	32.4	3.3	19.5	1.8
15% L929 CM	Bl			7.8	0.6	6.4	0.6	0.7	0.1	0.6	0.1
	P			6.9	0.6	20.9	1.8	2.0	0.2	0.6	0.1
	Imf			35.9	3.3	8.4	0.7	12.5	1.0	0.6	0.1
	M			14.8	1.1	1.4	0.1	2.5	0.3	3.3	0.2
	MM			24.0	2.3	6.4	0.5	32.3	3.4	10.4	0.8
	B			6.4	0.6	1.4	0.1	2.5	0.3	3.3	0.3
	S			2.9	0.2	11.9	0.7	3.3	0.3	2.6	0.3
	Mf			1.4	0.1	43.2	3.5	44.2	4.2	78.6	8.0
60 U/ml rGM-CSF	Bl			0.7	0.1	0	0	0	0	0	0
	P			18.3	1.4	13.0	1.5	0.7	0.1	6.8	0.6
	Imf			6.4	0.6	10.3	0.8	2.7	0.3	0.7	0.1
	M			8.4	0.9	34.2	3.2	0.7	0.1	12.9	0.9
	MM			35.2	3.4	10.3	0.8	21.5	2.0	23.8	2.1
	B			2.9	0.3	4.1	0.4	6.7	0.7	4.8	0.4
	S			0.7	0.1	18.0	1.5	39.5	4.1	18.3	1.6
	Mf			27.4	2.5	10.3	0.8	28.2	3.1	32.6	3.4
WEHI-3B CM + L929 CM	Bl			6.4	0.6	20.1	1.9	3.5	0.4	8.0	0.8
	P			11.9	0.9	22.2	1.9	13.9	1.2	52.7	4.8
	Imf			23.3	2.4	14.1	1.5	1.2	0.1	4.7	0.4
	M			19.8	1.7	5.4	0.5	10.2	0.9	2.6	0.3
	MM			31.0	3.2	2.1	0.2	9.2	0.9	7.4	0.8
	B			1.3	0.1	0	0	0.8	0.1	0.7	0.1
	S			0.7	0.1	11.4	1.0	3.6	0.4	8.0	0.9
	Mf			0	0	0	0	0	0	0	0
WEHI-3B CM + rGM-CSF	Bl			5.7	0.4	24.8	2.5	57.6	5.9	16.0	1.4
	P			2.5	0.2	7.4	0.8	1.4	0.1	2.1	0.2
	Imf			3.5	0.3	9.5	0.8	7.5	0.8	37.7	4.0
	M			5.0	0.4	25.0	2.6	0.7	0.1	3.4	0.3
	MM			14.7	1.5	12.9	1.1	6.2	0.5	2.8	0.2
	B			32.3	3.0	10.9	0.8	31.5	2.9	15.7	1.1
	S			2.8	0.3	4.1	0.4	3.4	0.3	0.7	0.1
	Mf			4.3	0.2	15.6	1.4	11.0	0.9	11.6	0.8
L929 CM + rGM-CSF	Bl			35.0	3.2	14.8	1.4	38.3	4.0	26.0	1.9
	P			2.1	0.1	10.0	0.6	4.7	0.3	6.7	0.5
	Imf			11.0	0.7	10.0	0.6	2.0	0.1	11.3	0.7
	M			10.3	1.2	16.2	1.2	0	0	17.3	1.4
	MM			16.1	1.2	10.0	0.8	10.9	0.9	2.7	0.1
	B			26.5	2.0	8.0	0.8	42.2	3.7	19.3	1.5
	S			4.4	0.2	0.0	0.0	2.1	0.1	0	0
	Mf			5.2	0.3	22.2	1.8	8.9	0.7	12.7	1.4
			24.2	2.6	23.6	1.9	29.2	2.5	30.0	2.5	

Appendix Table A3.7 SA8 leukaemic differential cell counts. SA8 bone marrow cells were cultured in FCS and optimal concentrations of WEHI-3B CM, L929 CM and rGM-CSF alone and in combination in the microtitre assay for eight hours, two, four and six days. Cytospins were prepared stained and the differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
FCS	Bl	1.12	0.15	1.00	0.15	0.00	0.00	0.07	0.01	0	0
	P	1.21	0.15	1.24	0.19	0.65	0.07	0.14	0.02	0.05	0.01
	Imf	0.00	0.00	0.52	0.08	0.42	0.06	0.07	0.01	0.00	0.00
	M	0.56	0.08	1.55	0.22	0.42	0.06	0.20	0.03	0.14	0.02
	MM	2.80	0.38	2.53	0.36	2.07	0.26	0.31	0.05	0.23	0.04
	B	0.18	0.03	0.37	0.05	0.46	0.06	0.02	0.00	0.00	0.00
	S	0.05	0.01	0.05	0.01	0.16	0.02	0.04	0.01	0.14	0.02
	Mo	0.56	0.08	0	0	0	0	0	0	0	0
Mf	0.05	0.01	0	0	1.39	0.18	1.14	0.17	1.15	0.19	
Total	cells	6.53	0.59	7.26	0.78	5.57	0.44	1.99	0.22	1.71	0.22
15% WEHI 3B CM	Bl			0.98	0.15	0.52	0.07	0.60	0.09	0.64	0.09
	P			1.56	0.22	1.96	0.28	5.72	0.79	11.08	1.46
	Imf			1.77	0.26	2.43	0.35	0.50	0.08	0	0
	M			0.37	0.05	0.91	0.13	0.50	0.08	1.11	0.15
	MM			2.08	0.29	0.59	0.09	1.51	0.20	4.25	0.59
	B			0.37	0.05	0.27	0.04	0.11	0.02	0.47	0.06
	S			0.16	0.02	0.52	0.07	1.10	0.15	1.42	0.17
	Mf			0.21	0.03	2.36	0.34	4.82	0.70	4.60	0.59
Total	cells			7.50	0.81	9.56	1.05	14.86	1.54	23.57	2.13
L929 CM	Bl			0.56	0.07	0.31	0.04	0.08	0.01	0.05	0.01
	P			0.50	0.07	1.02	0.14	0.22	0.03	0.05	0.01
	Imf			2.59	0.36	0.41	0.06	1.38	0.19	0.05	0.01
	M			1.07	0.14	0.07	0.01	0.28	0.05	0.26	0.03
	MM			1.73	0.25	0.31	0.04	3.56	0.55	0.83	0.11
	B			0.46	0.07	0.07	0.01	0.28	0.05	0.26	0.04
	S			0.21	0.03	0.58	0.07	0.36	0.05	0.21	0.03
	Mf			0.10	0.01	2.11	0.29	4.87	0.72	6.27	0.95
Total	cells			7.22	0.77	4.88	0.54	10.92	1.25	7.77	0.87
60 U/ml rGM-CSF	Bl			0.05	0.01	0	0	0	0	0	0
	P			1.32	0.18	1.15	0.17	0.09	0.02	0.59	0.08
	Imf			0.46	0.07	0.91	0.11	0.34	0.05	0.06	0.01
	M			0.61	0.09	3.03	0.40	0.09	0.02	1.12	0.14
	MM			2.54	0.38	0.91	0.11	2.73	0.40	2.06	0.28
	B			0.21	0.03	0.36	0.05	0.85	0.13	0.42	0.06
	S			0.05	0.01	1.60	0.20	5.02	0.77	1.59	0.22
	Mf			1.98	0.29	0.91	0.11	3.58	0.56	2.83	0.42
Total	cells			7.22	0.81	8.87	0.84	12.70	1.42	8.67	0.92

**Appendix Table A3.8** SA8 leukaemic bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in the microtitre assay. The total cell number per well was determined and cytopspins were prepared, stained and differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
WEHI-3B CM + L929 CM	BI	1.12	0.15	0.44	0.06	1.76	0.25	0.54	0.09	1.87	0.25
	P	1.21	0.15	0.82	0.10	1.94	0.27	2.13	0.30	12.3	1.58
	Imf	0	0	1.60	0.23	1.23	0.19	0.18	0.03	1.10	0.14
	M	0.56	0.08	1.36	0.18	0.47	0.07	1.56	0.22	0.61	0.09
	MM	2.80	0.38	2.13	0.31	0.18	0.03	1.42	0.21	1.73	0.24
	B	0.18	0.03	0.09	0.01	0	0	0.13	0.02	0.16	0.03
	S	0.05	0.01	0.05	0.01	1.00	0.14	0.55	0.09	1.87	0.27
	Mo	0.56	0.08	0	0	0	0	0	0	0	0
Mf	0.05	0.01	0.39	0.05	2.17	0.32	8.85	1.35	3.74	0.47	
Total	cells	6.53	0.59	6.88	0.71	8.75	0.94	15.36	1.74	23.31	2.10
WEHI-3B CM + rGM-CSF	BI			0.17	0.02	0.74	0.12	0.27	0.03	0.52	0.08
	P			0.24	0.03	0.95	0.14	1.46	0.21	9.49	1.53
	Imf			0.34	0.04	2.51	0.40	0.13	0.02	0.85	0.13
	M			1.00	0.14	1.29	0.19	1.20	0.15	0.67	0.10
	MM			2.20	0.29	1.09	0.16	6.12	0.82	3.96	0.55
	B			0.19	0.03	0.41	0.06	0.67	0.09	0.18	0.03
	S			0.29	0.03	1.56	0.24	2.13	0.27	2.92	0.41
	Mf			2.39	0.32	1.48	0.23	7.45	1.06	6.54	0.92
Total	cells			6.82	0.65	10.03	1.23	19.43	1.87	25.16	3.04
L929 CM + rGM-CSF	BI			0.14	0.02	0.85	0.11	0.76	0.10	1.28	0.18
	P			0.72	0.09	0.85	0.11	0.32	0.04	2.16	0.29
	Imf			0.67	0.11	1.37	0.19	0.00	0.00	3.31	0.48
	M			1.05	0.14	0.85	0.12	1.75	0.25	0.52	0.07
	MM			1.73	0.24	0.68	0.10	6.77	0.98	3.69	0.53
	B			0.29	0.04	0	0	0.33	0.04	0	0
	S			0.34	0.04	1.88	0.26	1.42	0.20	2.43	0.40
	Mf			1.58	0.25	2.00	0.28	4.69	0.67	5.73	0.84
Total	cells			6.52	0.75	8.48	0.97	16.04	1.85	19.12	2.31

Appendix Table A3.9 SA8 leukaemic bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in combination in the microtitre assay. The total cell number per well was determined and cytopins were prepared, stained and differentials counted.

GF	CELL	PERCENTAGE OF TOTAL CELLS ± STANDARD ERROR									
		SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
FCS	Bl	35.9	3.4	29.7	3.0	4.8	0.2	7.1	0.7	28.1	3.0
	P	7.7	0.6	8.5	0.9	18.1	1.5	42.2	3.9	31.0	3.0
	Imf	0	0	0.7	0.1	5.6	0.5	3.4	0.2	2.9	0.2
	M	4.5	0.5	7.1	0.6	2.1	0.2	11.1	0.9	2.9	0.3
	MM	48.8	4.5	51.7	4.9	41.3	3.6	12.5	0.9	1.3	0.1
	B	1.3	0.1	0.0	0.0	4.3	0.3	2.7	0.3	0.0	0.0
	S	1.3	0.1	2.1	0.1	8.4	0.7	3.4	0.4	4.2	0.3
	Mo	0.3	0.1	0.3	0.1	0	0	0	0	0	0
	Mf	0	0	0	0	15.4	1.2	17.6	1.6	29.7	2.6
15% WEHI-3B CM	Bl			13.6	1.5	7.9	0.7	14.7	1.6	8.1	0.8
	P			22.8	2.3	33.8	3.0	46.9	4.8	66.9	5.9
	Imf			2.2	0.1	4.3	0.4	4.2	0.3	4.0	0.3
	M			17.1	1.4	8.7	0.7	12.5	1.4	5.4	0.5
	MM			37.1	3.2	20.1	1.8	2.8	0.1	5.4	0.5
	B			4.3	0.4	3.7	0.3	0	0	0	0
	S			1.4	0.1	5.7	0.5	9.0	0.8	2.0	0.1
	Mf			1.4	0.1	15.9	1.4	9.8	0.8	8.1	0.7
15% L929 CM	Bl			5.2	0.4	10.0	0.7	13.4	1.0	4.8	0.3
	P			20.8	1.8	24.3	1.9	18.8	1.5	0.7	0.1
	Imf			4.5	0.5	2.9	0.2	8.0	0.7	4.1	0.5
	M			11.1	0.7	2.2	0.2	6.1	0.7	0.7	0.1
	MM			42.9	3.9	17.9	1.4	7.4	0.7	4.1	0.5
	B			3.8	0.4	3.5	0.4	0	0	1.4	0.1
	S			2.9	0.3	13.6	1.2	2.0	0.1	1.5	0.1
	Mf			8.9	0.7	25.7	2.0	44.2	4.0	82.9	6.5
60 U/ml rGM-CSF	Bl			13.0	1.2	18.2	1.4	15.9	1.3	44.1	4.0
	P			23.2	1.9	36.4	3.7	25.7	2.6	10.2	1.0
	Imf			3.0	0.2	0.7	0.1	11.7	0.7	7.5	0.7
	M			10.9	0.8	7.7	0.8	4.9	0.3	4.8	0.5
	MM			34.0	2.8	4.3	0.3	11.9	0.9	11.6	1.0
	B			0.7	0.1	3.5	0.3	1.4	0.1	0	0
	S			9.3	0.8	20.2	1.6	18.7	2.0	13.6	1.2
	Mf			5.9	0.5	9.0	0.6	9.8	0.8	8.2	0.9
WEHI-3B CM + L929 CM	Bl			12.8	1.0	6.4	0.5	31.3	2.6	39.3	4.0
	P			17.8	1.5	42.6	4.0	29.3	2.6	31.3	3.0
	Imf			1.4	0.1	1.4	0.1	3.3	0.3	3.3	0.3
	M			5.7	0.6	6.4	0.5	6.2	0.7	1.3	0.1
	MM			51.0	5.2	12.8	1.0	6.9	0.7	2.7	0.3
	B			2.9	0.3	2.9	0.1	0	0	0	0
	S			4.9	0.5	9.9	0.7	8.1	0.7	2.7	0.2
	Mf			0	0	0	0	0	0	0	0
WEHI-3B CM + rGM-CSF	Bl			3.5	0.3	17.8	1.3	14.9	1.6	19.4	1.6
	P			12.5	0.9	15.1	1.4	9.1	0.8	20.5	1.7
	Imf			17.9	1.8	31.6	3.2	34.2	3.1	23.3	1.9
	M			3.2	0.3	2.1	0.2	10.5	0.8	6.2	0.6
	MM			13.3	1.2	13.7	0.9	9.1	0.8	3.5	0.3
	B			34.3	3.0	9.6	0.9	4.2	0.4	4.7	0.2
	S			4.7	0.5	2.1	0.1	4.3	0.5	0.7	0.5
	Mf			5.6	0.5	13.7	1.5	13.9	1.5	9.6	0.7
L929 CM + rGM-CSF	Bl			8.6	0.5	12.3	1.4	14.8	1.5	31.5	3.3
	P			12.5	0.9	15.1	1.4	9.1	0.8	20.5	1.7
	Imf			17.9	1.8	31.6	3.2	34.2	3.1	23.3	1.9
	M			3.2	0.3	2.1	0.2	10.5	0.8	6.2	0.6
	MM			13.3	1.2	13.7	0.9	9.1	0.8	3.5	0.3
	B			34.3	3.0	9.6	0.9	4.2	0.4	4.7	0.2
	S			4.7	0.5	2.1	0.1	4.3	0.5	0.7	0.5
	Mf			5.6	0.5	13.7	1.5	13.9	1.5	9.6	0.7
L929 CM + rGM-CSF	Bl			6.9	0.5	12.3	1.1	11.1	0.6	14.1	1.2
	P			22.0	1.9	29.2	2.4	24.1	1.7	3.3	0.3
	Imf			2.3	0.1	8.0	0.7	5.4	0.5	13.4	1.2
	M			10.7	0.8	13.1	0.8	8.3	0.9	1.4	0.1
	MM			37.4	3.5	7.3	0.7	5.4	0.6	3.4	0.2
	B			2.3	0.2	2.2	0.2	2.1	0.2	2.0	0.2
	S			12.9	0.9	19.8	2.0	26.3	2.0	18.1	1.6
	Mf			5.4	0.3	8.0	0.5	17.2	0.5	44.3	4.0

Appendix Table A3.10 SA2 LP leukaemic differential cell counts. SA2 LP bone marrow cells were cultured in FCS and optimal concentrations of WEHI-3B CM, L929 CM and rGM-CSF alone and in combination in the microtitre assay for eight hours, two, four and six days. Cytospins were prepared stained and the differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
FCS	Bl	2.53	0.34	2.14	0.30	0.30	0.03	0.21	0.03	0.88	0.13
	P	0.54	0.07	0.61	0.09	1.14	0.15	1.25	0.18	0.97	0.14
	Imf	0	0	0.05	0.01	0.35	0.05	0.10	0.01	0.09	0.01
	M	0.32	0.05	0.51	0.06	0.13	0.02	0.33	0.04	0.09	0.01
	MM	3.43	0.46	3.72	0.50	2.60	0.35	0.37	0.05	0.04	0.01
	B	0.09	0.01	0.00	0.00	0.27	0.03	0.08	0.01	0.00	0.00
	S	0.09	0.01	0.15	0.02	0.53	0.07	0.10	0.02	0.13	0.02
	Mo	0.03	0.01	0.02	0.01	0	0	0	0	0	0
	Mf	0	0	0	0	0.97	0.12	0.52	0.07	0.93	0.13
Total	cells	7.03	0.69	7.20	0.68	6.29	0.64	2.96	0.32	3.13	0.32
15% WEHI 3B CM	Bl			0.94	0.15	0.92	0.14	1.25	0.19	1.25	0.19
	P			1.58	0.23	3.94	0.59	4.00	0.60	10.31	1.48
	Imf			0.15	0.02	0.50	0.08	0.36	0.05	0.62	0.08
	M			1.18	0.16	1.01	0.15	1.07	0.17	0.83	0.12
	MM			2.57	0.36	2.35	0.36	0.24	0.03	0.83	0.12
	B			0.30	0.04	0.43	0.06	0	0	0	0
	S			0.10	0.01	0.67	0.10	0.77	0.11	0.31	0.04
	Mf			0.10	0.01	1.85	0.28	0.84	0.12	1.25	0.18
Total	cells			6.92	0.75	11.7	1.42	8.53	0.94	15.40	1.74
60 U/ml rGM-CSF	Bl			0.37	0.05	0.83	0.10	0.72	0.10	0.42	0.05
	P			1.49	0.20	2.02	0.26	1.01	0.14	0.06	0.01
	Imf			0.32	0.05	0.24	0.03	0.43	0.06	0.36	0.06
	M			0.80	0.10	0.18	0.02	0.33	0.05	0.06	0.01
	MM			3.08	0.42	1.49	0.19	0.40	0.06	0.36	0.06
	B			0.27	0.04	0.29	0.04	0.00	0.00	0.12	0.02
	S			0.21	0.03	1.13	0.15	0.11	0.01	0.13	0.02
	Mf			0.64	0.08	2.14	0.27	2.38	0.35	7.31	0.97
Total	cells			7.18	0.74	8.32	0.84	5.38	0.62	8.82	0.95
60 U/ml rGM-CSF	Bl			0.92	0.13	2.20	0.30	1.37	0.18	6.15	0.85
	P			1.64	0.22	4.41	0.66	2.21	0.33	1.42	0.20
	Imf			0.21	0.03	0.09	0.02	1.01	0.12	1.04	0.15
	M			0.77	0.10	0.93	0.14	0.42	0.05	0.67	0.10
	MM			2.41	0.32	0.52	0.07	1.02	0.13	1.62	0.22
	B			0.05	0.01	0.42	0.06	0.12	0.02	0	0
	S			0.66	0.09	2.45	0.33	1.61	0.24	1.89	0.26
	Mf			0.42	0.06	1.09	0.14	0.84	0.11	1.14	0.17
Total	cells			7.08	0.75	12.1	1.34	8.60	0.92	13.93	1.45

Appendix Table A3.11 SA2 LP leukaemic bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in the microtitre assay. The total cell number per well was determined and cytopins were prepared, stained and differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
WEHI-3B CM + L929 CM	Bl	2.53	0.35	0.92	0.12	0.75	0.10	3.00	0.41	7.87	1.21
	P	0.54	0.07	1.28	0.17	5.01	0.71	2.81	0.40	6.27	0.94
	Imf	0	0	0	0	0.16	0.02	0.32	0.05	0.66	0.10
	M	0.32	0.05	0.41	0.06	0.75	0.10	0.59	0.09	0.26	0.04
	MM	3.43	0.46	3.67	0.54	1.50	0.20	0.66	0.10	0.54	0.09
	B	0.09	0.01	0.21	0.03	0.34	0.04	0	0	0	0
	S	0.09	0.01	0.35	0.05	1.16	0.15	0.78	0.11	0.54	0.07
	Mo	0.03	0.01	0.10	0	0	0	0	0	0	0
	Mf	0	0	0.25	0.03	2.09	0.27	1.43	0.22	3.89	0.55
	Total cells	7.03	0.69	7.19	0.75	11.76	1.26	9.59	1.06	20.03	2.30
WEHI-3B CM + rGM-CSF	Bl			0.83	0.11	2.02	0.29	1.00	0.14	4.68	0.65
	P			1.19	0.17	4.23	0.63	3.78	0.55	5.32	0.74
	Imf			0.21	0.03	0.28	0.04	1.16	0.16	1.41	0.21
	M			0.88	0.12	1.83	0.23	1.00	0.14	0.80	0.11
	MM			2.28	0.31	1.28	0.18	0.46	0.07	1.08	0.13
	B			0.31	0.05	0.28	0.03	0.47	0.08	0.16	0.12
	S			0.37	0.05	1.83	0.28	1.54	0.24	2.18	0.29
	Mf			0.57	0.07	1.65	0.26	1.63	0.25	7.19	1.10
	Total cells			6.64	0.71	13.40	1.45	11.04	1.26	22.82	2.56
L929 CM + rGM-CSF	Bl			0.56	0.07	1.40	0.20	1.08	0.15	3.02	0.45
	P			1.79	0.23	3.31	0.45	2.35	0.34	0.71	0.11
	Imf			0.19	0.02	0.91	0.13	0.53	0.08	2.87	0.44
	M			0.87	0.11	1.49	0.19	0.81	0.13	0.30	0.04
	MM			3.04	0.41	0.83	0.12	0.53	0.09	0.73	0.10
	B			0.19	0.02	0.25	0.04	0.20	0.03	0.43	0.07
	S			1.05	0.12	2.25	0.33	2.56	0.38	3.88	0.59
	Mf			0.44	0.05	0.91	0.11	1.68	0.22	9.50	1.45
	Total cells			8.13	0.78	11.35	1.23	9.74	1.23	21.44	2.64

Appendix Table A3.12 SA2 LP leukaemic bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in combination in the microtitre assay. The total cell number per well was determined and cytopspins were prepared, stained and differentials counted.

GF	CELL	PERCENTAGE OF TOTAL CELLS ±				STANDARD ERROR					
		SCS	8 HOUR		DAY 2		DAY 4		DAY 6		
FCS	Bl	19.3	2.0	35.0	4.0	6.7	0.5	0	0	0.9	0.1
	P	18.7	2.0	17.1	2.0	29.9	2.5	0	0	3.3	0.2
	Imf	0	0	10.3	0.8	4.0	0.3	1.5	0.1	1.4	0.1
	M	5.4	0.6	6.9	0.5	4.0	0.2	13.6	1.1	8.8	0.6
	MM	49.0	4.5	28.9	3.0	28.0	3.0	21.4	1.7	10.2	0.7
	B	2.8	0.3	0.7	0.1	4.0	0.2	1.5	0.1	0	0
	S	3.4	0.4	0.0	0.0	0.8	0.5	6.8	0.4	0	0
	Mo	1.3	0.1	0.5	0.1	0.2	0.1	0	0	0	0
	Mf	0	0	0.7	0.1	22.4	1.9	55.1	5.0	75.3	4.1
15% WEHI-3B CM	Bl			45.9	3.8	5.5	0.3	5.4	0.4	3.5	0.2
	P			21.0	1.7	27.4	1.7	22.6	1.9	33.5	2.7
	Imf			6.0	0.4	15.1	1.8	4.8	0.3	1.4	0.1
	M			12.2	0.9	9.6	1.2	10.3	0.7	3.6	0.4
	MM			10.8	0.9	13.6	1.2	27.4	2.1	17.5	1.5
	B			1.4	0.1	1.3	0.1	0	0	0.7	0.1
	S			0.7	0.1	2.8	0.1	6.9	0.8	7.7	0.5
	Mf			2.1	0.1	24.8	1.8	22.7	2.6	32.2	2.4
15% L929 CM	Bl			53.0	5.0	1.4	0.1	4.1	0.3	2.7	0.3
	P			17.1	1.0	34.0	2.6	6.7	0.3	0.7	0.1
	Imf			3.3	0.4	4.2	0.3	12.3	0.6	4.7	0.3
	M			9.5	1.1	4.2	0.3	2.0	0.1	1.3	0.1
	MM			13.7	1.1	13.8	1.5	7.4	0.9	10.1	0.5
	B			1.3	0.1	1.4	0.1	0.7	0.1	0	0
	S			0	0	5.5	0.4	1.4	0.1	2.0	0.1
	Mf			2.1	0.1	35.4	3.4	65.3	4.3	78.5	5.4
60 U/ml rGM-CSF	Bl			56.8	4.8	4.2	0.3	1.3	0.1	0	0
	P			14.2	1.5	37.5	3.2	6.9	0.5	1.3	0.1
	Imf			3.3	0.3	10.4	0.9	3.4	0.2	0	0
	M			6.1	0.7	10.4	0.9	18.6	2.0	2.8	0.2
	MM			14.9	1.3	4.8	0.3	35.9	3.0	17.0	1.5
	B			2.8	0.3	4.2	0.3	2.1	0.2	0.7	0.1
	S			2.0	0.1	6.2	0.5	4.1	0.3	8.8	1.0
	Mf			0	0	22.3	2.4	27.6	3.0	69.3	5.6
WEHI-3B CM + L929 CM	Bl			50.3	4.3	3.3	0.2	4.8	0.3	1.4	0.1
	P			22.4	3.0	37.3	4.1	23.6	1.8	22.7	2.4
	Imf			2.0	0.1	7.4	0.5	8.8	0.8	5.0	0.3
	M			7.5	0.5	4.8	0.3	6.8	0.8	10.7	0.8
	MM			10.3	0.6	2.7	0.3	10.1	0.8	10.6	0.8
	B			2.0	0.1	3.3	0.3	0	0	3.5	0.2
	S			0.7	0.1	11.4	0.9	8.1	0.8	4.2	0.2
	Mf			0	0	0	0	0	0	0	0
WEHI-3B CM + rGM-CSF	Bl			39.8	3.2	8.4	0.7	3.4	0.2	3.4	0.2
	P			19.6	2.0	43.2	3.6	27.0	2.0	27.6	3.0
	Imf			11.5	1.0	11.8	1.3	5.4	0.5	0.7	0.1
	M			10.8	0.9	2.8	0.1	10.1	0.7	6.8	0.5
	MM			13.6	0.9	4.2	0.3	15.6	1.2	21.3	1.5
	B			4.2	0.3	2.8	0.3	0	0	2.8	0.3
	S			0.7	0.1	12.1	1.0	8.8	1.0	9.6	0.5
	Mf			0	0	14.7	1.5	29.7	3.0	27.6	1.5
L929 CM + rGM-CSF	Bl			48.9	4.5	4.2	0.4	0	0	0.7	0.1
	P			25.5	2.5	44.8	4.2	25.5	2.2	7.7	0.6
	Imf			5.8	0.5	12.4	1.4	5.8	0.4	1.4	0.1
	M			8.7	0.5	9.6	1.0	15.3	1.2	5.7	0.6
	MM			8.1	0.5	2.8	0.1	11.0	1.2	18.4	1.5
	B			0.8	0.6	1.4	0.1	2.2	0.2	3.6	0.2
	S			2.2	0.2	13.2	1.0	34.4	2.9	8.5	0.6
	Mf			0	0	11.7	1.0	5.8	0.6	53.8	4.5

Appendix Table A3.13 SA2 HP leukaemic differential cell counts. SA2 HP bone marrow cells were cultured in FCS and optimal concentrations of WEHI-3B CM, L929 CM and rGM-CSF alone and in combination in the microtitre assay for eight hours, two, four and six days. Cytospins were prepared stained and the differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
FCS	Bl	1.31	0.17	2.59	0.40	0.43	0.06	0	0	0.02	0
	P	1.27	0.16	1.27	0.20	1.93	0.27	0	0	0.07	0.01
	Imf	0.00	0.00	0.76	0.10	0.26	0.03	0.05	0.01	0.03	0.00
	M	0.37	0.05	0.51	0.07	0.26	0.03	0.44	0.05	0.19	0.03
	MM	3.33	0.39	2.14	0.32	1.81	0.28	0.69	0.08	0.22	0.03
	B	0.19	0.02	0.05	0.01	0.26	0.03	0.05	0.01	0.00	0.00
	S	0.23	0.03	0.00	0.00	0.05	0.03	0.22	0.02	0.00	0.00
	Mo	0.09	0.01	0.04	0.01	0.01	0.01	0	0	0	0
Mf	0	0	0.05	0.01	1.45	0.20	1.78	0.22	1.62	0.21	
Total	cells	6.79	0.49	7.41	0.78	6.46	0.71	3.23	0.26	2.15	0.25
15% WEHI 3B CM	Bl			3.35	0.45	0.67	0.09	1.40	0.20	1.14	0.14
	P			1.53	0.21	3.35	0.47	5.80	0.86	11.08	1.52
	Imf			0.44	0.06	1.85	0.32	1.24	0.17	0.45	0.06
	M			0.89	0.12	1.17	0.21	2.64	0.37	1.18	0.19
	MM			0.79	0.11	1.67	0.26	7.04	1.01	5.78	0.81
	B			0.10	0.01	0.16	0.02	0	0	0.24	0.04
	S			0.05	0.01	0.34	0.05	1.77	0.30	2.53	0.32
	Mf			0.15	0.02	3.03	0.44	5.83	0.97	10.63	1.42
Total	cells			7.30	0.78	12.24	1.56	25.72	3.12	33.03	3.66
60 U/ml rGM-CSF	Bl			4.13	0.53	0.12	0.01	0.84	0.12	0.55	0.09
	P			1.33	0.14	2.91	0.34	1.38	0.18	0.14	0.03
	Imf			0.26	0.04	0.36	0.04	2.52	0.33	0.96	0.14
	M			0.74	0.11	0.36	0.04	0.42	0.06	0.27	0.04
	MM			1.07	0.13	1.18	0.17	1.53	0.27	2.05	0.28
	B			0.10	0.01	0.12	0.01	0.15	0.03	0	0
	S			0.00	0.00	0.47	0.05	0.29	0.04	0.41	0.06
	Mf			0.16	0.02	3.03	0.40	13.41	1.88	16.01	2.34
Total	cells			7.77	0.68	8.55	0.76	20.54	2.54	20.39	2.63
60 U/ml rGM-CSF	Bl			4.31	0.59	0.51	0.07	0.34	0.04	0	0
	P			1.08	0.16	4.57	0.63	1.76	0.21	0.16	0.02
	Imf			0.25	0.04	1.27	0.18	0.87	0.10	0	0
	M			0.46	0.07	1.27	0.18	4.73	0.68	0.34	0.05
	MM			1.13	0.16	0.58	0.07	9.13	1.15	2.10	0.31
	B			0.21	0.03	0.51	0.07	0.53	0.07	0.09	0.02
	S			0.15	0.02	0.76	0.10	1.05	0.13	1.09	0.18
	Mf			0.00	0.00	2.72	0.42	7.02	1.01	8.55	1.22
Total	cells			7.59	0.82	12.19	1.33	25.43	2.41	12.33	1.45

Appendix Table A3.14 SA2 HP leukaemic bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in the microtitre assay. The total cell number per well was determined and cytopspins were prepared, stained and differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
WEHI-3B CM + L929 CM	Bl	1.31	0.17	3.77	0.51	0.41	0.06	1.38	0.18	0.39	0.05
	P	1.27	0.16	1.68	0.29	4.59	0.76	6.85	0.92	6.36	0.99
	Imf	0	0	0.15	0.02	0.91	0.13	2.56	0.37	1.40	0.18
	M	0.37	0.05	0.56	0.07	0.59	0.08	1.97	0.32	2.99	0.41
	MM	3.33	0.39	0.77	0.09	0.33	0.05	2.94	0.40	2.96	0.41
	B	0.19	0.02	0.15	0.02	0.41	0.06	0	0	0.99	0.13
	S	0.23	0.03	0.05	0.01	1.41	0.21	2.35	0.35	1.19	0.15
	Mo	0.09	0.01	0	0	0.05	0	0	0	0	0
Mf	0	0	0.36	0.06	3.67	0.59	11.00	1.68	11.74	1.81	
Total	cells	6.79	0.49	7.49	0.78	12.32	1.52	29.05	3.21	28.02	3.21
WEHI-3B CM + rGM-CSF	Bl			3.05	0.40	1.10	0.15	1.13	0.13	1.11	0.12
	P			1.50	0.22	5.69	0.78	9.10	1.14	9.00	1.30
	Imf			0.88	0.12	1.56	0.24	1.81	0.25	0.24	0.04
	M			0.83	0.11	0.37	0.04	3.41	0.42	2.22	0.27
	MM			1.04	0.13	0.55	0.07	5.25	0.67	6.94	0.83
	B			0.32	0.04	0.37	0.06	0	0	0.91	0.13
	S			0.05	0.01	1.60	0.22	2.97	0.45	3.14	0.34
	Mf			0	0	1.93	0.29	9.99	1.43	9.00	0.99
Total	cells			7.67	0.79	13.17	1.42	33.66	3.41	32.56	3.12
L929 CM + rGM-CSF	Bl			4.16	0.56	0.50	0.07	0.00	0.00	0.18	0.03
	P			2.17	0.30	5.37	0.78	7.61	1.05	1.89	0.25
	Imf			0.49	0.06	1.49	0.23	1.73	0.22	0.34	0.04
	M			0.74	0.08	1.15	0.17	4.57	0.61	1.40	0.21
	MM			0.69	0.08	0.34	0.04	3.27	0.50	4.49	0.61
	B			0.07	0.05	0.17	0.02	0.65	0.09	0.88	0.11
	S			0.19	0.03	1.58	0.21	10.26	1.40	2.08	0.27
	Mf			0	0	1.40	0.20	1.73	0.26	13.13	1.79
Total	cells			8.51	0.84	12.00	1.32	29.82	3.21	24.39	2.63

Appendix Table A3.15 SA2 HP leukaemic bone marrow differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in combination in the microtitre assay. The total cell number per well was determined and cytopins were prepared, stained and differentials counted.

GF	CELL	PERCENTAGE OF TOTAL CELLS ±				STANDARD ERROR	
		SCS	8 HOUR	DAY 2	DAY 4	DAY 6	
FCS	Bl	99.0 0.3	79.6 1.8	50.0 2.5	84.4 0.6	36.5 2.1	
	P	1.0 0.3	20.4 1.8	12.5 0.9	7.3 1.7	14.1 1.2	
	Imf	0 0	0 0	36.5 1.7	8.3 0.9	49.4 1.0	
	M	0 0	0 0	0 0	0 0	0 0	
	MM	0 0	0 0	0 0	0 0	0 0	
	B	0 0	0 0	0 0	0 0	0 0	
	S	0 0	0 0	0 0	0 0	0 0	
	Mf	0 0	0 0	1.1 0.1	0 0	0 0	
15% WEHI-3B CM	Bl		46.8 1.4	88.9 3.0	90.8 3.3	70.6 2.4	
	P		53.2 1.4	10.1 3.0	5.1 1.5	14.1 1.2	
	Imf		0 0	1.0 0.1	4.1 2.0	15.3 1.2	
	M		0 0	0 0	0 0	0 0	
	MM		0 0	0 0	0 0	0 0	
	B		0 0	0 0	0 0	0 0	
	S		0 0	0 0	0 0	0 0	
	Mf		0 0	0 0	0 0	0 0	
15% L929 CM	Bl		64.5 1.3	72.7 1.9	82.0 2.2	66.3 2.5	
	P		34.8 1.3	27.3 1.9	12.0 1.2	23.9 1.3	
	Imf		0.8 0.1	0 0	6.0 1.0	9.8 1.3	
	M		0 0	0 0	0 0	0 0	
	MM		0 0	0 0	0 0	0 0	
	B		0 0	0 0	0 0	0 0	
	S		0 0	0 0	0 0	0 0	
	Mf		0 0	0 0	0 0	0 0	
60 U/ml rGM-CSF	Bl		46.9 1.7	76.0 3.4	79.5 2.6	48.3 3.3	
	P		53.1 1.7	16.7 2.0	3.4 0.9	13.8 1.4	
	Imf		0 0	5.2 1.6	17.1 1.8	37.9 2.0	
	M		0 0	0 0	0 0	0 0	
	MM		0 0	0 0	0 0	0 0	
	B		0 0	0 0	0 0	0 0	
	S		0 0	0 0	0 0	0 0	
	Mf		0 0	2.1 0.1	0 0	0 0	
WEHI-3B CM + L929 CM	Bl		58.6 2.8	93.8 1.4	75.5 3.4	75.8 2.7	
	P		40.4 2.7	5.2 1.2	13.3 1.6	11.0 1.6	
	Imf		0 0	1.0 0.3	11.2 1.9	13.2 1.2	
	M		0 0	0 0	0 0	0 0	
	MM		0 0	0 0	0 0	0 0	
	B		0 0	0 0	0 0	0 0	
	S		0 0	0 0	0 0	0 0	
	Mf		1.0 0.3	0 0	0 0	0 0	
WEHI-3B CM + rGM-CSF	Bl		58.7 1.4	86.7 3.3	89.0 2.1	61.5 3.7	
	P		38.1 1.1	7.2 2.1	1.1 0.3	16.7 1.3	
	Imf		3.2 0.4	6.1 1.2	9.9 1.9	21.9 2.7	
	M		0 0	0 0	0 0	0 0	
	MM		0 0	0 0	0 0	0 0	
	B		0 0	0 0	0 0	0 0	
	S		0 0	0 0	0 0	0 0	
	Mf		0 0	0 0	0 0	0 0	
L929 CM + rGM-CSF	Bl		45.6 2.6	62.6 1.3	92.5 1.7	30.1 2.4	
	P		51.1 2.8	33.3 1.3	4.3 1.3	5.5 0.6	
	Imf		0 0	4.0 0.3	3.2 0.7	64.4 1.9	
	M		0 0	0 0	0 0	0 0	
	MM		0 0	0 0	0 0	0 0	
	B		0 0	0 0	0 0	0 0	
	S		0 0	0 0	0 0	0 0	
	Mf		3.3 0.4	0 0	0 0	0 0	

Appendix Table A3.16 SA2 CL leukaemic differential cell counts. The SA2 CL was cultured in FCS and optimal concentrations of WEHI-3B CM, L929 CM and rGM-CSF alone and in combination in the microtitre assay for eight hours, two, four and six days. Cytospins were prepared stained and the differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
	Bl	6.21	0.40	8.42	0.78	18.57	2.21	46.58	5.04	21.00	2.50
	P	0.64	0.20	2.16	0.27	4.64	0.60	4.03	1.03	8.15	1.10
	Imf	0	0	0	0	13.54	1.60	4.60	0.50	28.51	3.03
FCS	M	0	0	0	0	0	0	0	0	0	0
	MM	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0
	S	0	0	0	0	0	0	0	0	0	0
	mf	0	0	0	0	0.39	0.06	0	0	0	0
Total cells		6.85	0.44	10.58	0.95	37.14	4.02	55.21	5.96	57.66	6.02
	Bl			4.46	0.50	34.17	3.93	51.60	5.63	40.21	4.06
	P			5.08	0.56	3.88	1.23	2.90	0.90	8.04	1.03
15%	Imf			0	0	0.39	0.04	2.32	1.16	8.71	1.07
WEHI	M			0	0	0	0	0	0	0	0
3B	MM			0	0	0	0	0	0	0	0
CM	B			0	0	0	0	0	0	0	0
	S			0	0	0	0	0	0	0	0
	mf			0	0	0	0	0	0	0	0
Total cells				9.54	1.03	38.44	4.23	56.82	5.84	56.96	5.42
	Bl			5.84	0.73	26.45	2.98	51.41	5.43	46.01	4.66
	P			3.15	0.40	9.92	1.29	7.52	1.08	16.59	1.80
15%	Imf			0.07	0.01	0	0	3.76	0.74	6.79	1.10
L929	M			0	0	0	0	0	0	0	0
CM	MM			0	0	0	0	0	0	0	0
	B			0	0	0	0	0	0	0	0
	S			0	0	0	0	0	0	0	0
	mf			0	0	0	0	0	0	0	0
Total cells				9.06	1.11	36.37	3.98	62.69	6.41	69.39	6.52
	Bl			4.77	0.62	28.19	2.78	41.28	4.65	27.38	3.45
	P			5.39	0.69	6.18	0.92	1.77	0.51	7.82	1.15
60	Imf			0	0	1.93	0.62	8.85	1.33	21.52	2.54
IU/mi	M			0	0	0	0	0	0	0	0
rGM-	MM			0	0	0	0	0	0	0	0
CSF	B			0	0	0	0	0	0	0	0
	S			0	0	0	0	0	0	0	0
	mf			0	0	0	0	0	0	0	0
Total cells				10.16	1.26	37.07	3.26	51.90	5.59	56.72	6.00

Appendix Table A3.17 The SA2 CL differential cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in the microtitre assay. The total cell number per well was determined and cytopspins were prepared, stained and differentials counted.

ABSOLUTE CELL NO./ WELL X 10<sup>4</sup> ± STANDARD ERROR

GF	CELL	SCS		8 HOUR		DAY 2		DAY 4		DAY 6	
WEHI-3B CM + L929 CM	Bl	6.21	0.40	5.75	0.66	33.33	3.50	44.07	5.01	47.69	5.20
	P	0.64	0.20	3.97	0.49	1.85	0.47	7.74	1.23	6.91	1.23
	Imf	0	0	0	0	0.37	0.12	6.55	1.30	8.29	1.14
	M	0	0	0	0	0	0	0	0	0	0
	MM	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0
	S	0	0	0	0	0	0	0	0	0	0
Mf	0	0	0	0	0	0	0	0	0	0	
Total	cells	6.85	0.44	9.82	1.03	35.55	3.69	58.36	6.09	62.89	6.43
WEHI-3B CM + rGM-CSF	Bl			5.83	0.46	31.65	2.84	47.55	4.19	38.77	4.64
	P			3.78	0.31	2.61	0.79	0.59	0.17	10.51	1.36
	Imf			0.32	0.05	2.23	0.47	5.28	1.11	13.80	2.22
	M			0	0	0	0	0	0	0	0
	MM			0	0	0	0	0	0	0	0
	B			0	0	0	0	0	0	0	0
	S			0	0	0	0	0	0	0	0
Mf			0	0	0	0	0	0	0	0	
Total	cells			9.93	0.75	36.49	2.96	53.42	4.53	63.08	6.52
L929 CM + rGM-CSF	Bl			4.32	0.45	23.78	2.63	54.71	4.93	16.16	1.97
	P			4.84	0.50	12.65	1.46	2.52	0.79	2.94	0.42
	Imf			0	0	1.53	0.20	1.89	0.45	34.52	3.33
	M			0	0	0	0	0	0	0	0
	MM			0	0	0	0	0	0	0	0
	B			0	0	0	0	0	0	0	0
	S			0	0	0	0	0	0	0	0
Mf			0.31	0.03	0	0	0	0	0	0	
Total	cells			9.47	0.82	37.96	4.13	59.12	5.21	53.62	4.93

Appendix Table A3.18 The SA2 CL leukaemic cell counts in absolute number of cells per well. Cells were cultured for eight hours, two, four and six days with optimal concentrations of WEHI-3B CM, L929 CM or rGM-CSF in combination in the microtitre assay. The total cell number per well was determined and cytopins were prepared, stained and differentials counted.

GF's	DAY	MATURATION INDEX				
		NBM	SA7	SA8	SA2 LP	SA2 HP
	SCS	2.0	0.3	1.3	0.9	1.3
FCS	8 HRS	2.1	0.4	0.7	1.2	0.4
	DAY 2	6.0	1.5	2.7	2.3	1.2
	DAY 4	24.3	2.3	1.0	0.6	5.6
	DAY 6	3.9	7.2	8.0	0.5	5.9
WEHI-3B CM	8 HRS	2.3	0.5	0.6	0.8	0.2
	DAY 2	1.0	1.1	0.6	0.8	0.7
	DAY 4	1.0	1.0	1.0	0.3	1.3
	DAY 6	0.4	1.5	0.8	0.2	1.4
L929 CM	8 HRS	3.9	0.2	0.5	1.4	0.2
	DAY 2	1.5	1.4	1.7	1.5	0.2
	DAY 4	3.4	3.0	4.6	1.1	3.0
	DAY 6	27.7	5.8	18.0	8.8	9.6
rGM-CSF	8 HRS	1.5	0.2	2.0	1.0	0.2
	DAY 2	1.2	0.8	0.7	0.6	0.6
	DAY 4	1.7	0.7	23.4	0.7	2.3
	DAY 6	6.3	4.3	3.9	0.5	23.7
WEHI-3B CM + L929 CM	8 HRS	2.9	0.7	0.6	1.8	0.2
	DAY 2	1.8	1.4	0.6	0.7	0.9
WEHI-3B CM + rGM-CSF	DAY 4	0.9	0.8	2.5	0.4	1.3
	DAY 6	0.3	0.9	0.5	0.3	1.5
WEHI-3B CM + rGM-CSF	8 HRS	2.9	0.9	2.9	1.1	0.2
	DAY 2	0.8	0.6	0.8	0.6	0.5
L929 CM + rGM-CSF	DAY 4	0.8	0.4	5.3	0.6	1.2
	DAY 6	0.4	1.6	1.2	0.9	1.6
L929 CM + rGM-CSF	8 HRS	1.7	0.8	1.5	1.4	0.1
	DAY 2	0.7	0.6	1.2	0.6	0.4
	DAY 4	2.7	0.6	4.7	1.0	1.1
	DAY 6	4.0	2.0	1.6	2.1	5.4

**Appendix Table A3.19** Differentiation study - Maturation Index. Cells were cultured for eight hours, two, four and six days with optimal concentrations of growth factor both singularly and in combination in the microtitre assay. The total number of cells per well were counted, cytopspins were prepared and differentials counted. The maturation index (MI) was determined from the differentials.

MI = Absolute number of mature cells / Absolute number of immature cells

Mature cells = metamyelocytes, bands, segmented, monocytes and macrophages

Immature cells = blasts, promyelocytes, myelocytes and immature monocytic cells

		PERCENTAGE No. OF PHAGOCYtic CELLS ± SE												
GF's	DAY	NBM		SA7		SA8		SA2 LP		SA2 HP		SA2 CL		
FCS	8 HRS	5.3	0.4	2.1	0.2	0	0	0	0	0.5	0.1	0	0	
	2	13.3	1.1	29.0	3.1	24.2	2.2	16.9	1.2	25.9	1.9	1.1	0.1	
	4	32.0	3.3	47.6	3.5	35.2	3.2	20.6	1.5	58.8	4.6	0	0	
	6	50.7	4.2	82.1	2.2	84.8	5.1	31.0	2.6	66.5	5.9	0	0	
WEHI	8 HRS	7.3	0.8	0.8	0.1	0.8	0.1	3.5	0.2	2.1	0.2	0	0	
	2	12.0	1.0	21.2	1.9	22.6	1.6	20.0	1.8	28.2	2.4	0	0	
	4	13.3	1.0	23.3	1.8	31.6	1.9	11.3	0.9	25.3	2.4	0	0	
	6	14.7	1.2	35.1	2.5	26.3	2.6	10.8	0.8	33.6	2.7	0	0	
L929	8 HRS	7.3	0.1	2.9	0.3	1.4	0.1	11.1	0.9	2.1	0.2	0	0	
	2	18.0	2.0	25.4	2.1	35.5	2.3	30.7	2.7	33.3	2.6	0	0	
	4	36.0	3.2	60.1	5.4	50.0	4.7	43.7	3.4	56.5	5.2	0	0	
	6	53.3	4.8	77.8	5.6	87.4	5.6	77.6	6.7	73.9	6.1	0	0	
rGM	8 HRS	7.4	0.8	3.7	0.4	34.5	2.9	8.1	0.7	0.7	0.1	0	0	
	2	16.0	1.5	19.3	2.1	17.8	1.2	18.8	1.5	25.8	1.9	2.1	0.1	
	4	14.7	1.5	15.8	1.0	36.2	2.6	16.6	1.5	26.3	2.4	0	0	
	6	33.4	3.5	43.3	3.9	39.3	3.4	19.5	5.6	69.3	5.8	0	0	
WEHI	8 HRS	12.6	1.0	6.9	0.5	5.7	0.4	6.3	0.5	4.8	0.3	1.0	0.1	
	2	15.4	1.2	23.0	1.8	23.5	2.0	19.1	1.6	33.1	2.9	0	0	
	L929	4	16.0	1.3	21.1	1.9	27.2	2.0	9.5	0.8	42.5	3.8	0	0
	6	8.0	0.7	37.7	2.5	20.0	2.0	20.0	1.7	42.5	3.6	0	0	
WEHI	8 HRS	7.3	0.8	3.0	0.3	44.9	3.5	12.4	1.1	0.7	0.1	0	0	
	2	9.4	1.0	16.7	1.7	20.9	1.7	19.1	1.7	22.4	1.6	0	0	
	rGM	4	8.7	0.7	12.4	0.9	41.1	3.6	19.0	1.6	29.7	1.8	0	0
	6	6.0	0.5	25.2	1.5	34.8	2.6	37.7	3.2	27.6	2.5	0	0	
L929	8 HRS	14.7	1.5	4.9	0.3	49.1	5.0	12.3	0.9	0.8	0.1	3.3	0.1	
	2	12.8	1.0	24.8	1.9	33.0	2.1	23.4	1.4	15.9	1.3	0	0	
	rGM	4	33.3	2.8	27.6	2.6	34.0	2.8	32.4	2.6	41.6	3.4	0	0
	6	26.1	2.7	44.8	4.2	45.3	3.6	60.4	5.2	62.4	5.1	0	0	

Appendix Table 3.20 Differentiation study - Absolute number of phagocytic cells. Normal and leukaemic bone marrow cells were cultured for eight hours, two, four and six days with optimal concentrations of growth factor singularly and in combination. 0.8 $\mu$  latex particles were added to the cultures for eight hours prior to preparing cytopins and determining the absolute cell number per well. The cytopins were stained with Jenner Giemsa and the percentage number of cells containing >10 latex particles was determined. At least 1000 cells were counted per slide.

GF's	DAY	ABSOLUTE No. OF PHAGOCYTYC CELLS / WELL ± SE												
		NBM		SA7		SA8		SA2 LP		SA2 HP		SA2 CL		
FCS	8 HRS	0.39	0.02	0.14	0.01	0	0	0	0	0.04	0	0	0	
	2	0.56	0.03	1.57	0.09	1.35	0.10	1.06	0.09	1.67	0.09	0.39	0.02	
	4	0.67	0.03	1.10	0.08	0.70	0.04	0.61	0.03	1.90	0.01	0	0	
	6	0.92	0.05	1.15	0.08	1.45	0.10	0.97	0.09	1.43	0.10	0	0	
WEHI	8 HRS	0.55	0.02	0.05	0	0.06	0	0.24	0.01	0.15	0.01	0	0	
	2	0.76	0.04	1.86	0.10	2.16	0.16	2.34	0.18	3.45	0.21	0	0	
	4	1.34	0.09	3.20	0.21	4.70	0.31	0.96	0.04	6.51	0.52	0	0	
	6	3.36	0.20	9.87	0.84	6.19	0.56	1.66	0.11	11.10	0.96	0	0	
L929	8 HRS	0.54	0.02	0.19	0.05	0.10	0	0.80	0.04	0.16	0.01	0	0	
	2	0.72	0.04	1.54	0.09	1.73	0.11	2.55	0.19	2.85	0.21	0	0	
	4	2.14	0.12	7.82	0.52	5.46	0.40	2.35	0.14	11.60	0.91	0	0	
	6	8.46	0.51	16.00	1.00	6.79	0.55	3.84	0.51	15.10	1.12	0	0	
rGM	8 HRS	0.60	0.05	0.28	0.01	2.49	0.15	0.57	0.02	0.05	0	0	0	
	2	0.89	0.08	1.50	0.09	1.58	0.11	2.27	0.18	3.15	0.25	0.77	0.03	
	4	1.16	0.09	2.15	0.15	4.60	0.40	1.43	0.09	6.68	0.56	0	0	
	6	2.98	0.27	5.19	0.48	3.41	0.25	8.70	0.75	8.55	0.74	0	0	
WEHI	8 HRS	0.90	0.09	0.48	0.03	0.39	0.02	0.45	0.02	0.36	0.02	0.10	0.01	
	2	0.96	0.09	1.75	0.09	2.06	0.19	2.25	0.19	4.08	0.31	0	0	
	L929	4	1.75	0.18	4.05	0.35	4.18	0.35	0.91	0.04	12.4	0.96	0	0
	6	2.48	0.22	10.70	0.89	4.68	0.39	4.01	0.29	11.9	0.85	0	0	
WEHI	8 HRS	0.55	0.02	0.21	0.01	3.06	0.28	0.82	0.04	0.05	0.01	0	0	
	2	0.65	0.03	1.45	0.08	2.10	0.16	2.56	0.20	2.95	0.22	0	0	
	rGM	4	1.07	0.59	2.71	0.20	7.98	0.68	2.10	0.16	10.00	0.89	0	0
	6	2.07	0.17	5.97	0.41	8.76	0.81	8.60	0.72	9.00	0.83	0	0	
L929	8 HRS	1.19	0.10	0.35	0.01	3.20	0.26	1.00	0.45	0.07	0.01	0.31	0.01	
	2	0.77	0.05	1.82	0.10	2.80	0.24	2.66	0.19	1.91	0.10	0	0	
	rGM	4	2.71	0.22	5.21	0.42	5.46	0.46	3.16	0.21	12.4	0.87	0	0
	6	5.66	0.49	9.68	0.84	8.66	0.82	13.00	0.98	15.2	1.15	0	0	

**Appendix Table A3.21** Differentiation study - Absolute number of phagocytic cells. Normal and leukaemic bone marrow cells were cultured for eight hours, two, four and six days with optimal concentrations of growth factor singularly and in combination. 0.8µm latex particles were added to the cultures for eight hours prior to preparing cytopspins and determining the absolute cell number per well. The cytopspins were stained with Jenner Giemsa and the percentage number of cells containing >10 latex particles was determined. At least 1000 cells were counted per slide.

**APPENDIX TABLES  
TO  
CHAPTER FOUR**

**PERCENTAGE OF CONTROL ± STANDARD ERROR**

SCS	Concentration of RA (M)									
	10 <sup>-9</sup>		10 <sup>-8</sup>		10 <sup>-7</sup>		10 <sup>-6</sup>		10 <sup>-5</sup>	
NBM	101	9	86	10	80	8	64	8	5	20
SA7	98	5	87	9	80	9	46	6	7	33
SA8	83	11	79	7	86	9	54	8	4	25
SA2 LP	83	6	74	17	33	6	8	13	5	19
SA2 HP	94	8	92	8	91	9	40	8	4	22
SA2 CL	98	7	89	8	64	8	25	19	14	25

**Appendix Table A4.1.** Single cell suspensions (SCS) of leukaemic and normal bone marrow cells and cells from the SA2 CL were cultured with varied concentrations of  $\beta$ atRA for four days in the microtitre assay to determine the concentration of  $\beta$ atRA required to decrease cellular proliferation. The growth factor dependent cells (NBM, SA7 HD, SA8 HD and the SA2 HD HP) were stimulated to proliferate with WEHI-3B CM and the growth factor independent cells (SA2 LP and the SA2 CL) were cultured with FCS alone. Proliferation was determined utilizing the uptake of 3H-TdR on day four of culture. The results from the scintillation CPM were expressed as 'Percentage of Control'. Percentage of Control = CPM of RA treated cells / CPM of WEHI-3B or FCS treated cells.

PERCENTAGE CELL COUNT / WELL ± SE

	FCS	+ 10 <sup>-6</sup> M RA	+ 10 <sup>-7</sup> M RA
<b>Bl</b>	0 0	0 0	0 0
<b>P</b>	0 0	0 0	0.8 0.1
<b>M</b>	4.2 0.3	0 0	4.8 0.3
<b>MM</b>	31.3 0.9	0.59 0.42	50.0 3.2
<b>B</b>	13.9 1.2	4.8 0.3	2.4 0.1
<b>S</b>	16.7 1.4	0 0	0 0
<b>Mo</b>	0 0	0 0	0 0
<b>Mf</b>	34.1 3.6	35.7 2.12	42.0 3.1

	WEHI-3B CM	+ 10 <sup>-6</sup> M RA	+ 10 <sup>-7</sup> M RA
<b>Bl</b>	1.4 0.1	2.1 0.1	3.5 0.2
<b>P</b>	43.4 3.4	30.3 2.4	41.3 3.6
<b>M</b>	9.0 0.7	7.7 0.6	9.9 0.8
<b>MM</b>	15.8 1.3	14.1 1.1	11.8 0.9
<b>B</b>	0.7 0.1	2.1 0.2	1.4 0.1
<b>S</b>	12.3 0.9	17.5 1.4	10.5 0.9
<b>Mo</b>	6.9 0.5	7.1 0.6	2.8 0.2
<b>Mf</b>	10.3 0.8	19.0 2.0	18.9 1.3

	L929 CM	+ 10 <sup>-6</sup> M RA	+ 10 <sup>-7</sup> M RA
<b>Bl</b>	3.4 0.2	0 0	0 0
<b>P</b>	17.3 1.2	0 0	0.8 0.1
<b>M</b>	3.5 0.3	0.7 0.1	0.8 0.1
<b>MM</b>	15.2 1.3	3.6 0.3	5.0 0.4
<b>B</b>	0.7 0.1	0 0	0.8 0.1
<b>S</b>	9.0 0.8	7.9 0.6	7.2 0.7
<b>Mo</b>	15.2 1.0	2.1 0.2	10.8 0.9
<b>Mf</b>	35.7 2.8	85.6 6.1	74.8 5.2

**Appendix Table A4.2** Normal bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used, 10<sup>-6</sup>M and 10<sup>-7</sup>M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM both. As a control cells were also cultured in FCS alone. On day four cytopins were prepared, stained and differentials counted. At least 500 cells were counted per slide.

ABSOLUTE CELL COUNT x 10 <sup>4</sup> / WELL ± SE						
	FCS		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	0	0	0	0	0	0
<b>P</b>	0	0	0	0	0.01	0
<b>M</b>	0.08	0.01	0	0	0.09	0.01
<b>MM</b>	0.66	0.05	1.03	0.09	0.91	0.10
<b>B</b>	0.29	0.03	0.08	0.01	0.04	0
<b>S</b>	0.35	0.04	0	0	0	0
<b>Mo</b>	0	0	0	0	0	0
<b>Mf</b>	<u>0.72</u>	<u>0.09</u>	<u>0.62</u>	<u>0.05</u>	<u>0.76</u>	<u>0.08</u>
Total Cells	2.10	0.15	1.73	0.10	1.81	0.15

	WEHI-3B CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	0.14	0.02	0.20	0.02	0.37	0.04
<b>P</b>	4.37	0.51	2.92	0.35	4.44	0.60
<b>M</b>	0.92	0.11	0.74	0.09	1.06	0.14
<b>MM</b>	1.59	0.19	1.36	0.16	1.27	0.16
<b>B</b>	0.07	0.01	0.20	0.03	0.15	0.02
<b>S</b>	1.24	0.14	1.69	0.20	1.13	0.15
<b>Mo</b>	0.70	0.08	0.69	0.08	0.30	0.04
<b>Mf</b>	<u>1.04</u>	<u>0.12</u>	<u>1.83</u>	<u>0.25</u>	<u>1.94</u>	<u>0.24</u>
Total Cells	10.08	0.88	9.65	0.85	10.76	1.10

	L929 CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	0.20	0.02	0	0	0	0
<b>P</b>	1.02	0.1	0	0	0.08	0.01
<b>M</b>	0.21	0.02	0.04	0.01	1.02	0.17
<b>MM</b>	0.90	0.10	0.21	0.03	0.08	0.01
<b>B</b>	0.42	0.07	0	0	0.47	0.08
<b>S</b>	0.53	0.06	0.47	0.06	0.08	0.01
<b>Mo</b>	0.90	0.09	0.13	0.02	0.68	0.09
<b>Mf</b>	<u>2.12</u>	<u>0.23</u>	<u>5.11</u>	<u>0.64</u>	<u>7.08</u>	<u>0.90</u>
Total Cells	5.94	0.43	5.97	0.61	9.49	1.02

**Appendix Table A4.3** Normal bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used, 10<sup>-6</sup>M and 10<sup>-7</sup>M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopspins were prepared, stained and differentials counted. At least 500 cells were counted per slide. The results were expressed as absolute cell numbers per well. Absolute cell number = Total Cell No. per Well x Percentage Cell No. per well.

PERCENTAGE CELL COUNT / WELL $\pm$ SE						
	FCS		$+ 10^{-6}$ M RA		$+ 10^{-7}$ M RA	
<b>Bl</b>	2.3	0.2	0.9	0.1	0	0
<b>P</b>	4.6	0.3	0	0	5.0	0.3
<b>Imf</b>	8.6	0.6	8.9	0.7	6.4	0.5
<b>M</b>	14.4	1.5	8.4	0.6	18.5	1.6
<b>MM</b>	14.4	1.5	27.1	2.0	38.6	3.2
<b>B</b>	4.5	0.3	1.7	0.1	0	0
<b>S</b>	2.3	0.2	3.4	0.3	2.9	0.3
<b>Mf</b>	48.9	4.3	49.6	5.2	28.5	1.9

	WEHI-3B CM		$+ 10^{-6}$ M RA		$+ 10^{-7}$ M RA	
<b>Bl</b>	4.9	0.4	3.1	0.2	3.5	0.4
<b>P</b>	29.4	2.3	13.1	1.1	16.8	1.3
<b>Imf</b>	3.5	0.3	8.2	0.6	16.8	1.3
<b>M</b>	12.3	1.0	7.1	0.6	7.0	0.6
<b>MM</b>	15.5	1.0	7.2	0.6	11.5	0.9
<b>B</b>	1.8	0.2	0.8	0.1	2.1	0.1
<b>S</b>	3.5	0.2	5.0	0.4	5.6	0.3
<b>Mf</b>	29.1	3.1	55.5	4.5	36.7	2.4

	L929 CM		$+ 10^{-6}$ M RA		$+ 10^{-7}$ M RA	
<b>Bl</b>	2.4	0.1	1.4	0.1	2.3	0.2
<b>P</b>	8.6	0.7	3.0	0.2	1.5	0.2
<b>Imf</b>	10.6	0.8	2.5	0.3	6.8	0.4
<b>M</b>	3.1	0.2	2.1	0.3	1.6	0.1
<b>MM</b>	4.4	0.3	1.7	0.1	3.7	0.2
<b>B</b>	0.3	0.1	0.3	0.1	0	0
<b>S</b>	1.8	0.1	1.8	0.2	3.1	0.2
<b>Mf</b>	68.8	0.5	87.2	8.2	81.1	0.7

**Appendix Table A4.4** SA7 leukaemic bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used,  $10^{-6}$ M and  $10^{-7}$ M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopins were prepared, stained and differentials counted. At least 500 cells were counted per slide.

ABSOLUTE CELL COUNT x 10 <sup>4</sup> / WELL ± SE						
	FCS		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>BI</b>	0.06	0.01	0.02	0	0	0
<b>P</b>	0.12	0.01	0	0	0.10	0.01
<b>IMf</b>	0.23	0.02	0.19	0.02	0.13	0.01
<b>M</b>	0.38	0.05	0.18	0.02	0.37	0.04
<b>MM</b>	0.38	0.05	0.50	0.05	0.78	0.09
<b>B</b>	0.12	0.01	0.05	0	0	0
<b>S</b>	0.06	0.01	0.07	0.01	0.06	0.01
<b>Mf</b>	<u>1.29</u>	0.13	<u>1.05</u>	0.13	<u>0.57</u>	0.06
Total Cells	2.65	0.15	2.12	0.14	2.01	0.16

	WEHI-3B CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>BI</b>	0.73	0.09	0.38	0.04	0.50	0.07
<b>P</b>	4.32	0.52	1.61	0.2	2.38	0.26
<b>IMf</b>	0.51	0.06	1.00	0.12	2.38	0.26
<b>M</b>	1.80	0.22	0.88	0.11	1.00	0.12
<b>MM</b>	2.27	0.25	0.89	0.11	1.63	0.18
<b>B</b>	0.26	0.04	0.10	0.02	0.30	0.03
<b>S</b>	0.51	0.05	0.62	0.08	0.79	0.08
<b>Mf</b>	<u>4.27</u>	0.6	<u>6.85</u>	0.84	<u>5.21</u>	0.53
Total Cells	14.67	1.32	12.33	1.14	14.19	1.12

	L929 CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>BI</b>	0.35	0.03	0.22	0.02	0.50	0.06
<b>P</b>	1.28	0.15	0.46	0.05	0.33	0.05
<b>IMf</b>	1.58	0.18	0.39	0.06	1.50	0.15
<b>M</b>	0.46	0.05	0.32	0.05	0.35	0.04
<b>MM</b>	0.65	0.07	0.26	0.03	0.82	0.08
<b>B</b>	0.04	0.01	0.05	0.02	0	###
<b>S</b>	0.21	0.02	0.28	0.04	0.68	0.07
<b>Mf</b>	<u>9.75</u>	0.87	<u>13.51</u>	1.69	<u>17.78</u>	1.49
Total Cells	14.93	1.33	15.49	1.28	22.07	1.84

**Appendix Table A4.5** SA7 leukaemic bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used, 10<sup>-6</sup>M and 10<sup>-7</sup>M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopspins were prepared, stained and differentials counted. At least 500 cells were counted per slide. The results were expressed as absolute cell numbers per well.

Absolute cell number = Total Cell No. per Well x Percentage Cell No. per well.

PERCENTAGE CELL COUNT / WELL ± SE						
	FCS		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	0.9	0.1	0	0	2.2	0.1
<b>P</b>	0	0	10.2	0.8	0	0
<b>Imf</b>	0	0	2.3	0.1	0	0
<b>M</b>	2.8	0.2	6.2	0.4	6.7	0.4
<b>MM</b>	37.9	2.5	2.8	0.1	12.2	0.6
<b>B</b>	1.9	0.1	0	0	0	0
<b>S</b>	9.3	0.6	2.8	0.1	1.1	0.1
<b>Mf</b>	47.2	3.2	75.7	1.0	77.8	1.3

	WEHI-3B CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	4.7	0.3	1.4	0.1	0.6	0.2
<b>P</b>	23.7	1.3	35.2	1.5	13.7	0.6
<b>Imf</b>	4.5	0.2	1.4	0.1	0	0
<b>M</b>	1.0	0.5	18.6	0.8	14.4	0.6
<b>MM</b>	21.1	1.3	8.3	0.8	21.9	1.7
<b>B</b>	4.0	0.2	1.4	0.1	4.8	0.2
<b>S</b>	5.0	0.3	0	0	4.1	0.2
<b>Mf</b>	36.1	2.1	33.8	2.5	40.4	2.3

	L929 CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	3.0	0.2	0	0	0	0
<b>P</b>	0	0	0	0	0	0
<b>Imf</b>	0	0	0	0	0.7	0.1
<b>M</b>	8.0	0.5	1.4	0.2	0	0
<b>MM</b>	24.0	1.2	7.6	0.8	3.5	0.4
<b>B</b>	0	0	0.7	0.1	0	0
<b>S</b>	2.0	0.2	0	0	0	0
<b>Mf</b>	63.0	2.3	90.3	2.1	95.8	2.4

**Appendix Table A4.6** SA8 leukaemic bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used, 10<sup>-6</sup>M and 10<sup>-7</sup>M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopins were prepared, stained and differentials counted. At least 500 cells were counted per slide.

ABSOLUTE CELL COUNT $\times 10^4$ / WELL $\pm$ SE						
	FCS		$+ 10^{-6}$ M RA		$+ 10^{-7}$ M RA	
<b>Bl</b>	0.02	0	0	0	0.04	0
<b>P</b>	0	0	0.18	0.02	0	0
<b>Imf</b>	0	0	0.04	0	0	0
<b>M</b>	0.06	0.01	0.11	0.01	0.12	0.01
<b>MM</b>	0.81	0.07	0.05	0	0.22	0.02
<b>B</b>	0.04	0	0	0	0	0
<b>S</b>	0.20	0.02	0.05	0	0.02	0
<b>Mf</b>	<u>1.01</u>	<u>0.09</u>	<u>1.34</u>	<u>0.11</u>	<u>1.40</u>	<u>0.12</u>
<b>Total Cells</b>	2.14	0.18	1.77	0.14	1.80	0.15

	WEHI-3B CM		$+ 10^{-6}$ M RA		$+ 10^{-7}$ M RA	
<b>Bl</b>	0.70	0.08	0.20	0.03	0.11	0.04
<b>P</b>	3.57	0.38	5.10	0.59	2.33	0.25
<b>Imf</b>	0.68	0.07	0.20	0.03	0	0
<b>M</b>	0.15	0.08	2.70	0.31	2.45	0.26
<b>MM</b>	3.17	0.34	1.20	0.17	3.73	0.46
<b>B</b>	0.60	0.06	0.20	0.03	0.81	0.08
<b>S</b>	0.75	0.08	0	0	0.70	0.08
<b>Mf</b>	<u>5.43</u>	<u>0.58</u>	<u>4.90</u>	<u>0.64</u>	<u>6.87</u>	<u>0.77</u>
<b>Total Cells</b>	15.05	1.35	14.50	1.55	17.00	1.63

	L929 CM		$+ 10^{-6}$ M RA		$+ 10^{-7}$ M RA	
<b>Bl</b>	0.33	0.04	0	0	0	0
<b>P</b>	0	0	0	0	0	0
<b>Imf</b>	0	0	0	0	0.08	0
<b>M</b>	0.87	0.12	0.17	0.02	0	0
<b>MM</b>	2.62	0.26	0.92	0.11	0.43	0.05
<b>B</b>	0	0	0.08	0.01	0	0
<b>S</b>	0.22	0.02	0	0	0	0
<b>Mf</b>	<u>6.89</u>	<u>0.68</u>	<u>10.89</u>	<u>1.43</u>	<u>11.66</u>	<u>1.29</u>
<b>Total Cells</b>	10.93	0.98	12.06	1.31	12.17	1.15

**Appendix Table A4.7** SA8 leukaemic bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used,  $10^{-6}$ M and  $10^{-7}$ M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopspins were prepared, stained and differentials counted. At least 500 cells were counted per slide. The results were expressed as absolute cell numbers per well.

Absolute cell number = Total Cell No. per Well  $\times$  Percentage Cell No. per well.

PERCENTAGE CELL COUNT / WELL $\pm$ SE						
	FCS		$+ 10^{-6}$ M RA		$+ 10^{-7}$ M RA	
Bl	7.0	0.5	10.8	1.2	25.7	2.4
P	41.9	4.2	14.1	1.2	18.0	1.5
Imf	3.5	0.4	7.0	0.5	5.5	0.3
M	11.2	0.8	9.7	0.5	2.8	0.3
MM	12.6	0.8	10.8	0.8	24.3	1.5
B	2.8	0.1	0.5	0.1	2.8	1.5
S	3.5	0.2	13.5	0.8	4.9	0.5
Mf	17.5	1.3	33.5	2.5	15.9	0.9

	WEHI-3B CM		$+ 10^{-6}$ M RA		$+ 10^{-7}$ M RA	
Bl	14.7	1.5	5.6	0.3	11.1	0.7
P	46.9	3.5	26.7	2.3	29.9	2.5
Imf	4.2	0.3	7.7	0.5	5.5	0.5
M	12.6	0.8	7.1	0.5	8.3	0.5
MM	2.8	0.2	6.3	0.5	8.3	0.5
B	0	0	1.4	0.1	0	0
S	9.0	0.5	8.5	0.7	7.0	0.5
Mf	9.9	0.5	36.7	3.5	29.9	3.0

	L929 CM		$+ 10^{-6}$ M RA		$+ 10^{-7}$ M RA	
Bl	13.4	0.9	2.3	0.2	1.4	0.1
P	18.8	1.9	0	0	0	0
IMf	8.0	0.7	13.8	0.7	6.7	0.7
M	6.0	0.5	0	0	0.8	0.1
MM	7.4	0.5	2.3	0.2	8.9	0.7
B	0	0	0	0	0	0
S	2	0.2	6.9	0.5	2.0	0.2
Mf	44.3	3.5	74.6	5.3	80.0	6.5

Appendix Table A4.8 SA2 LP leukaemic bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used,  $10^{-6}$ M and  $10^{-7}$ M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopspins were prepared, stained and differentials counted. At least 500 cells were counted per slide.

ABSOLUTE CELL COUNT x 10 <sup>4</sup> / WELL ± SE						
	FCS		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>BI</b>	0.22	0.02	0.20	0.03	0.75	0.08
<b>P</b>	1.32	0.15	0.26	0.04	0.52	0.05
<b>IMf</b>	0.11	0.01	0.13	0.02	0.16	0.01
<b>M</b>	0.35	0.03	0.18	0.02	0.08	0.01
<b>MM</b>	0.39	0.03	0.20	0.03	0.70	0.06
<b>B</b>	0.08	0.01	0.01	0	0.08	0.04
<b>S</b>	0.11	0.01	0.25	0.03	0.14	0.02
<b>Mf</b>	<u>0.53</u>	<u>0.05</u>	<u>0.62</u>	<u>0.08</u>	<u>0.46</u>	<u>0.04</u>
<b>Total Cells</b>	3.12	0.22	1.85	0.20	2.90	0.15

	WEHI-3B CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>BI</b>	1.31	0.16	0.49	0.04	0.92	0.12
<b>P</b>	4.19	0.44	2.36	0.25	2.49	0.34
<b>IMf</b>	0.38	0.04	0.68	0.06	0.46	0.07
<b>M</b>	1.13	0.11	0.63	0.06	0.69	0.09
<b>MM</b>	0.25	0.03	0.56	0.06	0.69	0.09
<b>B</b>	0	0	0.12	0.01	0	0
<b>S</b>	0.81	0.07	0.75	0.08	0.58	0.08
<b>Mf</b>	<u>0.88</u>	<u>0.08</u>	<u>3.23</u>	<u>0.37</u>	<u>2.49</u>	<u>0.37</u>
<b>Total Cells</b>	8.95	0.65	8.83	0.55	8.32	0.91

	L929 CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>BI</b>	0.73	0.10	0.19	0.03	0.12	0.01
<b>P</b>	1.02	0.16	0	0	0	0
<b>IMf</b>	0.44	0.06	1.13	0.13	0.54	0.08
<b>M</b>	0.33	0.05	0	0	0.06	0.01
<b>MM</b>	0.40	0.05	0.19	0.03	0.72	0.09
<b>B</b>	0	0	0	0	0	0
<b>S</b>	0.11	0.02	0.56	0.07	0.18	0.02
<b>Mf</b>	<u>2.40</u>	<u>0.33</u>	<u>6.09</u>	<u>0.77</u>	<u>6.46</u>	<u>0.8</u>
<b>Total Cells</b>	5.42	0.62	8.16	0.85	8.08	0.76

**Appendix Table A4.9** SA2 LP leukaemic bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used, 10<sup>-6</sup>M and 10<sup>-7</sup>M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopins were prepared, stained and differentials counted. At least 500 cells were counted per slide. The results were expressed as absolute cell numbers per well.

Absolute cell number = Total Cell No. per Well x Percentage Cell No. per well.

PERCENTAGE CELL COUNT / WELL ± SE						
	FCS		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
B1	0	0	0	0	0	0
P	0	0	0	0	1.4	0.1
IMf	1.5	0.1	3.5	0.4	3.6	0.4
M	13.7	0.9	17.9	0.7	8.7	0.4
MM	21.4	1.7	7.7	0.7	21.00	1.6
B	1.6	0.1	1.7	0.2	2.9	0.3
S	6.9	0.5	5.1	0.2	1.4	0.1
Mf	54.9	3.5	64.1	5.6	60.9	5.3

	WEHI-3B CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
B1	5.4	0.5	3.5	0.3	1.3	0.1
P	22.6	1.8	21.1	1.8	28.5	1.7
IMf	4.8	0.5	9.8	0.8	4.1	0.3
M	10.3	0.5	4.2	0.3	11.5	0.7
MM	27.4	2.8	9.2	0.5	22.4	1.8
B	0	0	0.7	0.1	5.5	0.5
S	6.9	0.5	14.1	0.8	6.8	0.5
Mf	22.7	1.8	37.3	3.5	19.8	2.3

	L929 CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
B1	4.1	0.2	0	0	0	0
P	6.7	0.5	0.7	0.1	0	0
IMf	12.2	0.9	7.8	0.6	12.3	1.3
M	2.0	0.1	1.4	0.1	3.5	0.3
MM	7.4	0.6	10.7	0.8	5.4	0.5
B	0.8	0.6	0.7	0.1	0	0
S	1.4	0.1	5.0	0.4	2.1	0.2
Mf	65.4	5.4	73.6	6.3	76.7	6.3

Appendix Table A4.10 SA2 HP leukaemic bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used, 10<sup>-6</sup>M and 10<sup>-7</sup>M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopspins were prepared, stained and differentials counted. At least 500 cells were counted per slide.

ABSOLUTE CELL COUNT x 10 <sup>4</sup> / WELL ± SE						
	FCS		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>B1</b>	0	0	0	0	0	0
<b>P</b>	0	0	0	0	0.06	0.01
<b>IMf</b>	0.06	0.01	0.12	0.02	0.14	0.02
<b>M</b>	0.51	0.05	0.63	0.08	0.33	0.03
<b>MM</b>	0.79	0.08	0.27	0.04	0.82	0.1
<b>B</b>	0.06	0.01	0.06	0.01	0.11	0.02
<b>S</b>	0.25	0.02	0.18	0.02	0.06	0.01
<b>Mf</b>	<u>2.03</u>	<u>0.19</u>	<u>2.24</u>	<u>0.32</u>	<u>2.38</u>	<u>0.3</u>
<b>Total Cells</b>	3.70	0.25	3.50	0.40	3.90	0.35

	WEHI-3B CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>B1</b>	1.05	0.17	0.36	0.06	0.25	0.1
<b>P</b>	4.39	0.51	2.18	0.3	5.44	0.25
<b>IMf</b>	0.94	0.05	1.01	0.08	0.78	0.05
<b>M</b>	1.99	0.12	0.44	0.07	2.20	0.07
<b>MM</b>	5.33	0.03	0.95	0.06	4.27	0.08
<b>B</b>	0	0	0.08	0.02	1.05	0
<b>S</b>	1.34	0.09	1.46	0.08	1.30	0.06
<b>Mf</b>	<u>4.41</u>	<u>0.11</u>	<u>3.84</u>	<u>0.43</u>	<u>3.77</u>	<u>0.35</u>
<b>Total Cells</b>	19.44	1.76	10.31	0.97	19.06	1.53

	L929 CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>B1</b>	0.63	0.07	0	0	0	0
<b>P</b>	1.04	0.12	0.05	0	0	0
<b>IMf</b>	1.89	0.05	0.49	0.13	1.09	0.08
<b>M</b>	0.32	0.03	0.09	0	0.31	0.01
<b>MM</b>	1.15	0.05	0.68	0.02	0.48	0.1
<b>B</b>	0.11	0	0.05	0	0	0
<b>S</b>	0.22	0.01	0.32	0.06	0.18	0.03
<b>Mf</b>	<u>10.09</u>	<u>0.29</u>	<u>4.65</u>	<u>0.73</u>	<u>6.79</u>	<u>0.86</u>
<b>Total Cells</b>	15.45	1.33	6.32	0.53	8.85	0.93

**Appendix Table A4.11** SA2 HP leukaemic bone marrow differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used, 10<sup>-6</sup>M and 10<sup>-7</sup>M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopspins were prepared, stained and differentials counted. At least 500 cells were counted per slide. The results were expressed as absolute cell numbers per well.

Absolute cell number = Total Cell No. per Well x Percentage Cell No. per well.

PERCENTAGE CELL COUNT / WELL ± SE						
	FCS		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	84.4	5.4	79.8	6.5	74.8	3.6
<b>P</b>	7.3	0.5	9.1	0.3	13.1	0.3
<b>Imf</b>	8.3	0.5	11.1	0.3	12.1	0.3

	WEHI-3B CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	90.8	7.6	82.3	8.3	82.7	8.0
<b>P</b>	5.1	1.3	6.3	0.3	11.2	1.7
<b>Imf</b>	4.1	0.3	11.4	0.3	6.1	1.7

	L929 CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	82	7.5	79.6	6.4	63.6	5.6
<b>P</b>	12	1.5	10.7	0.6	12.3	1.3
<b>Imf</b>	6	1.5	9.7	0.6	23.2	1.7

ABSOLUTE CELL COUNT × 10 <sup>4</sup> / WELL ± SE						
	FCS		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	18.48	2.42	8.64	1.03	8.82	0.76
<b>P</b>	1.60	0.21	0.98	0.09	1.53	0.12
<b>Imf</b>	<u>1.83</u>	<u>0.24</u>	<u>1.20</u>	<u>0.11</u>	<u>1.43</u>	<u>0.11</u>
Total Cells	21.90	2.50	10.83	0.95	11.80	0.85

	WEHI-3B CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	22.63	2.72	9.03	1.37	10.63	1.52
<b>P</b>	1.27	0.34	0.68	0.08	1.44	0.27
<b>Imf</b>	<u>1.02</u>	<u>0.12</u>	<u>1.26</u>	<u>0.15</u>	<u>0.78</u>	<u>0.23</u>
Total Cells	24.92	2.15	10.98	1.25	12.85	1.35

	L929 CM		+ 10 <sup>-6</sup> M RA		+ 10 <sup>-7</sup> M RA	
<b>Bl</b>	14.46	1.83	8.63	1	7.84	1.09
<b>P</b>	2.12	0.32	1.17	0.12	1.62	0.24
<b>Imf</b>	<u>1.06</u>	<u>0.28</u>	<u>1.05</u>	<u>0.11</u>	<u>2.86</u>	<u>0.37</u>
Total Cells	17.63	1.55	10.85	0.90	12.33	1.32

**Appendix Table A4.12** The SA2 CL differentiation study with  $\beta$ atRA. Cells were cultured in the microtitre assay with or without  $\beta$ atRA. Two concentrations of  $\beta$ atRA were used, 10<sup>-6</sup>M and 10<sup>-7</sup>M total concentration per well. Cells were stimulated to proliferate with WEHI-3B or L929 CM. As a control cells were also cultured in FCS alone. On day four cytopspins were prepared, stained and differentials counted. At least 500 cells were counted per slide. The results were expressed as absolute cell numbers per well. Absolute cell number = Total Cell No. per Well × Percentage Cell No. per well.

PERCENTAGE PHAGOCYtic CELLS / WELL $\pm$ SE										
	NBM		SA7		SA8		SA2 LP		SA2 HP	
FCS	33.3	2.6	49.4	3.6	61.2	4.3	20.4	1.4	58.8	4.3
+ 10 <sup>-6</sup> M RA	33.3	1.3	39.8	1.5	75.7	5.6	33.3	1.5	57.3	2.6
+ 10 <sup>-7</sup> M RA	39.6	1.3	22.5	1.5	67.2	4.6	14.6	1.5	56.5	3.3
WEHI-3B CM	13.8	0.9	20.6	1.6	31.3	2.3	11.2	0.9	25.4	1.4
+ 10 <sup>-6</sup> M RA	19.8	1.7	45.1	2.7	22.7	2.3	33.1	1.6	29.6	1.3
+ 10 <sup>-7</sup> M RA	18.1	1.7	34.4	2.7	34	2.6	30.6	2.6	19.2	2.3
L929 CM	37.2	1.5	47.2	5.0	50.0	3.6	43.7	3.3	56.5	4.6
+ 10 <sup>-6</sup> M RA	85.7	4.3	50.4	3.5	63.3	4.8	54.6	2.6	69.3	5.3
+ 10 <sup>-7</sup> M R	70.7	4.3	53.8	2.6	53.3	4.8	55.6	3.6	69.3	4.5

ABS No. PHAGOCYtic CELLS / WELL $\times 10^4 \pm$ SE											
	NBM		SA7		SA8		SA2 LP		SA2 HP		
FCS	0.70	0.07	1.31	0.12	1.31	0.14	0.64	0.06	2.17	0.22	
+ 10 <sup>-6</sup> M RA	0.60	0.04	0.84	0.06	1.34	0.15	0.61	0.07	2.00	0.25	
+ 10 <sup>-7</sup> M RA	0.70	0.06	0.45	0.05	1.21	0.13	0.42	0.05	2.20	0.24	
WEHI-3B CM	1.39	0.15	3.03	0.36	4.71	0.55	1.00	0.11	4.93	0.52	
+ 10 <sup>-6</sup> M RA	1.91	0.23	5.56	0.61	3.29	0.48	2.92	0.23	3.05	0.32	
+ 10 <sup>-7</sup> M RA	1.95	0.27	4.88	0.54	5.78	0.71	2.55	0.35	3.66	0.53	
L929 CM	2.22	0.18	7.04	0.97	5.47	0.63	2.37	0.32	8.73	1.03	
+ 10 <sup>-6</sup> M RA	5.09	0.58	7.81	0.84	7.64	1.01	4.46	0.51	4.38	0.5	
+ 10 <sup>-7</sup> M R	6.71	0.83	11.86	1.14	6.49	0.85	4.49	0.51	6.13	0.76	

Appendix Table A4.13 Differentiation study. Leukaemic and normal cells were cultured for four days in the microtitre assay. On day four the cells were incubated with 8 $\mu$  latex particles for eight hours. Cytospins were prepared, stained and the percentage and absolute number of cells containing greater than 10 particles were determined.

Absolute number = Total Cell No. per Well  $\times$  Percentage Phagocytic Cells per well.

PERCENTAGE SURVIVAL									
DAY	FCS					FCS + 10 <sup>-6</sup> M RA			
	Number of Cells Injected					10 <sup>5</sup>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>
	10 <sup>5</sup>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>	10				
1 to 10	100	100	100	100	100	100	100	100	100
11	70	100	100	100	100	20	100	100	100
12	10	70	100	100	100	20	80	100	100
13	0	70	90	100	100	20	0	100	100
14		30	70	90	100	20		100	100
15		20	60	90	100	20		80	100
16		10	50	90	100	20		20	100
17		6	50	90	100	20		20	100
18		0	50	90	100	20		20	100
19			50	90	100	20		20	100
20			40	90	100	20		20	100
21			40	90	100	20		20	100
22			40	90	100	20		20	100
23			40	80	100	20		20	100
24			40	80	100	20		20	100
25			40	80	100	20		20	100
26			40	80	100	20		20	100
27			40	80	100	20		20	100
28 to 90			30	80	100	20		20	100

**Appendix Table A4.14 *In Vivo* Clonogenic Cell Assay.** Single cell suspension of SA7 HD leukaemic bone marrow cells were cultured in the microtitre assay with FCS  $\pm 10^{-6}$  M  $\beta$ atRA. On day four of culture the cells were harvested with a pasteur pipette, the number of viable cells counted and cells at varied concentrations were injected into syngeneic recipients to determine the number of clonogenic leukaemic cells remaining in culture after each treatment. Five mice were injected per test group and percentage survival was monitored for 90 days. Results are the means of three experiments and the standard errors were not greater than 5% for each treatment.

PERCENTAGE SURVIVAL								
DAY	WEHI-3B CM				WEHI-3B CM + $10^{-6}$ M RA			
	Number of Cells Injected							
	$10^5$	$10^4$	$10^3$	$10^2$	$10^5$	$10^4$	$10^3$	$10^2$
1 to 11	100	100	100	100	100	100	100	100
12	70	100	100	100	50	100	100	100
13	30	100	100	100	10	100	100	100
14	30	60	90	100	0	90	80	90
15	20	60	80	100		50	80	90
16	0	50	80	100		50	80	90
17		50	80	100		50	80	90
18		50	70	100		50	80	90
19		50	70	100		50	80	90
20		30	70	100		50	70	90
21		30	70	100		50	70	90
22		20	70	100		40	70	90
23		10	70	100		40	70	90
24		10	70	100		40	70	90
25		10	70	100		40	70	90
26 to 90		10	70	100		30	70	90

L929 CM								
DAY	L929 CM				L929 CM + $10^{-6}$ M RA			
	Number of Cells Injected							
	$10^5$	$10^4$	$10^3$	$10^2$	$10^5$	$10^4$	$10^3$	$10^2$
1 to 11	100	100	100	100	100	100	100	100
12	60	100	100	100	100	100	100	100
13	0	100	100	100	50	100	100	100
14		50	90	100	40	100	100	100
15		40	90	100	40	70	90	100
16		20	90	100	10	70	90	100
17		20	90	100	10	70	90	100
18		20	90	100	10	70	90	100
19		20	90	90	10	70	90	100
20		20	90	90	10	70	90	100
21		20	90	90	0	70	90	100
22		20	90	90		70	90	100
23		20	90	90		70	90	100
24		20	90	90		70	90	100
25		20	90	90		70	90	100
26		20	90	90		70	90	100
27		20	90	90		70	90	100
28		20	90	90		70	90	100
29 to 90		20	90	90		60	90	100

**Appendix Table A4.15** *In Vivo* Clonogenic Cell Assay. Single cell suspension of SA7 HD leukaemic bone marrow cells were cultured in the microtitre assay with WEHI-3B CM  $\pm 10^{-6}$  M  $\beta$ atRA and L929 CM  $\pm 10^{-6}$  M  $\beta$ atRA. On day four of culture the cells were harvested with a pasteur pipette, the number of viable cells counted and cells at varied concentrations were injected into syngeneic recipients to determine the number of clonogenic leukaemic cells remaining in culture after each treatment. Five mice were injected per test group and percentage survival was monitored for 90 days. Results were the means of three experiments and the standard errors were not greater than 5% for each treatment.

**APPENDIX TABLES  
TO  
CHAPTER FIVE**

		PERCENTAGE OF CONTROL ± STANDARD ERROR					
CONC RA (M)		CONCENTRATION ARA-C (M)					
		0	10 <sup>-10</sup>	10 <sup>-9</sup>	10 <sup>-8</sup>	10 <sup>-7</sup>	10 <sup>-6</sup>
N	0	1.2 8	114 15	89 11	86 8	61 9	1.4 16
	10 <sup>-9</sup>	101 9	94 9	83 7	74 9	57 11	0.9 15
B	10 <sup>-8</sup>	86 10	92 10	118 8	110 8	55 12	1.5 24
M	10 <sup>-7</sup>	80 8	130 8	120 10	111 13	62 10	1.8 13
	10 <sup>-6</sup>	64 8	78 14	70 10	75 8	47 11	1.4 18
	10 <sup>-5</sup>	5 20	4 24	5 28	6 26	5 14	0.9 22
S	0	0.7 15	113 9	88 13	87 11	62 10	0.9 25
	10 <sup>-9</sup>	98 5	85 13	76 12	75 9	59 13	1.2 32
A	10 <sup>-8</sup>	87 9	92 11	119 9	109 9	54 11	1.4 24
7	10 <sup>-7</sup>	80 9	121 15	123 12	112 13	63 10	0.8 14
	10 <sup>-6</sup>	46 6	82 12	72 7	75 9	47 11	0.9 18
	10 <sup>-5</sup>	7 5	6 10	5 7	4 8	7 8	7 6
S	0	1.2 33	104 10	99 9	97 10	48 11	3 24
	10 <sup>-9</sup>	83 11	55 6	36 10	34 6	31 12	1.5 21
A	10 <sup>-8</sup>	79 7	51 11	37 9	41 9	30 9	2.4 33
8	10 <sup>-7</sup>	86 9	44 14	34 12	54 10	39 7	1.7 16
	10 <sup>-6</sup>	54 8	74 7	44 5	52 8	36 10	2.2 33
	10 <sup>-5</sup>	4 25	4 8	3 17	4 8	5 12	4 9
S	0	100 5	43 7	52 5	3 2	0.5 18	1.3 16
A	10 <sup>-9</sup>	42 7	19 2	16 5	15 8	2 2	0.7 12
2	10 <sup>-8</sup>	43 2	19 6	17 4	19 15	3 19	0.6 18
P	10 <sup>-7</sup>	29 5	17 3	13 6	11 6	3 6	0.5 15
3	10 <sup>-6</sup>	12 12	6 3	5 8	5 10	1.5 2	0.7 10
6	10 <sup>-5</sup>	3 5	3 13	2 5	3 26	1 4	1 23
S	0	100 8	113 11	78 8	88 9	26 13	1.3 17
A	10 <sup>-9</sup>	56 13	66 12	58 11	40 7	10 9	1.5 19
2	10 <sup>-8</sup>	47 10	59 15	33 8	40 15	11 9	1.6 20
P	10 <sup>-7</sup>	35 12	17 9	23 12	22 14	6 8	1.5 8
3	10 <sup>-6</sup>	6 11	8 9	8 5	8 13	4 7	1 14
9	10 <sup>-5</sup>	2 9	3 24	2 20	3 9	2 14	2 11
S	0	3 6	84 8	49 6	46 7	22 9	0.6 7
A	10 <sup>-9</sup>	35 12	25 15	9 11	9 7	16 6	0.8 19
2	10 <sup>-8</sup>	39 9	26 12	9 10	9 7	5 10	1.1 26
P	10 <sup>-7</sup>	50 9	25 8	11 13	15 16	7 2	3 21
5	10 <sup>-6</sup>	39 6	32 9	28 8	30 8	11 9	0.6 8
5	10 <sup>-5</sup>	3 5	4 8	5 3	3 9	4 6	3 5
S	0	2 15	109 8	61 8	63 7	26 9	1 8
A	10 <sup>-9</sup>	63 8	89 8	21 8	32 8	11 9	1 8
2	10 <sup>-8</sup>	72 8	106 9	29 9	37 8	22 8	3 20
P	10 <sup>-7</sup>	64 8	86 8	55 8	55 8	23 9	4 16
5	10 <sup>-6</sup>	40 8	47 8	33 8	33 8	13 8	1 11
9	10 <sup>-5</sup>	5 9	5 4	3 7	6 11	7 14	8 5
S	0	100 9	84 9	97 8	92 8	37 9	2 25
A	10 <sup>-9</sup>	98 7	103 6	102 8	94 7	44 6	2 25
2	10 <sup>-8</sup>	89 8	89 8	100 8	98 6	34 6	1 24
	10 <sup>-7</sup>	64 8	85 6	70 8	76 8	24 6	1 33
C	10 <sup>-6</sup>	30 13	34 6	30 6	36 6	12 7	2 10
L	10 <sup>-5</sup>	14 9	7 14	6 14	9 8	4 7	2 11

Appendix Table A5.1. Single cell suspensions of normal and leukaemic bone marrow and cells from the SA2 CL were cultured in the microtitre assay with combinations of  $\beta$ atRA and ara-C at varying concentrations. Growth factor dependent cells were stimulated to proliferate with WEHI-3B CM and growth factor independent cells were cultured with FCS alone. On day four of culture the cells were pulsed for eight hours with 3H-TdR. The scintillation CPM were converted to 'Percentage of Untreated Control'.

## LIST OF ABBREVIATIONS

AML	acute myeloid leukaemia
APL	acute promyelocytic leukaemia
ara-C	cytosine arabinoside
C	Celsius (degrees)
CM	conditioned medium
CR	complete remission
CSF's	colony stimulating factors
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EBV	Epstein Barr virus
eg	example
FAB	French American British collaborative for the classification of leukaemia
g	gram
GBq	giga Bequerel
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
GM-CFU	granulocyte macrophage colony forming unit
G6PD	glucose-6-phosphate dehydrogenase
Gy	Gray (Dose of radiation)
IL-3	interleukin-3
<sup>3</sup> H-TdR	tritiated thymidine
ip	intraperitoneal
iv	intravenous
kBq	kilo Bequerel
kD	kilo daltons
l	litre
L-CFU	leukaemic-colony forming unit
LAI	leukaemia associated inhibitor
LIA	leukaemia inhibitory activity
MDS	myelodysplastic syndrome
ml	microlitre
ml	millilitre
mmol	millimolar
μ	micron
NBM	normal bone marrow
NBT	nitroblue tetrazoleum
ppm	parts per minute
rGM-CSF	recombinant granulocyte macrophage-colony stimulating factor
βatRA	β-all <i>trans</i> retinoic acid
13- <i>cis</i> RA	13- <i>cis</i> retinoic acid
rpm	rotations per minute
SCS	single cell suspension
Vit	vitamin
v/v	volume per volume
w/v	weight per volume

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