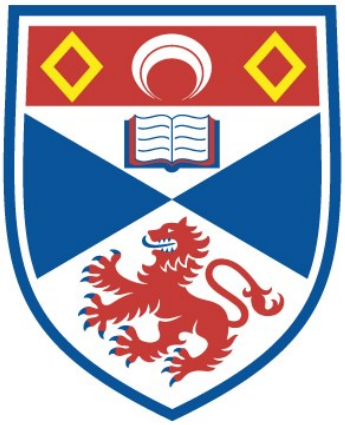
THE ROLE OF LEUKAEMIA INHIBITORY FACTOR AND A LEUKAEMIC ASSOCIATED INHIBITOR IN THE CONTROL OF THE PROLIFERATION OF HAEMATOPOIETIC STEM CELLS

Alan Taylor

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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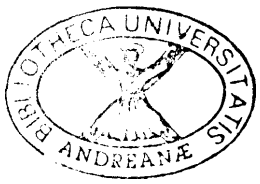
THE ROLE OF LEUKAEMIA INHIBITORY FACTOR AND A LEUKAEMIC ASSOCIATED INHIBITOR IN THE CONTROL OF THE PROLIFERATION OF HAEMATOPOIETIC STEM CELLS.

Thesis submitted for the degree of Doctor of Philosophy to the University of St. Andrews

by

ALAN TAYLOR



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I, Alan Thomas Mcturk Taylor , hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Alan Taylor Date 13/3/96

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12, and as a candidate for the degree of Ph.D. on 1st October, 1992.

Signed Alan Taylor Date 13/3/96

Certificate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

Signature of Supervisor Date 13/3/96

Dr. Andrew C. Riches

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This Thesis is dedicated to my family:
my wife Yvonne;
my mother and my father;
Gwenda and Richard;
and of course Uncle William.

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4.1 Femoral marrow

DIRECT

INDIRECT

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ABBREVIATIONS

5FU :	5 FLOURO URACIL
ARA C :	CYTOSINE ARABINOSIDE
BFU-E :	BURST FORMING UNIT ERYTHROCYTE
CFU-S :	COLONY FORMING UNIT SPLEEN
CNTF :	CILLIARY NEUTROPHIC FACTOR
GEMM-CFC :	GRANULOCYTE , ERYTHROCYTE , MACROPHAGE , MEGAKARYOCYTE COLONY FORMING CELL .
GM-CFC :	GRANULOCYTE-MACROPHAGE COLONY FORMING CELL
GM-CSF :	GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR
HPP-CFC :	HIGH PROLIFERATIVE POTENTIAL COLONY FORMING CELL
IFN-β :	INTERFERON BETA
IL-1α	INTERLEUKIN 1 ALPHA
IL-1β	INTERLEUKIN 1 BETA
IL-3 :	INTERLEUKIN THREE
IL-4 :	INTERLEUKIN FOUR
IL-6 :	INTERLEUKIN SIX
IL-8 :	INTERLEUKIN EIGHT
IL-11 :	INTERLEUKIN ELEVEN
L929cm :	L929 CONDITIONED MEDIUM
LAI :	LEUKAEMIA ASSOCIATED INHIBITOR
LAIA :	LEUKAEMIAASSOCIATED INHIBITORY ACTIVITY
LIF :	LEUKAEMIA INHIBITORY FACTOR
LPP-CFC :	LOW PROLIFERATIVE POTENTIAL COLONY FORMING CELL .
LTBMc :	LONG TERM BONE MARROW CULTURE
M-CSF :	MACROPHAGE COLONY STIMULATING FACTOR
MIP :	MACROPHAGE INFLAMMATORY PROTEIN
MIP-1-α :	MACROPHAGE INFLAMMATORY PROTEIN 1 ALPHA
MIP-1-β :	MACROPHAGE INFLAMMATORY PROTEIN 1 BETA
MIX-CFC :	MIXED COLONY FORMING CELL
NBM :	NORMAL BONE MARROW
OSM :	ONCOSTATIN M
RBM :	REGENERATING BONE MARROW

RH123 :	RHODAMINE 123
SA2JMB1cm:	SA2JMB1 CONDITIONED MEDIUM
TGF-β -	TRANSFORMING GROWTH FACTOR BETA
TNF-α -	TUMOUR NECROSIS FACTOR ALPHA
WEHIcm :	WEHI CONDITIONED MEDIUM

ABSTRACT

Activities associated with, or interacting with, leukaemic cell populations were assayed for the ability to influence *in vitro* haematopoiesis.

The first of these, the glycoprotein leukaemia inhibitory factor (LIF), has a role in aspects of murine, non human primate and human haematopoiesis. It is thought to be particularly important in the development of megakaryocytes and is also known to induce the terminal differentiation of certain leukaemic cell lines.

LIF was assayed both for direct and indirect effects on the proliferation of haematopoietic precursor cell populations *in vitro*. As a direct acting agent in semi-solid agar culture of haematopoietic cell populations derived from normal bone marrow or 15 day foetal liver, LIF was unable to support colony formation. In cultures of cells derived from normal bone marrow stimulated with single, or combinations of, growth factors, the addition of LIF had no statistically significant effect on the level of colony formation. In cultures of cells derived from foetal liver, stimulated with particular growth factor combinations (medium conditioned by the Wehi3B leukaemic cell line + medium conditioned by the lung fibroblast cell line, L929); GM-CSF + M-CSF; IL-1α + IL-3 + M-CSF), LIF, was shown to decrease the level of colony formation.

LIF did not directly alter the proportion of the population in DNA synthesis in cell populations derived from normal femoral marrow, 15 day foetal liver or γ- irradiated femoral marrow.

As an indirect acting agent LIF failed to block the synthesis of a stem cell stimulator, or it's action, on a population of high proliferative potential colony forming cells derived from normal femoral marrow, cloned in the presence of Wehicm+L929cm. (HPP-CFC (Wehicm + L929cm))

LIF's actions on clones of a murine myeloid leukaemia (SA2JMB1) were also assessed. LIF had no statistically significant effect on colony formation or the level of DNA synthesis in populations of SA2JMB1 leukaemic cells.

A second group of associated activities was produced by the X-irradiation induced murine myeloid leukaemia (SA2JMB1). Medium conditioned by the leukaemic cells was assayed *in vitro* both for direct and indirect effects on the proliferation of haematopoietic cells derived from femoral marrow.

As a direct acting agent in 7 and 14-day semi-solid agar culture of femoral marrow, leukaemic conditioned medium alone stimulated limited colony formation. In 7 and 14 day cultures stimulated with single and combinations of specific colony stimulating factors: (rmGM-CSF, rhM-CSF, rhIL-1α) a significant increase in colony number was noted in all cases when cultures were supplemented with leukaemic conditioned medium.

SA2JMB1cm was shown to support the proliferation of an IL-3 dependent cell line (FDCP-A4 cells). The colony enhancing ability of SA2JMB1cm was shown to be blocked by pre-treatment with antibodies to IL-3. This suggested that SA2JMB1 conditioned medium contained IL-3 or an IL-3 like activity, as one of its components.

The conditioned medium failed to directly alter the level of DNA synthesis in a population of HPP-CFC (Wehicm+L929cm) derived from normal bone marrow or γ- irradiated bone marrow.

As an indirect acting agent the conditioned medium did block the action of a stem cell proliferation stimulator on normal bone marrow derived HPP-CFC (Wehicm+L929cm).

This leukaemia associated activity was shown to be larger than 50KD, sensitive to heat treatment and able to act in a different manner to the stem cell inhibitor MIP-1-α.

Thus this novel activity may be important in blocking stimulator action in haematopoietic stem cells and thus contribute to the haematopoietic insufficiency seen in leukaemia.

CHAPTER 1: Introduction

1.1 Haematopoiesis

1.1.1 General

The process of haematopoiesis, or blood cell formation, has to be continuous throughout the life-span of an individual-most blood cells have a limited life-span, from hours (neutrophilic granulocytes) to upwards of one hundred days (erythrocytes), thus they need to be constantly replenished. The system thus must be able to produce an output, but at the same time, maintain the cellular integrity necessary to generate that output. It must be specific yet adaptable, that is: it must be able to produce a given quantity of each of the eight types of blood cell but at the same time be able to selectively alter the level of production with demand. (Moore, 1991; Metcalf, 1992; Ogawa, 1993; Ogawa, 1994). When we consider that the average daily turnover of blood cells in a 70kg man is thought to be approximately 1 trillion (Ogawa, 1993), the need for precise regulation within this system becomes obvious.

The first part of this thesis will take a brief overview of haematopoiesis looking at the different categories of haematopoietic cells. It will examine the assays used to study them and the manner in which early haematopoietic cells are regulated, with particular reference to inhibitory activities. The second part focuses on factors associated with leukaemic cell populations, examining their actions on various aspects of *in*

vitro haematopoiesis. The first of these is the haematopoietic growth factor: Leukaemia inhibitory factor. The second, an unknown activity(ies) produced by a cloned leukaemic cell line.

1.1.2 The haematopoietic tissues

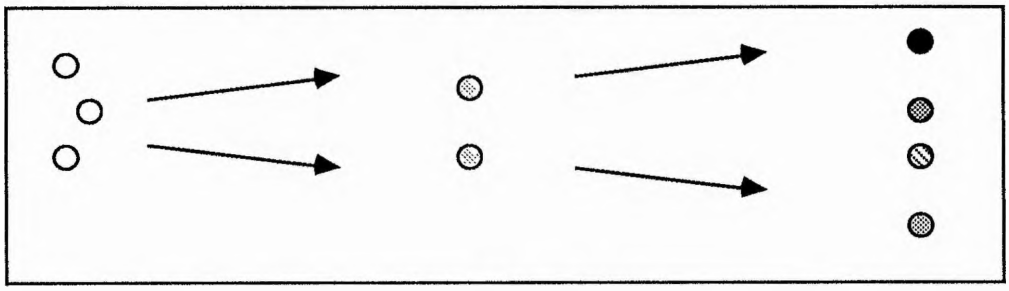
These tissues are essentially haematopoietic factories in which blood cells are formed, undergo differentiation and maturation before migrating into the circulation, and, in some cases, on into other tissues (Ogawa, 1994). The tissue is made up of cells at progressive levels of differentiation, the organisation of which is not completely random, (Schofield *et al* 1979; Spangrude *et al* 1991; Trentin, 1970), but equally is not neatly arranged into anatomical compartments in the way of dynamic systems such as the skin or intestinal wall. It has thus been with the development of techniques such as the purification of growth factors (Review Nicola, 1989; Arai *et al* ,1990; St Georgiev and Albright, 1993), *in vitro* clonogenic assays (Review Testa and Molineux, 1993), recognition of specific cell surface markers (Review Moore, 1991) and cell sorting (Review Lord and Marsh, 1993; Visser and Bekkum, 1990) that a picture of haematopoietic tissue as three sequential yet continuous cell compartments has emerged (Tubiana *et al*, 1993).

The first compartment ,the most primitive, consists of pluripotent quiescent **stem cells** (Lajtha *et al*, 1969). In normal steady state haematopoiesis the size of the stem cell pool is maintained by a balance between self-renewal and differentiation. The next compartment consists of so called

committed progenitor cells. These are restricted to development along one or more lineages and respond to a range of regulators known as cytokines. The stem cell compartment is essentially a continuum of cells whose maturation is characterised by decreasing pluripotency and an increased likelihood of proliferation. These feed into the progenitor cell compartment where proliferation and amplification occurs. Derived from these is the third compartment, the **end cell** compartment, made up of cells which have differentiated along a given lineage and have lost their capacity for proliferation. (Lajtha, 1979; Dexter, 1987; Brown *et al*, 1985) .(Diagram 1)

NORMAL BLOOD CELL FORMATION (Haematopoiesis)

TAKES PLACE WITHIN BONE MARROW



STEM CELLS
-The majority are quiescent bathed in a stem cell inhibitor.

PROGENITOR CELLS
-Transitional cell type.

FUNCTIONAL END CELLS
-Red and white blood cells

This is the case in normal healthy individuals

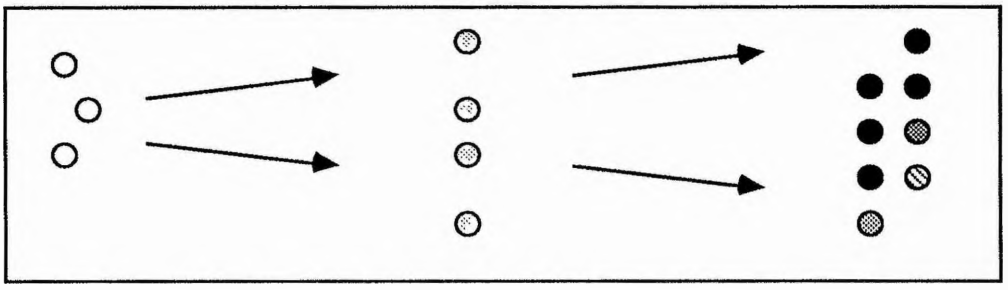
DIAGRAM 1 SCHEMATIC APPRECIATION OF NORMAL BLOOD CELL FORMATION

The nature of the progression of a daughter stem cell from a point of maximal self-renewal and pluripotency towards commitment to a particular cell lineage may occur randomly (stochastic) (Ogawa , 1983), may be rigidly determined (Nicola and Johnson, 1982) or might be influenced by growth factor and micro-environmental cues (Trentin, 1970).

This is an idealised but helpful picture of the balance that exists in normal haematopoiesis. Should the system be perturbed the level of proliferation within compartments one and two can alter by a series of subtle cellular and humoral regulatory cues to accommodate increased needs. (Moore, 1991; Ogawa, 1993; Ogawa, 1994; Ali *et al*, 1989; Dawood *et al*, 1990; Robinson and Riches, 1991) (Diagram 2)

HAEMATOPOIESIS AFTER STRESS TO THE SYSTEM

TAKES PLACE WITHIN BONE MARROW



STEM CELLS	PROGENITOR CELLS	FUNCTIONAL END CELLS
-More of these come into cycle switched on by a stem cell stimulator .	-Get greater numbers of this transitional cell type.	-The result is greater numbers of these end cells thus replenishing the system.

DIAGRAM 2 BLOOD CELL FORMATION IN INDIVIDUALS SUFFERING LONG-TERM ILLNESS OR RECOVERING FROM HAEMATOPOIETIC STRESS (mild irradiation or chemotoxicity.)

1.1.3 Studying Haematopoiesis-Assaying Stem cells and progenitor cells

Stem cells are few in number and difficult to distinguish morphologically. The ultimate definition of a stem cell is *"that cell in a tissue which under normal circumstances, maintains its own population, undiminished in function and size, and furnishes daughters to provide new functionaL-cells of that tissue* ." (Schofield, 1983) These can only be truly identified by their ability to reconstitute all blood cells (both myeloid and lymphoid) in irradiated animals .

1.1.3.1 The spleen colony assay-(CFU-S)

In 1961 Till and McCulloch (in work fundamental to the study of haematopoiesis) demonstrated that mouse haematopoietic tissues contained cells (colony forming unit-spleen (CFU-S)) at a frequency of about 1 in 10^4 cells which when injected into a lethally irradiated syngeneic recipient could give rise to splenic colonies of haematopoietic cells. Each colony was composed of multiple end-cell lineages as well as cells capable of generating more spleen colonies when injected into new recipients. Thus CFU-S not only showed multi-potency and an ability for extensive proliferation but also an ability to self-renew. (Siminovitch *et al* 1963; Becker *et al*, 1963; Vogel *et al*, 1968; Schofield *et al*, 1980). With the observation that some colonies were transient it became clear that the CFU-S was a heterogenous age-structured cell compartment. The so called early CFU-S (day 7-10) (CFU-SII) were apparently more mature progenitor cells and gave rise to colonies containing cells of mainly one lineage with little or no ability to self-renew. Late CFU-S(day 12) (CFU-S I) appeared to be more primitive, giving rise to colonies containing cells of more than the one lineage along with cells which showed a self-renewal ability in secondary assays. (Worten *et al*, 1969; Schofield and Lajtha, 1973; Morley and Blake, 1974; Morley *et al*, 1975; Magli *et al*, 1982; Johnston and Nicola, 1984). The nature of the ageing associated with the CFU-S heterogeneity was explained in the Niche hypothesis (Schofield, 1978; Schofield, 1983). This hypothesis not only addressed many of the anomalies associated with CFU-S ageing but also importantly proposed

that they were not totipotent haematopoietic stem cells. In essence it states that the CFU-S can be thought of as part of a stem cell pool, internally heterogeneous, hierarchically arranged according to self-renewal ability. At the head of this pool is a stem cell whose defining characteristic is it's totipotency and its ability for maximum self-renewal.

This notion is confirmed by various lines of evidence including the lack of lymphoid cells found in CFU-S colonies (Paige *et al*, 1979), the emergence of a pre-CFU-S (Hodgson and Bradley, 1979) and the ability of cells not forming spleen colonies to facilitate long term marrow re-population. (Ploechmacher *et al*, 1989; Bertoncello *et al* 1992). The Pre-CFU-S population is thought to be that population from which CFU-S are derived. They are also thought to be the cells responsible for recovery of the haematopoietic system following X-irradiation, this property being referred to as a radio protective ability (RPA). In fractionation studies of haematopoietic tissue, a cell population consisting of populations of day 12 CFU-S and pre-CFU-S could be separated from other cells by virtue of their wheat germ agglutinin + fluorscien isothiocyanate binding. The injection of 80 of these enriched cells, per animal, to a group of lethally irradiated mice resulted in 50% survival. 10^4 whole bone marrow cells would be required in injection to attain the same result (Ploechmacher and Brons, 1989). RH123 is a vital dye which was initially thought to stain mitochondria preferentially and therefore be a marker for metabollicaly active cells. However it has been shown that low levels of RH123 staining of human haematopoietic progenitor cells may

be the result of the expression of P-glycoprotein which confers the multidrug resistance phenotype. Although the mechanism of low level RH123 staining in these cells is not known it is apparent that RH123 permits separation of the primitive cell population into two subsets: RH123high and RH123low (Fleming, 1993). Fractionation of this cell population along these lines yields two fractions: RH123low with a high preCFU-S/low CFU-S 12 content and RH123high with a higher CFU-S 12/low pre-CFU-S content. It is the RH123low fraction which is responsible for the recovery seen in these animals. Other separation techniques provide similar evidence for the existence of a pre-CFU-S population. (Bertoncello *et al* 1992; Wright and Pragnell, 1992)

The work on CFU-S should not be dismissed however, it was fundamental in establishing a picture of a stem cell pool, a possible hierarchy and ultimately still represents a valuable *in vivo* tool for examining early haematopoietic cells.

1.1.3.2 *In vitro* Colony Assays

In vitro colony assays of progenitor cells (colony forming cells) able to proliferate, differentiate and mature in semi-solid agar culture were first reported by Pluznik and Sachs (1965) and Bradley and Metcalf (1966). Initially only cells of the granulocyte and macrophage lineage were seen. The cells giving rise to these were termed granulocyte-macrophage colony forming cells (GM-CFC) In later work colonies with granulocutes, erythrocytes, eosinophils, macrophages and

megakaryocytes (GEMM-CFC/MIX-CFC) were demonstrated. (Johnston and Metcalf, 1977; Fuasser and Messner, 1978; Metcalf, 1988). Stimulation of colony formation in these assays was produced by media conditioned by the growth of another tissue. As the factors responsible for this stimulation were identified an explosion in *in vitro* colony assays took place.

Colony stimulating factors-a class of haematopoietic growth factor

Haematopoietic growth factors are a group of acidic glycoproteins (collectively known as cytokines) which play a crucial role in the survival and development of haematopoietic cells through interaction with specific receptors on target cells. (Kaushansky and Karplus, 1993). Originally known as the colony stimulating factors, to date at least 20 factors (each encoded by it's own unique gene) with proliferative effects on haematopoietic cell populations have been produced in recombinant form. (Moore, 1991; Metcalf, 1992; Metcalf, 1993; Adamson, 1993). Although they show little amino acid sequence homology structurally they have a similar molecular weight of 13-21KD (this can vary however due to high glycosylation variability) adopting a four helix bundle tertiary fold structure. Most exist as monomers (IL-1α and β, IL-3 ,LIF) although there are dimers (IL-8) and trimers TNF α and β. Several cytokines exist both as soluble and membrane bound forms which may reflect the paracrine and autocrine nature of their actions. They are also often highly conserved between

species (Nicola, 1989; Bazan, 1990). They interact with specific high affinity receptors which may exist as more than the one sub-unit and exhibit homology in their extracellular domains. (Hirano *et al*, 1994; Gearing , 1993) Frequently there appears to be co-evolution of cytokines and receptors in more than the one system. (Bazan, 1991; Yamamori and Sarai, 1992).

Curiously cytokines can show different activities (they are plieotropic) on cells of different lineages or on cells of the same lineage at different points in maturation, with expression of relevant receptors on both cell types (Nicola, 1989). This is compounded by the fact that many of the cytokines appear to have overlapping functions, a phenomenon known as redundancy. (Metcalf, 1992; Metcalf, 1993; Chiarugi *et al*, 1993). In essence the principle of redundancy refers to the fact that there appear to be more regulators with similar or overlapping functions than would seem necessary to achieve cell proliferation (Metcalf, 1993). In support of this observation are several strands of evidence including: common proliferative actions; common biological actions; shared receptor sub-units and co-expression of receptors on the same cell type. (Metcalf 1992, Metcalf 1993). Careful interpretation of this evidence, however, suggests that the relationship between these factors may be more complicated than it first appears. An insight into the potentially subtle relationship that exists between factors comes from studies using gene knockout mice. In these mice genes for haematopoietic regulators known to share functions with other cytokines can be deleted, and the effects of this deletion studied. Results show that such deletions result in

mice unable to develop normally. (Escary *et al*, 1993; Metcalf, 1993; Dumenil *et al*, 1993). This suggests that although, *in vitro*, many cytokines may appear identical in terms of function, in the finely balanced *in vivo* environment they may have a role which is far more subtle than previously noted. This principle has been termed growth factor " subtlety". The principle of subtlety is further substantiated by the observation that growth factors "synergise". That is: growth factors able to stimulate a proliferative response in a cell population are able to stimulate a more than additive response when used together. The fact that such a response is more than merely the sum of the parts strongly suggests that there is some subtle interplay between growth factors. In later discussions on LIF the phenomena of redundancy and subtlety are considered more fully.

For the purposes of this introduction it is sufficient to appreciate that the use of growth factors in assays has made comprehensive *in vitro* studies of progenitor and more primitive haematopoietic cells possible.

Several reviews of haematopoietic growth factors are available (Nicola, 1989; Arai *et al*, 1990; Devalia and Linch, 1991).

Colony Forming Cells with differing proliferative potentials.

Low proliferative potential colony forming cells (LPP-CFC)

Several of the cytokines which have been molecularly cloned and purified to homogeneity (e.g GM-CSF, G-CSF, M-CSF (CSF-1), IL-3 (MULTI-CSF), EPO) have direct stimulatory effects on colony forming progenitor cells with a limited lineage developmental potential and a **low proliferative potential.** These are low proliferative potential colony forming cells (LPP-CFC) e.g GM-CFC/MIX-CFC. (Metcalf, 1989)
However, more cytokines have emerged, in particular several of the interleukins (Heyworth *et al*, 1990; Moore, 1991) which acting alone have only a very limited effect on progenitor cells but in association with other factors exert a synergistic stimulatory effect on the proliferation of multi-potent progenitor cells.

High proliferative potential colony forming cells (HPP-CFC)

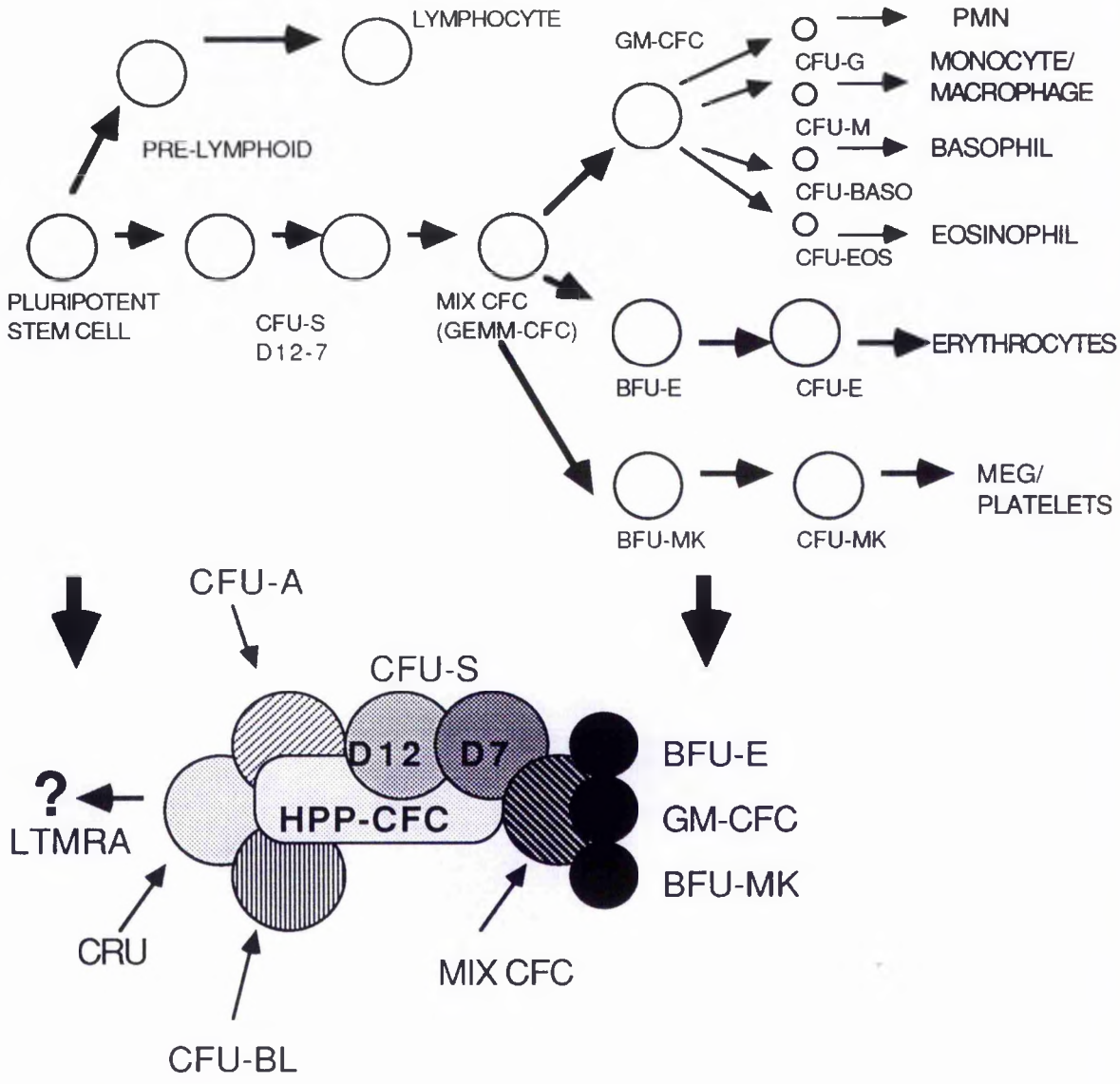
The **High proliferative potential** colony forming cell (HPP-CFC) was originally reported in cultures of murine bone marrow by Bradley and Hodgson (1979). They demonstrated a relatively primitive, quiescent population with a resistance to 5

fluorouracil (5FU) (a cytotoxic agent which targets more mature cycling progenitors) higher than that seen in LPP-CFC. These cells required more than the one growth factor in order to proliferate, and gave rise to colonies made up of more than 50,000 cells per colony.

The HPP-CFC surviving 5 fluorouracil (5FU) treatment were rare with a frequency of 1 in 400 NBM cells. They showed a pattern of regeneration similar to pre-CFU-S, were present in low density RH123 dull fractions of normal bone marrow and could be detected in clonal agar culture only in the presence of IL-1α + IL-3 + M-CSF. HPP-CFC shared many phenotypic characteristics with early haematopoietic cells. These HPP-CFC could give rise to CFU-S day13, cells with short term Marrow repopulating ability, erythroid and megakaryocyte progenitors as well a second class of HPP-CFC which require only IL-3 + M-CSF to proliferate in culture. (Bradley *et al*, 1980; Bradley *et al*, 1985; Bertoncello *et al*, 1985; Mcniece *et al*, 1987; Bartelmez *et al*, 1989; Bertoncello, 1992).

The second class of HPP-CFC is postulated to be a more mature cycling population on the basis of it's sensitivity to 5FU treatment (99% depletion) (Bradley *et al*, 1985). They are found in the RH123 bright fraction of low density bone marrow (Hodgson *et al*, 1989; Bertoncello *et al*, 1991). In replating experiments they generated only LPP-CFC cells, which, as outlined in 1.3.2.3, respond to lineage specific single factors (Mcniece *et al*, 1990). This suggested a hierarchical scheme of progressively maturing progenitor populations, from HPP-CFC stimulated with IL-1-α+IL-3+M-CSF to HPP-CFC stimulated

with IL-3 + M-CSF to CFC stimulated with lineage restricted factors (LPP-CFC). These were designated HPP-CFC1, 2 and 3 (LPP-CFC) (Bertoncello, 1992) (Diagrams 3 and 4 show the heterogenous HPP-CFC pool and how it relates to other haematopoietic cell types .Some of the markers used to detect cell populations are also indicated).



KEY:

LTMRA-LONG TERM MARROW REPOPULATING ABILITY
CRU-COMPETITIVE REPOPULATING UNIT
CFU A- COLONY FORMING UNIT
CFU-BL-BLAST COLONY FORMING UNIT
HPP-CFC-HIGH PROLIFERATIVE POTENTIAL COLONY FORMING CELLS
CFU-S-COLONY FORMING UNIT SPLEEN
MIX-CFC-MIX COLONY FORMING CELLS
BFU-E-BURST FORMING UNIT ERYTHROCYTES
GM-CFC-GRANULOCYTE-MACROPHAGE COLONY FORMING CELLS
BFU-MK-BURST FORMING UNIT MEGAKARYOCYTE

DIAGRAM 3 SCHEMATIC SHOWING CELL DEVELOPMENT IN HAEMATOPOIESIS WITH VENN DIAGRAMM SHOWING CLOSE RELATIONSHIP IN EARLY CELL POPULATIONS .

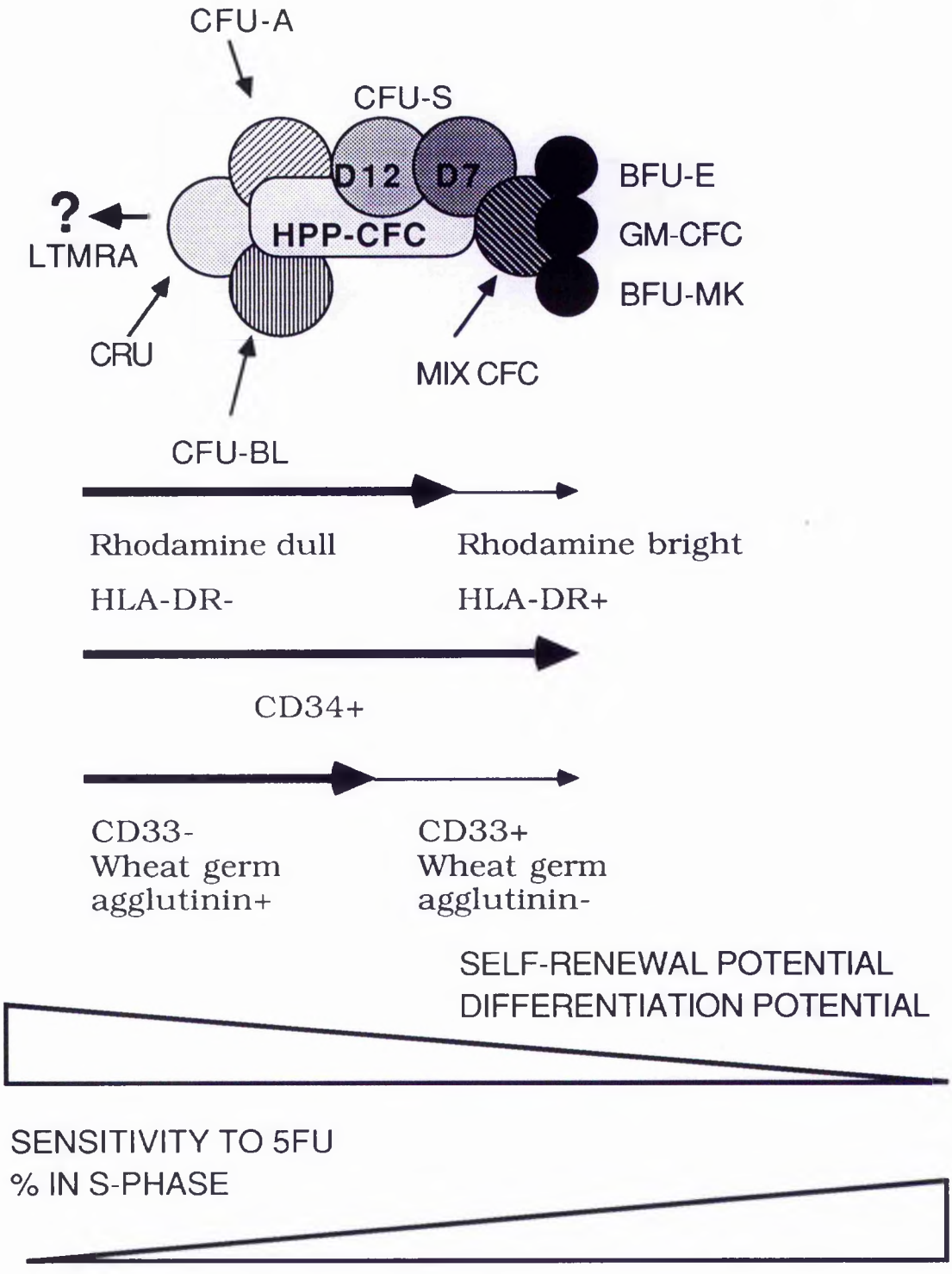


DIAGRAM 4 SCHEMATIC SHOWING CELL DEVELOPMENT IN HAEMATOPOIESIS WITH GRADIENTS HIGHLIGHTING THE ALTERATION IN SELF-RENEWAL AND PROLIFERATIVE STATUS .

In querying the validity of this scheme it is worth noting that Fleming *et al*, (1993) equates heterogeneity within early progenitor cell populations with cell cycle status. Using propidium iodide staining, Hoeschst dye and tritiated thymidine labelling they determined the fraction of phenotypically defined murine stem cells (Thy 1.1 lo, LIN-/lo, SCA-1+) which were in DNA synthesis. The fraction was estimated to be 18-22%. Further they used RH123 to fractionate this stem cell population. RH123 permits separation of the primitive cell population into two subsets: RH123high and RH123low. Of these cells only 2.9% of RH123 lo are in S/G2/M where 30% RH123high are in S/G2/M. These cell fractions were separated, injected into lethally irradiated recipients and survival monitored over thirty days. The injection of 100 G0/G1 cells (RH123low) rescued 90% of lethally irradiated mice in contrast to 100S/G2/M cells(RH123high) which rescued only 25% of lethally irradiated recipients. Long term donor derived multi-lineage reconstitution of the peripheral blood was observed in recipients of RH123low but not RH123high. The data not only confirm heterogeneity within a stem cell pool but also confirm that with active proliferation comes a reduced stem cell activity. This data is in agreement with the 5FU and other findings for HPP-CFC.

A variety of other growth factor combinations which stimulated HPP-CFC and were ablated by 5FU treatment emerged (Falk and Vogel, 1988; Bertoncello, 1992). A study by Kriegler *et al* (1994) set out to define the inter-relationship of these and various other HPP-CFC populations. Their conclusions

are complex but in brief they state that the HPP-CFC compartment is extremely heterogenous, both in terms of consisting of five major HPP populations, but also within those populations themselves. They envisage a complicated cell compartment in which gradual changes in growth factor requirements occur as HPP-CFC mature. At the head of the pool is a primitive HPP-CFC (more primitive than HPP-CFC1) stimulated by at least two combinations of four factors. (Other work by Lowry *et al* (1991) suggested possibly up to six factor combinations but Kriegler could not repeat this work) Bertoncello (1992) quotes two other examples of work in which four factors stimulate more RH123 dulL-cells than three factor combinations but only speculates as to their place in the HPP-CFC hierarchy.

According to the model proposed by Kriegler *et al* (1994), as the compartment progresses we find HPP-CFC1 a heterogenous sub-pool stimulated by IL-1α+Il-3+M-CSF. However they also suggest several other three factor combinations able to stimulate HPP-CFC1, but which do **not** produce the same levels of self renewal as seen with the IL-1α + IL-3 + M-CSF combination. This leads them to conclude that these combinations stimulate more mature HPP-CFC1. Two two factor combinations are also proposed to stimulate these later HPP-CFC1.

Next is the similarly heterogenous HPP-CFC 2, in which earlier cells are stimulated by IL-3 + M-CSF, and later by other two factor combinations which may overlap with the single factor combinations stimulating LPP-CFC or HPP-CFC 3. To an extent

this work is supported by the findings of Yoder *et al* (1993) who confirm an increased growth factor requirement in earlier HPP-CFC. Using counterflow centrifugal elutriation they report that HPP-CFC are heterogenously elutriated on the basis of differences in size or density. They separate HPP-CFC into six subsets differing in growth factor responsiveness. HPP-CFC elutriated at the lower flow rates (lower density) differ in their growth factor requirement for cloning as compared to HPP-CFC elutriated at higher flow rates (higher density). Lower density fractions require more growth factors. The lower density fraction contained no CFU-S day 12 where the higher density fraction did. This further suggests that the low densityfraction consists of cells more primitive than those in the high density fraction. These could possibly be developmentally linked to the pre-CFU-S which were found in a similar fraction. This confirms the notion of a highly heterogenous stem cell compartment. Interestingly Han (1994) reports a new HPP-CFC sub-population able to form colonies containing HPP-CFC 2, GEMM-CFC and megakaryocytes. They term it HPP-mCFU-MK and postulate that it may in fact be HPP-CFC1 expressing its potential to develop magakaryocytes in the presence of correct levels of the relevant factor. This contributes to the notion of heterogeneity existing within the overall heterogeneity of the cell pool.

It is clear that the HPP-CFC compartment is heterogenous and that sub-populations can be identified by analyzing the kinetics of cell regeneration and the specific cellular requirements for cytokines. (Diagram 5 summarises this complex compartment).

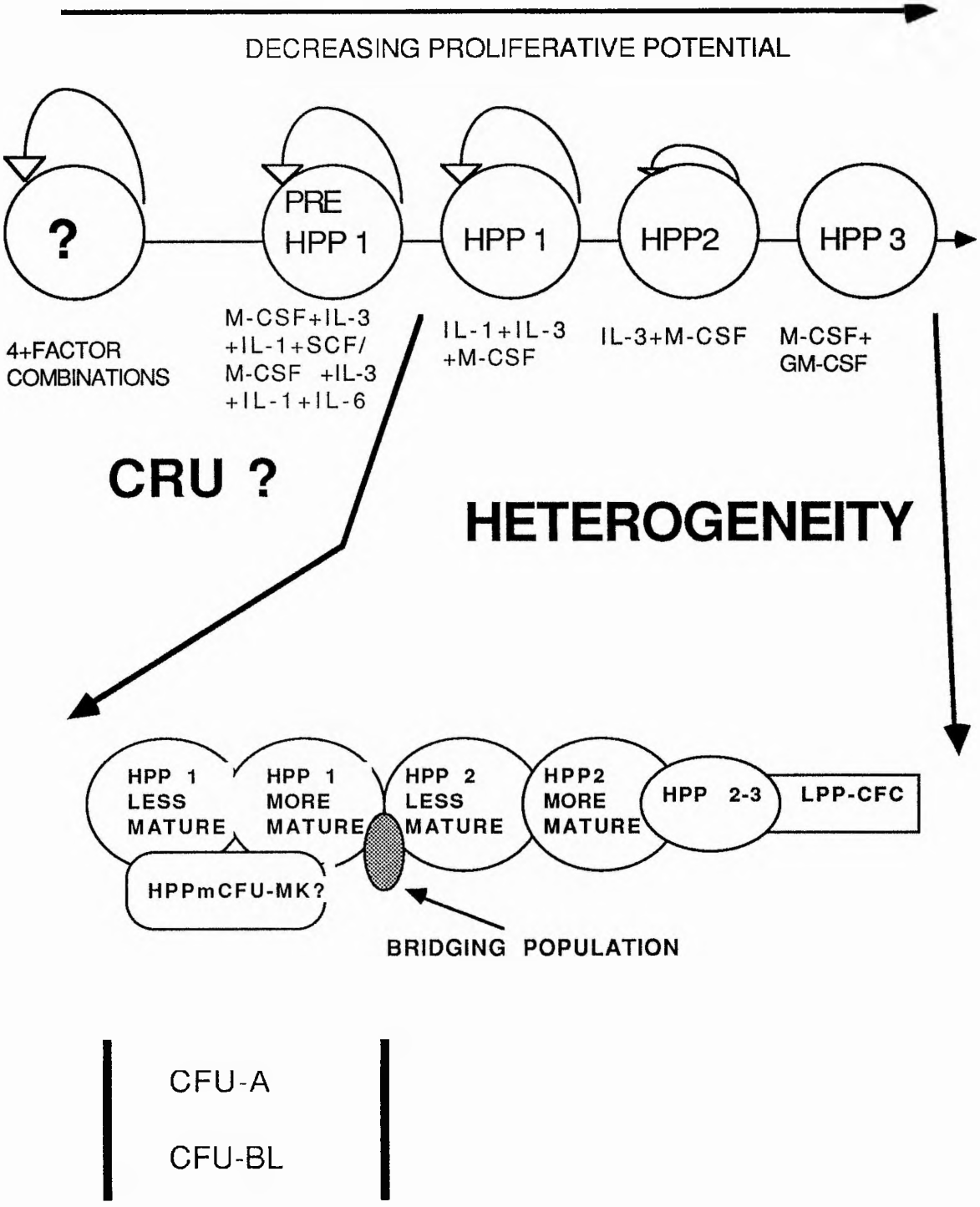


DIAGRAM 5 SCHEMATIC SHOWING THE EXCESSIVE HETEROGENEITY WITHIN THE HPP-CFC POOL .

From the above it would seem that there was evidence relating CFU-S to HPP-CFC, however the exact relationship can at present remain only largely anecdotal. Interestingly Bertoncello (1992) notes that HPP-CFC1 do not form an accurate index of Long term marrow repopulating ability in haematopoietic tissue suggesting that they may not be pre-CFU-S noted to have this ability. It may be that one of the more primitive combinations Kriegler *et al* (1994) report will emerge with this ability.

Importantly these studies confirm that a very primitive heterogenous cell population with a reported human counterpart (Gordon, 1993) can be studied *in vitro*.

Other early progenitor cells *in vitro*

HPP-CFC do not represent the only *in vitro* assay for early haematopoietic progenitor cells. Nakahata and Ogawa identified a population present in both human and mouse, producing undifferentiated blast cell colonies at 16 days in culture. (Nakatha and Ogawa, 1982) A human counterpart is also reported (Leary *et al*, 1988). Replating of these cells revealed colonies containing secondary blasts and GEMM-CFC. This suggests that CFU-BL are more primitive than the late GEMM-CFC. There is considerable evidence including the above observation (Suda *et al* 1983; Ikebuchi *et al*, 1988; Wong *et al*, 1988; Bertoncello, 1992) that CFU-BL may well be closely related to HPP-CFC1.

Similarly Pragnell *et al* (1988) describe an early progenitor named the CFU-A. Stimulated to colony formation with 10% L929cm (as a source of M-CSF) and 10% AF1-19Tcm (a source of GM-CSF) and incubated at 37°C for 11 days these cells bear many similarities to HPP-CFC-1. Not least of these is the difficulty in separating one cell type from the other in enriched bone marrow populations. (Graham *et al*, 1990; Kriegler *et al* 1994; Bertoncello, 1992).

Other early populations reported include the CRU(competitive repopulation unit) and the CAFC(Cobblestone area forming cell) (Review Wright and Pragnell, 1992)

As yet the cell populations discussed above have not been integrated into one model and although evidence infers that they may be closely related, an accurate picture of how one may relate to another would require more data.

Summary

In vitro progenitor cell assays have established several potentially overlapping cell populations which are comparable in organisation and developmental potential to the CFU-S assayed *in vivo*. These are potentially the members of the stem cell pool responsible for haematopoietic homeostasis and as such these assays provide an excellent controlled medium in which to examine the effects of exogenous factors on early haematopoietic events.

1.2 PROLIFERATION REGULATION

1.2.4 Proliferation regulation within the stem cell pool

Haematopoiesis can be viewed as a complex multi-stage process with haematopoietic tissue broadly divided into several compartments of progressively more mature cell types. (Moore, 1991; Wright and Lord, 1992) The manner in which normal haematopoiesis is regulated has recently been elucidated and it has become clear that it is controlled by a balance between positive and negative regulators. (Lord, 1986; Lord 1988; Axelraad, 1990; Han and Caen, 1994)

Haematopoietic stem cells (as represented by CFU-S) have long been considered to be quiescent with regard to proliferation with 85-95% of the population in the G0 phase of the cell cycle. (Lajtha *et al* 1969; Becker *et al* 1965). CFU-S derived from normal non-stressed foetal liver tissue show a high proportion in S-phase (30-40%) (Riches *et al*, 1981; Dawood *et al*, 1989). Following depletion of CFU-S from normal marrow by physiological insult the proportion of CFU-S in S-phase is increased to 30-50%. (Lahiri and Van Putten, 1972; Byron, 1975).

Local variations in the proliferative status of CFU-S suggested that this alteration in proliferative status was under the control of locally produced factors. (Gidali and Lajtha, 1972; Rencricca *et al* 1970; Wright and Lord, 1977; Ali *et al*, 1989) which act specifically on CFU-S as opposed to later progenitors (Tarejo *et*

al, 1984) and were non-species specific in their action (Wright *et al*, 1980; Cork *et al*, 1986).

A stem cell inhibitor associated with normal bone marrow tissue (Lord *et al*, 1977; Wright *et al*, 1980; Cork *et al*, 1981) and a stem cell ` associated with bone marrow recovering from physiological stress or foetal liver tissue (Lord *et al*, 1977; Croizat and Frindell, 1980; Dawood *et al*, 1990) were demonstrated. These activities were shown to be different in terms of molecular weight with the stimulator in a 30-50KD banding, the inhibitor in a 50-100KD banding and no opposing activity in either banding.(Lord *et al*, 1977; Graham *et al*, 1990). They were shown to be produced by separate populations of cells within the monocyte-macrophage lineage (Wright *et al* 1980; Wright *et al* 1982; Simmons and Lord, 1985; Wright and Lorrimore, 1987; Ali *et al* , 1989; Robinson and Riches, 1991) whose distribution within the marrow was different and which could be separated by virtue of their different densities (Wright and Lord, 1979; Robinson *et al* 1991) Regardless of the proliferative status of the tissue both producer cell populations were present. (Wright and Lord, 1979; Lord, 1986). A variety of macrophage cell lines were screened and an activity derived from medium conditioned by J774.2 macrophages shown to act in the same way as the stem cell inhibitor. The activity was subsequently shown to be a protein species that migrated as a doublet on SDS PAGE gel electrophoresis. The doublet was found to be two previously described cytokines macrophage inflammatory protein -1-β (MIP-1-β) and macrophage inflammatory protein-1-α (MIP-1-

α). By using antibodies directed against MIP-1-α the inhibitory activity was subsequently shown to reside with this part of the doublet. (Graham *et al*, 1990; Wright and Pragnell, 1992) One of a family of at least ten basic heparin binding polypeptides sometimes referred to as chemokines, it is a peptide of 694 amino acids with a molecular weight of 8-10Kd which was originally identified by its pro-inflammatory capacity after injection into the footpads of C3H/HeJ mice. (Moore, 1991; Broxmeyer *et al*, 1993; Lord *et al*, 1993) It has an identified human counterpart " LD78". (Lord *et al*, 1993)

1.2.4.1 Macrophage inflammatory protein-1-α

With the finding that MIP-1-α corresponded to the normal bone marrow associated stem cell inhibitor came much interest in its actions within haematopoiesis. The picture which has emerged demonstrates a molecule which is part of a family of chemokines with a confusing array of functions. (Broxmeyer *et al*, 1993)

Broxmeyer *et al* (1989) showed that MIP-1 (containing both 1-α and 1-β) was synergistic with certain colony stimulating factors (GM-CSF and M-CSF but not G-CSF) for the promotion of enhanced GM-CFC colony formation *in vitro*. Whilst MIP-1 was shown to have no significant effect on erythroid progenitor cell colony formation, MIP-1-α alone was shown to inhibit colony formation by the erythroid burst forming unit. Interestingly this inhibition could be removed by the addition of MIP-1-β, implying that MIP-1-β is antagonistic to the effects of MIP-1-α.

Work on CFU-A, an *in vitro* colony assay similar to the HPP-CFC assay and thought to be representative of the *in vivo* CFU-S cell population (Dunlop *et al*, 1992; Maltman *et al*, 1993), showed that MIP-1-α could inhibit the formation of CFU-A colonies whilst MIP-1-β, at least at the concentrations tested, was ineffective in this respect. (Pragnell *et al,* 1988; Lorrimore *et al* 1990; Wright and Pragnell 1992; Lord *et al,* 1993; Broxmeyer *et al,* 1993). This finding is further complicated by the fact that there is evidence to suggest that both molecules act through the same receptor .(Graham and Pragnell 1992). Graham *et al* (1990) have shown that MIP-1-α was effective in reversibly inhibiting the cycling of CFU-A with regenerating marrow as a source of these stem cells. Both *in vitro* liquid suspension culture of factor stimulated immature stem cell populations supplemented with MIP-1-α (Bodine *et al*, 1991) and *in vivo* administration of MIP-1-α to mice (Dunlop *et al*, 1992; Lord *et al*, 1992) demonstrated MIP-1-α's ability to suppress proliferation within, and, consequently be haemoprotective for, early stem cell populations (Broxmeyer *et al*, 1993). Mayani *et al* (1995) confirm this finding, testing MIP-1-α on populations of highly purified cord blood cells. MIP-1-α is also known to show an inhibitory activity for non haematopoietic cells. It is active in inhibiting the proliferation of epidermal keratinocytes and may be produced locally within the epidermis from epidermal Langerhans cells (Graham and Pragnell, 1992; Graham *et al*, 1994).

As will be subsequently discussed other haemoregulatory inhibitory activities exist one of which is Transforming growth

factor beta (TGF-β). This inhibitor appears to have similar functions to MIP-1-α. (Maltman *et al*, 1993), and it is noted that both inhibitors are able to decrease proliferation within CFU-A populations to the same extent. Interestingly they find that TGF-β can down-regulate expression of the MIP-1-α gene in bone marrow derived macrophages. The expression of MIP-1-β is inhibited in a similar manner. In the murine macrophage cell line RAW264.7, 24hrs after treatment with TGF-β western blotting showed that there was a reduction in the level of MIP-1-α protein production. On FDCP-mix cells TGF-β can down regulate MIP-1-α receptor expression. Although based on *in vitro* findings the extent of this down regulation is such that they question the significance of MIP-1-α in the presence of TGF-β *in vivo*. Several points are worth considering here. Firstly the work of Mayani *et al* (1995) suggests that the mode of action of MIP-1-α and TGF-β is perhaps more subtle than this finding suggests with each molecule acting in a slightly different way on different but overlapping primitive cell populations. Secondly problems such as the aggregation of MIP-1-α which pose a significant problem *in vitro*, leading to loss of inhibitory activity, are far less problematic *in vivo*. (Lord *et al*, 1993; Graham *et al*, 1994). Indeed the fact that the molecular weight of the originally described NBM associated inhibitor was 50-100KD is probably attributable to the aggregation of MIP-1-α and may be necessary for localising *in vivo* activity or ligand-receptor interaction. ((Graham and Pragnell, 1992; Graham *et al*, 1994). The precise relationship between these inhibitors remains to be elucidated. A report by

Verfaillie *et al* (1994) discusses the effect of MIP-1-α addition to a unique LTBMc set-up. They conclude firstly that the addition of MIP-1-α alone to cultures has no significant effect on the number of primitive cells in culture after eight weeks. They suggest that this may indicate that MIP-1-α does not have a direct action and that for inhibitory action MIP-1-α requires interaction with other cytokines. Interestingly when they co-culture IL-3 and MIP-1-α they find that cultures produce more primitive progenitors capable of initiating long-term cultures than with either molecule alone. This is in contrast to findings for IL3+TGF-β where cultures showed reduced numbers of long term culture initiating cells. They hypothesize that the IL-3+MIP-1-α result may be attributable to an increased proliferation without differentiation. MIP-1-α may act as a survival agent here rather than an inhibitory signal. They demonstrate that the action of MIP-1-α and IL-3 requires the release of undefined diffusible factors from stroma but that the two molecules interact directly with primitive progenitor cells and not the stroma. A system such as this whose dynamics are not fully defined is a vivid example of the complex interplay that exists between cytokines "*in vivo*". The strength of the TGF-β data above is surely open to debate.

In summary members of the MIP family show a range of haematopoietic effects with MIP-1-α showing direct suppressing activities and a possible role as a survival agent for immature progenitors.

1.2.4.2 The interaction of stimulator and inhibitor

The nature of the interaction between stimulator and inhibitor providing the balance of haematopoiesis is thought to be governed by a number of parameters. Firstly it has been demonstrated that the two factors do not interact with one another in a simple on-off switching mechanism but that they interact with the other's producer cell to regulate the level of production. (Lord and Wright, 1982; Riches and Cork, 1987). Further, there is a differential sensitivity to these factors within the stem cell pool. The earlier (more primitive) a cell the greater it's sensitivity to inhibitor the later the greater it's sensitivity to stimulator. (Wright *et al*, 1985). This could be a consequence of stem cell pool heterogeneity or indeed it's cause. Fleming *et al* (1993) note that stem cell heterogeneity is well correlated with cell cycle status suggesting that more quiescent cells exhibit greater stem cellness, as judged by their marrow reconstituting ability. It may be that a greater sensitivity to stimulator and a consequential movement into cycle represents stem cell ageing.

The balance between stimulator or inhibitor production has also been proposed to be a consequence of the size of the CFU-S pool. It is postulated that CFU-S themselves produce a feedback signal to producer cells which effectively prevents the production of stimulator. The isolation from stem cell conditioned medium of a 30-50KD fraction termed 'stem cell feedback factor' able to actively suppress stimulator production

seemed to be confirmation of this hypothesis. (Lord, 1986) However there are situations where stem cell numbers are high but stimulator is still being produced such as foetal *al* liver (Dawood *et al*, 1989). This forces a reassesment of the system proposed by Lord. It may well be that the production of stem cell regulators is not only related to the size of the CFU-S pool.

1.2.4.3. A variety of negative haemoregulatory activities.

Besides those discussed above other negative and positive haemoregulatory activities exist (review Guigon and Najman , 1988; Axelraad, 1990; Moore, 1991; Wright and Pragnell, 1992; Mayani *et al*, 1995). Recently with the desire for haemoprotection an integral consideration in cancer therapy (Moser and Paukovits, 1991; Tubiana *et al*, 1993; Guigon and Bonnet, 1995) there has been much interest in negative regulators which act in a reversible way to arrest DNA synthesis or to prevent entry into S-phase, in early stem cells. Several inhibitory molecules other than MIP-1-α have emerged (again raising Metcalf's (1993) question of redundancy in growth factors) (Guigon and Bonnet, 1995).

These include the large molecules Transforming growth factor beta (TGF-β) and TNF-α (Ruscetti *et al*, 1991; Moore 1991; Maltman *et al*, 1993) Leukaemia associated inhibitors LAI (Olofsson and Olsson, 1980 a ,b and c.) and Leukaemia associated inhibitory activities LAIA (acidic isoferratin) (Bognacki *et al*, 1981; Broxmeyer *et al*, 1981) and two small

chemically unrelated peptides: the penta peptide PEEDCK (HP5B) and the tetrapeptide AcSDKP (Axelraad, 1990; Lord and Dexter, 1992; Moser and Paukovits, 1991; Wright and Pragnell, 1992; Robinson *et al*, 1993; Tubiana *et al* 1993; Guigon and Bonnet, 1995). The majority of these have an often confusing array of plieotrophic and overlapping functions with some e.g. TNF-α and PEEDCK exhibiting both positive and negative activities. The effects of many of these inhibitors are to be found at the level of progenitor cells however at least one of these the tetrapeptide ACSDKP has been shown to be a proliferation inhibitor for haematopoietic stem cells.

1.2.4.4. The haematopoietic inhibitory peptide ACSDKP

GENERAL BIOLOGY

The tetrapeptide AcSDKP was first isolated as a crude extract from foetal calf bone marrow (Frindel and Guigon, 1977) and foetal calf liver (Guigon *et al*, 1984). It was purified as a 487 dalton peptide which was non-species specific, being active in both human and mouse, and could be chemically synthesized (Termed "Seraspenide"). (Lenfant *et al*, 1989; Guigon *et al*, 1990). AcSDKP is degraded by the enzyme plasma angiotensin-1 converting enzyme (ACE) in a reaction mediated *in vivo* by the N-terminal end of the enzyme. (Rousseau *et al*, 1995). Both native and synthetic peptide inhibit the entry into S-phase of CFU-S following haematopoietic stress. (Frindel and Guigon

1977,; Lenfant *et al 1989*; Guigon *et al*, 1990). In contrast to MIP-1-α the tetrapeptide is thought only to be active at the Go/G1 point in the cell cycle i.e it does not alter the cycling status of cells already in the cell cycle. (Monpezat and Frindell, 1989; Wright and Pragnell, 1992). *In vitro* work by Robinson *et al* (1993) demonstrated the ability of AcSDKP to inhibit the cycling of a murine HPP-CFC population induced by a stem cell stimulator. They attribute the inhibitory activity to the SDK sequence of the molecule. Godden *et al* (1993) showed the same effect on murine CFU-A induced to enter S-phase in response to a stimulator. On enriched populations of human stem cells (CD34+HLA-DRlow) thought to contain the primitive stem cells capable of initiating long term culture, AcSDKP reversibly inhibited proliferation and colony generation. (Bonnet *et al*, 1992) The molecule is also active on some later progenitor populations (Guigon *et al*, 1990). They report that 24hr incubation of human mono-nuclear cells with seraspenide induces growth inhibition in GM-CFC, BFU-E and CFU-E. Unlike MIP-1-α, TNF-α and TGF-β AcSDKP has never exhibited stimulatory activity, and importantly, for a role in cancer therapy, it does not act on leukaemic cells. (Guigon and Bonnet, 1995). The peptide sequence is found in TNF-α and thymosin-β-4 where it represents the N-terminal sequence (Lenfant *et al*, 1989). Speculation that AcSDKP may exist as a latent part of a higher molecular weight molecule cleaved to release the active tetrapeptide was given credence by the finding that thymosin-β-4 could be enzymatically cleaved to release AcSDKP. (Grillon *et al*, 1990). However Pradelles *et al* (1990) note that the

distribution of thymosin-β-4 and AcSDKP in mouse tissue did not support this theory. (Guigon and Bonnet, 1995)

The inhibitory ability of AcSDKP appears not to be limited to the haematopoietic system. Adult rat hepatocytes are normally quiescent being triggered into cycle only in response to partial hepatectomy. Tubiana *et al* (1993) report work by Lombard *et al* (1990) in which AcSDKP significantly reduced hepatocyte proliferation following partial hepatectomy. The fact that AcSDKP prevents the cycling of a non-haematopoietic cell population suggests that it may act by interfering with a general growth regulatory mechanism rather than a process associated with one cell type.

Mechanism of action

The mechanisms of action by which an inhibitor exerts its activity include blocking or down regulating receptors for positive stimulatory factors; interfering with the actions of genes activated by positive regulators or blocking the synthesis of positive regulators (Moore, 1991). In the case of AcSDKP the mechanism of action is unclear. (Tubiana *et al*, 1993; Guigon and Bonnet, 1995).

Robinson *et al* (1993) showed the ability of AcSDKP to block the action of a stem cell stimulator on an HPP-CFC sub-population. Since the stimulator had already been synthesized this would imply a role in either stimulator destruction, some form of receptor down-regulation or active blocking/interference with the normal intracellular signaling pathway. The fact that

receptors for AcSDKP itself have not yet been identified (Guigon and Bonnet, 1995) may add weight to the first of these suppositions

Tubiana (1993) and Guigon and Bonnet, (1995) review data from Aizawa *et al* (1992) and Lenfant *et al* (1991) on the role of AcSDKP in stem cell to stromaL-cell adherence. They note that the peptide enhanced the adherence of CFU-S to stromaL-cells and that binding of this nature could be lost in the presence of anti-AcSDKP antibodies. With the assumption being that haematopoietic cells bound to the stroma respond to stromaL-cell produced factors does this suggest that AcSDKP interacts to alter the micro-environment. If this is the case then how does this relate to the observation that AcSDKP directly interacts with CD34+ cells? (Bonnet *et al*, 1993) It is perhaps worth considering the work of Verfaillie *et al* (1994) in which it was demonstrated that stromal released soluble factors were essential for the IL3+MIP LTBMC maintenance but that these molecules themselves directly interacted with early progenitor cells. AcSDKP could be altering properties of the microenvironment by interacting with factors produced by it whilst not itself stimulating the microenvironment to produce such factors.

An engaging report on the protection of normal human haematopoietic progenitors against photoforin-11 mediated phototherapy suggests several possible mechanisms of protective action (Coutton *et al*, 1994). The killing effect itself relies on the action of a sensitizer (photoforin 11) to induce the oxidation of biological material within a cell in response to

light. Although the mechanisms by which the cell killing is induced are not fully understood it is clear that AcSDKP prevents the effect on progenitor cells in this instance. This is particularly interesting as AcSDKP's protective effect is not extended to either of the leukaemic cell populations tested. They discuss four possible explanations for this finding. Firstly they postulate that AcSDKP may maintain some progenitors within the target population out of DNA synthesis thus preventing the initial uptake of the killing agent. They are reluctant to believe that this alone could account for the reduced killing effect. Unlike the work of Robinson *et al* (1993) discussed earlier, peptidic analogues have not been tested in these systems and thus the activity cannot be attributed to the SDK sequence. Secondly they discuss the possibility that AcSDKP actively dissociates the killing agent but add that their methods should discount this possibility. Their final two proposals are linked in that they suggest the effect of AcSDKP is mediated through the production of other factors. Firstly in inhibiting apoptosis, but no relevant cytokines could be found, and secondly in producing cytokines which in turn cause the release of anti-oxidative enzymes, again no relevant cytokines could be found. Both of these findings concur with work by Bonnet *et al* (1992) which showed no cytokine induction by AcSDKP in human long-term bone marrow cultures.

Recently work on inhibitory cytokines has focussed on comparing their actions with the intention of elucidating some general inhibitory mechanism.

Bonnet *et al* (1995) in studies which assayed and compared the effects of AcSDKP, MIP-1-α, TNF-α and TGF-β on *in vitro* populations of purified CD34+ cells conclude that the mechanism of action of these molecules is likely to be different. Differential inhibitory responses illicited from varying CD34+ cell subsets suggest that these four molecules may target different but overlapping cell populations within the CD34+ cell population. Such a subtle regulatory mechanism would in some way address Metcalf's question of redundancy. The heterogeneity of the cell pool may require several activities to succesfully regulate itself. Most interestingly their work indicates that although TNF-α contains the SDK peptidic sequence its effects are different to that of AcSDKP. TNF-α induced a large reduction in CFU-GM(GM-CFC) numbers from the CD34+HLA-DRhigh cell fraction. It was shown to act in a dose dependant fashion where AcSDKP was only moderately inhibitory for this cell fraction and was completely ineffective at higher and lower concentrations. This bell shaped response curve for ACSDKP is similar to that seen for the pentapeptide pEEDCK (Guigon and Bonnet, 1995; Mouser and Paukovits, 1991). The reason for the pattern of this response is unclear. Earlier studies which demonstrated that TNF-α but not AcSDKP acted by modulating cytokine receptor expression (Bonnet *et al*, 1993; Khoury *et al*, 1994) further suggest that these molecules act in a different way. This begs the question "What is the significance of the SDK sequence in TNF-α ?".

In comparing AcSDKP to MIP-1-α Bonnet *et al* note a similarity in that both molecules require daily addition to cultures to

remain effective. However rather than a genuine functional overlap related to some common structural or genetic target this finding is likely to be explained in terms of small peptide degradation (AcSDKP) (Rouseau *et al*, 1995) and possible polymerisation with consequential lack of function (MIP-1-α) (Graham *et al*, 1992; Graham *et al*, 1994). Of greater interest here are the differences between the two molecules. Specifically there is the fact that they operate differently in their inhibition of stem cell cycling. There is also MIP's greater ability to slow down differentiation in the CD34+HLA-DRlow cell sub-set (could this relate to Verfaille's theory that MIP-1-α may be a survival factor in certain instances?). As touched on above it could be argued here that AcSDKP may prove to have a similar action on another cell-subset in which MIP-1-α would be less active. A case for subtlety over redundancy? The possibility that AcSDKP acts to induce MIP-1-α (based on its antagonism by MIP-1-β) (Cashman *et al* 1994) seems discountable given that MIP-1-α and AcSDKP act differently and that levels of MIP-1-α found in experimental systems did not support this theory. Again this ties up with observations discussed above on cytokine induction by AcSDKP.

In summary the mechanism(s) of action of AcSDKP cannot be fully elucidated and indeed the molecule may operate through several different mechanisms. The importance of a small sequence within the peptide has been demonstrated although the mechanism by which it executes its function is not understood nor is its relevance in explaining the effects of AcSDKP in other systems.

The physiological significance of AcSDKP

In attempting to define the physiological significance of AcSDKP as an inhibitor of haematopoietic stem cells, strategies have been to either look (Both *in vivo* and *in vitro*) for its synthesis or to examine the effects of its withdrawal. Several delicate experiments along these lines have been undertaken. Perhaps the most convincing evidence for a physiological role rests with the fact that AcSDKP is synthesized both *in vitro* and *in vivo*. Wdzieczak-Bakala *et al* (1990) demonstrated the formation of AcSDKP both *in vitro*, in LTBMC's, and *in vivo*, following injection of mice with radio-labelled precursors (tritiated proline). Work initially by Lauret *et al* (1989) using an anti-serum with cross-reactivity for AcSDKP results in an increase in the percentage of CFU-S in S-Phase with the total number of CFU-S unchanged. (Frindel *et al*, 1992; Tubiana *et al*, 1993).

Using an enzyme immunoassay to show the distribution of AcSDKP in man it was demonstrated that red-blood cells and granulocytes possessed only low levels whereas lymphocytes, plasma and serum appeared to possess high levels. (Pradelles *et al*, 1990; Loizon *et al*, 1993).

There does appear therefore to be significant evidence for a physiological role for AcSDKP.

Summary

In summary, it is the possibility of reversibly blocking the cycling of normal stem cells which presents an enticing prospect for chemotherapy. Guigon and Bonnet (1995) report the ability of AcSDKP to protect haematopoietic stem cells against a variety of cytotoxic chemicals. Protection against photopurging has also been demonstrated (Coutton *et al*, 1994). Unlike many of the other inhibitory activities, AcSDKP has a limited range of actions and would seem a good candidate for a haemoprotective agent which does not cause many other side-effects.

It was primarily the potential of AcSDKP to regulate haematopoietic stem cells and the finding that the SDK sequence conferred this ability on the molecule which stimulated interest in Leukaemia inhibitory factor (a cytokine known to contain the -SDK-sequence) in this lab.

1.3: LEUKAEMIA INHIBITORY FACTOR

1.3.5 LEUKAEMIA INHIBITORY FACTOR: GENERAL

Since cytokines became available in recombinant form, it has become obvious that few are specific to one target cell and fewer still are unique in function (Gearing, 1993). The plieotropy associated with the molecule leukaemia inhibitory factor exemplifies this perhaps better than any other cytokine. (Review Gough and Williams, 1989; Metcalf, 1991; Villiger *et al*, 1992; Hilton, 1992; Gearing, 1993; Metcalf, 1994).

Biology of LIF

As an agent effective on leukaemic cell populations LIF was initially characterised as a factor associated with Krebs 11 Ascites tumour conditioned medium able to induce the differentiation of a mouse leukaemic cell line M1(Metcalf *et al*, 1988). It was purified to homogeneity (Hilton *et al*, 1988) and a cDNA encoding active LIF was isolated (Gearing *et al*, 1987) A human genomic clone encoding LIF was subsequently identified (Gough *et al*, 1988). The M1 cell line can be induced to differentiate by a variety of agents among them G-CSF, a differentiation factor (D factor) (Tomida *et al*, 1984) purified from L-cell conditioned medium and a differentiation inducing factor (DIF-A) from human monocytic cells (Abe *et al*, 1989). It was subsequently shown by Lowe *et al* (1989) that D-factor, DIF A and LIF were all identical. Other similar factors such as the macrophage-granulocyte inducer MG1-2B showed similarities to LIF but on sequencing were shown to be other molecules(IL-6). (Hilton *et al*, 1988; Metcalf, 1991).

Many of LIF's activities were similar to molecules which had been independently purified and cloned by a variety of groups working in other fields (Hilton, 1992). It emerged that these molecules were all identical to LIF and as a consequence LIF is known under a variety of names often reflecting the variety of its functions. These are summarised in table I1.

NAME	ACRONYM	
DIFFERENTIATION INDUCING FACTOR	D-FACTOR DIF	LOWE ET AL 1989 TOMIDA ET AL 1984

DIFFERENTIATION INHIBITING ACTIVITY	DIA	SMITH ET AL 1988
HUMAN INTERLEUKIN FOR DA CELLS	HILDA	MOUREAU ET AL 1988
HEPATOCYTE STIMULATING FACTOR 111	HSF111	BAUMANN AND WONG 1989
CHOLINERGIC NEURONAL DIFFERENTIATION FACTOR	CNDF	YAMAMORI ET AL 1989
MELANOMA-DERIVED LIPO-PROTEIN LIPASE INHIBITOR	MLPL1	MORI ET AL 1989

TABLE 11: Other names for LIF.

Perhaps most notable are DIA (Smith *et al*, 1987; Smith *et al*, 1988; Williams *et al*, 1988) where LIF actually shows an ability to INHIBIT differentiation and HSF-111 where LIF stimulates the release of acute phase proteins from hepatocytes (Metcalf, 1991). (Review diagram 6)

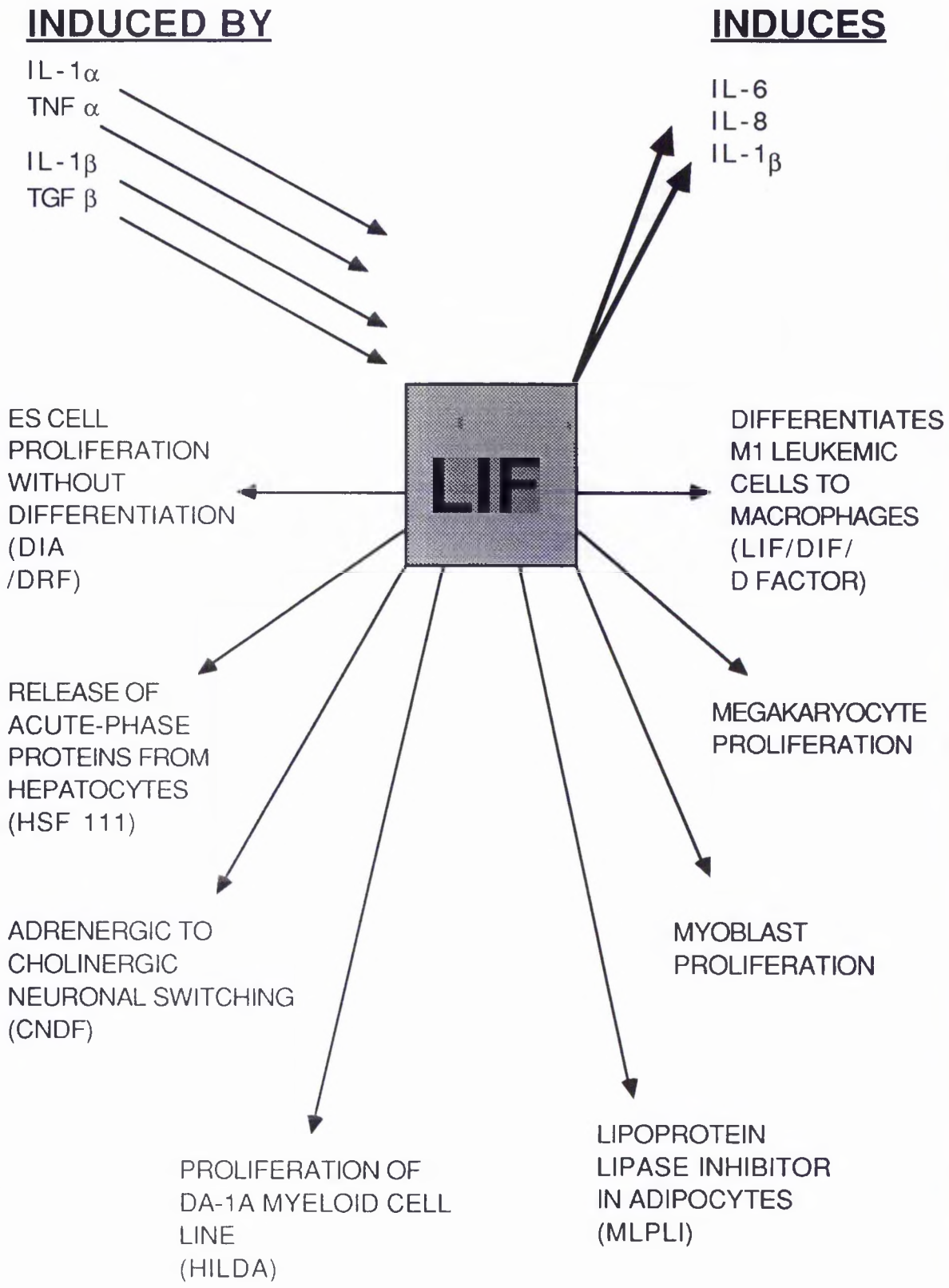


DIAGRAM 6 SUMMARY SCHEMATIC OF LIF'S ACTIONS ON VARIOUS TYPES OF TISSUE

Molecular biology of LIF

Human and murine LIF exhibit 78% sequence homology within the 179 residues making up the molecule (Gough *et al*, 1988) and in both cases LIF is a highly glycosylated single chain (monomeric) polypeptide, a glycoprotein. It has a molecular weight of 58KD (Hilton *et al*, 1988) (although other sources quote 45-52KD(Metcalf 1991) and 38-67KD (Gough and Williams, 1989)) and de-glycosylated of 20-30KD(Hilton, 1992; Gearing, 1993) concurring with the size predicted from the cDNA by Gearing *et al* (1987). The variability noted in size is due to differential glycosylation associated with tissue source of LIF (Metcalf, 1991). It appears that *in in vitro* the level of glycosylation does not relate to function. (Hilton, 1992; Gearing, 1993). It may be that *in vivo* LIF is vulnerable to catabolism and heavy glycosylation may act to prevent this. Disulphide bridging of cysteine residues within the molecule is necessary for activity .(Metcalf, 1991). In both human and mouse, LIF appears to be encoded by a unique gene located at chromosome 22q12 in human (Sutherland *et al*, 1989) and 11 in mouse (Gough and Williams, 1989; Gearing, 1993). In mouse LIF exists in two forms (Gearing *et al*, 1987; Estrov *et al*, 1992; Hilton 1992; Gearing, 1993). The LIF gene is composed of three exons, in mouse an alternative LIF transcript arises due to their being an alternative copy of the first exon associated with a distinct promoter region. The two resultant LIF proteins, one diffusible another membrane bound, differ only in their amino

terminal portion of the leader sequence ie they have a variant signal peptide but identical mature proteins. This difference results in a form of LIF which is incorporated into the extracellular matrix (Rathjen *et al*, 1990). In other species no analogous protein has been found.

The SDK sequence and LIF

The importance of the SDK sequence in AcSDKP biology has been suggested. A study by Wilson *et al* (1992) to define structural and functionally important regions of the LIF gene and protein revealed regions of conserved sequence in the LIF protein from Murine, rat, human ,ovine and porcine species. They conclude that LIF is a highly conserved molecule from 74% sequence homology between mouse and sheep to 92% between rat and mouse. Importantly examination of the cross-species comparison of protein sequence revealed large areas of the molecule which are completely conserved suggesting the sequence arrangement to be of fundamental importance. They further note that all six cysteine residues found in murine LIF are conserved at identical positions in the four other species. This further suggests that intramolecular disulphate bonds are vital to the molecule (Gough *et al*, 1992). Of interest here is the observation that the sequence SDK appears in the murine LIF protein sequence. Comparison with the other species reveals that in all cases the ser and lys ends of this short sequence are always conserved whilst the asp residue appears only in the murine form. In the case of human, ovine and porcine LIF it

has been substituted with 'gly'. With such emphasis given to those sequences which are preserved the fact that this sequence is not would imply that its functional significance is not great. However two questions arise from this finding. Firstly the SDK sequence is present in the mouse is it thus able to give murine LIF an inhibitory role like that associated with AcSDKP ? Perhaps this role is fulfilled by another molecule in the other species. Secondly is the substituted residue significant ? In three cases the substitute is 'gly' why is this particular sequence conserved in three species ? Robinson *et al* (1993) showed that SDK was likely to be the significant component of the AcSDKP molecule. Is it that this substitute sequence could show a similar role ?

Sources of LIF

As with many cytokines the cell types which have the potential to produce LIF are many and varied. However the *in vivo* significance of an inducible *in vitro* response must always be questioned.

It is thus with some caution that LIF has been noted to be a potential product of many cell types including: T lymphocytes (Gearing *et al*, 1987) activated monocytes (Anegon *et al*, 1990) human lung fibroblasts and human umbilical vein endotheliaL-cells (Lubbert *et al*, 1991) Buffalo rat liver cells (Smith and Hooper, 1987) Krebs 11 ascites cells (Hilton *et al*, 1988) the melanocytic cell line THP-1(Abe *et al* 1989)Human bone marrow stromaL-cells (Wetzler *et al*, 1991; Derigs and Boswell,

1993; Wetzler *et al*, 1994) human carcinoma cell lines (Kamohara *et al*, 1994) peripheral blood mononucleur cells in anaemia (Bagnara *et al*, 1993)human articular chondrocytes and cartilage (Campbell *et al*, 1993) human synovial fibroblasts (Hamilton *et al*, 1993) human dermal fibroblast cultures (Lorenzo *et al*, 1994) embryonic blastocyst cells (Metcalf, 1991).

Work by Brown *et al* (1994) in which the *in vivo* expression of LIF is assayed in 24 normal adult mouse tissues revealed that LIF was expressed at low levels in most tissues. They postulate that such low levels of LIF expression suggest either that this finding is not biologically significant or represents the subtle paracrine nature of LIF's action. Such a scheme would sit well with the problem of containing a molecule exhibiting such a high degree of plieotropy. This work taken in conjunction with the work of Weztler *et al* above is important in suggesting that LIF may have a role to play in normal haematopoiesis.

The pattern of induction of LIF expression suggests some factors which may play a common role in stimulating LIF production. Campbell *et al* (1993) note that LIF production by human articular chondrocytes and cartilage is induced by IL-1α and TNF-α. The same factors induce LIF expression in human synovial fibroblasts (Hamilton *et al*, 1993). IL-1α is noted to induce substance P in sympathetic ganglia through the induction of LIF(Schadiack *et al*, 1993). This latter example highlights not only the induction of LIF by cytokines but also the importance of immune cytokines in the nervous system (For review consult Bazan, 1991; Yamamori *et al* 1992). TNF-α

stimulates LIF production in human dermal fibroblast cultures (Lorenzo *et al*, 1994). TNF-α has also been shown to stimulate proliferation of mouse primordial germ cells in culture. In the presence of LIF this stimulatory action is enhanced (Kawase *et al*, 1994) It may be that TNF-α acts to produce sub-optimal levels of LIF here and although it is noted that TNF-α is more effective in inducing proliferation than the soluble form of LIF the possibility that TNF-α acts indirectly through a fibroblastic feeder layer is worth considering. It has been demonstrated by Hamilton *et al* (1993) and Lubbert *et al* (1991) that fibroblasts can produce LIF. Are they producing a matrix associated form in this instance ? Further studies by Dolci *et al* (1994) looking at the effects of LIF on the proliferation and survival of primordial germ cells concluded that this is governed both by contact dependent mechanisms and diffusible factors. Other evidence to confirm this exists (Yoshida *et al*, 1994). Alexander *et al* (1992) showed that a single dose of human LIF completely protected mice from E coli endotoxin (lipopolysaccharide) with only a short pre-treatment interval. TNF-α and IL-1-α have been shown to provide similar protection but only after a longer pre-treatment interval suggesting that they may mediate this response through LIF.

IL-1α,β,TNF-α and TGF-β have all been shown to increase the expression of LIF by both human and murine bone marrow stromaL-cell cultures. (Wetzler *et al*, 1991; Derigs and Boswell, 1993) Given the importance of TGF-β as a potential haematopoietic inhibitor this observation may be extremely important in implicating LIF in haematopoeitic regulation. IL-4

has been demonstrated to reduce LIF protein levels in human stromaL-cell cultures. (Wetzler *et al*, 1994). Interestingly IL-4 also inhibits the LIF induced differentiation of M1 cells (Kasukabe *et al*, 1991) which would suggest that IL-4 acted both on LIF (or its action) and its manufacture. The fact that Kasukabe *et al* (1994) report that IL-4 interferes with a LIF activated target gene, c-myc, suggests that IL-4 is at least capable of acting on the target.

LIF as an inducer of other cytokines

Since the biological effects of cytokines are determined by their interactions with other cytokines. It is important to consider not only those cytokines which induce LIF production but also those which are induced by LIF. A common pattern of cytokine induction in various tissue types emerges. Human articular chondrocytes express IL-1β, IL-6 and IL-8 mrna. It is worth noting that IL-8 can be induced by IL-1α (Villiger *et al*, 1993). Is this secondary activation through LIF? A similar pattern of cytokine induction by LIF is found in human blood monocytes, synovocytes and both epithelial and neuronaL-cell lines (Villiger *et al*, 1993). It is proposed by Abdollahi *et al* (1991) in studies looking at the expression of IRF-1, a positive transcription factor for expression of the IFN-β gene, that LIF acts (in differentiating M1 cells) to induce the expression of IFN-β. During an acute inflammatory response cytokines released by different cell types (monocytes / fibroblasts / endotheliaL-cells) stimulate the synthesis and secretion of a set

of plasma proteins-acute phase proteins by the liver. Mayer *et al* (1993) note that LIF can induce the production of acute phase proteins in non-human primates. Although uncertain as to whether this is an instance where LIF acts directly it is worth noting that Wegenka *et al* (1993) discuss the finding that a factor essential to the action of acute phase proteins is known to be activated by IL-6 a molecule with an ever growing list of similarities to LIF. As discussed above LIF can induce the expression of IL-6 mRNA. Is this a further example of LIF stimulating the release of a secondary cytokine ?
In summary LIF has the potential to modulate inflammatory and immune responses through its pattern of induction and the pattern of secondary cytokines it can induce. (Diagram 6)

LIF and its receptor: An explanation of LIF's functional similarity to other cytokines

Perhaps one of the most significant developments in LIF biology over the past five years has been the observation that it possesses many functional similarities to other cytokines. IL-6, Oncostatin M (OSM), and to a lesser extent , Ciliary neutrophic factor (CNTF) and IL-11 share similarities with LIF (Hilton 1992; Metcalf, 1993; Robinson *et al*, 1994). All of these exhibit over lapping functions in the immune response, haematopoiesis, the nervous system and acute phase protein regulation (Hirano et al, 1994). A point has been reached where attention has focussed on whether LIF and related cytokines are functionally equivalent and thus a classic example of the

redundancy postulated by Metcalf. In order to address this question it is first necessary to consider the LIF receptor.

Two LIF receptor types have been identified, one a high affinity receptor the other a low affinity receptor. (Metcalf, 1991; Hilton, 1992 ; Gearing *et al*, 1991; Gearing, 1993; Robinson *et al*, 1994). The difference between the two has been shown to be due to the rates of dissociation, which is much higher from the low affinity receptor. (Hilton and Nicola, 1992). A human low affinity receptor has been cloned (Gearing *et al*, 1991) and found to be a 200KD glycoprotein. It is a member of the cytokine binding family of receptors characterised by a conserved pattern of cysteine residues and a "trp-sex-x-trp-sex " amino acid motif in the extracellular 200 amino acids unit of the structure. (Cosman D, 1993; Robinson *et al*, 1994). Receptors for IL 2, 3, 4, 5, 6, 7, 9, GM-CSF, G-CSF and CNTF have all been shown to be members of this family (Gearing, 1993). Murine cDNA's encoding soluble LIF receptors and showing 70% sequence homology with the human sequence(Gearing *et al*, 1991) were detected. This soluble form was demonstrated at high levels in the serum of mice and rats but no equivalent was detected in humans. (Layton *et al*, 1992). Importantly this soluble LIF can block the binding of LIF to its membrane bound receptor *in vitro* suggesting perhaps a role for the receptor in localising LIF. The consequences of LIF binding to its receptor have been noted for various cell types with the majority of data derived from M1. These include phosphorylation of heat shock protein HSP27, induction of INF-1 and tyrosine phosphorylation of of a 160KD protein. (Abdollahi *et al*, 1991

;.Lord *et al*, 1991; Michishita *et al*, 1991; Gearing 1993). Feldman *et al* (1994) report that LIF binding to its receptor induces the tyrosine phosphorylation of p91, a transcription factor which leads to the assembly of protein complexes essential for the function of the enhancers of two genes. It is noted (Yamamori *et al*, 1991) that LIF binding does lead to enhanced transcription of genes such as c-fos and jun-b. Yin *et al* (1994) identify a 130KD tyrosine phosphorylated protein induced by LIF in 3T3 cells as JAK 2 tyrosine kinase which is intimately associated with the signal transducing region of the LIF receptor. Similarly Ernst *et al* (1994) show that a tyrosine kinase HCK in ES cells was associated with the signal transducing region of the LIF receptor and showed increased activity in response to LIF binding. Studies by Boulton *et al* (1994) on a variety of cell lines conclude that LIF binding to its receptor results in tyrosine kinase phosphorylation and ultimately in the triggering of genes within the cell. (Hirano *et al*, 1994).

The receptor itself has been found to be related structurally to the G-CSF receptor as well as to gp 130, the transmembrane signal transducing region of the IL-6 protein. Gp 130 associates with IL-6rα, an 80KD membrane bound molecule, when IL-6 binds to IL-6rα. (Gearing *et al*, 1991; Hirano *et al*, 1994). At a glance this similarity immediately suggests an explanation for the large functional overlap seen between IL-6 and LIF. (For a review see Gearing, 1993; Hirano *et al*, 1994). However the observation that there were both high and low affinity receptors could not be explained by this model. The picture

which emerged revealed the LIF receptor as consisting of at least two subunits (Gearing and Bruce, 1992). A low affinity LIF receptor, designated LIFrβ, forms functional high affinity receptors in combination, i.e. by heterodimerization, with gp 130. (Davis *et al*, 1993) It is envisaged that LIF may interact with an α sub-unit, like that for IL-6, with which the β unit may be associated and the gp 130 would come to be associated. Gp 130 is a common receptor subunit shared between IL-6 and LIF suggesting a common signal transduction pathway between these two receptors. Through this work and other studies it emerged that other members of this cytokine family had receptors which shared the gp 130 molecule and were essentially variations on a theme. Most notably amongst these was OSM. This is a 28 KD glycoprotein produced by activated monocyte and T lymphocytes known to be linked to LIF in terms of primary amino acid sequence and predicted secondary structures (Rose and Bruce, 1991; Robinson *et al*, 1994) the location of their genes on human chromosone 22 within 19 kbp of each other (Jeffrey *et al*, 1993) and the ability of both to differentiate M1 cells at similar concentrations .(Gearing, 1993). Gp130 could bind OSM probably after the binding of OSM to an a sub-unit with low affinity (Gearing and Bruce, 1992). In the presence of the LIFrβ sub-unit a high affinity receptor emerged (Hirano *et al*, 1994). In the case of CNTF a 22KD glycoprotein with structural similarities to LIF and IL-6 (Richardson, 1994) the receptor complex is composed of an 80KD CNTFrα receptor sub-unit (with 30%sequence homology to the IL-6rα) anchored to the cell surface. This becomes associated with LIFrb and

gp130 sub-units for biological action. (Richardson, 1994; Hirano *et al*, 1994; Gearing, 1993; Davis *et al*, 1993). (These receptor subunits are summarised in diagram 7.)

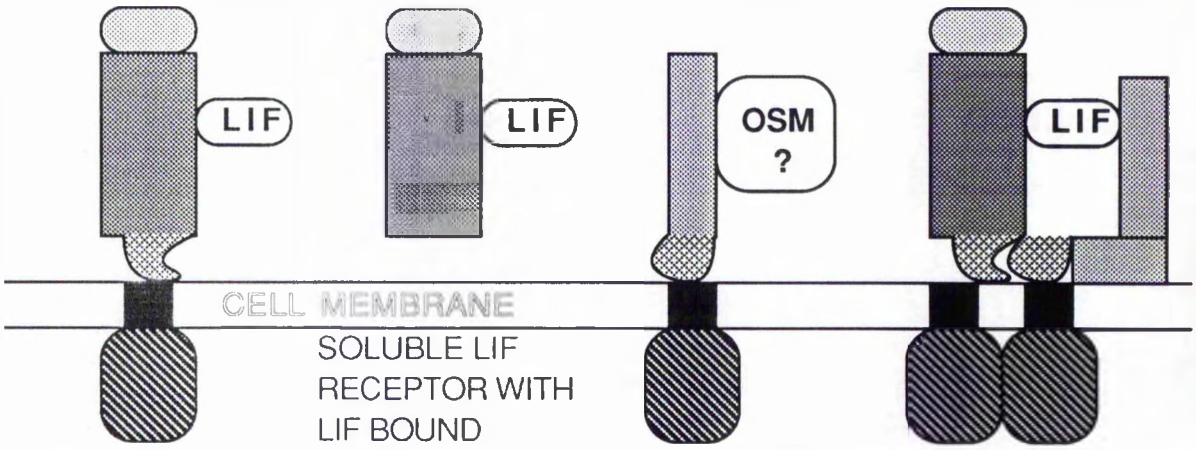


DIAGRAM 7 SCHEMATIC REPRESENTATION OF THE HIGH AND LOW AFFINITY LIF RECEPTOR AND THEIR RELATIONSHIP WITH GP130 . OSM AND CNTF RECEPTORS ARE THOUGHT TO BE BASED ON SIMILAR SUB-UNIT PAIRING WITH FRACTIONS OF THE HIGH AFFINITY LIF RECEPTOR POSSIBLY INVOLVED IN THE CONSTRUCTION OF EACH OF THE OTHER TYPES OF RECEPTOR .

Functional similarity: subtlety or redundancy?

The question of redundancy or subtlety remains difficult to resolve. The shared receptor structure discussed for the above cytokines appears to offer an explanation as to how the cytokines induce similar functions. However it does not answer the question WHY? Perhaps a clue as to the solution may lie in considering not those functions which cytokines have in common but those which they do not.

For example in the case of LIF and IL-6 where there is significant functional overlap in more than the one cell type (Review Gearing, 1993) it is known that some actions common to both, such as the activation of a gene in the H35 rat hepatoma cell line, can be modified independently and differentially for each cytokine. (Bauman *et al*, 1992) This suggests that there is a genuine subtlety in the way in which these cytokines are involved in regulating this gene. Although at face value it appears that the gene is activated by both, the control of that activation appears subtly different -perhaps modified by the specific receptor subunit? An equally illuminating example of this can be found in work by Pellorce *et al* (1994) in which LIF and IL-6 were shown to stimulate heptaglobin and fibrinogen production in HEPG2 hepatoma cells. Although both stimulate production the dose response curves for the two are radically different. The same is true for the differentiation of M1 leukaemic cells. (Metcalf and Gearing, 1989).

Oncostatin M is known to share a variety of functions with LIF (Gearing, 1993; Jeffrey *et al*, 1993; Thoma *et al*, 1994). Scrutiny of these shared responses occasionally reveals a differential nature within an apparently identical response. For example LIF and OSM both stimulate the release of acute phase proteins from hepatocytes however the response is larger with OSM than LIF(Richards *et al*, 1992). Of greater importance is the observation that OSM has functions not shared with LIF. These include inhibition of growth of A375 melanoma cells (Zarling *et al*, 1986) stimulation of plasminogen activity in aortic endotheliaL-cells. (Gearing, 1993). These findings also suggest the presence of a unique non-LIF binding OSM receptor. As yet no murine OSM has been detected . The discovery of this would strengthen the case for subtlety over redundancy. A report by Tanigawa *et al* (1995) looking at the macrophage differentiation of M1 cells in response to LIF, OSM and IL-6 concludes that these factors induce a differential pattern of differentiation. The gp130 heterodimers LIF and OSM are shown to decrease levels of a transcription factor from a gene (SCL) which is a crucial step in the differentiation process. IL-6, a gp130 heterodimer, appears to act differently, as, although equally capable of decreasing the levels of the transcription factor from the SCL gene, enforced SCL expression does not inhibit IL-6 induced differentiation of M1 cells. Differentiation with OSM and LIF is inhibited .

CNTF and IL-11 both exhibit examples of functions shared with LIF but regulated in different ways and functions not shared with LIF. (Review Pellorce *et al*, 1994; Davis *et al*, 1993;

Richardson, 1994; Robinson 1994; Gearing 1993; Hilton, 1992; Gough and Williams, 1989; Metcalf, 1991).

It seems logical to deduce from these few examples that the apparent redundancy in cytokine action may represent an *in vivo* subtlety which is not obvious within the relatively crude restrictions of *in vitro* assay. Gearing argues that this functional overlap represents a synergistic mechanism which protects against toxicity. The fact that a molecule such as LIF has been conserved in so many systems shows significant physiological economy. If this is possible why would there be such a wasteful dynamic as so many identical molecules ? Despite the advances in *in vitro* culture techniques and the ability to use panels of anti-bodies against possible secondary cytokines the *in vivo* environment represents a delicate signalling network which may well be regulated in a manner which simply precludes the problem of redundancy. It may well be an *in vitro* artefact. The homology seen between these molecules may represent a common ancestor which has diversified to perform specific functions with suitable alterations to the molecular structure. It is worth noting that in the case of other related molecules showing high sequence homology, e.g. MIP-1-α and β, this does not itself guarantee functional similarity. MIP-1-α is a potent stem cell inhibitor, MIP-1-β, which shares 67% sequence homology with the former, is not. Identification of the functionally relevant regions of each of the above cytokines could resolve the redundancy question revealing that far from wasteful, the

system exhibits great economy.with fine tuning of a core molecule for specific tasks.

LIF in haematopoiesis.

As outlined in the early part of this introduction haematopoiesis is a balance between differentiation and self-renewal(proliferation and survival). The success of haematopoiesis relies on maintenance of this delicate balance. The importance of a molecule which can induce differentiation in one system (M1 leukaemic cells) and promote proliferation in another (ES cells (Smith *et al*, 1988) as a potential regulator in haematopoiesis is considerable. With the discovery that the SDK sequence was present in murine LIF, elucidating a role for LIF within haematopoiesis became important. Noting its relevance in AcSDKP function and the importance of other tetrapeptides such as AGR-GLY-ASP(RGD) (the sequence within fibronectin which mediates attachment (D'souza *et al*, 1991) it seemed possible that murine LIF could interact with stem cell stimulator.

LIF has been shown to induce the differentiation of M1 leukaemic cells to macrophage like cells as has IL-6 (although LIF is 16-25 times more effective) (Metcalf and Gearing, 1989). IL-6 is constitutively expressed by M1 cells and this expression increases in the presence of LIF. It is unlikely however that LIF acts through IL-6 as the increase is slight and the factors inducing IL-6 do not induce a response like that seen for LIF. Interestingly macrophages are known to express IL-6 so this

may be a consequence of the cells differentiating rather than the cause. The LIF induced differentiation could be enhanced by addition of G-CSF or M-CSF to cultures but not by GM-CSF or IL-3. (Metcalf *et al*, 1988; Metcalf, 1989) Work by Maekawa *et al* (1990) notes that human myeloid leukaemic cell lines (HL60 and U937) showed reduced clonogenicity in the presence of combinations of LIF/IL-6/G-CSF and GM-CSF but not to LIF alone. LIF was synergistic in this respect with G-CSF and GM-CSF. Wang *et al* (1990) report that LIF prolongs the doubling time of a clonogenic population of newly established acute myeloblastic cell lines in liquid culture. In normal haematopoiesis IL-6 and IL-3 act synergistically to support the proliferation of haematopoietic cells by decreasing the G0 period of individual stem cells. (Ikebuchi *et al*, 1987; Leary *et al*, 1988). Takanashi *et al*, (1993) studied the effects of LIF on blast cell progenitors from human acute myeloblastic leukaemia (AML) in serum free culture. In stark contrast to the work of Metcalf and Maekawa they conclude that when used in combination with GM-CSF, IL-3 or IL-6 LIF increases AML blast proliferation and does not induce differentiation. The responses of various leukaemic models to LIF are heterogenous not all respond and in those which do both stimulation and inhibition are seen.

Metcalf *et al*, (1988) note that LIF had no colony stimulating activity for normal granulocyte-macrophage progenitor cells and did not alter their responsiveness to other cytokines (IL-6 and G-CSF) Metcalf, (1989). Several groups however suggest that LIF may have a role to play in the production of normal

blood cells. Fletcher *et al*, (1990) undertook a study to test the effects of LIF on recovery and retroviral vector infection of CFU-S13 *in vitro*. The frequency of CFU-S13 infection was increased in groups of murine bone marrow cultured on irradiated virus-producing fibroblasts in the presence of LIF. As infection is thought to require the host to transit the cell cycle, it appeared that LIF either induced CFU-S proliferation or promoted their survival. The fact that there was no detectable alteration in the differentiation potential of the CFU-S suggests that the latter may be the case. Leary *et al*, (1990) report the ability of LIF, independently of IL-6, to act as a secondary cytokine augmenting IL-3 dependant proliferation of early haematopoeitic progenitors (CD34+ cells). Verfaillie and McGlave, (1991) take this work further concluding that LIF excerpts its activity by direct interaction with CD34 cells and does not require accessory cells. A study by Escary *et al*, (1992); Dumenil *et al*, (1993) using LIF deficient mice derived by gene targeting techniques shows these mice to have dramatically decreased numbers of stem cells in spleen and bone marrow. Two points of fundamental importance arise here. Firstly remaining stem cells retain pluripotency showing that if stem cell survival is a function of LIF it is not unique to LIF alone. Secondly the number of committed progenitors are reduced in spleen but not in marrow suggesting a differential role for LIF in haematopoiesis between these two. In brief LIF thus appears to be important for maintenance of a complete stem cell pool. It is worth considering that if LIF were really a redundant cytokine then surely its action in knockout mice

would be taken up by another molecule and this pathology would not result. Schaafsma *et al* (1992) report that in LTBMC's LIF did not alter the proliferation and differentiation of committed haematopoietic progenitor cells.

The past five years has seen the emergence of LIF as an agent promoting megakaryopoiesis both *in vitro* (Metcalf *et al*, 1991) and *in vivo*(Metcalf *et al*, 1990) (Review Waring *et al*, 1993). LIF injection to mice results in an increase of spleen megakaryocytes and circulating platelets (Metcalf *et al*, 1990) and in combination with IL-3 potentiates megakaryocyte production in cell culture (Metcalf *et al*, 1991). It is suggested that the CD34+ cell stimulation with IL-3 and enhanced by LIF may actually be elevated megakaryocytopoiesis. (Debilli *et al*). Does this relate to the HPP-CFC meg population seen by Han (1994) ?

The pattern emerging from this work suggests that LIF in fact acts at two stages in haematopoiesis. Initially it may have a role in maintaining early stem cells. Later it may effect megakaryocytopoiesis. (Diagram 8)

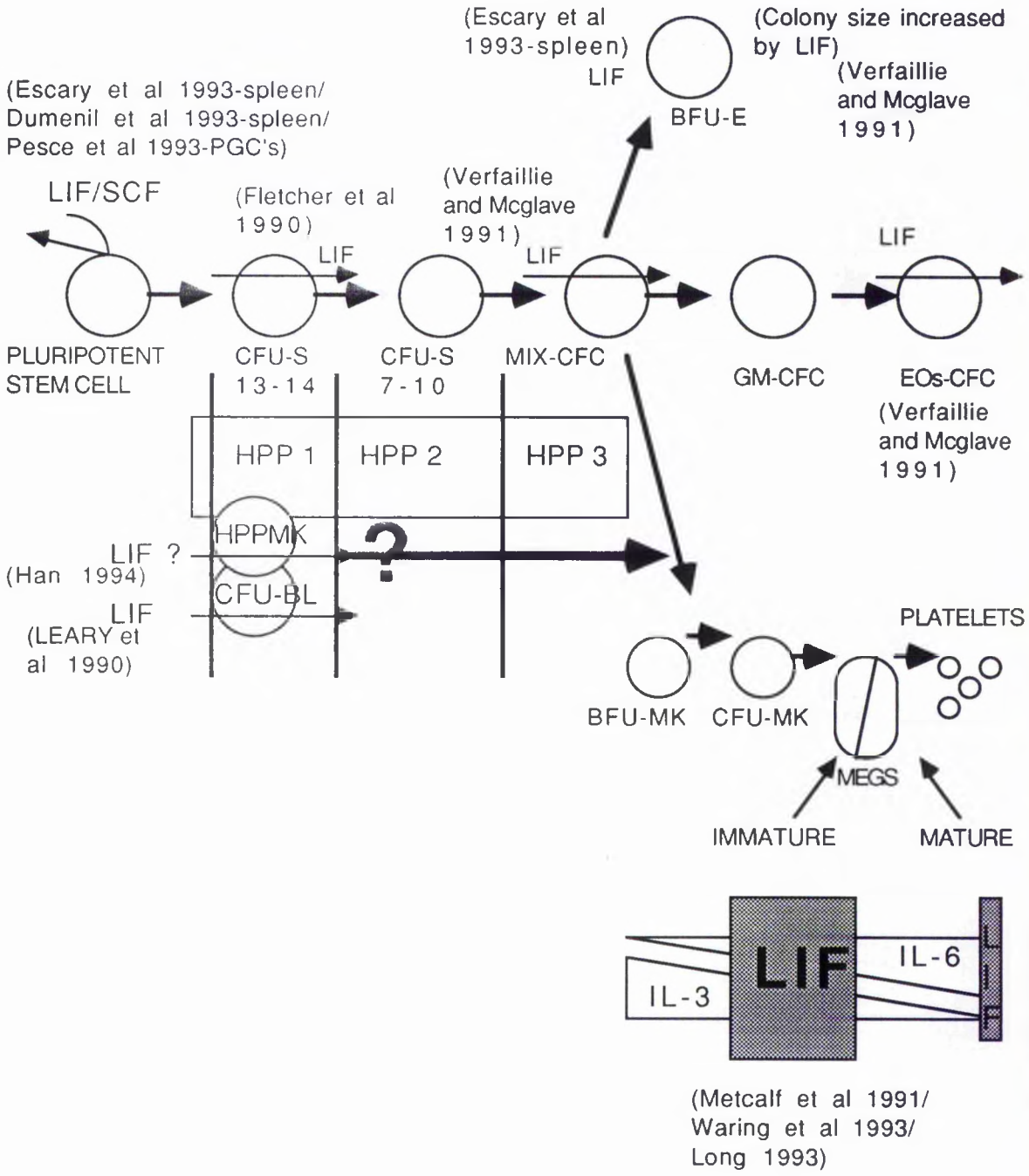


DIAGRAM 8 THE INTERACTION OF LIF WITH HAEMATOPOIETIC CELLS . (It should be noted that some of these interactions are from human tissue whilst others are associated with murine haematopoiesis . The diagram represents a possible scheme of LIF's action should these activities emerge as identical between murine and human tissue) .

Summary

LIF is a highly plieotrophic molecule with a staggering array of often contradictory functions. It is known most famously for its role in leukaemic cell differentiation but in fact this may prove a misnomer as this is a far from consistent finding. In haematopoiesis it may play at least a two stage role and in the murine form it contains the SDK sequence.

1.4 Aims

This study was primarily concerned with elaborating the role of factors associated with, or interacting with, leukaemic cell populations, in the control of the proliferation of normal haematopoiesis.

Firstly Leukaemia inhibitory factor: LIF. The primary aim was to determine LIF's ability to regulate proliferation in populations of primitive stem cells and committed progenitor cells derived from femoral marrow and foetal liver, *in vitro*.

A second aim was to assay LIF's effects on the responses induced by other cytokines in these cell populations.

The ability of LIF to influence clonogenicity and DNA synthesis in a leukaemic cell population was also assessed.

Thereafter work focussed on activities associated with an X-irradiation induced murine myeloid leukaemia (SA2 JMB1).

The primary aim was to determine how this leukaemic cell population may interact with normal haematopoiesis. Thus

conditioned medium derived from the leukaemic cells was assessed for its ability to regulate proliferation in populations of primitive stem cells and committed progenitor cells, *in vitro* As in studies with LIF, the effect of this conditioned medium on responses induced by other cytokines in these cell populations was also assessed. A further aim was to identify the activity(ies) which was/were associated with this conditioned medium.

CHAPTER 2: METHODS AND MATERIALS

2.1: MATERIALS

2.(1.1) Medium

Two types of medium were used in the course of this work:

a) Dulbecco's 10 x (Gibco) supplemented with: 50 IU/ml benzyl penicillin(Sigma); 50 µg/ml streptomycin sulphate(Sigma); 2mM L-glutamine (Flow); $NaHCO_3$ (BDH) according to manufacturer's recommendation; 20%(v/v) horse serum, unless otherwise quoted. (Globepharm Ltd)
This is referred to as 'Dulbecco's 20%'.

b) Fischer's 10 x (Gibco) supplemented with: 50 IU/ml benzyl penicillin; 50 µg/ml streptomycin sulphate; 2mM L-glutamine; $NaHCO_3$ (BDH) according to manufacturer's recommendation; 10%(v/v) foetal calf serum unless otherwise quoted
This is referred to as 'Fischer's 10%'

All media were prepared under conditions of absolute sterility in a Laminar horizontal flow hood (John Bass, Southampton).
As well as cell culture media a variety of conditioned media was used, details may be found below.

2(1.2) Growth factors

The following growth factors were used throughout the course of this work:

RECOMBINANT

r mu Leukaemia inhibitory factor (LIF) (Gibco);

r hu Interleukin-1α (IL-1α) (Immunex);

r mu Interleukin-3 (IL-3) (Immunex);

r hu Macrophage colony stimulating factor (M-CSF) (Immunex);

r mu Granulocyte-Macrophage colony stimulating factor (GM-CSF) (Immunex).

CONDITIONED MEDIUM AS A SOURCE OF GROWTH FACTOR:

WEHI 3D myelomonocytic Leukaemic cell line

-source of IL-3 (Ihle *et al* (1982))

L929 fibroblast cell line - source of M-CSF (Stanley and Heard (1977))

2(1.3) Tissue

All mice were housed in controlled animal house conditions and fed on a diet of chlorinated water, RM1 chow (Special Dietary Services, Edinburgh) for general maintenance, or RM3 chow (SDS, Edinburgh) during breeding.

Tissue used was derived from four sources:

a) Femoral marrow from 9-12 week old CBA/H mice (Harwell);

b)15 day foetal liver tissue from 9-12 week old CBA/H mice.

c) Femoral marrow from 9-12 week old CD1 mice (Charles River).

d) SA2 JMB1 cells - a cloned cell line from an X-irradiation induced myeloid leukaemia (Hepburn *et al*, 1987).

Mice were killed by ether inhalation overdose followed by cervical dislocation. The appropriate tissue was then removed from the animal.

2(1.3.1) Femoral marrow

In the case of femoral marrow the femur was removed to a petri dish (Sterilin). In the sterile laminar horizontal flow hood excess muscle tissue was scraped away, and the end of the hip joint removed by single scalpel incision. With the cleaned femur held between a pair of sterile forceps a 1ml syringe (Becton Dickinson) with blue tip microlance needle (23G (0.6mm x 25 mm)) (Becton Dickinson) and containing 0.5ml Dulbecco's medium was used to gently bore a hole through the epiphyseal cartilage plate (knee) just into the marrow cavity. This done the medium was gently forced into the femur through the syringe, washing the marrow out into a 5ml plastic vial scalpel. The femur was then inverted and, by gently inserting the needle at the level of the cut pelvic joint, washed through in the opposite direction with an equal volume of Dulbecco's 20%. The femoral shaft was washed through in this manner several times. This process was repeated for each femur.

Single cell suspension

To obtain a single cell suspension the collected marrow was gently sucked up and down the 1ml syringe, firstly through the blue tipped microlance needle and secondly through an orange tipped microlance needle (25G (0.5mm x 16mm)) (Becton Dickinson). Cells were then transferred to a10 ml red-top conical centrifuge tube (Sterilin), made up to a final volume of 8mls with Dulbecco's 20% and washed by spinning at 1000 r.p.m. for ten minutes in a Centaur 2 centrifuge (Fisons -MSE) . The medium was then poured off, cells resuspended in 1ml Dulbecco's by gently sucking them up and down a P1000 Pipettman (Gilson's) with blue c200v pipette tip (Greiner), made up to a final volume of 8ml with Dulbecco's 20% and spun as before. Again the medium was then poured off and the cells resuspended in 1-3ml Dulbecco's 20%. The process is summarised in diagram 9

2(1.3.2) Foetal liver

15 days after the appearance of a vaginal mucous plug, pregnant mice were killed as described above 2(1.3). Tearing of the ventral skin followed by dissection of the abdominal cavity revealed the uteri containing the foetuses. This was removed by cutting the horns of the uterus at the lateral margins using sterile scissors, and lifting the foetuses clear with sterile forceps. The tissue was then placed in a petri dish (Sterilin) lined with gauze (Smith and Nephew. Foetuses were

removed firstly from the uterus, and then the amniotic sac, by scalpel dissection, decapitated and laid out ventral side up. The large, red, haematopoietically active liver could clearly be seen in the foetal abdominal cavity. The tips of Sterile watchmaker forceps were laid across the abdominal cavity with the liver in between. By applying gentle pressure to the forceps the liver was forced from the abdominal cavity onto the forceps tips and could be removed. This method was useful in that it decreased the likelihood of non-experimental tissue being removed with the liver.

Single cell suspension

To obtain a single cell suspension the liver was first sucked up into a 1ml syringe (without needle) thus disrupting the structure. The resultant tissue was then treated exactly as outlined for femoral marrow in 2 (1.3.1).

The process is summarised in diagram 9

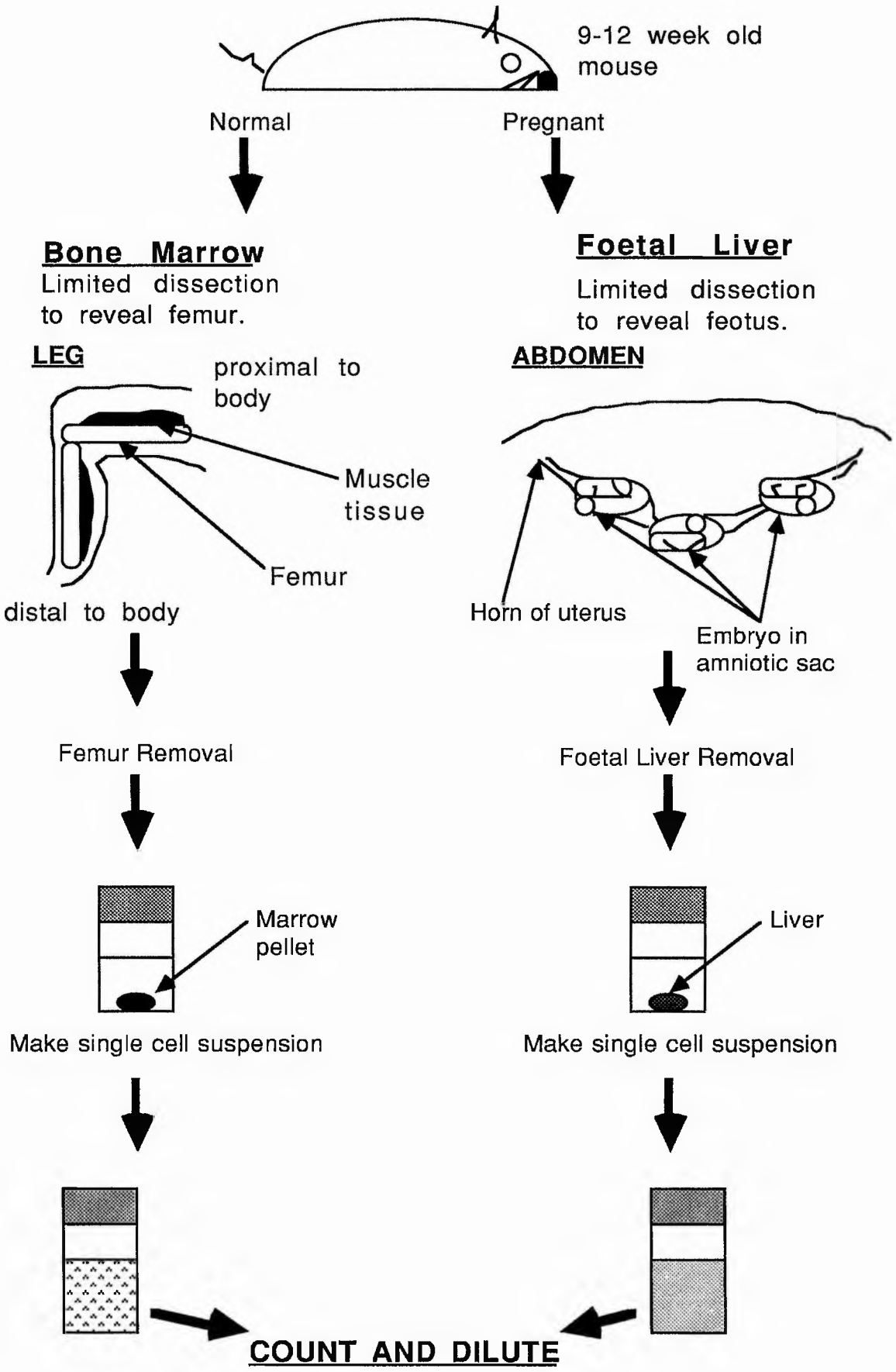


DIAGRAM 9 EXTRACTION OF BONE MARROW AND FOETAL LIVER TISSUE .

2(1.3.3) SA2 JMB1 Cells

A clone of an X-irradiation induced murine myeloid leukaemia (SA2 JMB1) which grows *in-vitro* in the absence of exogenous growth factors (Hepburn *et al*, 1987) was used to investigate the effects of myeloid leukaemia on normal haematopoiesis. The Leukaemic potential of the clones had been verified in CBA mice by intraperitoneal injection of 5 mice with 10^5 cells per mouse. After 23 days all mice were dead .Autopsy revealed enlarged infiltrated spleen.

SA2 JMB1 cells were diluted in Fischer's 10% to 2 x 10^4 cells per ml. This medium was best for optimal proliferation of the cells over the desired incubation period. It sustained levels of proliferation which did not result in overcrowding of the culture flask, depletion of nutrients and/or accumulation of toxic products detrimental to the cells. 10 ml of this cell dilution was transferred to a 25ml vent topped cell culture flask (Nunclon) and incubated for 3 days in a Leec incubator at 37°C with 5% CO_2 and a fully humidified atmosphere. With a doubling time of approximately twenty-four hours under these conditions, cells proliferated to numbers approximating 1million per ml.(Diagram 10)

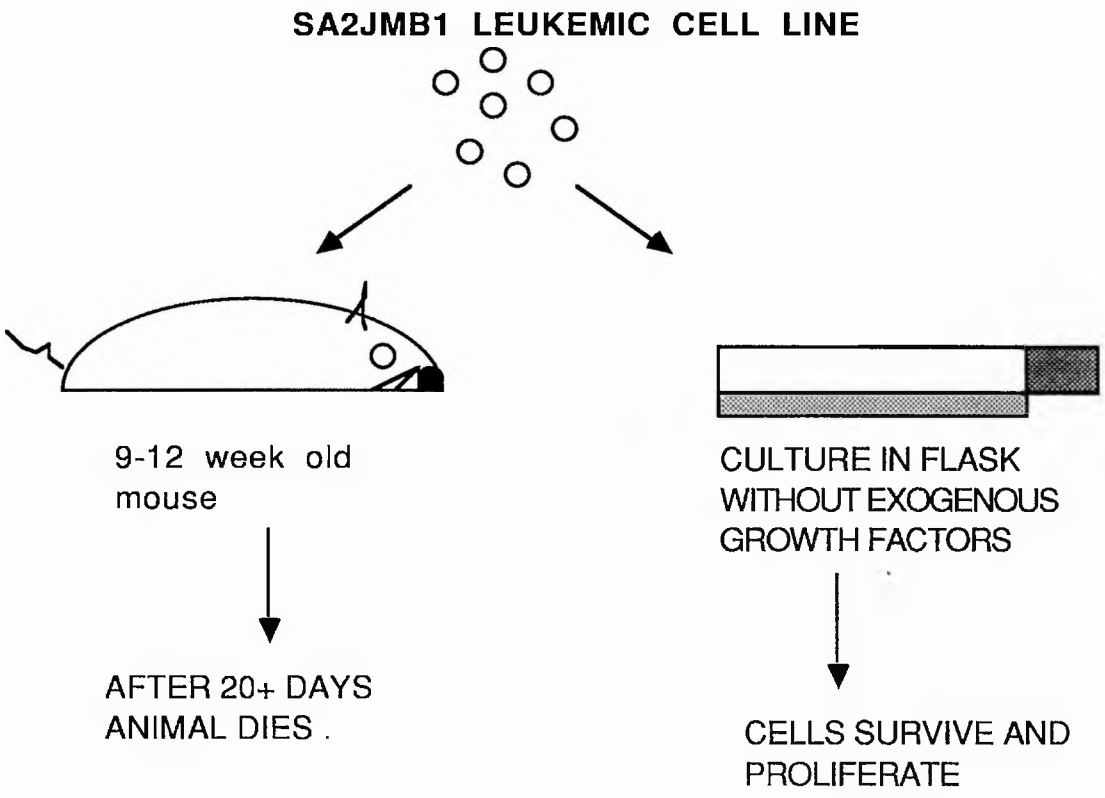


DIAGRAM10 SUMMARY OF SA2 CELL CULTURE

Single cell suspension

After three day incubation, cells were transferred to a 10ml conical bottom red topped centrifuge tube and centrifuge spun at 1000rpm for 10 minutes. The medium was then poured off and discarded. The cells were resuspended in 8ml fresh medium by firstly, gently sucking them up and down a 10ml glass pipette, then a 5ml glass pipette, and finally a 2ml glass pipette (All John Poultern Ltd Essex). They were then spun as in 2(1.3.1). After spinning excess medium was again poured off and the cells resuspended in 3ml fresh medium.

Viablility of the cells was determined by transferring 1ml of cell suspension to a 5ml teklab, to which was added 50ul of fast

green (Sigma) (2%(v/v) in 100ml 0.9%(v/v) NaCl filtered through a 0.2µm millipore filter) (Bosanquet *et al* 1983). This was then incubated in a water bath at 37°C for 15 minutes. Fast green is a stain excluded by live cells. Using a 145mm glass pasteur pipette (John Poultern ltd, Essex) one-two drops of stained cell suspension was placed on a 76mmx26mm microscope slide(BDH), and a 22x22mm cover slip (Chance Proper Ltd, England) gently laid on top. The suspension could then be screened under the light microscope (Ernst Leitz Weltzer, Germany) for the presence of dead cells, which were stained green. Viability of greater than 75% was considered suitable for further cell passage.

2(1.3.4) FDCP-A4 Cells

In the 1980's Spooncer *et al* described how haematopoietic cell lines could be isolated and cloned from murine long term bone marrow cultures (LTBMC's) These were dependent on IL-3 for their survival and proliferation and had a limited differentiation potential in that under the correct conditions they could become neutrophils or macrophages. These lines were designated FDCP-factor dependent cell lines Patterson. Whereas these lines were derived from normal LTBMC's it was also found that following injection of bone marrow with a retrovirus carrying the SRC oncogene, growth factor dependent cell lines could be generated which were multipotent. These were able to produce cells of the erythroid, megakaryocyte, neutrophil, macrophage, eosinophil, mast cell and osteoclast

lineages. These were designated FDCP-mix cells because of this property. In the presence of IL-3 these cell lines undergo self-renewal without differentiation. They are dependent absolutely on IL-3 for their survival and proliferation. At points in the latter part of this work it was neccessary to assay for the presence fof IL-3, FDCP-mix A4 cells (One FDCP-mix clone) were used routinely in this work.

General protocol

In a 30ml universal cells were diluted in F20% horse serum+10%WEHIcm, to a concentration of 6 x 10^4 cells/ml. 10ml of this dilution was prepared. This was transferred to a 25ml vent topped cell culture flask(Nunclon) and incubated for 2-3 days in a Leec incubator as in 2(1.3.3) Cultures generally became static at 8-10 x 10^5 cells/ml.

2(1.3.5)Determining cellularity

Cellularity was determined by diluting a known volume of cell suspension in a known volume of Isoton (Coulter Euro Diagnostics), lysing the red cell population using three drops of Zaponin (an acetic acid based lysing agent) (Coulter electronics Ltd) and counting on a Coulter counter model ZM (Coulter electronics Ltd). In the case of SA2 JMB1 and FDCP/A4 cells zaponin addition was not necessary as no red cell population was present. Previous work had determined that the optimal Coulter settings for accurate cellularity readings were:

TISSUE	*Femur/ Foetal liver*	*Sa2 cells*	*FDCPA4*
PARAMETER			
current	130	700	700
Lower threshold	30	21	15
Full Scale	10	1	1
Polarity	+	+	+
Attention	1	1	1
Preset gain	2	1	6

Table 1M: Cell Counting

2 (1.4) PREPARATION OF CONDITIONED MEDIA

2 (1.4.1) SA2 JMB1 Conditioned media

SA2 JMB1 cells were diluted to a concentration of 10^5 cells/ml in Fischer's 10%. 10 ml of this dilution was transferred to a 25ml vent topped cell culture flask (Nunclon). This was gassed with CO_2 for 15-20 secs and incubated for three days in an incubator at 37°C, 5% CO_2 in air, fully humidified atmosphere. Thereafter the cell suspension was transferred to a 10ml red topped conical bottomed centrifuge tube and centrifuge spun at 1000rpm for ten minutes. This process resulted in a firm cell pellet being precipitated at the base of the centrifuge tube. By a quick single tip of the centrifuge tube the medium could be transferred to a second identical centrifuge tube leaving the cell pellet behind. The pellet was then either discarded or

retained for the preparation of more conditioned medium. The medium was centrifuge spun at 3000 rpm for a further ten minutes. This led to a small opaque pellet, presumed to be cell debris, being precipitated at the base of the centrifuge tube. Again a single swift tip of the centrifuge tube allowed the medium to be poured off into a sterile 50ml glass beaker leaving the pellet behind to be discarded. The 'clean' medium was then aliquoted into 5ml plastic vials , labelled, frozen and stored at -20°C until use .

2 (1.4.1.1) Ultrafiltration in the Centricon microconcentrator

SA2JMB1 conditioned medium was screened by molecular weight in this work. In order to attain different molecular weight fractions it was necessary to filter the medium. This was accomplished on a Centricon micro-concentrator.

General Protocol

Concentration was achieved by ultrafiltering a sample of conditioned medium through a Centricon10 or Centricon 50 microconcentrator (Amicon). The filter unit consists of three parts. Firstly the sample reservoir containing the important 10Kd filter and into which a known volume of the solution to be filtered is placed. This is connected, at its base, to the second part: the filtrate cup. The third part, the retentate cup, is then placed on top of the sample reservoir. (Diagram 11).

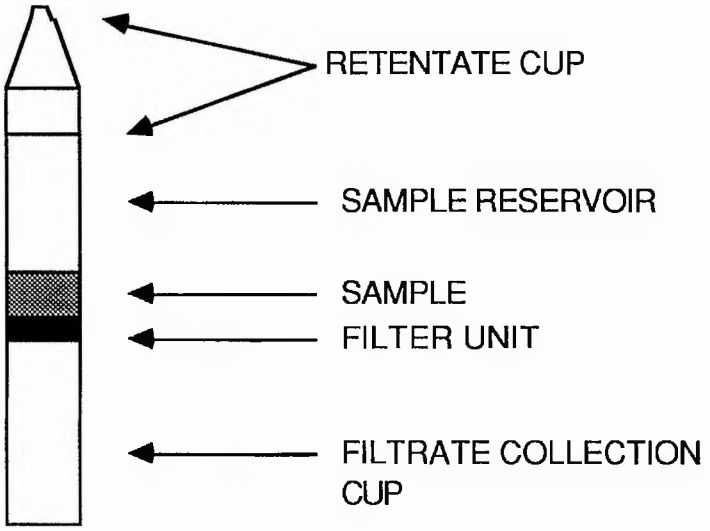


DIAGRAM 11 STANDARD AMICON ULTRAFILTRATION UNIT

The entire device is then centrifuged. Centrifugal force drives the solution in the sample reservoir through the filter and into the filtrate cup. The size of the filter means that only molecules smaller than 10Kd will pass through the filter thus concentrating the larger molecules on the other side. By ceasing centrifugation after a known volume has passed into the filtrate cup the level of concentration may be known. After initial centrifugation the filtrate cup may be separated from the sample reservoir. The sample reservoir is then inverted and centrifuge spun thus driving the remaining solution into the retentate cup. Both filtrate and retentate may then be stored and assayed for relevant activity.

Sterilising apparatus

In line with manufacturers recommendations all parts of the filter unit were placed into the sterile hood and treated with 70% alcohol for 10minutes, then vigorously washed with sterile water. The apparatus was shaken dry. This meant that the solutions recovered from the filter could be used in bioassays without fear of contamination.

Ultrafiltration In the sterile hood

2ml of medium was aliquoted into the sample reservoir. The filtrate cup was then added and, in place of the Retentate cup, a piece of sterile foil tightly wrapped around the top of the sample reservoir. (The retentate cup did not fit in the centrifuge). The unit was now ready for the ultra-centrifuge. Units were placed in rubber inserts, foil end up. The inserts were then placed into the J-20 rotor (Beckman) and balanced. The rotor was then transferred to the J2-21 M/E ultra-centrifuge (Beckman) at 4^0C and spun at 6500rpm for 30mins-1hour. At the end of this time the filter units were carefully removed from the inserts, placed on ice and transferred to the sterile hood. The filtrate cup now contained a solution (1.6-1.8ml) with those molecules smaller than 10KD. The sample reservoir contained a solution (200-400ul) with those molecules larger than 10KD. The foil was removed from the sample reservoir and 1.8ml sterile distilled water added to the volume in the sample reservoir. The contents of the filtrate cup were transferred to a 2ml epindorph the foil cap replaced on the sample reservoir and the unit spun as before. This process was repeated twice .Thereafter the unit was topped up to 2ml

with SA2cm and filtered as before. This was repeated three times. At the end of the third spin the foil was replaced with the retentate cup. The capped sample reservoir was then removed from the filtrate cup inverted and reconnected via the retentate cup. The unit was then placed into the Centaur centrifuge and spun for 10 mins at 3000rpm. Thereafter the unit was returned to the sterile hood and the filtrate cup was separated from the retentate cup. 1ml sterile water was added to sample reservoir. The solutions from both cups were aliquoted into separate plastic vials, refrigerated at +4oC or frozen and stored at -20^0c. Fractions were then assayed for activity as outlined in 2(3.1.5) (Diagram 12)

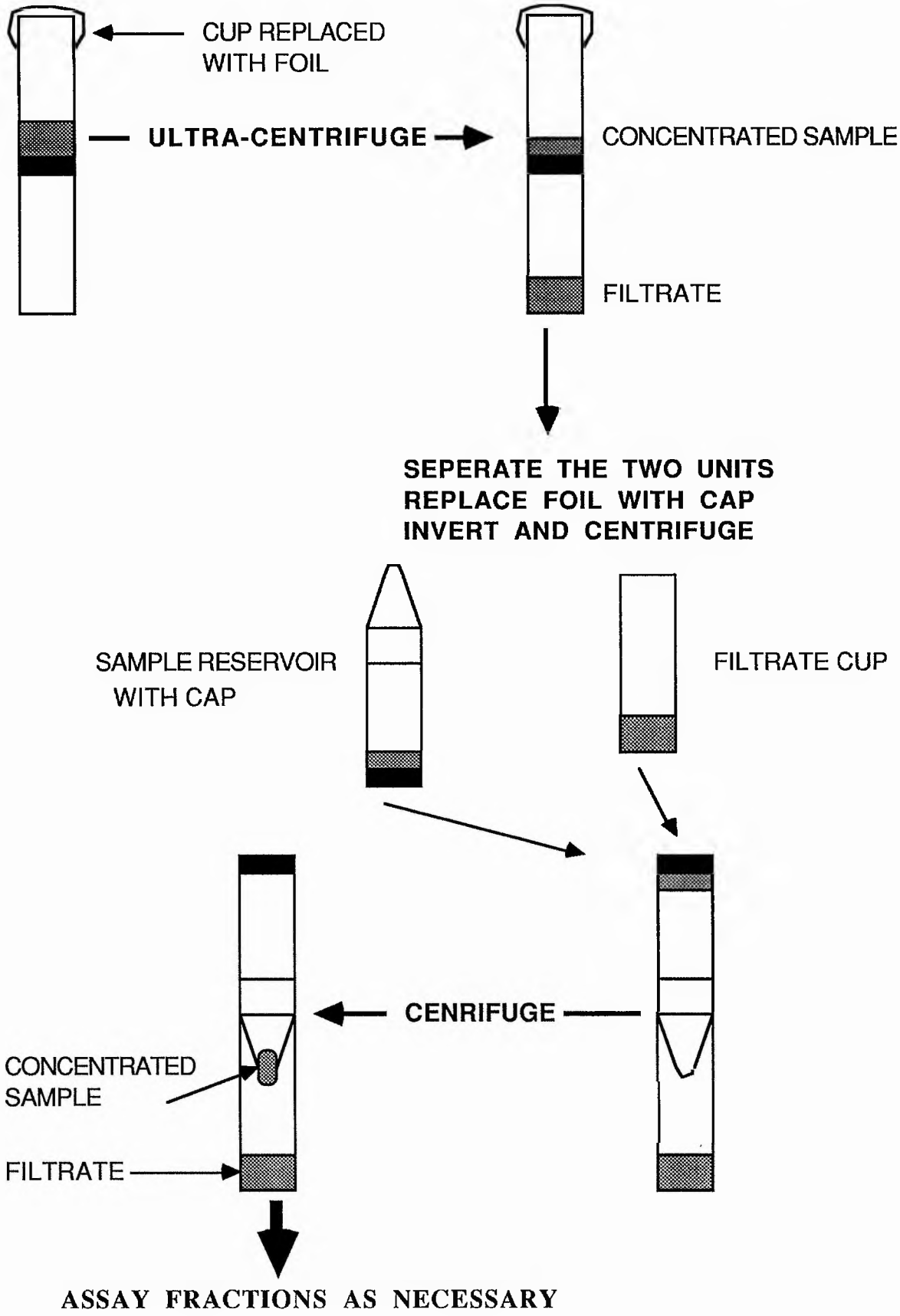


DIAGRAM 12 SUMMARY OF METHOD USED IN ULTRAFILTRATION

2 (1.4.2) Source of a haematopoietic stimulator-medium conditioned by regenerating bone marrow.

Following haematopoietic damage the normally quiescent stem cell compartment is 'switched on', primitive cells come into cycle generate progeny and the system is regenerated. (Becker *et al* (1965)). A consequence of this regeneration is thought to be the production by mononuclear phagaocytes of a stem cell *specific* cell stimulator. It has been determined that medium exposed to regenerating marrow will become rich in this stimulator and can then be used to stimulate quiescent 'stem cells'. (Lord *et al* (1977), Wright and Lord (1978), Wright *et al* (1982)).

To obtain stimulator CD1 mice were given 4.5Gy whole body γ-irradiation (CIS Biointernational IBL 437C 137Cs γ-radiation source with a dose rate of 4.66 Gy/min). Seven days later they were killed their femurs extracted and a single cell suspension of regenerating femoral marrow in Dulbecco's 20% made as described in 2 (1.3.1) (Robinson *et al* (1994)). Cellularity was then determined, adjusted to 15-25 x 10^6 cells/ml and the cell suspension transferred to a 30ml universal (Sterilin) . The universal was incubated in a water bath at 37°C for 3 hours. Thereafter the cell suspension was centrifuged, aliquoted and stored according to the protocol laid out for SA2 JMB1 cells in 2 (1.3.3). When thawed the medium was filtered through a 0.22μm pore filter (Millipore) into a 5ml plastic vial prior to use.

2.2: ASSAYS

2(2.1) COLONY FORMING CELL ASSAYS

2 (2.1.1) Cell culture in semi-solid agar

Much of the present knowledge concerning haematopoiesis has come about as a result of techniques allowing *in-vitro* cell study. In this work use is made of clonogenic assays for progenitor and more primitive cells in semi-solid culture medium stimulated with a variety of growth factors or conditioned medium. (e.g. L929 fibroblast cell line - a crude source of M-CSF / CSF-1 or WEHI 3B myelomonocytic Leukaemic cell line- a crude source of IL-3). The growth factor requirements and incubation times of individual assays may be manipulated to select for specific haematopoietic cell populations .

2 (2.1.2) Assay for the Granulocyte-Macrophage colony forming cell (GM-CFC)

This assay allows the development of colonies containing mature cells from two distinct myeloid lineages from the granulocyte-macrophage progenitor cells. (See introduction) In brief the assay involves single layer semi-solid agar culture of haematopoietic cells with growth factors, for 7 days. (Heyworth and Spooncer (1993)).

General protocol

A bottle of 3% (v/v) agar (Bactoagar, Difco) was suspended in a boiling water bath. In the sterile horizontal flow hood Dulbecco's 20% and growth factors in appropriate volumes were Pipetted into a 30ml universal. This was warmed for approximately five minutes in a 37^0c water bath. Eight 10ml triple vent non-tissue culture grade petri-dishes (Sterilin) were laid out in the hood and labelled. Femoral marrow or foetal liver cells extracted as outlined above were diluted to 5 x 10^5 /ml in Dulbecco's 20%. The warmed universal was then removed from the water bath swabbed and placed in the sterile hood. 1ml of the cell suspension was pipetted into the universal using the Gilson P1000. This was thoroughly mixed by gentle inversion of the universal. 1ml of the now boiling 3%(v/v) agar was added to the universal using a 1ml disposable sterile syringe (Becton Dickinson). This was thoroughly mixed by repeatedly drawing it up and down a 1ml c200v blue tip pipette on a P1000 Gilson Pipettman. 1ml of the mixture was then pipetted into each of the petri-dishes .Dishes were gently swirled around to spread the mixture out over the entire surface of the plate. The layer, which contained 0.3%(v/v) agar, relevant growth factors and 5 x 10^4 cells/ml (5 x 10^4 cells total), was then allowed to set. This took approximately ten minutes.

Incubation

Set cultures were stored in an alcohol sterilised plastic container and incubated in a 37^0c, 5% CO_2 in air, fully

humidified incubator for 7 days. The short life span of the cells within colonies means that after this time they will die and the colonies disperse.

Counting

After seven days dishes were removed from the incubator. Using a stereoscopic zoom microscope (Kyowa, Tokyo) at magnification setting 1.5, colonies were counted using a tally counter(ENM England). In this assay a colony is taken to be a group of 50 or more cells. Three types of colony may appear: the macrophage colony (appears as a large dispersed group of cells); the granulocyte colony (appears as a tighter 'ball' of cells) and the mixed granulocyte-macrophage colony (an inner dense centre with a periphery of more dispersed cells) (Heyworth and Spooncer (1993)). Although this is subjective where relevant it is possible to note the number of each colony type per dish. This can be checked by cytological studies on any individual colony. Differential counting of this sort was not routinely used throughout this work. The emphasis was directed towards alterations in overall colony numbers. A summary of the protocol is shown in diagram 13

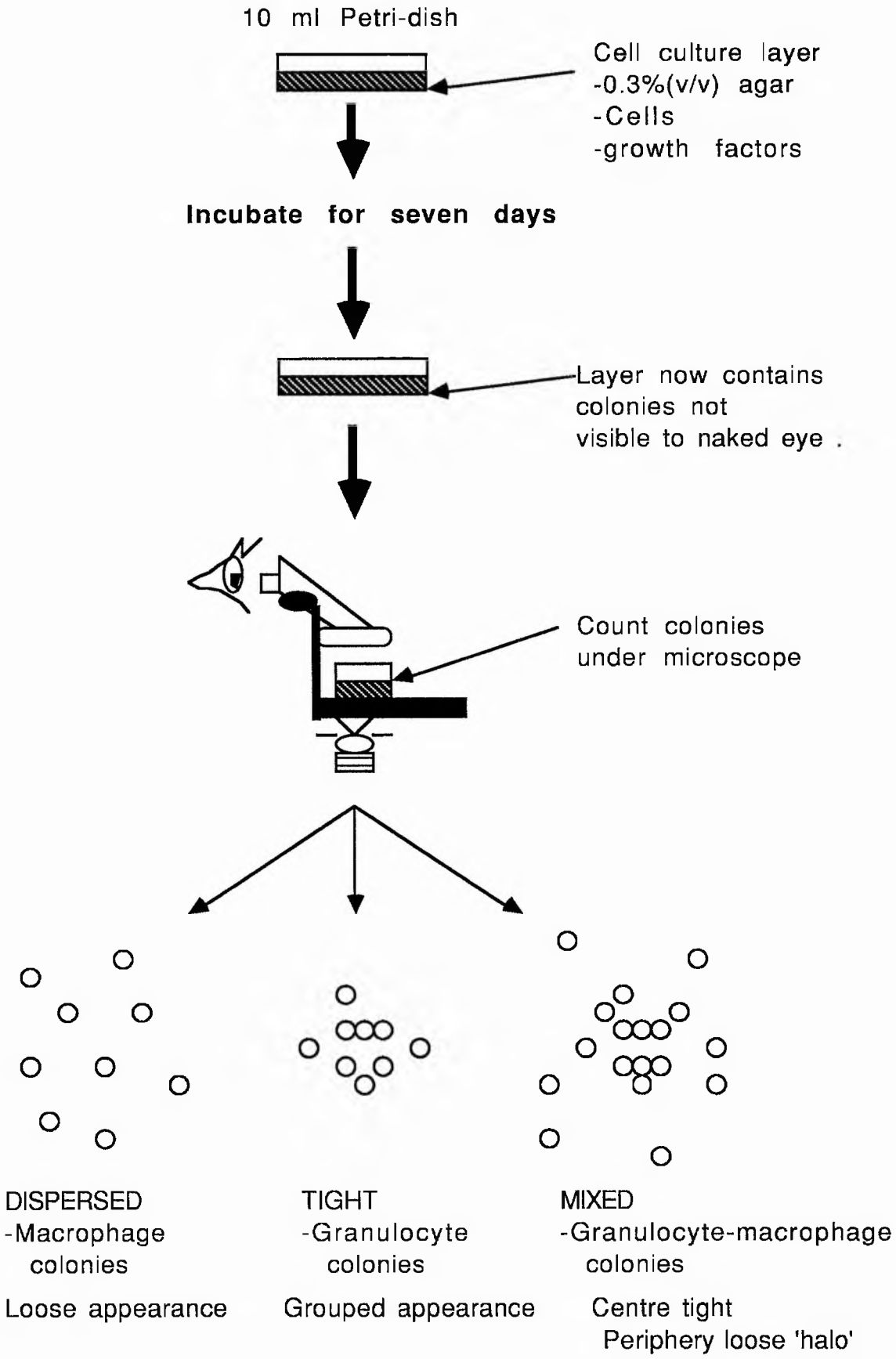


DIAGRAM 13 SUMMARY OF THE GM-CFC ASSAY

2 (2.1.3) Assay for the High Proliferative Potential Colony-Forming Cell (HPP-CFC)

In the HPP-CFC assay cells more primitive than those seen in the GM-CFC assay may be stimulated to form colonies with a combination of growth factors. (Bradley and Hodgson (1979)) (See introduction). Briefly the assay involves semi-solid agar bi-layer culture of haematopoietic cells. The basal layer the 'feeder layer' is loaded with defined growth factors and 0.5% (v/v) agar (Bactoagar, Difco). This makes it an excellent growth factor supply, but, due to its high agar content is itself unable to support colony formation. A second layer, the 'culture layer', containing the cells and 0.3%(v/v) agar overlays the 'feeder layer'. The lower agar content of this 'culture layer' means that cells within it responding to growth factors leached from the 'feeder layer' will be able to proliferate to form colonies. As the HPP-CFC compartment is heterogeneous altering the growth factors in the feeder layer will alter the cell type responding in the culture layer. (See chapter 1) Thus different HPP-CFC populations may be assayed.

General protocol

Feeder layer

A bottle of agar 5%(v/v) (Bactoagar, Difco) was suspended in a boiling water bath. In the sterile horizontal flow-hood Dulbecco's 20% and relevant growth factors in appropriate

volumes (see below) were pipetted into a 30ml universal.This was warmed for approximately 5 minutes in a 37^0C water-bath. Four 50ml diameter triple vent non-tissue culture grade petri-dishes (Sterilin) were laid out in the sterile hood and labelled. The universal was removed from the water bath swabbed with 70%(v/v) alcohol and placed into the hood. Immediately 1ml of the now melted 5%(v/v) agar was added to the universal via a 1ml disposable, sterile syringe (Becton Dickinson). The mixture was thoroughly mixed by repeatedly drawing it up and down a 1ml pipette on a P1000 Gilson Pipettman 2ml of this mixture was then immediately plated into each of the petri-dishes using the Pipettman. The dishes were then allowed to set (approximately five minutes).

Culture layer

A bottle of 3% (v/v) agar (Bactoagar, Difco) was suspended in a boiling water bath. In the sterile hood 8ml Dulbecco's medium was Pipetted into a 30ml universal. This was warmed for approximately five minutes in a 37^0c water bath. The petri-dishes with feeder layers were either ready or, if they had been placed in an incubator to avoid a pH swing, were laid out in the sterile hood. Cells extracted as outlined were diluted to a concentration of 2 x 10^5 cells ml in Dulbecco's (This cell number was selected because previous work indicated that it contained sufficient HPP-CFC to count comfortably without overcrowding of the dish). The warmed universal was then removed from the water bath swabbed and placed in the sterile hood. 1ml of the cell suspension was pipetted into the

universal using the Gilson P1000. This was thoroughly mixed by gentle inversion of the universal. 1ml of the now boiling 3%(v/v) agar was added to the universal using a 1ml disposable sterile syringe. This was thoroughly mixed as outlined above. Using the Gilson P1000 , 2ml of the mixture was delicately pipetted over the feeder layer, particular care being taken not to puncture the set feeder layer. It was found that the best way to avoid this was to pipette in a spiral motion very near to the plate. The layer, which contained 0.3%(v/v) agar and 2 x 10^4 cells/ml (4 x 10^4 cells total), was then allowed to set. This took approximately ten minutes.

Incubation

Set cultures were stored in an alcohol sterilised plastic container and incubated in a 37^0c, 5% CO_2 in air, fully humidified incubator for 14 days.

Staining

Approximately 12 hours before assay cultures were removed from the incubator and 1ml of an autoclaved solution of 1mg 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazoliumchloride (INT)/ml/)0.9%(w/v) NaCl (BDH) was added to each culture. The plates were then returned to the incubator. Over the next twelve hours the INT was metabolised by viable cells to a red tetrazolium salt. This was visible as a dark staining within colonies.

Counting

After twelve hours further incubation the cultures were removed from the incubator and colony numbers per plate assessed. This was accomplished by placing a 1mm acetate grid beneath a plate, scanning for colonies of a given size and counting them.

The protocol for counting were :

a) For cultures stimulated with conditioned media as a source of growth factor HPP-CFC derived colonies were those greater than **2mm** in diameter.

b) For cultures stimulated with recombinant growth factors HPP-CFC derived colonies were those greater than **1mm** in diameter.

The protocol is summarised in diagram 14. Photographs of stained HPP-CFC colonies are shown in figure PH1.

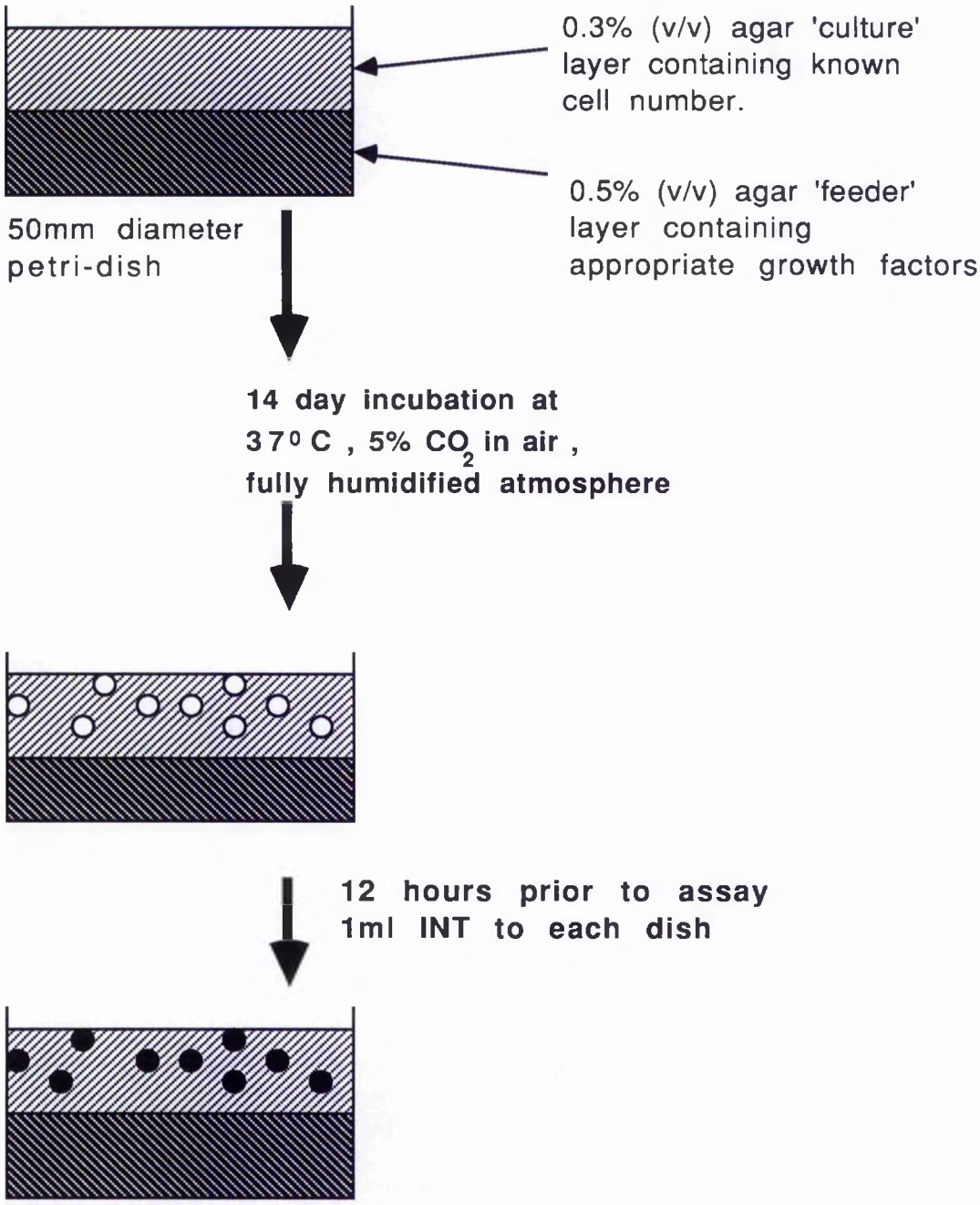
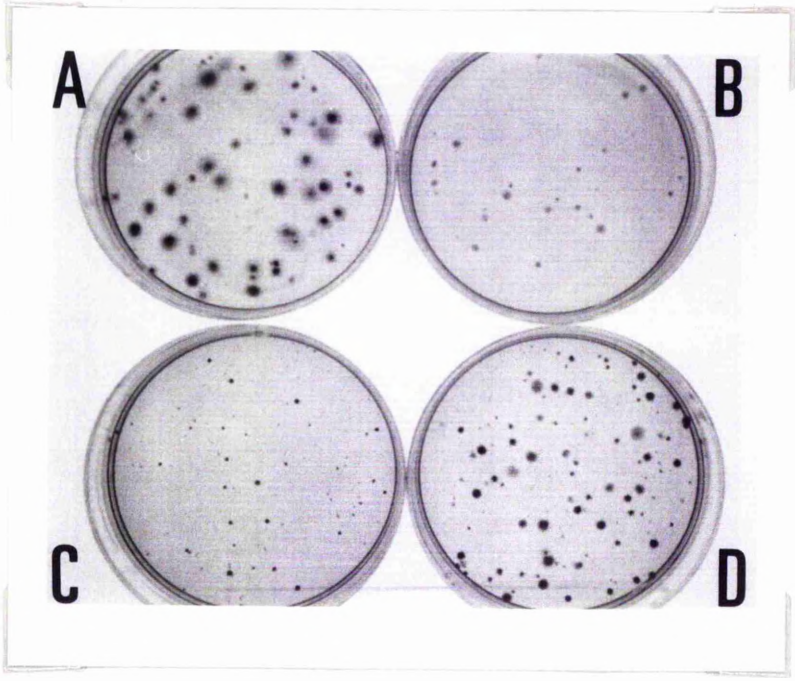


DIAGRAM 14 A SUMMARY OF THE HPP-CFC ASSAY

WEHIcm/L929cm **GM-CSF/M-CSF**

A **B**



IL-1α / IL-3 **IL-1α /IL-3/M-CSF**

C **D**

FIGURE PH1 : The effect of varying growth factor regimes on colony formation in 14 day semi-soild agar assays of NBM .

2(2.1.4) Assay for SA2JMB1 clonogenicity in the absence of growth factors

In brief this assay involved 7-day single layer semi-solid agar culture of a leukaemic cell line in the presence or absence of growth factors.

General protocol

A bottle of 3% (v/v) agar (Bactoagar, Difco) was suspended in a boiling water bath. In the sterile horizontal flow hood 8ml Dulbecco's 20% was pipetted into a 30ml universal. This was warmed for approximately five minutes in a 37^0C water bath. Eight 10ml triple vent non-tissue culture grade petri-dishes (Sterilin)were laid out in the hood and labelled. A single cell suspension of SA2JMB1 cells prepared as outlined above was adjusted to 2 x 10^4/ml in Dulbecco's 20%. The warmed universal was then removed from the water bath swabbed and placed in the sterile hood. 1ml of the cell suspension was pipetted into the universal using the Gilson P1000. This was thoroughly mixed by gentle inversion of the universal. 1ml of the now boiling 3%(v/v) agar was added to the universal using a 1ml disposable sterile syringe (Becton Dickinson). This was thoroughly mixed by repeatedly drawing it up and down a 1ml c200v blue tip pipette on a P1000 Gilson Pipettman. 1ml of the mixture was pipetted into each of the petri-dishes .Dishes were gently swirled around to spread the mixture out over the entire surface of the plate. The layer, which contained

0.3%(v/v) agar, and 2 x 10^3 cells/ml (5 x 10^3 cells total), was then allowed to set. This took approximately ten minutes.

Incubation

Set cultures were stored in an alcohol sterilised plastic container and incubated in a 37^0c, 5% CO2 in air, fully humidified incubator for 7 days. The short life span of the cells within colonies meaning that after this time they will die and consequently the colonies would disperse.

Counting

After seven days dishes were removed from the incubator. Using a stereoscopic zoom microscope (Kyowa, Tokyo) at magnification setting 1.5 colonies were counted using a tally counter(ENM England). In this assay a colony is taken to be a group of 50 or more cells. The colonies are very different to those seen in the GM-CFC assay being much tighter, denser centres of proliferation. This is convenient as it means that should leukaemic clones become differentiated any differences in colony type which may arise as a result would be obvious. Such observations could be confirmed by cytological studies.

2(2.1.5) Assay to measure the effect of preincubation with exogenous factors on the size of a colony forming cell pool

It was important not only to determine the effect of exogenous factors on colony formation by haematopoietic cell populations ,

but also to determine the effect on these populations of preincubation with factors, prior to assay. Femora were removed from mice and a single cell suspension prepared in Dulbecco's 20% as outlined in 2(1.3.1).Cellularity was determined and adjusted to 5 x 10^6 cells/ml. The required volume, dependent on the number of exogenous fcators under assay, of this dilution was prepared. One red-topped conical bottomed centrifuge tube for each test condition, was swabbed with 70% alcohol and placed into the sterile hood. Using the P1000 Gilson Pipettman and C200v tips, 1ml aliquots of cell dilution were pipetted into each tube. Using C20v tips appropriate dilutions of relevant factors were aliquoted into each tube. The pipet tip was brought just into the 1ml cells to ensure that the entire aliquot was delivered. A control tube, received an aliquot of Dulbecco's 20% only. The tubes were then incubated in a 37°C water bath for 2 hours with occasional vortex mixing. At the end of this time 7ml Dulbecco's 20% was added to all tubes. Tubes were then washed twice by centrifuge spinning in the manner described in 2(1.3.1). Thereafter each washed cell pellet was resuspended in 1ml Dulbecco's 20% taking a fresh pipette tip for each tube. Cellularity was determined for each tube .2(1.3.4)

GM-CFC

The cells in each tube were diluted to a concentration of 5x 10^5/ml. 2mls of this dilution was prepared. A GM-CFC assay stimulated with the relevant growth factor was carried out for each tube. 2(2.1.2)

HPP-CFC

The cells in each tube were diluted to a concentration of 2 x 10^5 cells/ml. 4mls of this dilution was prepared for each tube. Assays for HPP-CFC stimulated with relevant factor combinations were performed for each tube .2(2.1.3)

2 (2.1.6) Assay to measure the uptake of tritiated thymidine by femoral bone marrow in limited volume liquid suspension culture

In this experiment samples of whole bone marrow were cultured in limited volume liquid suspension culture with a known or unknown growth factor (s). Tritiated thymidine is taken up by cells in DNA synthesis. If a population of cells is treated with tritiated thymidine for a given time then washed any remaining radioactivity not washed away is a measure of thymidine uptake and is therefore an indication as to the proportion of a cell population in DNA synthesis over the period for which the thymidine label was available. By adding tritiated thymidine to limited volume cultures stimulated with growth factor(s) it was thus possible to determine the effect of this growth factor on the proportion of the cell population in S-phase.

General protocol

Femora were removed and a single cell suspension prepared as noted in 2(1.3.1) above. Cellularity was determined and

adjusted to 5 x 10^5 cells/ml. A 96 well microtitre plate (Nunc) and lid (Nunc) were placed into the sterile hood and removed from packaging. The plate was divided up as necessary depending on the required number of test conditions. Using the P200 Gilson Pipettman and C20V tips, 100uL of cell suspension was pipetted into each of the well. Aliquots of test growth factors were pipetted into each well to a final well volume of two hundred ml.

Four replicate plates were prepared one for each of four time points.

Plates were then placed in four vented plastic containers sterilised with 70% alcohol and placed in the incubator at 37^0C with 5% CO_2 in air and a fully humidified atmosphere.

At twenty four hours

Twenty four hours after incubation the first plate was removed from the incubator to the sterile hood. Tritiated thymidine (Amersham LTD) was diluted from a stock of 37 Mega Bequerels to 300KBq/ml. 25ul of this was added to all wells in both blocks. The plate was replaced in the plastic container and returned to the incubator for 18 hours.

Harvesting the cells

The cells were harvested using a Titerteck cell harvester (Skatron Norway). The equipment consists of three major parts: a cell suction head; a filter holder and a wash disposal system. The cells suction head has 12 pairs of small pipes mounted in a plastic casing which line up with the 12 wells in any one row of

the microtitre plate. When activated one pipe carries the wash fluid (in this case saline-0.9%v/v NaCl) into each well, the other aspirates the wash and cells from each well .The filter holder consists of 12 O-rings mounted on a plastic casing which when locked onto a filter paper form 12 separate filter discs. Each disc corresponds to a well on the microtitre plate. The wash disposal system consists of a switch to control the wash cycle of cells and a collecting vessel in vacuum. When the switch is activated cells from the microtitre plate are washed to the filter paper where they are deposited, the remaining wash being sucked through into the collecting vacuum vessel. A separate switch to draw in air to 'dry' the filter paper after washing is also present.

Method

Having established a Vacuum in the collecting vessel a piece of glass fibre filter paper (Skatron) was inserted into the rear of the filter holder smooth side up. Notches on the filter paper allow it to be lined up with guides on the filter holder to optimize the number of wells sucked through one filter paper. The filter holder was then locked down onto the filter paper. The suction head was placed into an empty row of wells in a separate microtitre wash plate. The wash switch was pressed down (to operate) for 5 seconds. This pre-wet the filter paper in line with manufactures recommendations for optimal cell recovery. The wash plate was replaced with the experimental plate. The suction head was placed over the first row of wells to be harvested and the wash switch depressed for 10-15

seconds. The air switch was then depressed for 10-15 seconds. The filter clamp was gently (to prevent filter discs sticking to the filter clamp) released and the filter paper moved forward to the next filter point by carefully lifting it on a paper towel. The clamp was then locked down on the new section of filter paper and the entire process repeated for the next set of wells . After all wells had been washed the filter paper was gently placed in an oven (Macfarlane Robson LTD) at 60^0C for 1-2 hours, to dry.

Measuring Thymidine uptake

Thymidine uptake was measured using a 1214Rackbeta scintillation counter (LKB Loughborough). Using plain tweezers dry filter discs were pushed out into labelled counting vials, an accurate note of this process was made. 2ml of a liquid scintillation fluid 'Optiphase safe' (LKB) was aliquoted into each tube. Tubes were then placed into racks and inserted into the scintillation counter which was set to measure activity from a tritiated source. Briefly the machine measures very low levels of light. These are generated by decaying radioactive material liberating radiation (in this case Beta) which reacts with scintillant to give photon emissions. The level of light emission is translated by the counter to a number. The greater the number the greater the light emissions.

This whole process was repeated for each time point.

2 (2.1.7) Assay to measure the proportion of GM-CFC and HPP-CFC in DNA synthesis- The S-phase suicide assay.

Measuring the proliferative activity of GM-CFC or HPP-CFC requires a manipulation of the colony assay techniques outlined above. Cells which possess a colony forming potential will express that potential when plated in semi-solid agar supplemented with appropriate colony forming signals. This gives a measure of the clonogenicity of that population but does not tell us about the proportion of that clonogenic population in DNA synthesis at any one time. However, if, prior to plating, the cells are treated with an agent which will selectively destroy those cells in DNA synthesis, this killing effect would translate as a loss of colony-forming capacity in a colony assay. Thus there would be a decrease in the overall number of colonies which when compared to a control (which had not been treated with a killing agent) would give an indication as to the percentage of that population in S-phase. Such an S-phase specific killing agent is cytosine arabinoside (1-b-D-arabinofuranosylcytosine (ARA-C)). This agent is treated by the cell as cytosine being incorporated into the newly synthesized DNA. Unlike cytosine however, ARA-C sterically hinders the binding of subsequent bases to the DNA chain preventing successful replication and ultimately leading to cell death. Within normal femoral marrow *in-vitro* the HPP-CFC population is generally not cycling with a relatively low background value of 10-15% S-phase. The level of cycling in

the GM-CFC population however is higher with 20-30% in S-phase (Robinson *et al* 1993). Thus it is possible to test an exogenously added factor as a possible stimulatory signal for normal HPP-CFC and as an inhibitor for normal GM-CFC .

General protocol

A known dilution of cells is aliquoted into paired red topped conical bottomed centrifuge tubes, one pair for each exogenous factor under study plus one control pair .The exogenous factor under study is then added to both tubes in each pair. Tubes are then incubated in a water bath for a given number of hours dependent on whether a factor is being tested as an inhibitor (4hr) or stimulator (2hr). At the end of this incubation Ara-C (the S-phase specific killing agent) is added to one tube in each pair and all tubes returned to the water bath for a further one hour incubation. At the end of this time cells are washed resuspended, counted, diluted and assayed using either the HPP-CFC or GM-CFC assays described above.

2 (2.1.7.1) Manipulation of the percentage HPP-CFC in S-phase

The assay outlined above is limited in that it can examine only the effect of potential stimulators on HPP-CFC. It is often desirable to test the potential of an exogenous factor as an inhibitor for HPP-CFC cycling. In order to do this it is first necessary to bring the quiescent HPP-CFC into cycle. This can be accomplished in two ways:

Direct

1 By introducing an 'insult' to the femoral marrow.

In this case mild irradiation was used to push the HPP-CFC population into cycle (Robinson *et al* 1993).

Regenerating femoral bone marrow.

CBA mice were given 2Gy whole body g-irradiation (CIS Biointernational IBL 437C 137Cs γ-radiation source with a dose rate of 4.66 Gy/min). Three days later they were killed their femurs extracted and a single cell suspension of regenerating femoral marrow in Dulbecco's medium made as described above. (Note that the cells were NOT washed by centrifuge spinning). Cellularity was determined, adjusted to 5 x 10^6 cells/ml. Exogenous factors could then be tested as an inhibitor of HPP-CFC cycling in this population following the protocol in 2(2.1.4.2.)

Indirect

Normal femoral marrow can be 'switched on' by the addition of a factor which brings the quiescent HPP-CFC population into DNA synthesis. One such stimulator of DNA synthesis in the primitive stem cell pool is the conditioned medium derived from 7 day post 4.5 grey gamma irradiation mice as outlined in 2(1.4.2). Normal femoral marrow cells are pre-treated with this stimluator in the presence or absence of exogenous factors. An S-phase suicide assay for HPP-CFC is then performed as

outlined in(2(2.1.4.2)). The level of S-phase kill can then be taken to show whether exogenously added factors have acted to inhibit the stimulation of HPP-CFC.

2(2.1.8) Assay to measure the ability of exogenous factors to interfere with the action of a stem cell stimulator- the 'stimulator-block assay'

This assay is designed to study the effects of exogenous factors on the action of a stem cell stimulator defined above (2(2.1.5.2)). It is essentially a refinement of the stimualtor assay.

General protocol

A known dilution of cells is aliquoted into paired red topped conical bottomed centrifuge tubes, one pair for each exogenous factor under study plus one control pair. 1ml of 'stimulator' was then aliquoted into all but the control tubes to which 1ml of Delbecco's 20% medium is added. The exogenous factor under study is then added to both tubes in one pair, to a second pair and the control pair an equal volume of medium is added. The tubes are then incubated in a water bath for 2hr. Thereafter the assay continues as a standard S-phase suicide assay 2(2.1.7).

Assays for HPP-CFC stimulated with relevant factors are performed for each tube.

2(2.1.9) Assay to measure the ability of exogenous factors to interfere with the production of a stem cell stimulator- the 'stimulator production block assay'

Exogenous factors could prevent cell cycling by interfering with the action of cell stimulator or the manufacture of cell stimulator. This technique assays the ability of factors to block the synthesis of stem cell stimulator.

General protocol

To obtain stimulator the protocol in 2(1.4.2) was used. Post femur extraction cells were centrifuge washed twice 2(1.3.1). Cellularity was determined and adjusted to 25 x 10^6 cells/ml. As much as possible of this dilution was made- ideally twelve ml or more. For every four ml of cell dilution 1 red-topped conical bottomed centrifuge tubes was swabbed with 70% alcohol and placed into the sterile hood. Using the Gilson Pipettman 4ml aliquots of cell dilution were pipetted into each tube. The exogenous factor under study was then added to each relevant tube and an equal volume of medium to the control tubes. The tubes were then incubated in a water bath at 37°C for 3 hours. Thereafter the cell suspensions were centrifuged aliquoted and stored according to the protocol laid out for conditioned media, above 2(1.4.2). When thawed the medium was filtered through a 0.22µm pore filter (Millipore) into a 5ml plastic vial prior to use.

The medium was then used as a source of stimulator in a the stimulator assay laid out in 2(2.1.7.1).

2.3: DATA

2(3.1) STATISTICS

2(3.1.1) Comparing data

Data was always normalised as a percentage of the control. That is in any experiment where results were not quoted as a percentage (e.g colony assays) the control was set as 100%. All other data within the experiment was expressed as a percentage value relative to this control .

For example consider specimen experiment 1:

	CONTROL	CONDITION A	CONDITION B
MEAN COLONY NUMBER	24	24	20
AS A PERCENTAGE	100%	24/24 X 100 100%	20/24 X 100 83%

This meant that data between experiments could be compared as it was expressed in absolute terms.

Data was evaluated with respect to controls by comparing the mean of the treatment condition data with the mean of the control data via the Student's T-test.

This is a parametric test for assessing whether the means of two populations can be regarded as equal. The initial assumption is that the two means are the same (the null

hypothesis). If it turns out that there is a very low probability of obtaining the results when the null hypothesis is true we reject it in favour of the alternative hypothesis. This is significance testing and the critical level of probability used to reject the hypothesis is known as the significance level (P value). This is set at 5% (p=0.05) to avoid errors known as type 1 and type 2. The assumption is that given a normal distribution with a critical value of 5%, 2.5 % of the distribution lies in the upper tail of the curve and 2.5% in the lower tail. Hence we use a two-tailed t-test with a critical value of 5% (p=0.05). If the P value is smaller than 0.05 then the data under comparison are said to be significantly different. If P is greater than 0.05 then the opposite is true.

2(3.1.2)Determining the percentage in S-phase

Post assay colony numbers for each condition were known. For any one condition comparing the colony numbers in the cultures derived from cells which were treated with the S-phase specific killing agent, ARA-C, with colony numbers derived from those treated only with Dulbecco's 20% or relevant media gave an indication as to the percentage of the population in S-phase. Comparing the percentage in S-phase in the control group with the percentage S-phase in the treatment groups indicated whether or not treatment had altered the fraction of the population in DNA synthesis.

2(3.2) STAINING AND PHOTOGRAPHY

2(3.2.1) Blood films

A drop of blood was placed in the centre of a labelled 76 x 26mm glass slide (BDH) about 1-2cm from the end. An identical slide was then placed just in front of the drop on the first slide at an angle of forty five degrees. The second slide was then moved back to touch the drop and spread quickly along the length of the first slide drawing out the blood. The sample was then air dried.

2(3.2.2) Agar colony plates

Post counting the colony plates were stored at 4^0C for at least 1 hour. A shallow plastic container was filled with cold Isoton solution. The fact that this is Isotonic prevents the cells in colonies from lysing and stops colony degradation. Colony plates were removed from the fridge and gently submerged in the Isoton. Using a dissecting needle the agar disc containing the colonies was gently teased from the dish to be free floating in the Isoton. A 76 x 51mm microscope slide was submerged in the Isoton and used to catch the agar disc. The slide with the disc on top was delicately withdrawn from the Isoton. A 4.5cm piece of filter paper (Whatman) was placed on top of the agar disc and everything then placed on a drying plate (Hearson) at 50^0C for 2-4 hours. Thereafter the filter paper was removed from the agar disc which had now dried onto the slide. The slide was ready for staining .

2(3.2.3) Jenner-Giemsa staining

The selective staining of components within the cell relies on differences in chemical structure. With Romanowsky dyes such as Jenner and Giemsa acidic and alkaline differences in chemical composition are exploited to allow selective differential staining. Jenner's stain is the most complex of the Romanowsky stains Giemsa the most primitive. When used in conjunction they give results superior to those achieved when using either singly, or indeed, any of a variety of other stains.

After preparation, slides were immersed in methanol (BDH) for 10 mins to fix the cells. They were then transferred to a staining jar containing 168ml Jenner's stain/ 132ml water for 5 mins. The slides were then removed and rinsed in water before being submerged in a second staining jar containing 45ml Giemsa / 225 ml water. The slides were left there for 10mins before being rinsed. They were then sat on end and left to air dry. The slides could then be examined under the microscope.

CHAPTER 3: LEUKAEMIA INHIBITORY FACTOR

3.1: FEMORAL MARROW

In this section Leukaemia Inhibitory Factor was assessed *in vitro* as an agent influencing certain aspects of haematopoiesis. This fell into two broad categories: **direct action,** where LIF's actions on cells derived from femoral marrow were assessed; **indirect action**, where LIF's actions on agents which act on cells derived from femoral marrow were assessed. In all experiments (unless otherwise noted) three concentrations of LIF were tested: 1000units/ml; 500 units/ml and 100/units ml. These were prepared by diluting LIF in Dulbecco's 20% to concentrations of 25,000 units/ml; 12,500 units/ml and 2500 units/ml .Identical volumes (40µl) of each dilution added per1ml of culture fluid gave 1000units/ml; 500 units/ml and 100 units/ml respectively. The concentrations chosen were based on previously published data (Metcalf, 1991; Fletcher, 1990; Metcalf, 1989).

All results for individual experiments were normalised as a percentage of the control. This validated the comparison of data between individual experiments and also meant that data could be pooled as a mean.

An unpaired two-tailed t-test was used to analyse data. (See 2(3.1.1)) P values are shown below means. The means of data for individual experiments are given below the P value.

DIRECT ACTION

3(1.1) The effect of Leukaemia Inhibitory Factor (LIF) on the colony forming potential of GM-CFC in semi-solid agar culture.

To determine the role LIF may play in the development of lineage restricted progenitor cell populations LIF was assayed in vitro for colony stimulating activity on populations of GM-CFC (7 day semi-solid agar cultures).

3(1.1.1) PROCEDURE

In a standard GM-CFC assay varying concentrations of LIF were assessed both as an agent acting alone, and as a synergistic agent with the following growth factors:

GROWTH FACTOR	FINAL CONCENTRATION
GM-CSF	40U/ml
M-CSF	50U/ml
IL-3	80U/ml

TABLE 3.1: *Summary of factors used to stimulate 7-day cultures.*

In all cases three replicate experiments were performed.

3(1.1.2)RESULTS

Table 3R.1 and figure G3.1 show the effect of LIF on colony formation by populations of GM-CFC. In all cases (except for IL-3 stimulated colony formation supplemented with 100 U/ml LIF) the addition of LIF at any of the test concentrations had no

significant effect on colony formation when compared to controls. (p always > 0.05)

In the case of IL-3 stimulated colony formation + 100U/ml LIF, there is a statistically significant **reduction** in colony formation, compared to the control. (p<0.005) The reduction is not great: 94% ±1.2 with LIF, compared to 100% without. However it is present and is consistent throughout the three expreiment from which the mean value was derived (96%, 92% and 95%) .

TABLE 3R.1: The effect of Leukaemia Inhibitory Factor on the colony forming potential of GM-CFC from NBM in semi-solid agar culture.

LIF CONC. (U/ml)	**0**	**100**	**500**	**1000**
		COLONY FORMATION (%)		
FACTOR				
/	0	0	0	0
GM-CSF (40U/ml)	**100.0** 19.0±4.2 22.5±1.5 14.5±2.5	**114.0±8.0** 0.05<p<0.1 N=3 19.0±1.3 25.5±5.5 18.0±0.2	**99.6±6.0** p>0.4 N=3 17.2±5.3 22.5±5.5 16.0±7.0	**109.0±8.0** 0.1<p<0.375 N=3 22.5±5.5 21.0±2.0 17.0±3.0
M-CSF (50U/ml)	**100.0** 21.5±2.5 13.5±5.5 11.0±1.0	**106.0±17.5** 0.1<p<0.375 N=3 15.5±7.5 17.5±1.5 13.0±6.0	**122.0±31.9** 0.1<p<0.375 N=3 21.5±0.5 25.0±6.0 9.0±3.0	**118.0±17.0** 0.1<p<0.375 N=3 27.0±3.0 19.5±0.5 9.5±2.5
IL-3 (80U/ml)	**100.0** 15.5±0.5 16.3±2.5 11.2±1.4	**94.0±1.2** p<0.005 N=3 15.3±1.4 15.1±3.5 11.5±0.5	**120.0±12.8** 0.05<p<0.1 N=3 17.0±1.0 16.0±5.2 17.3±0.5	**88.0±12.9** 0.1<p<0.375 N=3 15.0±0.4 17.3±3.5 7.0±5.3

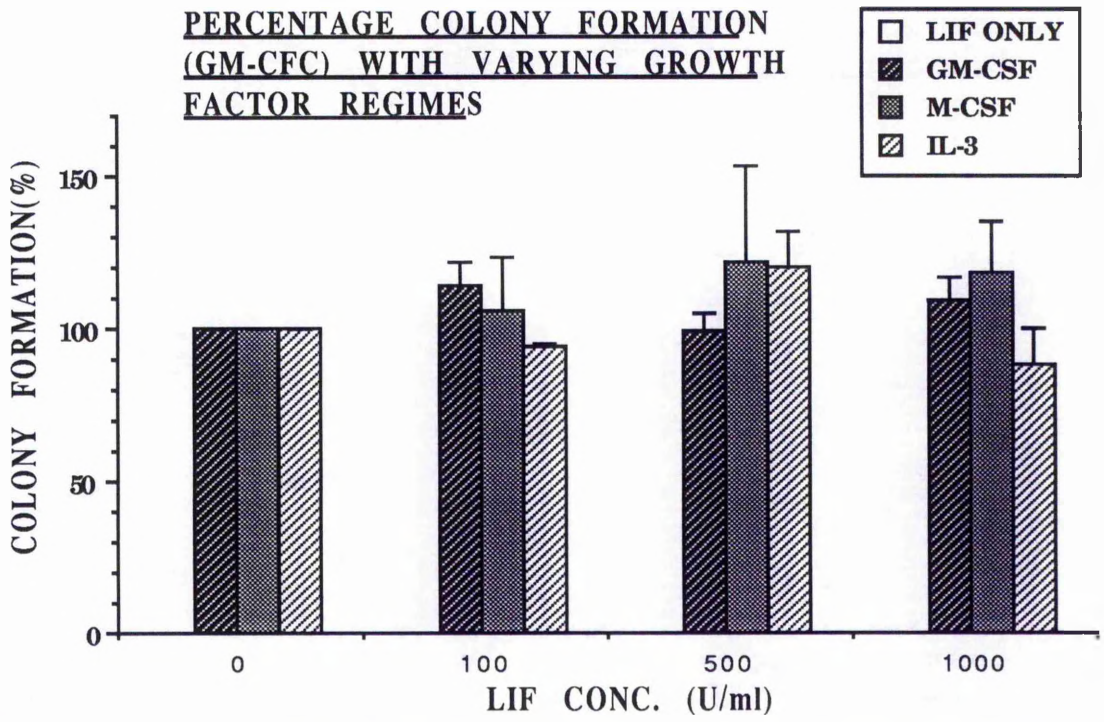


FIGURE G3.1: Colony formation (expressed as percentage of control 0U/ml LIF) in 7-day semi-solid agar cultures of NBM stimulated with varying growth factor regimes in the presence or abscence of varying concentrations of LIF.

3(1.2) The effect of LIF *in vitro* on the colony forming potential of HPP-CFC in semi-solid agar culture.

To determine the role LIF may play in the development of early stem cell populations, LIF was assayed *in vitro* for colony stimulating activity on populations of HPP-CFC (14 semi-solid agar cultures).

3(1.2.1) PROCEDURE

In a standard HPP-CFC assay LIF was assessed both as an agent acting alone and as an agent acting synergistically to promote HPP-CFC colony formation. HPP-CFC populations were stimulated with the following growth factor combinations:

In all cases three replicate experiments were performed.

GROWTH FACTORS	FINAL CONCENTRATION
L929 CM/	10%V/V
WEHI CM	10%V/V
GM-CSF	40U/ml
M-CSF	50U/ml
IL-3	80U/ml
M-CSF	50U/ml
IL-1α	10U/ml
IL-3	80U/ml
M-CSF	50U/ml
IL-1α	10U/ml
IL-3	80U/ml
IL-1α	10U/ml
M-CSF	50U/ml

TABLE 3.2: *Summary of factors used to stimulate 14 day HPP-CFC cultures.*

3(1.2.2)RESULTS

Table 3R.2 and figure G3.2 show the effect of LIF on colony formation by populations of HPP-CFC. In all cases the addition of varying concentrations of LIF has had no statistically significant effect on colony formation in 14 day culture. (P always > 0.05) .

Thus LIF does not act directly to support the proliferation of an HPP-CFC population, nor is it synergistic with other growth factors for the proliferation of HPP-CFC sub-populations.

TABLE 3R.2: The effect of Leukaemia Inhibitory Factor on the colony forming potential of HPP-CFC from NBM in semi-solid agar culture.

LIF CONC. (U/ml)	0	100	500	1000
		COLONY	*FORMATION*	(%/control)
FACTOR				
...../.....	0	0	0	0
L929/WEHI	**100.0** 26.5±2.8 19.8±1.1 23.6±1.2	**103.0±10.0** 0.375<p<0.4 N=3 25.8±1.2 23.5±0.6 21.3±1.4	**101.3±7.0** p>0.4 N=3 26.4±2.1 23.0±1.1 23.5±0.7	**92.3±5.7** 0.1<p<0.375 N=3 25.8±2.0 19.7±2.0 19.3±1.8
GM-CSF/ M-CSF	**100.0** 4.5±1.4 11.8±1.1 15.5±2.3	**125.0±28.0** 0.1<p<0.375 N=3 8.3±0.75 11.0±1.2 15.0±3.0	**124.0±26.0** 0.1<p<0.375 N=3 7.0±1.6 11.8±1.5 14.5±1.5	**111.0±12.2** 0.1<p<0.375 N=3 5.8±0.5 14.2±1.2 13.0±4.0
IL-3/ M-CSF	**100.0** 38.5±0.5 23.0±2.0 25.3±0.5	**98.0±2.0** 0.1<p<0.375 N=3 36.0±0.0 23.3±1.0 25.8±1.5	**92.0±5.0** 0.1<p<0.375 N=3 32.0±3.2 23.5±1.5 23.3±1.5	**93.0±5.1** 0.1<p<0.375 N=3 31.5±2.5 22.0±1.0 25.5±0.7
IL-1/IL-3/ M-CSF	**100.0** 9.5±0.5 12.0±1.0 14.5±2.5	**112.0±22.0** 0.1<p<0.375 N=3 13.5±2.5 15.0±1.0 10.0±2.0	**107.0±19.0** 0.1<p<0.375 N=3 13.5±2.5 12.5±0.5 11.0±1.0	**98.0±11.0** p>0.4 N=3 11.0±4.0 12.5±1.5 11.0±2.0
IL-1/IL-3	**100.0** 5.3±4.3 5.8±2.0 5.0±7.1	**119.6±18.0** 0.1<p<0.375 N=3 6.5±2.5 5.0±0.0 7.5±0.5	**100.6±9.2** p>0.4 N=3 6±3.0 4.8±2.4 5.3±1.0	**86.6±12.1** 0.1<p<0.375 N=3 4.3±2.4 4.0±1.8 5.5±2.0
IL-1/ M-CSF	**100.0** 15.3±3.5 15.0±1.0 11.5±0.5	**102.0±23.0** P>0.4 N=3 9.5±1.0 15.5±4.5 16.5±3.5	**81.7±12.0** 0.1<P≤0.375 N=3 8.8±1.0 14.5±1.5 10.5±2.5	**96.0±11.5** 0.375<P≤0.4 N=3 14.5±1.8 11.5±0.5 13.5±2.5

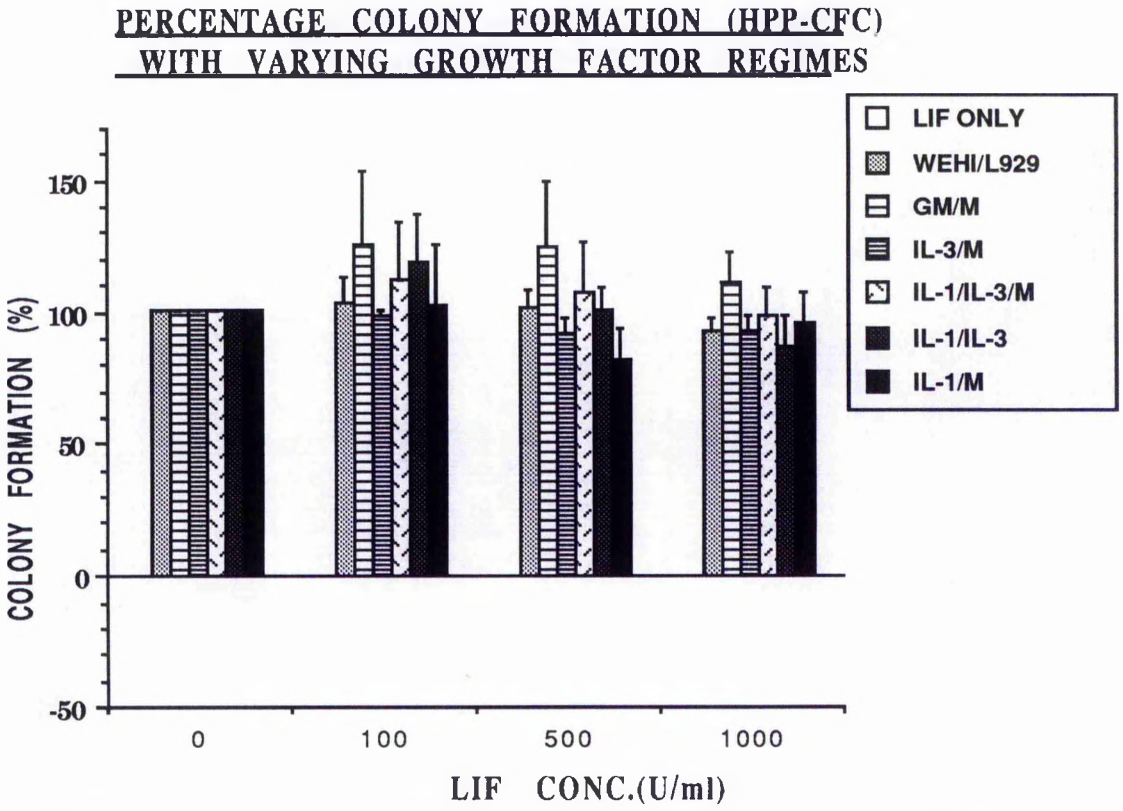


FIGURE G3.2: Colony formation (expressed as a percentage of the control 0U/ml LIF) in 14 day semi-solid agar cultures of NBM stimulated with varying growth factor combinations in the presence or absence of varying concentrations of LIF

3(1.3) The effect of LIF as a short term incubation agent in liquid suspension culture on the colony forming potential of GM-CFC and HPP-CFC.

The colony assay techniques above study the effect of non-toxic concentrations of LIF on a seeded colony forming cell population of given size. That is, in a cell population a given fraction will be a specific HPP-CFC subpopulation. The colony assays above pick out these HPP-CFC sub-populations without altering the size of the population .In contrast this experiment looked at the effect of pre-incubating femoral marrow for a

short time with non toxic concentrations of LIF to determine if this altered the size of the colony forming cell pool prior to plating. Washing the marrow after short term incubation ensured that LIF was only available to the cell population for a brief period and thus any alteration in colony forming cell pool size was a direct result of LIF.

In other words can LIF pre-incubation alter the size of HPP-CFC and/or GM-CFC populations ?

3(1.3.1) PROCEDURE

In a standard pre-incubation assay, cells were incubated with LIF for two hours. Thereafter assays for GM-CFC stimulated with 10% WEHIcm, and HPP-CFC, stimulated with WEHI/L929 conditioned media, GM-CSF/M-CSF (HPP 3) and IL-1α/IL-3 / M-CSF (HPP 1), were performed for each tube. The effect of LIF pre-incubation on colony formation in these assays could thus be assessed.

Three replicate experiments were performed.

3(1.3.2)RESULTS

In the case of GM-CFC (Table 3R.3.1/figure G3.3) pre-incubation with varying concentrations of LIF has had no statistically significant effect on colony formation in all cases ($p>0.05$), except cultures stimulated with IL-3 + 100U/ml LIF. ($p<0.05$). In the case of these cultures the difference is not great: 109%±3 with LIF, compared to 100% without. As LIF was not present in cultures but available only before assay this **increase** in colony formation suggests that LIF increased the size of the colony forming pool available to IL-3.

In all other cases LIF does not alter the size of the colony forming cell pool available to the growth factor stimuli.

In the case of HPP-CFC (Table 3R .3.2/figure G3.4) the addition of LIF has had no statistically significant effect on the level of colony formation from a variety of HPP-CFC populations. ($p>0.05$) Thus LIF does not alter the size of the HPP-CFC cell pool available to growth factor stimuli .

TABLE 3R.3.1: The effect of Leukaemia Inhibitory Factor as a short term pre-incubation agent on the colony forming potential of GM-CFC.

LIF CONC. (U/ml) PRE-INC	**0**	**100**	**500**	**1000**
		COLONY	***FORMATION***	**(%/control)**
FACTOR				
/	0	0	0	0
M-CSF (50U/ml)	**100.0** 19.8±1.1 17.0±2.0 18.0±1.1	**120.0±19.0** 0.1<p<0.375 N=3 18.3±1.3 27.8±3.5 20.0±3.0	**104.0±5.0** 0.1<p<0.375 N=3 22.5±3.0 18.0±2.8 17.0±1.8	**101.0±3.0** 0.1<p<0.375 N=3 18.5±3.0 18.8±1.4 19.0±2.0
IL-3 (80U/ml)	**100.0** 28.5±2.0 16.5±2.1 29.3±1.4	**109.0±3.0** 0.025<p<.05 N=3 32.8±4.8 18.5±2.6 30.0±1.1	**97.0±6.0** 0.1<p<0.375 N=3 31.3±5.4 15.5±0.5 26.0±1.0	**103.0±13.9** 0.375<p<0.4 N=3 29.5±2.4 21.0±3.7 23.8±7.0

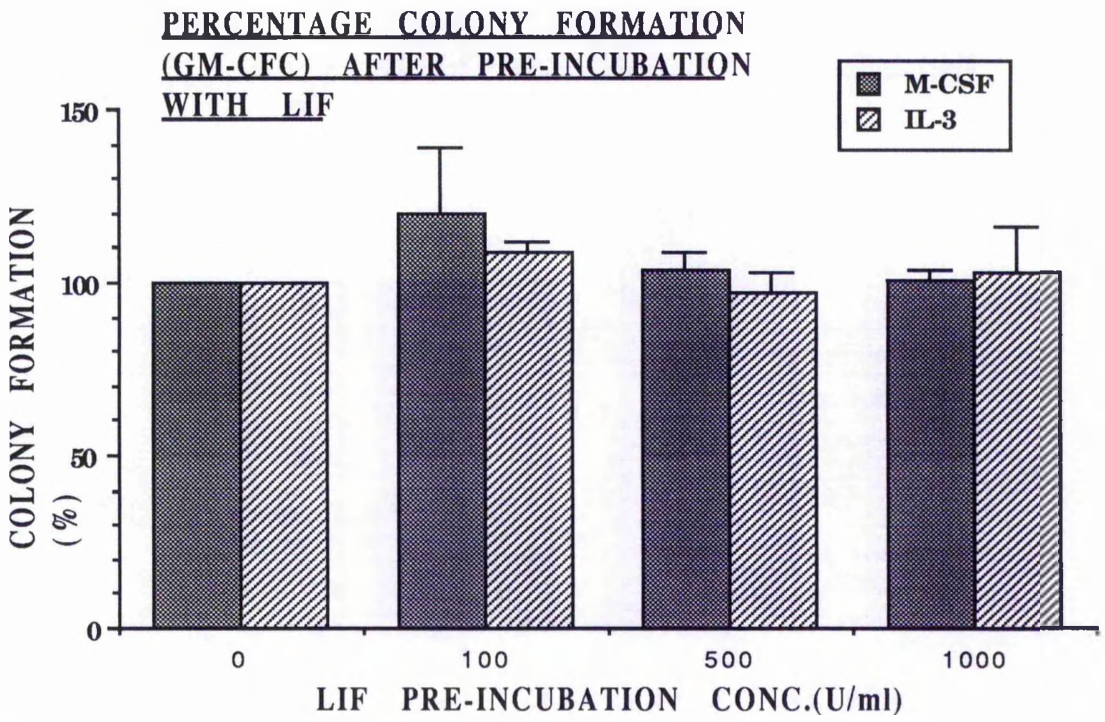


FIGURE G3.3: Colony formation (expressed as percentage of the control 0U/ml LIF) in 7-day semi-solid agar cultures of NBM stimulated with varying growth factor regimes after preincubation with varying concentrations of LIF.

TABLE 3R.3.2: The effect of Leukaemia Inhibitory Factor as a short term pre-incubation agent on the colony forming potential of HPP-CFC in semi-solid agar culture.

LIF CONC. (U/ml) PRE-INC.	0	100	500	1000
		COLONY	*FORMATION*	(%/control)
FACTOR				
...../.....	0	0	0	0
L929/WEHI	**100.0** 27.0±1.7 25.8±2.4 22.8±1.0	**102.0±3.0** 0.1<p<0.375 N=3 26.5±2.4 25.8±2.3 25.0±1.9	**108.3±14.8** 0.1<p<0.375 N=3 27.0±4.1 22.5±1.0 31.3±6.0	**107.0±7.3** 0.1<p<0.375 N=3 25.8±1.1 27.8±5.5 27.8±1.2
GM-CSF/ M-CSF	**100.0** 7.0±1.5 5.8±1.1 3.3±0.6	**106.0±13.4** 0.1<p<0.375 N=3 5.8±1.3 7.5±1.9 3.5±0.9	**101.8±13.9** p>0.4 N=3 5.5±1.5 7.3±1.1 3.3±0.9	**98.0±13.0** p>0.4 N=3 6.5±1.0 5.0±1.3 3.8±1.0
IL-3/IL-1 M-CSF	**100.0** 12.0±1.3 11.0±1.4 10.0±0.9	**115.3±17.7** 0.1<p<0.375 N=3 10.0±2.9 14.3±1.4 12.3±1.9	**86.0±18.7** 0.1<p<0.375 N=3 12.5±1.6 11.8±1.5 5.0±1.9	**103.0±11.0** 0.375<p<0.4 N=3 10.8±1.1 10.5±1.6 12.8±1.8

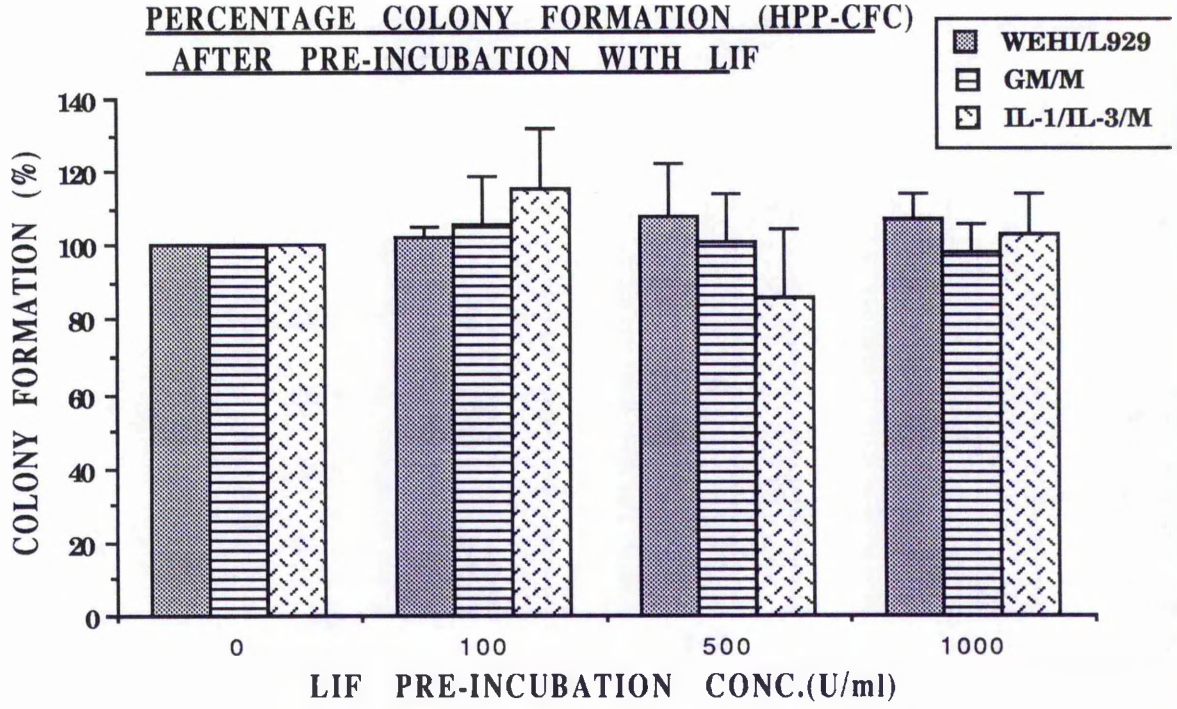


FIGURE G3.4: Colony formation (expressed as apercentage of the control) in 14 day semi-solid agar cultures of NBM stimulated with varying growth factor combinations after preincubation with varying concentrations of LIF

3(1.4) The effect of LIF *in vitro* on the proportion of GM-CFC and HPP-CFC in DNA synthesis-S-phase suicide assay

These experiments were designed to determine whether LIF could stimulate or inhibit DNA synthesis in populations of haematopoietic cells.

3(1.4.1) PROCEDURE

1000units LIF, 500 units LIF and 100 units LIF were assayed as potential stimulators and inhbitors of DNA synthesis in a modification of 2(2.1.5.2).

3(1.4.1.1)As a potential stimulator of DNA synthesis

HPP-CFC

In NBM the HPP-CFC population is essentially quiescent with only a small fraction ,10-15%, in S-phase. Thus it was possible to assess LIF as a stimulator of DNA synthesis in HPP-CFC populations .All tubes were incubated in a 37°C water bath for 2 hours and Cytosine arabinoside (ARA-C) used in a standard suicide assay 2(2.1.5).

The cells in each centrifuge tube were diluted to a concentration of 2 x 10^5 cells/ml. 4mls of this dilution was prepared for each tube. Assays for HPP-CFC stimulated with WEHI/L929 conditioned media, were performed for each tube. Three replicate experiments were performed.

3 (1.4.1.2) As a potential inhibitor of DNA synthesis

GM-CFC

In NBM a significant proportion of the GM-CFC population is in S-pase (30-40%). Thus it was possible to assess LIF as an inhibitor of DNA synthesis in this population.

The procedure was exactly as that outlined to test LIF as a stimulator in 3(1.4.1.1) with the exception that cells are incubated for three hours prior to the one hour ARA-C treatment. A standard GM-CFC assay with WEHIcm as a source of growth factor was carried out for the cells in each tube.

Four replicate experiments were performed.

HPP-CFC

To determine whether HPP-CFC can be switched out of cycle by LIF it is necessary to firstly bring the HPP-CFC into cycle. This is accomplished by introducing an 'insult' to the femoral marrow 2(1.3.1).

CBA mice were given 2Gy whole body γ-irradiation. Three days later femoral marrow was removed from mice and LIF was assessed as an agent able to inhibit DNA synthesis in HPP-CFC stimulated to colony formation with WEHI/L929.

Four replicate experiments were performed.

(Summary of protocol is shown in diagram 15)

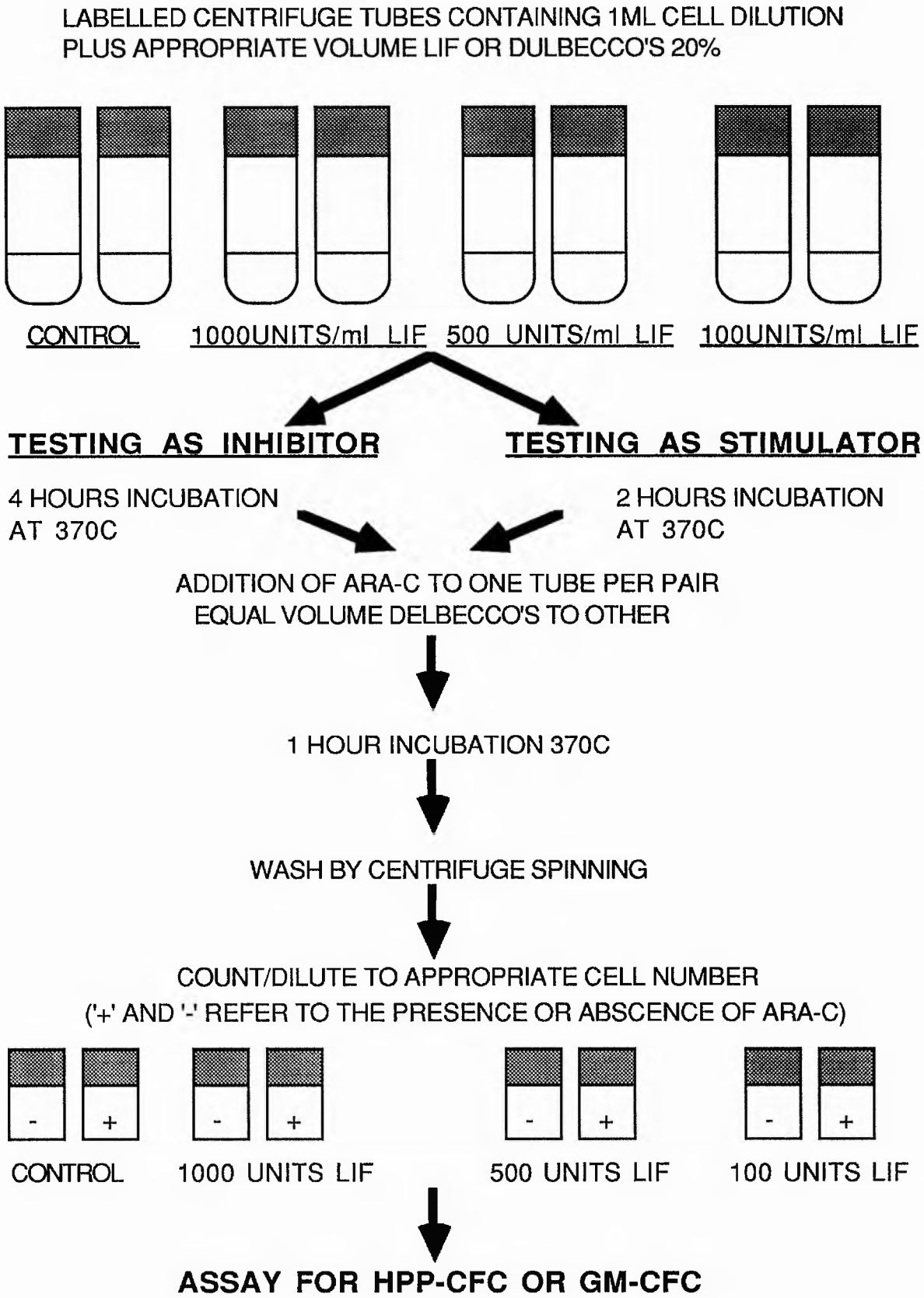


DIAGRAM 15 SUMMARY OF ASSAY USED TO TEST THE EFFECT OF LIF ON DNA SYNTHESIS

3(1.4.3) RESULTS

AS A POTENTIAL STIMULATOR

Table 3R.4.1and figure G3.5 show the effect of LIF on the proportion of the HPP-CFC population in S-phase. At all concentrations tested LIF has had no statistically significant effect on the proportion of HPP-CFC (WEHI/L929) in S-phase. ($P>0.05$).

AS A POTENTIAL INHIBITOR

Table 3R.4.2 and figure G3.6 show the results for GM-CFC treated with LIF. At all concentrations tested LIF has had no statistically significant effect on the proportion on GM-CFC (WEHI) in S-phase. ($p>0.05$).

Table 3R.4.3 and figure G3.7 show the results for HPP-CFC treated with LIF. At both concentrations tested LIF has had no statistically significant effect on the proportion of HPP-CFC in S-phase.

These results indicate that LIF does not stimulate or inhibit DNA synthesis in any of the cell populations tested.

The effect of Leukaemia Inhibitory Factor of GM-CFC(WEHI) and HPP-CFC(WEHI/L929) in DNA synthesis.

AS A STIMULATOR OF DNA SYNTHESIS

TABLE 3R.4.1: HPP-CFC

LIF CONC. (U/ml)	0	100	500	1000
	S-PHASE (%)			
FACTORS (HPP-CFC)				
...../.....	0	0	0	0
L929/WEHI	9.4±1 11.00 7.63 9.71	9.0±2.8 N=3 p>0.4 13.85 9.27 4.08	11.4±1.2 N=3 0.375<p<0.4 9.00 13.00 12.28	9.6±1.5 N=3 p>0.4 10.00 7.00 12.00

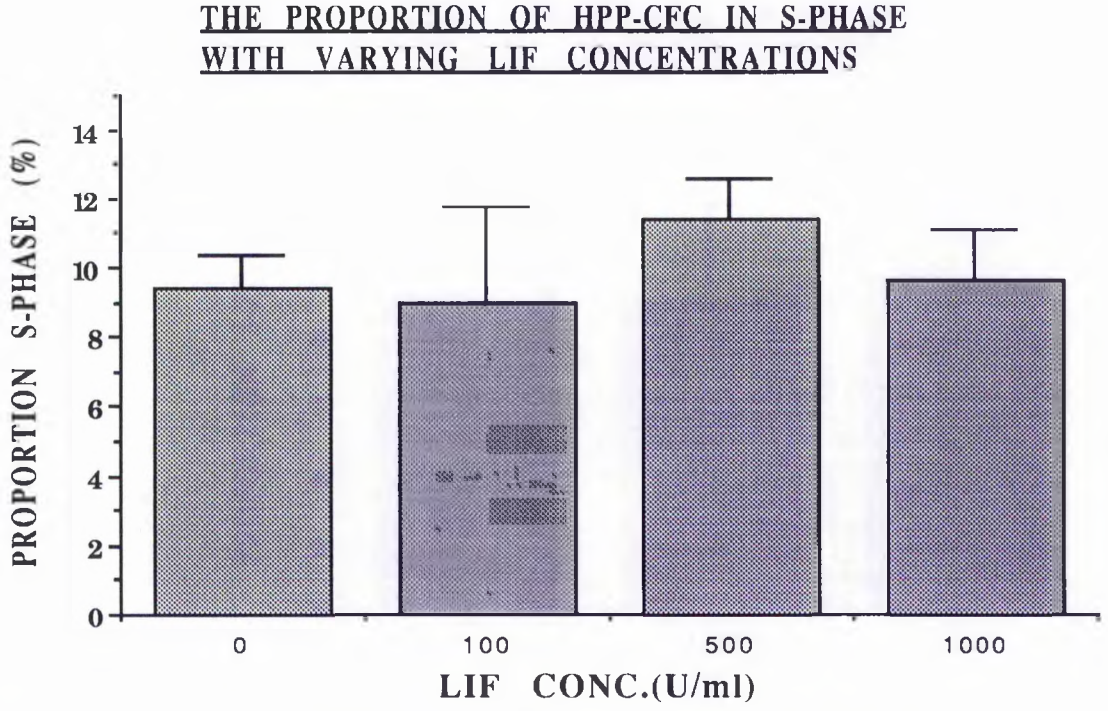


FIGURE G3.5: The proportion of HPP-CFC (WEHI/L929) from NBm in S-phase (%) following incubation with varying concentrations of LIF

AS A POTENTIAL INHIBITOR OF DNA SYNTHESIS

TABLE 3R.4.2: GM-CFC

LIF CONC. (U/ml)	0	100	500	1000
	S-PHASE (%)			
FACTOR (GM-CFC)				
...../.....	0	0	0	0
WEHI	**22.0±4.0**	**24.0±3.0**	**26.0±4.2**	**22.0±4.0**
		p>0.4	0.1<p<0.375	p>0.4
		N=4	N=4	N=4
	25.04	29.00	21.09	28.01
	18.05	30.00	17.08	31.07
	35.89	17.07	32.65	18.00
	13.70	20.13	35.30	11.00

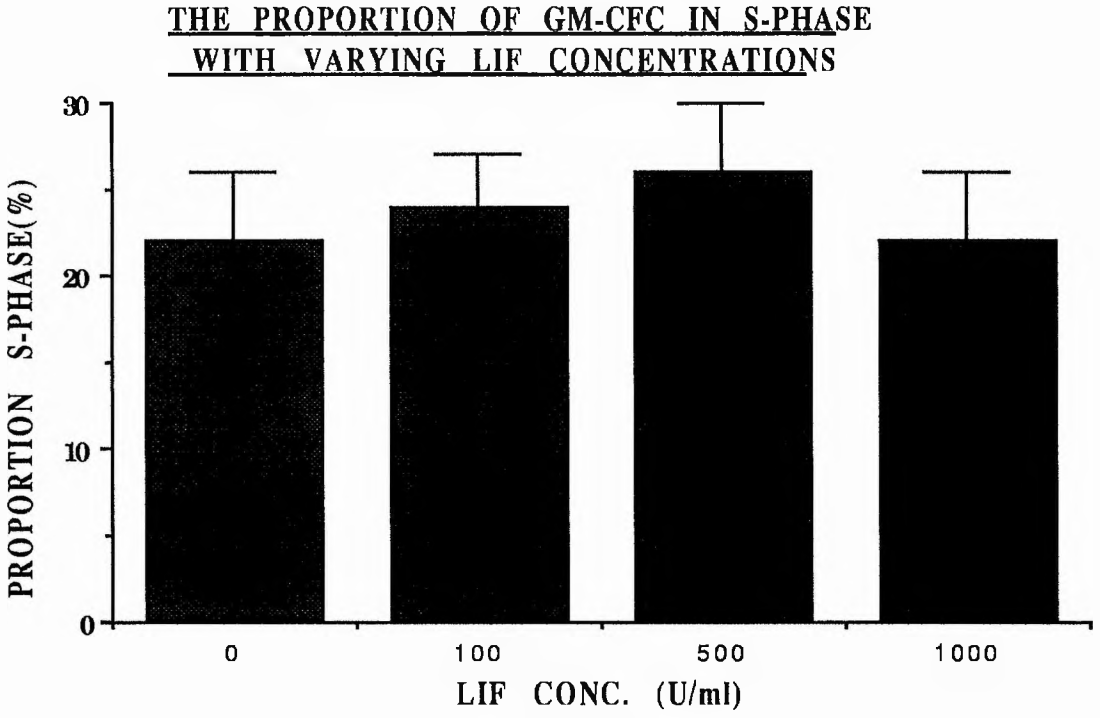


FIGURE G3.6: The proportion of GM-CFC (WEHI) from NBM in S-phase (%) following incubation with varying concentrations of LIF

TABLE 3R.4.3: HPP-CFC

LIF CONC. (U/ml)	0	100	1000
	S-PHASE (%)		
FACTOR (HPP-CFC)			
...../.....	0	0	0
L929/WEHI	**30.6±2.2**	**27.9±6.0**	**27.4±4.0**
		0.1<p<0.375	0.1<p<0.375
		N=4	N=4
	36.50	33.55	15.32
	25.60	9.76	32.73
	29.70	32.90	27.36
	30.83	35.80	34.48

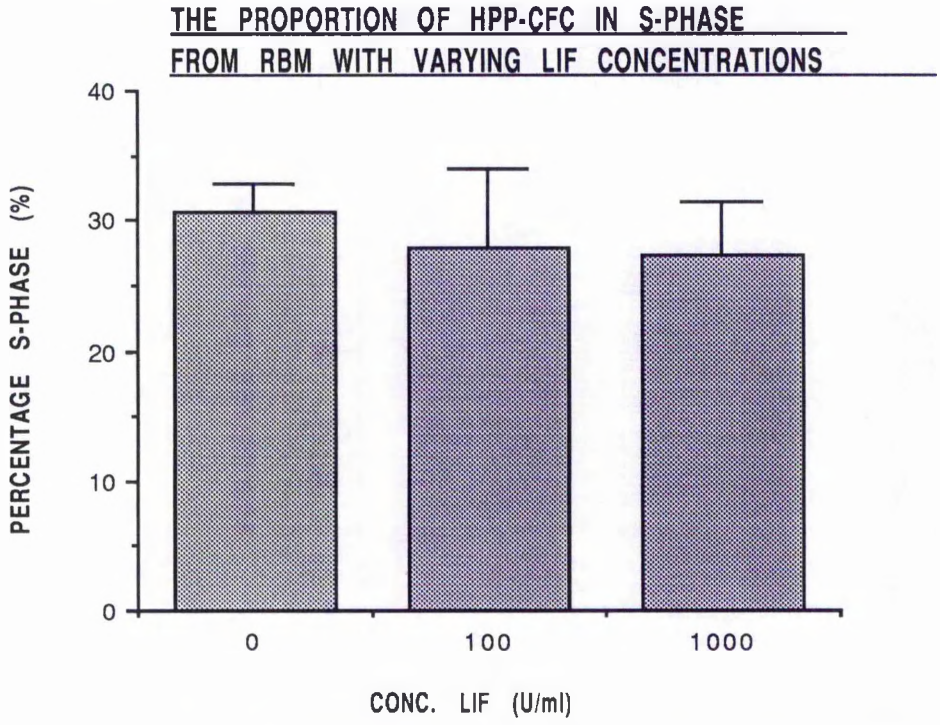


FIGURE G3.7: The proportion of HPP-CFC (WEHI/L929) from regenerating bone marrow in S-phase (%) following incubation with varying concentrations of LIF

3(1.5) Does LIF alter the uptake of tritiated thymidine by femoral bone marrow in limited volume liquid suspension culture ?

It is possible to examine DNA synthesis by a population of cells in more than one way. In this experiment samples of whole bone marrow were cultured in limited volume liquid suspension culture with a known growth factor (GM-CSF), in the presence or absence of LIF. Trititated thymidine is taken up by cells in DNA synthesis. If a population of cells is treated with tritiated thymidine for a given time, then washed, any remaining radioactivity not washed away is a measure of thymidine uptake. This is an indication as to the proportion of a cell population in DNA synthesis over the period for which the thymidine label was available. By adding tritiated thymidine to limited volume cultures stimulated with LIF alone, or with GM-CSF in the presence or absence of LIF, it was thus possible to determine the effect of this growth factor on the proportion of the cell population in S-phase.

3(1.5.1) PROCEDURE

This protocol was a modification of the standard thymidine assay 2(2.1.4) Plates were set up as follows:

3 (1.5.1.1) LIF only

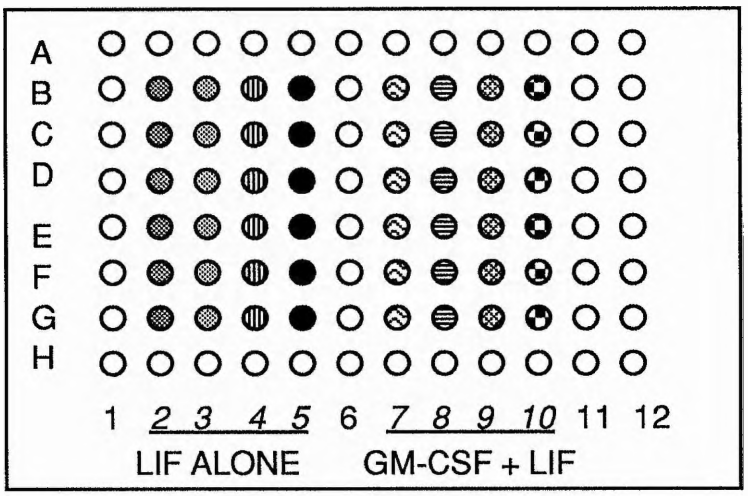
The plate was divided into four blocks of six wells. The first six wells, the control wells had no factor present. 100µl of Dulbecco's 20% was pipetted into each of the six wells. In the subsequent groups 40µl of the appropriate LIF dilution was added to each of the six wells. 60µl Dulbecco's 20% was then added. This was repeated for each of the three LIF dilutions.

3 (1.5.1.2) GM-Stimulated cells

The plate was divided up into four groups of six wells. GM-CSF had been diluted to a concentration of 800units/ml. 50µl of this was pipetted into each of the twenty four wells. The first six wells, the control wells had only GM-CSF present. 50µl of Dulbecco's 20% was pipetted into each of the six wells. In the subsequent groups 40 µl of the appropriate LIF dilution was added to each of the six wells. 10µl Dulbecco's 20% was then added. This was repeated for each of the three LIF dilutions.

Four replicate plates were prepared one for each of four time points.

Two replicate experiments were performed .A summary of the microtitre plate is shown in diagram 16.



96 well microtitre plate

INCUBATION

ADDITION OF TRITIATED THYMIDINE

FURTHER 18 HOUR INCUBATION

HARVEST CELLS

COUNT IN SCINTILATION COUNTER

DIAGRAM 16 MICROTITRE ASSAY PLATE : LIF ON NBM CELLS

3(1.5.2)RESULTS

In this instance results are compared to the control result for the same day. (i.e. day 2 lif 100 U/ml is compared to day2 control etc).

Table 3R.5 and figure G3.8 show the results for populations of bone marrow cells treated with LIF alone. At day 1 there is a statistically significant difference between the level of thymidine uptake observed with 500 U LIF as compared to the control ($0.025<p\leq0.05$). However by day 2 this has disapeared and does not re-appear in day 3 ($0.1<p\leq0.375$ in both cases). This would suggest that this initial difference, although stastistically significant, was not important and that this concentration of LIF did not contribute to sustained altered proliferation within the population.

For all other conditions there is no statistically significant alteration in thymidine uptake at any time point. ($P>0.05$ in all cases). LIF has not altered the level of proliferation within populations of bone marrow cells at any of the test concentrations.

Table 3R.5.2 and figure G3.9 show the results for populations of bone marrow cells stimulated with GM-CSF ± LIF. As above, in one instance, (Day 0 100U/ml LIF) there appears to be a statistically significant difference between this result and that seen in the control ($0.0005<p<0.05$). Again this difference dissapears by day two of the experiment and does not re-appear.

In all other cases there is no statistically significant difference in thymidine uptake compared to controls at any time point with any concentration of LIF. ($0.1<p\leq0.375$ in all cases)
LIF does not alter the level of proliferation seen in cultures of bone marrow cells stimulated with GM-CSF.

TABLE 3R.5: The effect of varying concentrations of LIF on the uptake of thymidine by a limited volume liquid suspension culture of Normal bone marrow cells.

CONDITION (NBM)	MEAN THYMIDINE UPTAKE (%/control) ± STANDARD ERROR
DAY 1 CONTROL	**100.0** 306.53±54.35 375.89±94.83
DAY 2 CONTROL	**76.0±1.0** 231.72±4.900 292.13±26.5
DAY 3 CONTROL	**88.5±47.5** 127.90±17.53 514.12±74.27
DAY 1 100 ULIF	**95.5±6.5** 0.1<p<0.375 271.49±14.12 383.62±93.02
DAY 2 100 U LIF	**118.0±55.0** 0.1<p<0.375 194.36±23.16 653.09±117.69
DAY 3 100 U LIF	**74.0±36.0** P>0.4 118.94±4.94 414.28±24.97
DAY 1 500 U LIF	**117.5±5.5** 0.025<P<0.05 345.07±61.83 465.91±122.78
DAY 2 500 U LIF	**93.5±17.5** 0.1<p<0.375 234.15±35.72 417.69±21.68
DAY 3 500 U LIF	**67.5±27.5** 0.1<p<0.375 125.49±9.59 360.07±34.50
DAY 1 1000 U LIF	**156±34** 0.1<p<0.375 375.40±21.82 714.05±184.10
DAY 2 1000 U LIF	**70.5±16.5** 0.375<P<0.4 165.11±11.88 325.8±29.31
DAY 3 1000 U LIF	**89.5±13.5** P>0.4 233.16±23.43 388.37±39.00

In all cases N = 2

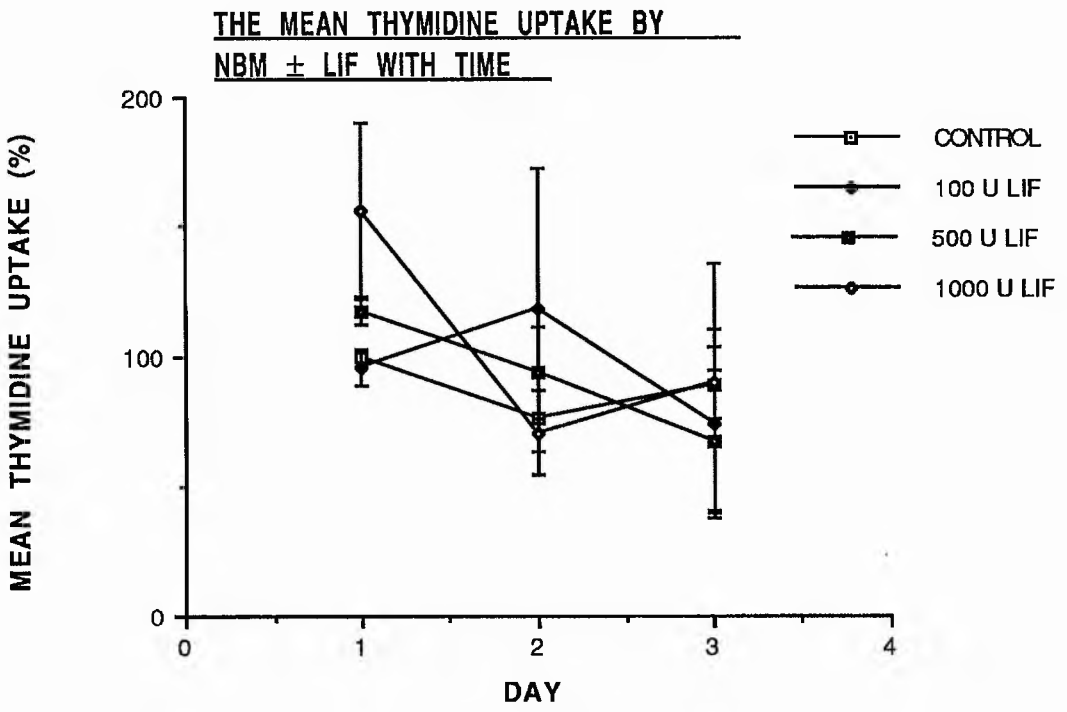


Figure G3.8: Mean thymidine uptake (expressed as a percentage of the appropriate control) by NBM over three days in the presence or absence of varying concentrations of LIF.

TABLE 3R.5.2: The effect of varying concentrations of LIF on the uptake of thymidine by a limited volume liquid suspension culture of Normal bone marrow cells .stimulated with GM-CSF.

CONDITION (NBM±40U/ml GM-CSF)	MEAN THYMIDINE UPTAKE(%control) ±STANDARD ERROR
DAY 1 CONTROL	**100** 2577.12±259.37 2458.61±233.67
DAY 2 CONTROL	**267.5±76.5** 4941.91±288.34 8448.21±472.89
DAY 3 CONTROL	**469±15** 11705.90±818.11 11896.18±358.76
DAY 4 CONTROL	**321.5±130.5** 11650.84±579.26 4695.61±356.78
DAY 1 100 U LIF	**108.5±0.5** 0.0005<P<0.005 2826.52±144.37 2669.64±88.89
DAY 2 100 U LIF	**328±44** 0.1<p<0.375 7339.49±730.57 9161.84±623.82
DAY 3 100 U LIF	**498±13.5** 0.1<p<0.375 13197.98±1460.01 11933.46±951.6
DAY 4 100 U LIF	**249.5±110.5** 0.1<p<0.375 9298.87±535.76 3425.19±514.86
DAY 1 500 U LIF	**106±7** 0.1<p<0.375 2923.76±400.35 2436.90±61.88
DAY 2 500 U LIF	**321±25** 0.1<p<0.375 7653.39±303.80 8496.55±258.41
DAY 3 500 U LIF	**482.5±28.5** 0.1<p<0.375 11699.32±981.57 12567.90±659.59
DAY 4 500 U LIF	**209.5±64.5** 0.1<p<0.375 7066.07±671.57 3564.88±149.60
DAY 1 1000 U LIF	**100.5±0.5** 0.1<p<0.375 2567.24±147.90 2506.36±89.71
DAY 2 1000 U LIF	**338.5±18.5** 0.1<p<0.375 8225.58±400.90 8775.64±279.87
DAY 3 1000 U LIF	**513.5±55.5** 0.1<p<0.375 14651.49±932.32 11254.59±393.44
DAY 4 1000 U LIF	**196±67** 0.1<p<0.375 6788.13±231.84 3173.85±139.45

In all cases N=2

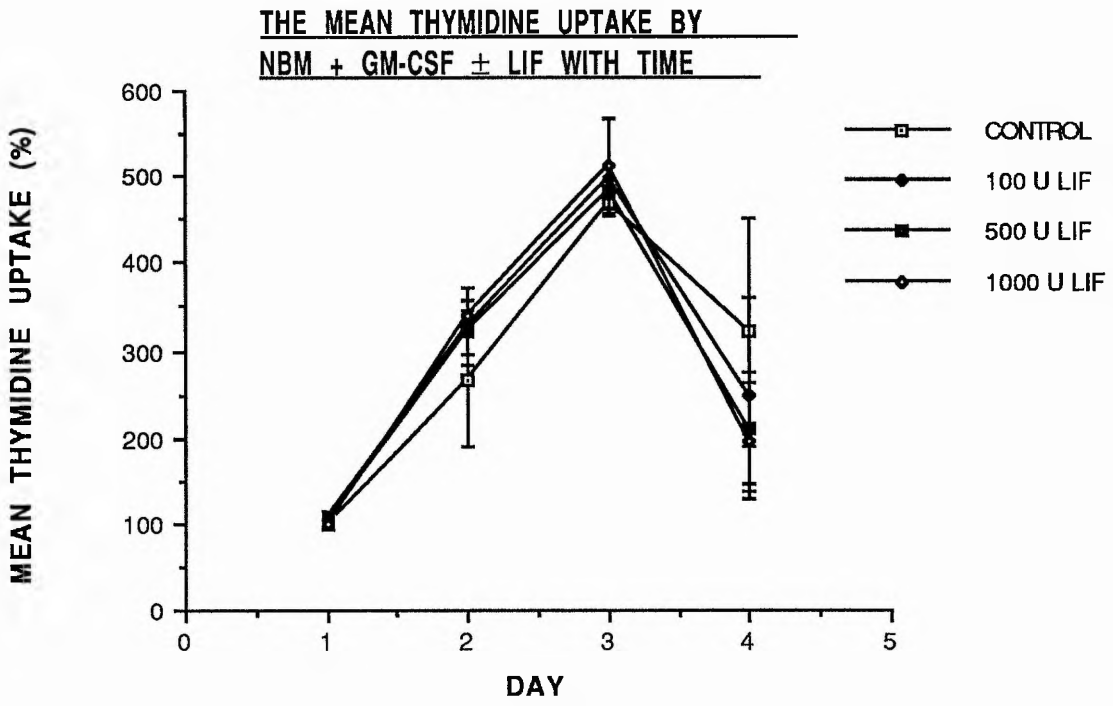


Figure G3.9: Mean thymidine uptake (expressed as a percentage of the relevant control) by NBM over three days in the presence or absence of varying concentrations of LIF in cultures stimulated with GM-CSF.

INDIRECT ACTION

3 (1.6) Does LIF alter the action of a haematopoietic stimulator on a defined HPP-CFC subpopulation *in vitro* ?

Normal femoral marrow is in a resting state of haematopoiesis. It can be 'switched on' however by the addition of a factor which brings the quiescent HPP-CFC population into DNA synthesis. One such stimulator of DNA synthesis in the primitive stem cell pool is the conditioned medium derived from 7day post 4.5 gy gamma irradiated mice 2(1.4.2). This

experiment is designed to test whether 1000U/ml LIF interferes with the ability of this stimulator to act on HPP-CFC stimulated to colony formation with WEHIcm + L929cm.

3(1.6.1) PROCEDURE

This was a modifictaion of the stimulator-S-phase suicide assay 2(2.1.5.2) 2000units LIF (80ul of the appropriate dilution) was aliqouted into two centrifuge tubes, 80µl Dulbecco's 20% into all others.others.

The assay then progressed as a standard stimulator/S-phase suicide assay with cytosine arabinoside .

Assays for HPP-CFC stimulated with WEHI/L929 conditioned media were performed for each tube.

Three replicate experiments were performed.

Summary of the protocol used is shown in diagram 17.

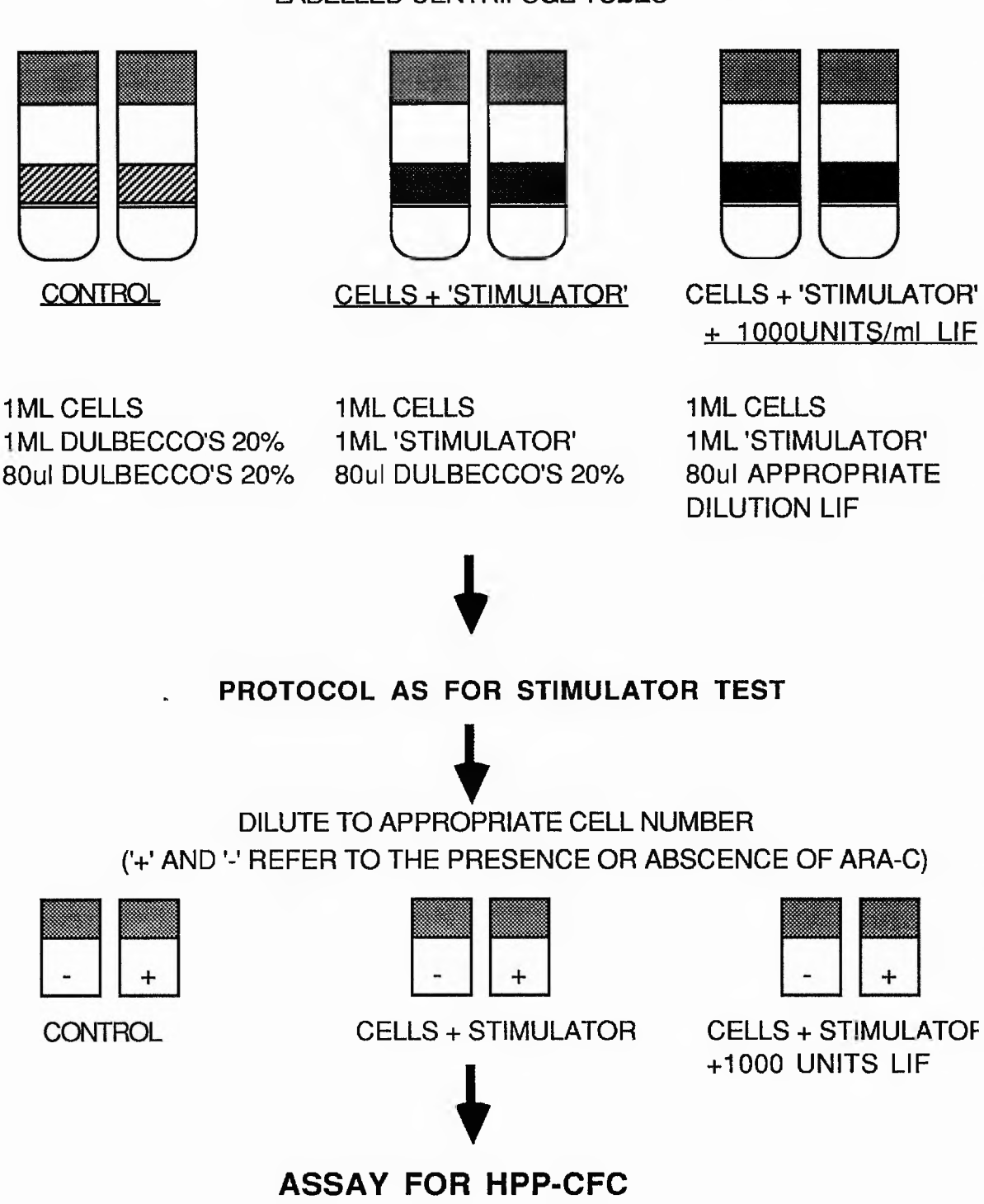


DIAGRAM 17 THE EFFECT OF LIF ON THE ACTION OF A STEM CELL STIMULATOR

3(1.6.2) RESULTS

Table 3R.6 and figure G3.10 show the results for the effect of 1000U/ml LIF on the action of a stem cell stimulator. A significant increase in the proportion of HPP-CFC(WEHI/L929) in S-phase is seen in the presence of a stem cell stimulator ($0.01<p\leq0.025$). 1000U/ml LIF fails to inhibit the action of this stimulator with the level of HPP-CFC cycling remaining significantly higher than in the control ($0.05<p\leq0.1$)

Thus LIF does not indirectly block stem cell proliferation by interfering with the action of a stem cell stimulator.

TABLE 3R.6: The effect of LIF on the action of a stem cell stimulator.

LIF CONC. (U/ml)	0 NBM	0 NBM/STIM	1000 NBM/STIM
	S-PHASE (%)		
FACTOR (HPP-CFC)			
L929/WEHI	**10.61±2.30**	**34.52±9.22**	**29.90±10.1**
		0.01<P<.025	0.05<P<0.1
		N=4	N=4
	11.18	28.16	21.76
	4.12	23.73	17.80
	15.52	61.90	59.20
	11.63	23.65	17.19

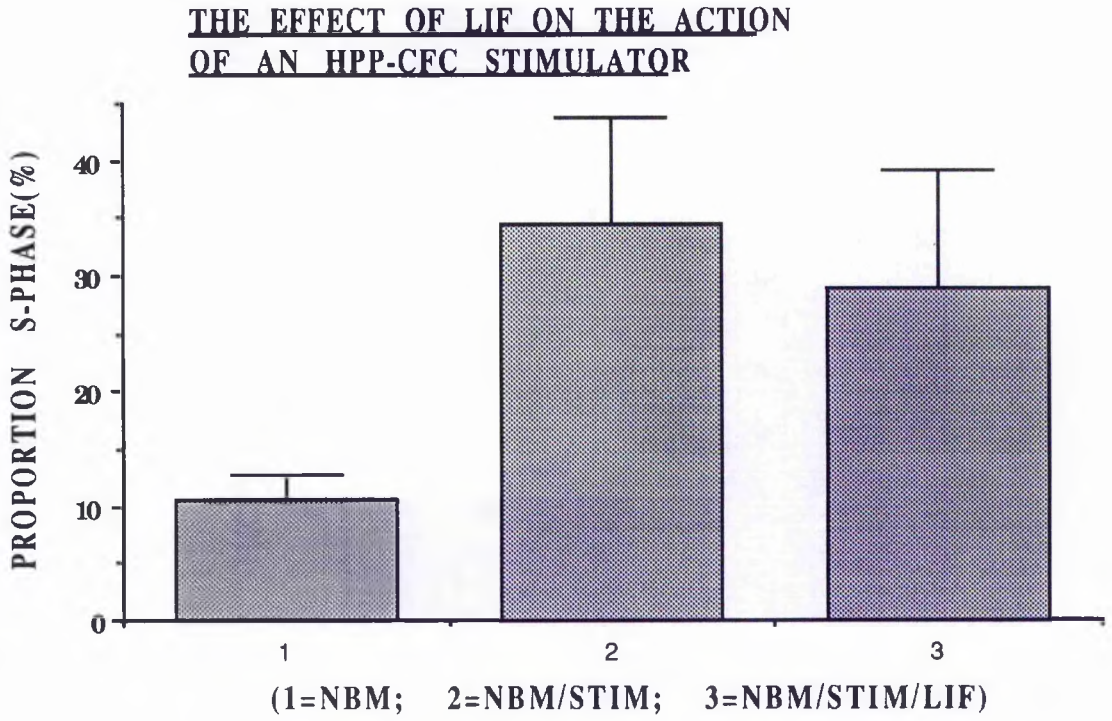


Figure G3.10: The proportion HPP-CFC (WEHI/L929) from NBM in S-phase (%) after treatment with a stem cell stimulator in the presence or absence of LIF.

3 (1.7) Does LIF influence the production of a haematopoietic stimulator *in vitro?*

As outlined previously it is possible to generate a stimulator for primitive stem cells. Negative regulators of cell proliferation could act indirectly by blocking the action or manufacture of this stimulator. This experiment is designed to determine if 1000U/ml or 100U/ml LIF interferes with the production of this stimulator.

3(1.7.1) PROCEDURE

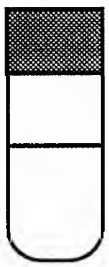
This was a modificiation of the protocol used to generate stem cell stimulator 2(1.4.2). 4000units LIF (160µl of the appropriate

dilution) was aliqouted into one centrifuge tube. In the same way 400 units LIF was aliqouted into the second tube. The third tube, the control tube, received an aliquot of 160µl Dulbecco's 20% .The assay then progressed as a standard assay to determine the effect of exogenous factors on stimulator production.

Assays for HPP-CFC stimulated with WEHI/L929 conditioned media were performed for each tube

Three replicate experiments were performed. A summary of the protocol is shown in diagram 18.

LABELLED CENTRIFUGE TUBES

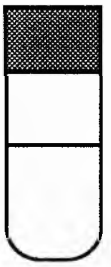


CONTROL

4ml CELLS
160uL DELBECCO'S 20%

1000UNITS/ml LIF

4ml CELLS
160uL LIF AT APPROPRIATE DILUTION



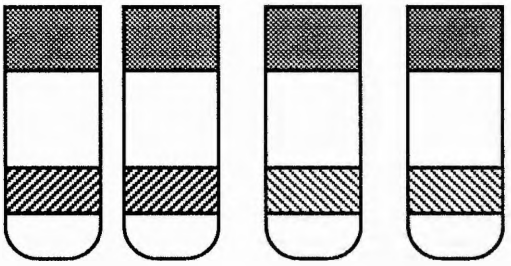
100 UNITS/ml LIF'

4ml CELLS
160uL LIF AT APPROPRIATE DILUTION

INCUBATE FOR THREE HOURS AT 370C

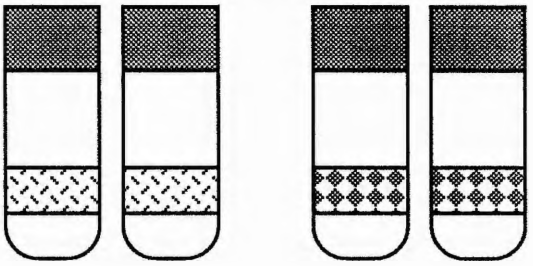
CENTRIFUGE SPIN TO REMOVE CELLS AND DEBRIS
FILTER THROUGH 0.22uM PORE FILTER

PREPARE CENTRIFUGE TUBES FOR STIMULATOR SUICIDE ASSAY
1ML CELLS PLUS 1ML DULBECCO'S OR APPROPRIATE STIMULATOR



CONTROL

'STIMULATOR' DULBECCO'S



'STIMULATOR' 1000 UNITS/ml LIF

'STIMULATOR' 100 UNITS/ml LIF

FOLLLOW PROTOCOL FOR STIMULATOR SUICIDE ASSAY

DIAGRAM 18 THE EFFECT OF LIF ON THE PRODUCTION OFA STEM CELL STIMULATOR

3(1.7.2) RESULTS

Table 3R.7 and Figure G3.11 show the results for the level of DNA synthesis in an HPP-CFC population treated with stimulator manufactured in the presence or absence of LIF. In all cases there is a statistically significant increase in the proportion of HPP-CFC in S-phase in the presence of stimulator ($p<0.05$).

Thus the presence of LIF has not prevented the production of stimulator.

TABLE 3R.7: The effect of varying concentrations of LIF on the production of a stem cell stimulator.

CONDITION LIF CONC. DURING STIM PROD. (U/ml)	NBM /	NBM/STIM 0	NBM/STIM 100	NBM/STIM 1000
			S-PHASE	(%)
HPP-CFC (WEHI/ L929)	**11.75±1.09** N=3 13.24 12.39 9.62	**31.99±4.04** .005<P<.0005 N=3 37.16 24.04 34.78	**23.59±3.82** 0.01<P<.025 N=3 21.74 18.10 30.94	**31.69±0.85** P<0.0005 N=3 30.49 33.33 31.25

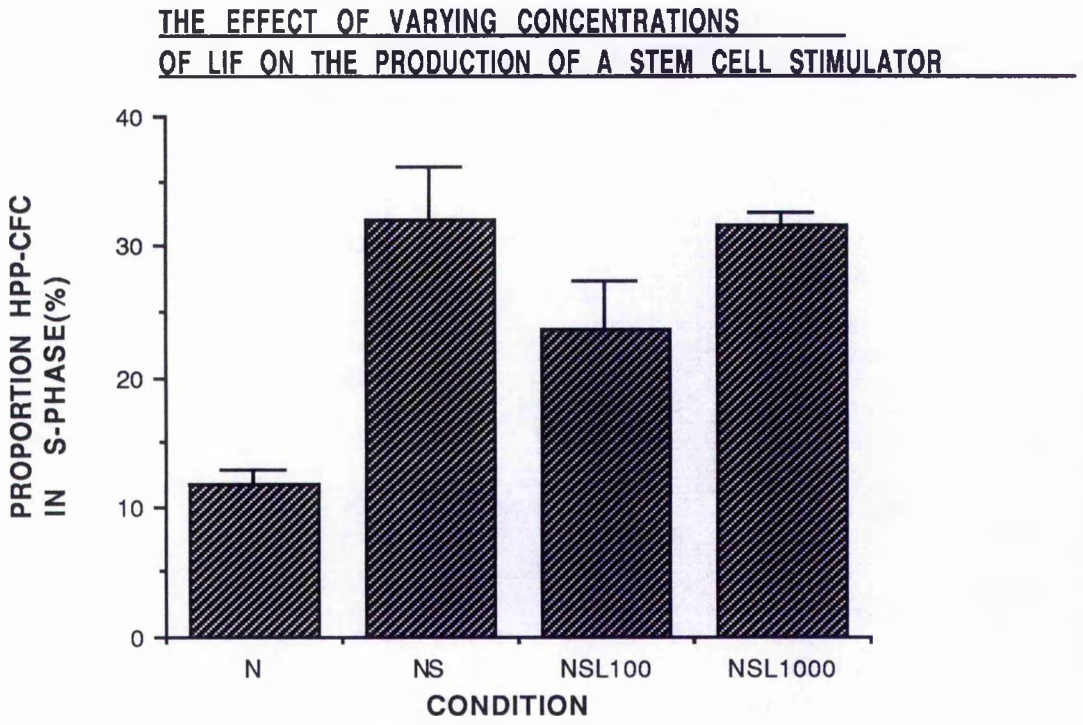


Figure G3.8 :The proportion HPP-CFC from NBM in S-phase (%) after incubation with a stem cell stimulator produced in the presence or absence of LIF.

3.2: SA2 CELLS

LIF's interaction with a variety of human and a murine Leukaemic cell line are documented. No general pattern of responsiveness to treatment with LIF is observed in these populations. In this section LIF's actions *in vitro* on a cloned cell line (SA2JMB1) from an X-irradiation induced murine myeloid leukaemia were investigated.

3 (2.1) The effect of Leukaemia Inhibitory Factor (LIF) on the clonogenicity of SA2JMB1 cells in semi-solid agar culture.

When plated in Semi-solid agar in the absence of exogenous growth factors and cultured at 37^0C for 7-days SA2JMB1 cells will form distinctive colonies In this experiment it was decided to examine the effect of LIF alone, and as a synergistic agent with GM-CSF, on the colony forming potential (the clonogenicity) of SA2JMB1 cells in 7-day culture.

3 (2.1.1) PROCEDURE

SA2JMB1 colony formation in the presence of LIF alone

8 petri-dishes were laid out in the sterile hood and labelled. 40µl of the appropriate LIF dilution was added directly into each of the petri-dishes. This protocol was repeated for each LIF dilution. A control was prepared by pipetting 40µl of Dulbecco's 20% into each of eight petri-dishes. A standard assay for SA2 clonogenicity was then carried out as in 2(2.1.8).

SA2JMB1 colony formation in the presence of GM-CSF ± LIF

8 petri-dishes were laid out in the sterile hood labelled and 40µl of the appropriate LIF dilution added directly into each of the petri-dishes. A control was prepared by pipetting 40µl of Dulbecco's 20% into each of eight petri-dishes. A 30ml universal was prepared containing 7ml Dulbecco's20%, 1ml diluted cells, 1ml GM-CSF at a concentration of 400u/ml and just prior to plating 1ml melted 3%(v/v) agar. (One for each condition).

Suspension was plated as in 2(2.1.8).

In both instances three replicate experiments were performed.

3(2.1.2) RESULTS

Table 3R.8 and figure G3.12 show the results for SA2 clonogenicity in the presence or absence of LIF.

In all cases the addition of LIF to cultures does not alter the level of colony formation compared to that seen in the control. (P>0.05).

TABLE 3R.8: The effect of Leukaemia Inhibitory Factor on the colony forming potential of SA2 JMB1 cells in semi-solid agar culture.

LIF CONC. (U/ml)	0	100	500	1000
		COLONY	*FORMATION*	(%/control)
FACTOR ADDED				
/	**100.0**	**114.5±16.9**	**116.4±24.6**	**100.8±21.3**
		0.1<p<0.375	0.1<p<0.375	P>0.4
		N=3	N=3	N=3
	78.0	90.6	93.6	66.1
	82.1	69.5	59.2	61.2
	42.2	60.1	65.94	60.1
GM-CSF (40U/ml)	**100.0**	**103.1±3.0**	**108.2±5.0**	**99.1±4.1**
		0.1<p<0.375	0.05<P<0.1	P>0.4
		N=3	N=3	N=3
	72.8±6.3	78.3±6.3	76.5±7.1	72.5±6.5
	69.3±8.3	72.3±6.5	81.8±4.1	75.5±5.0
	58.0±1.9	56.5±5.9	57.3±6.3	54.7±8.0

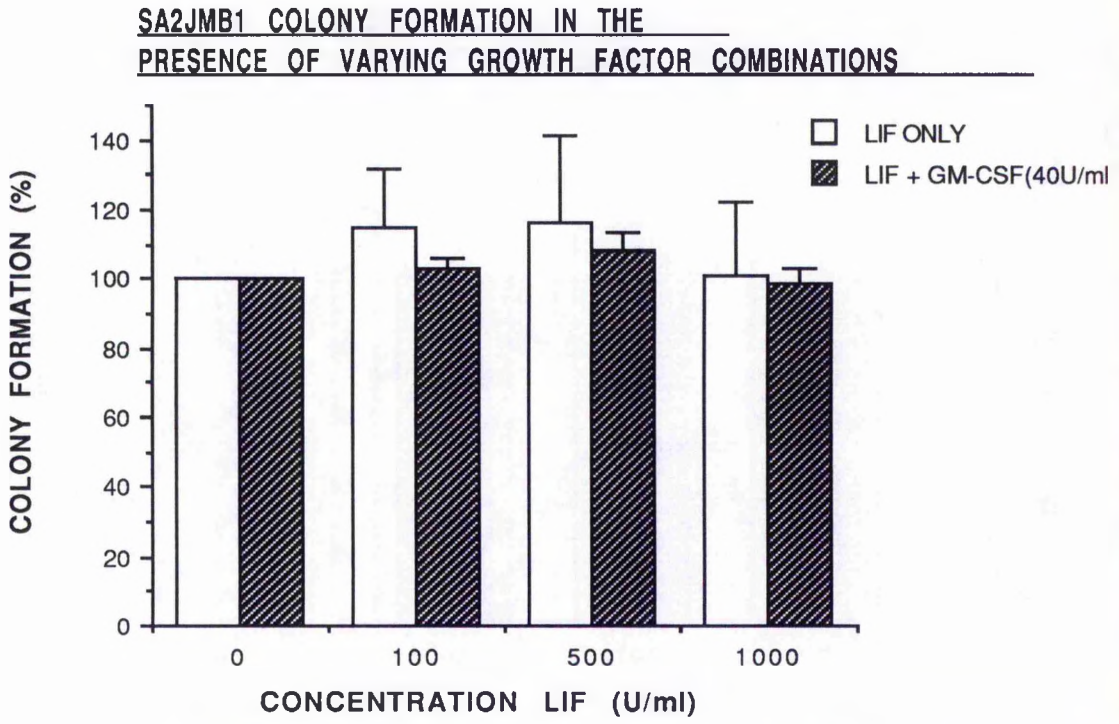


Figure G3.12: The effect (expressed as a percentage of the control 0U/ml LIF) of varying concentrations of LIF on the colony forming potential of SA2 JMB1 cells in semi-solid agar culture.

3 (2.2) The effect of LIF as a short term pre-incubation agent on the clonogenicity of SA2JMB1 cells

In this experiment LIF was assessed as an agent able to alter clonogenicity prior to plating. In the above experiment LIF is present throughout the 7 day incubation period. Here it is available only for a short time prior to plating .LIF was assessed both as an agent acting alone and as a synergistic agent, in association with GM-CSF.

3(2.2.1) PROCEDURE

This was a modification of the standard preincubation assay 2(2.1.5).

Centrifuge tubes were supplemented with 1000U/ml LIF, 500U/ml LIF and 100U/ml LIF in the presence or absence of 40U/ml GM-CSF. Controls were also prepared.

For cultures containing LIF alone three replicate experiments were performed. For those containing GM-CSF two replicate experiments were performed.

3(2.2.2)RESULTS

Table 3R.9 and figure G3.12 show results for the clonogenicity of SA2JMB1 cells following preincubation with LIF ± GM-CSF.

In all cases except one (GM-CSF + 100U/ml LIF) the preincubation of SA2JMB1 cells with LIF has had no effect on the clonogenicity of the cells ($P>0.05$ in all cases). In the case of GM-CSF +100U/ml LIF there is a statistically significant increase in the level of colony formation seen as compared to controls (Control 100% against GM-CSF + 100U/ml LIF 112.0%±3.1).

Thus LIF at one concentration appears to alter the size of the colony forming cell pool available to GM-CSF.

TABLE 3R.9: The effect of preincubation with varying concentrations of Leukaemia Inhibitory Factor on the colony forming potential of SA2 JMB1 cells in semi-solid agar culture .

LIF CONC. (U/ml) (PRE-INC)	0	100	500	1000
		COLONY	*FORMATION*	(%/control)
FACTOR ADDED (PRE-INC)				
/	**100.0** 101.0±2.0 85.5±5.5 82.7±1.8	**94.8±1.8** 0.01<P<.025 N=3 97.2±6.3 83.0±7.5 75.5±1.8	**96.9±1.1** 0.01<P<.025 N=3 97.0±5.2 84.2±4.9 79.2±5.1	**99.3±2.4** P<0.4 N=3 104.2±4.2 85.7±4.0 78.2±9.5
GM-CSF (40U/ml)	**100.0** 72.8±3.2 66.5±5.0	**112.0±3.1** 0.025<P<.05 N=2 83.7±3.9 72.7±5.8	**96.3±2.4** 0.1<p<0.375 N=2 71.8±3.8 70.7±10.0	**108.5±14.5** 0.1<p<0.375 N=2 68.5±5.39 81.7±3.59

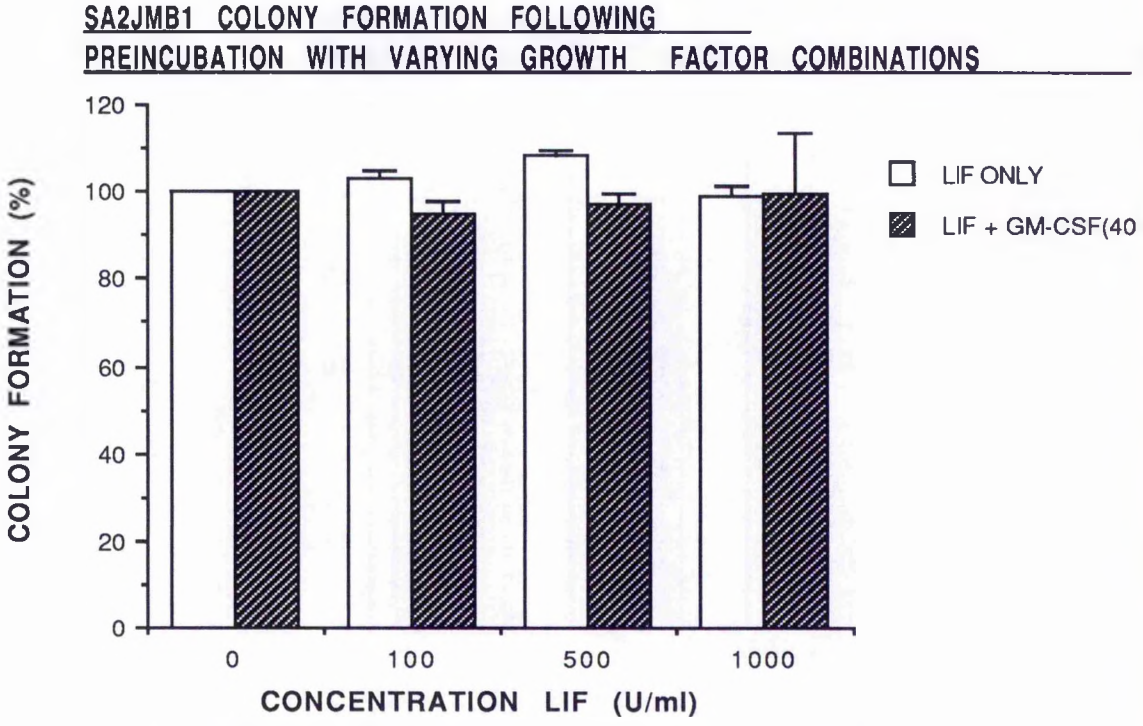


Figure G3.13: Colony formation(expressed as a percentage of the appropriate control 0U/ml LIF ± GM-CSF) by SA2JMB1cells following pre-incubation with varying concentrations of Leukaemia Inhibitory Factor on the colony forming potential of SA2 JMB1 cells in semi-solid agar culture .

3 (2.3) The effect of LIF on the proportion of SA2JMB1 in DNA synthesis.

A high proportion of the Leukaemic cell population is in DNA synthesis (approximately 60%). It is thus possible to assay exogenous factors as potential inhibitors of DNA synthesis in this cell population. For LIF this is a particularly relevant experiment as one manner in which a leukaemia could be stripped of its potency is if it were to be prevented from proliferating further. This experiment was designed to assay the ability of LIF to inhibit DNA synthesis in SA2JMB1 cells.

3(2.3.1) PROCEDURE

This technique was a variation of the standard Inhibitor/S-phase suicide assay 2(2.1.5). A single cell suspension was prepared as in 2(1.3.3) and adjusted to $5x10^5$/ml in Dulbecco's 20%. Cells were treated with 1000units LIF, 500 units LIF and 100 units in the presence of controls.

A standard S-phase suicide assay using cytosine arabinoside was performed.

Assays for SA2JMB1 colony formation in the absence of growth factors were performed for the cells derived from each tube.

Four replicate experiments were performed.

3(2.3.2)RESULTS

Table 3R.10 and figure G3.14 show the level of clonogenicity in SA2JMB1 cells following incubation with LIF. In the control it can be seen that a high proportion of the cell population is in S-phase (74.73%±5.43) treatment of cells with varying concentrations of LIF has had no statistically significant effect on the proportion of the cell population in S-phase when compared to the control. (100U/ml LIF 69.50%±6.37 / 1000U/ml LIF 72.67%±5.35; $p>0.05$ in both cases)

Thus LIF does not inhibit the DNA synthesis of SA2JMB1 cells.

TABLE 3R.10: The effect of Leukaemia Inhibitory Factor on the proportion of SA2JMB1 in DNA synthesis.

LIF CONC. (U/ml)	0	100	1000
S-PHASE (%)	**74.73±5.43**	**69.50±6.37**	**72.67±5.35**
		0.1<p<0.375	0.375<P<0.4
		N=4	N=4
	84.03	81.60	77.42
	83.59	79.13	85.50
	62.22	61.22	62.90
	69.01	56.10	64.87

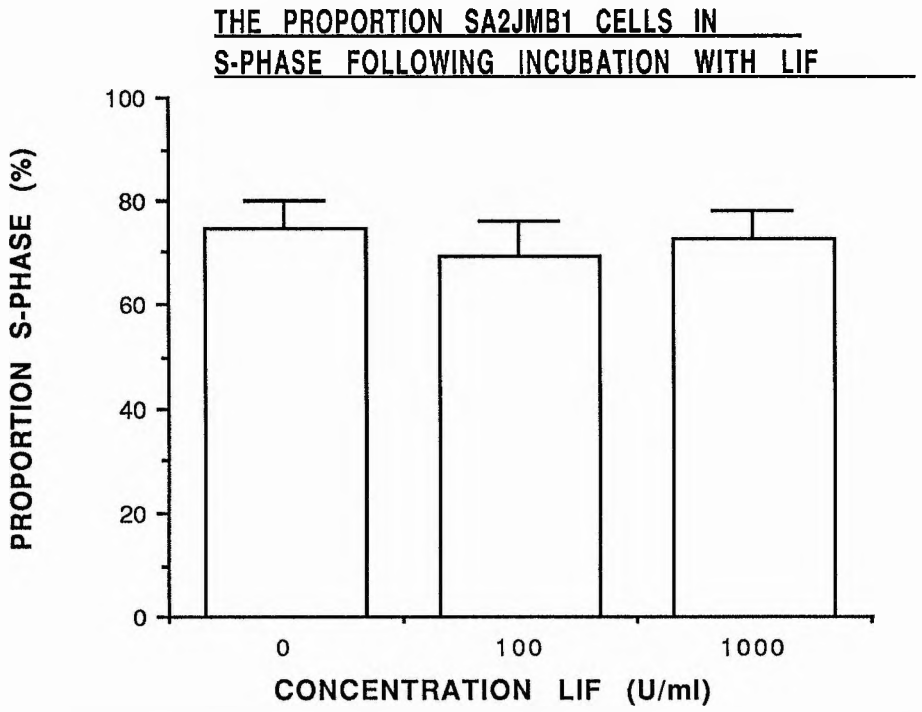


Figure G3.14: The proportion of SA2JMB1 in DNA synthesis (%) in the presence or absence of LIF.

3(2.4) Does LIF alter the uptake of tritiated thymidine by SA2 JMB1cells in limited volume liquid suspension culture?

In this experiment SA2JMB1 cells were cultured in limited volume liquid suspension culture in the presence or absence of LIF. The principle of thymidine labelling is exactly the same as outlined for femoral marrow in 3(1.5). The experiment represented a second manner in which to investigate the effects of LIF on DNA synthesis in SA2JMB1 cells.

3(2.4.1) PROCEDURE

This was a modification of the standard tritiated thymidine uptake assay. 2(2.1.4). Cellularity was determined and adjusted to $2x10^3$ cells/ml. A microtitre plate was divided into four groups of six wells. 100ul cells was added to all wells. 1000U/ml LIF, 500U/ml LIF, 100U/ml LIF were assayed for the ability to alter the level of thymidine uptake as compared to a control.

The protocol was exactly as outlined for femoral marrow above 3(1.5.3).

Four replicate plates were prepared one for each of four time points.

Three replicate experiments were performed.

3(2.4.2) RESULTS

Table 3R.11 and figure G3.15 show the effect of LIF on the uptake of tritiated thymidine by SA2JMB1 cells. The pattern of results here is interesting. Specifically at day three, in all cases, (i.e. 100U/ml LIF, 500U/ml LIF and 1000U/ml LIF) there is a statistically significant difference in the level of tritiated thymidine uptake when compared to the relevant controls. ($P<0.05$ in all cases). Examining the data it is obvious that these differences are not large. These findings are discussed more fully in 3D.4.

TABLE 3R.11: The effect of varying concentrations of LIF on the mean thymidine uptake (%) by SA2JMB1 cells over three days

CONDITION (SA2 CELLS)	MEAN THYMIDINE UPTAKE (%/control) ± STANDARD ERROR	
DAY 1 CONTROL	**100.00** 602.32±69.85 868.50±26.07 931.64±92.71	
DAY 2 CONTROL	**275.33±8.97** 1355.01±69.38 2501.99±72.68 2615.19±116.22	
DAY 3 CONTROL	**530.00±163.44** 1466.02±87.40 7024.71±526.51 5191.94±348.85	
DAY 1 100 U LIF	**94.87±3.96** 600.87±69.38 856.97±14.07 806.18±46.75	0.1<p<0.375
DAY 2 100 U LIF	**302.67±35.35** 1508.77±37.18 3236.04±516.04 2379.87±116.22	0.1<p<0.375
DAY 3 100 U LIF	**680.67±80.31** 3774.57±169.16 7276.54±368.83 5349.8±348.85	0.1<p<0.375
DAY 1 500 U LIF	**89.67±8.35** 564.2±106.85 861.22±48.67 686.12±35.77	0.1<p<0.375
DAY 2 500 U LIF	**280±15.53** 1554.33±65.34 2761.67±193.44 2530.65±46.85	0.1<p<0.375
DAY 3 500 U LIF	**626±22.03** 3989.70±149.97 5475.90±440.72 5456.65±253.15	0.1<p<0.375
DAY 1 1000 U LIF	**87.33±6.01** 502.95±51.25 858.56±11.53 732.56±41.07	0.05<P<0.1
DAY 2 1000 U LIF	**293±33.65** 1466.02±115.99 3102.46±51.96 2605.38±88.08	0.1<p<0.375
DAY 3 1000 U LIF	**580.33±41.13** 3528.61±112.57 5624.21±593.78 4713.86±333.60	0..375<p<0.4

In all cases N=3

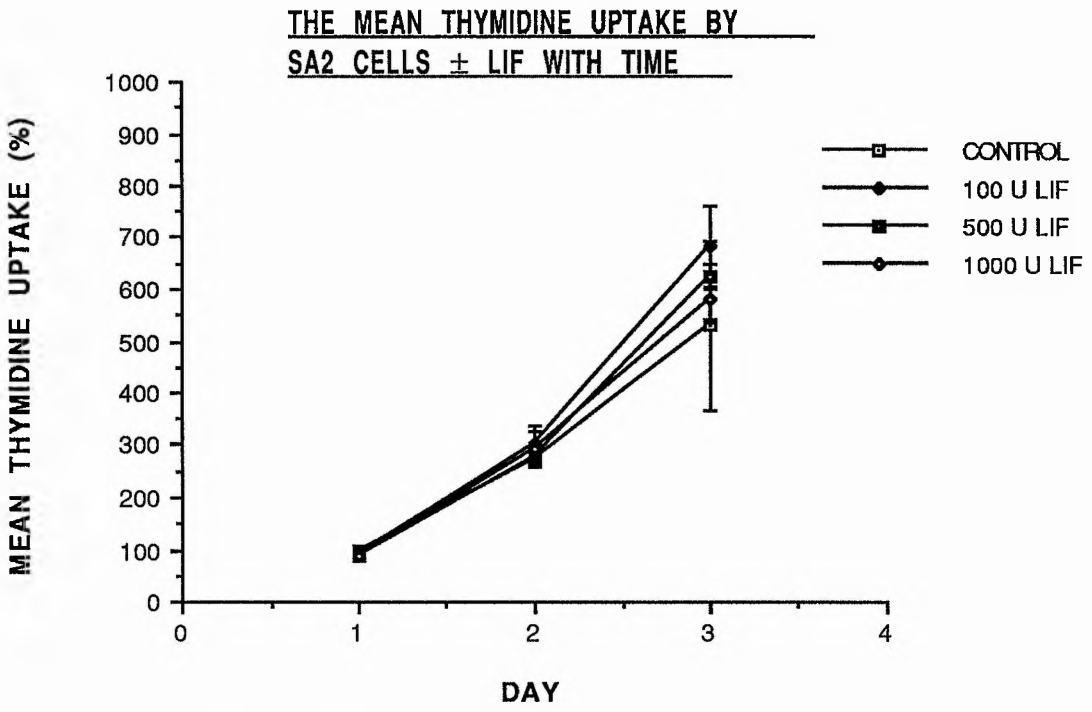


Figure G3.15: Mean thymidine uptake (expressed as percentage of relevant control) by SA2JMB1 cells over three days in the presence or absence of varying concentrations of LIF

3.3: 15 DAY FOETAL LIVER

15 day foetal liver represents an active haematopoietic environment. As LIF is noted to exhibit different functions between different haematopoietic environments (Escary 1993) it was decided to assay the effects of LIF on cell populations derived from this haematopoietic tissue.

3 (3.1) The effect of LIF on the colony forming potential of HPP-CFC in semi-solid agar culture

This experiment was designed to assay the effect of LIF on the number of colonies formed by an HPP-CFC subpopulation.

3(3.1.1) PROCEDURE

LIF was assessed *in vitro* as an agent able to influence colony formation by HPP-CFC. It was assayed both as an agent able to stimulate colony formation, and as a synergistic agent on populations of HPP-CFC stimulated with:

- 10% WEHI cm + 10% L929 cm;
- GM-CSF + M-CSF (HPP 3);
- IL-3 + M-CSF (HPP 2);
- IL-1a + IL-3 + M-CSF (HPP 1);

The protocol used was exactly as outlined for femoral marrow (3(1.2)) but with foetal liver tissue.

Three replicate experiments were performed in the case of the first two subpopulations. Two replicate experiments were performed for the latter.

3(3.1.2)RESULTS

Table 3R.12 and Figure G3.16 show the effect of LIF on the number of colonies formed by populations of HPP-CFC.

The pattern of results found here is interesting for a number of reasons. Firstly the effect of LIF on colony formation appears to be more notable here than in any other instance, but no discernable consistent pattern emerges.

In the case of HPP-CFC stimulated with WEHI/L929 + 100 U/ml LIF there is a statistically significant decrease in the level of colony formation compared to controls. ($0.025<p\leq0.05$). However neither of the two other LIF concentrations have a similar effect on this HPP-CFC population ($0.1<p\leq0.375$ in both cases).

In the case of HPP-CFC stimulated with GM-CSF/M-CSF + 500 U/LIF there is a statistically significant increase in colony numbers as compared to controls. ($0.05<p\leq0.1$). However neither of the two other LIF concentrations show a similar effect on this HPP-CFC population. ($0.1<p\leq0.375$ in both cases) .

In the case of HPP-CFC stimulated with IL-3/M-CSF LIF does not alter the level of colony formation in any instance ($P>0.05$ in all cases)

In the case of HPP-CFC stimulated with IL-1a/IL-3/M-CSF +500U/ml or 1000U/ml LIF there is a statistically significant decrease in colony formation($p<0.05$ in both cases). However at the lower concentration of 100 U/ml LIF there is no effect on colony formation ($0.1<P\leq0.375$).

These results are discussed fully in 3D.5

TABLE 3R .12: Colony formation by HPP-CFC from foetal liver (expressed as percentage of control) in the presence or absence of varying concentrations of LIF.

LIF CONC. (U/ml)	0	100	500	1000
		COLONY	*FORMATION*	(%/control)
FACTOR				
...../.....	0	0	0	0
L929/WEHI	**100.0**	**83.8±7.3**	**108.0±14.8**	**96.0±5.8**
		0.025<P<.05	0.1<p<0.375	0.1<p<0.375
		N=3	N=3	N=3
	20.5±1.3	19.5±7.5	27.2±1.5	22.0±1.3
	29.2±0.8	25.2±2.0	23.9±1.8	25.5±2.9
	31.7±3.5	22.5±2.1	35.0±4.14	29.7±2.3
GM-CSF/ M-CSF	**100.0**	**88.5±12.6**	**114.6±8.4**	**112.5±15.7**
		0.1<p<0.375	0.05<P<0.1	0.1<p<0.375
		N=3	N=3	N=3
	14.8±1.6	15.5±3.1	15.2±1.9	14.5±2.4
	11.3±2.1	7.7±0.7	14.9±1.4	16.3±0.7
	11.7±1.0	11.2±1.6	13.0±2.3	11.2±1.6
IL-3/ M-CSF	**100.0**	**84.5±15.5**	**96.5±11.5**	**88.5±7.5**
		0.1<p<0.375	0.375<P<0.4	0.1<p<0.375
		N=2	N=2	N=2
	26.5±1.7	26.8±0.3	22.5±2.6	25.8±3.4
	26.0±1.4	18.8±1.9	28.0±1.6	21.8±1.9
IL-1/IL-3/ M-CSF	**100.0**	**93.0±18.0**	**76.5±5.5**	**73.5±6.5**
		0.1<p<0.375	0.01<P<.025	0.025<P<.05
		N=2	N=2	N=2
	32.0±3.4	24.3±6.3	22.7±1.9	25.7±3.8
	35.0±3.1	31.5±4.9	28.8±1.8	23.5±3.5

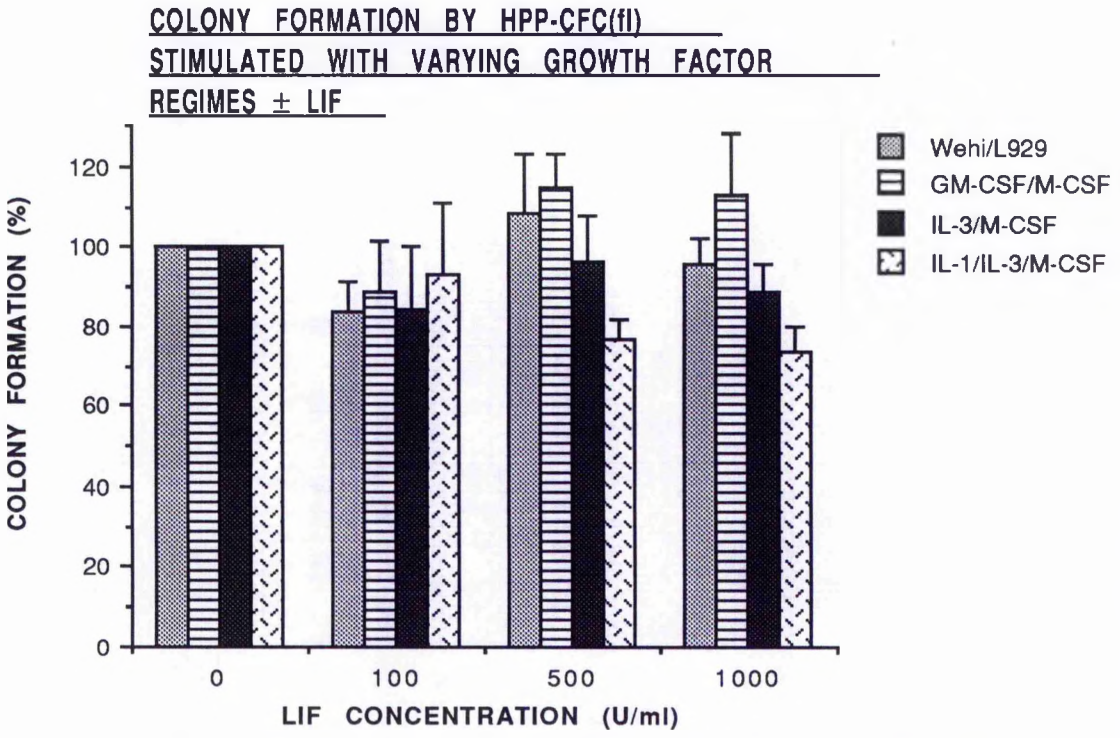


Figure G3.16: Colony formation by HPP-CFC from foetal liver (expressed as a percentage of the control) in the presence or absence of varying concentrations of LIF .

3 (3.3) The effect of LIF *in vitro* on the proportion of HPP-CFC in DNA synthesis.

Whereas normal femoral marrow was relatively quiescent, foetal liver is haematopoietically very active. In HPP-CFC derived from foetal liver approximately 20-30% of the population are in cycle. Thus it is possible to test LIF as an inhibitor of an innately cycling HPP-CFC population in this tissue.

3(3.3.1) PROCEDURE

With the exception that the tissue was foetal liver and did not require prior irradiation, the protocol was identical to that used

to test LIF as an inhibitor of HPP-CFC in femoral marrow 2(2.1.4).

Assays were performed for HPP-CFC stimulated to colony formation with WEHIcm and L929cm.

Three replicate experiments were performed.

3(3.3.2) RESULTS

Table 3R.13 and figure G3.17 show the effect of LIF on the proportion of HPP-CFC stimulated to colony formation with WEHIcm and L929cm, in S-phase. There is a relatively large proportion of the HPP-CFC population in S-phase (21.34%±2.81). Neither concentration of LIF has had a significant effect on the level of DNA synthesis within the HPP-CFC population (100U/ml LIF 23.66%±4.09/ 1000U/ml LIF 24.15%±3.31 where $0.1<p\leq0.375$ in both cases).

TABLE 3R.13: The proportion HPP-CFC (WEHI/L929) from foetal liver in S-phase in the presence or absence of varying concentrations of LIF.

LIF CONC. (U/ml)	0	100	1000
	S-PHASE (%)		
FACTORS (HPP-CFC)			
...../.....	0	0	0
L929/WEHI	21.34±2.81	23.66±4.09	24.15±3.31
		0.1<p<0.375	0.1<p<0.375
		N=3	N=3
	25.29±16.99	31.78±7.98	25.42±5.11
	22.89±6.47	20.51±9.79	29.13±10.19
	15.91±8.52	18.69±7.95	17.89±8.24

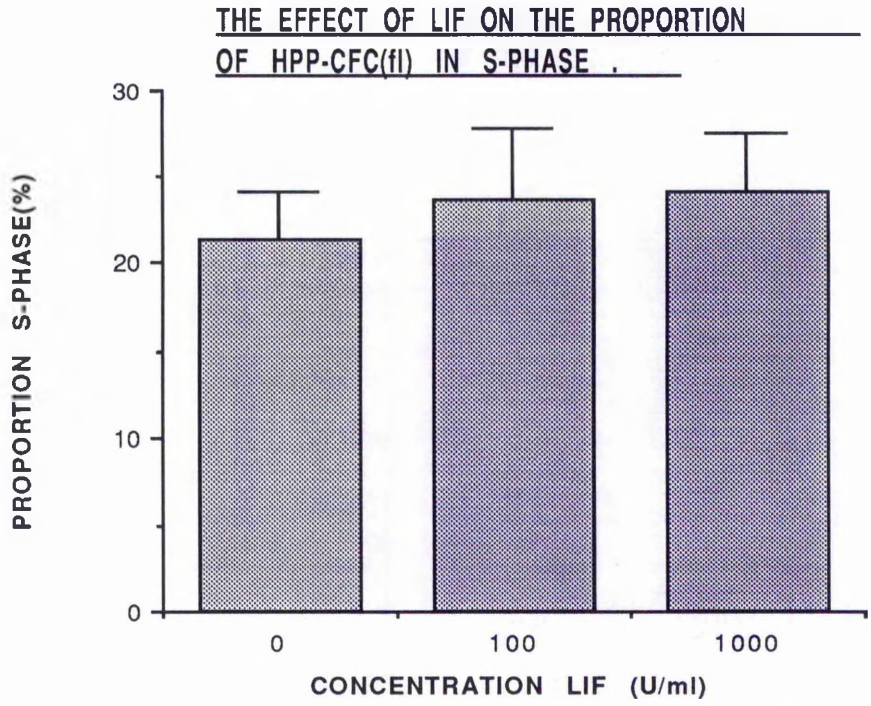


Figure G3.17: The proportion HPP-CFC (WEHI/L929) from foetal liver in S-phase in the presence or absence of varying concentrations of LIF.

3D:DISCUSSION

3D.1: The effect of LIF on Colony formation in semi-solid agar culture

In all cases LIF has failed to support the proliferation of haematopoietic cell populations in semi-solid agar culture. In almost all cases, whether as a direct acting agent in the dish, or as a pre-incubation agent prior to culture, LIF has had no effect on the number of HPP-CFC or GM-CFC stimulated to colony formation by a variety of growth factor combinations.

The two cases in which LIF was shown to alter the level of colony formation as compared to controls were perplexing. In the first of these (IL-3 stimulated colony formation) the addition of 100 U/ml LIF to cultures results in a **decrease** in the level of colony formation. In the second case, the addition of 100U/ml LIF to a population of whole bone marrow cells prior to assay for GM-CFC stimulated with IL-3 results in **increased** colony formation. Thus a picture of colony stimulation and inhibition emerges. In addressing this anomaly several points must be considered. Firstly in both cases the shift in the level of colony formation is not great (94%±1.2 against 100%; 109%±3 against 100%, respectively). This begs the question-What would be the physiological significance of a change of this magnitude ? This is best answered by considering data from comparable experiments. In work by Metcalf *et al* (1990) where LIF is shown to alter the level of

murine megakaryocyte colony formation stimulated by IL-3, the change in colony numbers is not great. (Control :16 megakaryocyte colonies per 50,000 cells plated, against 21 colonies per 50,000 cells in the presence of 1000 U/ml LIF). In other work such as that by Verfaillie and McGlave (1991), LIF mediates substantial increases in colony number for given human cell populations, as compared to controls. For example the LIF mediated increase in colony numbers of MIX-CFC is 5 times that seen in the control (560% against 100%). There is evidence therefore that LIF can induce both a substantial and a subtle alteration in the level of colony formation with precedents for accepting the validity of both.

There is considerable evidence from other groups (Metcalf *et al*, 1988; 1989; Verfaillie and McGlave, 1991) that LIF is unable to alter the level of human GM-CFC colony formation either alone, or in combination with other factors (IL-3 and GM-CSF). The majority of the data from tables 3R.1 and 3R.3 concur with this finding. Furthermore the fact that the change in levels of colony formation is so small and not observed with other concentrations of LIF strongly suggests the apparent stimulation and inhibiton noted in this work may in fact be unimportant. It is perhaps worth noting, however ,that in the work of Verfaillie and McGlave, foetal calf serum is used in assays where here horse serum is used. Could there be a factor in horse serum which interacts with LIF thus allowing it to alter the level of colony formation seen in GM-CFC stimulated with IL-3? Again this seems unlikely as the majority of the data in this work do not support this notion.

The ability of LIF to enhance the level of murine megakaryocyte colony formation stimulated by IL-3 is noted (Metcalf *et al*, 1990; Debilli *et al*, 1993) The same is true for human megakaryocyte colony formation. (Burnstein *et al*, 1990). The possibility that LIF is enhancing an IL-3 stimulated megakaryocyte colony formation should be considered. This seems unlikely for a number of reasons. Firstly the MEG-CFC assay is subtly different to the GM-CFC assay and thus this possibility should be discountable. Secondly LIF enhances colony formation in those assays where here, in one instance, it inhibits. Thirdly if LIF as a pre-incubation agent were increasing the size of the Meg-CFC pool then concentrations of LIF able to enhance the IL-3 megakaryocyte colony formation should exceed 100U/ml. Therefore the increase in colony formation seen in this assay should have been larger at the higher concentrations.

Perhaps most intriguing is the fact that LIF has acted both to stimulate and inhibit at identical concentrations on an identical progenitor cell population. The difference lies in the assay technique. In the first instance LIF (at non-toxic concentrations) is acting in synergy with IL-3 to reduce GM-CFC colony formation. In the second instance LIF is increasing the size of the GM-CFC pool either by an active increase or by increasing the level of cell survival over the incubation period prior to assay with IL-3. Is LIF increasing survival of this cell pool ? In considering this it is interesting to note that Escary *et al* (1993) implicate LIF in the maintenance of GM-CFC numbers in the spleen and to a much lesser extent in the marrow. This

implies that LIF is capable of performing a role in maintaining cell populations. However, it suggests that such a role is not seen in femoral marrow cell populations. This would seem to confirm that this result is unimportant.

From the data it seems reasonable to assume that LIF does not alter levels of colony formation in populations of GM-CFC, either as an agent in the dish, or as a short term pre-incubation agent.

HPP-CFC colony formation.

LIF failed to support colony formation by HPP-CFC. This is unsurprising as HPP-CFC populations are at least partly defined by the dependence of their proliferation on multiple growth factor combinations. The failure of LIF to alter the level of colony formation seen in any of the HPP sub-populations tested is curious. Verfaillie and McGlave (1991) showed that LIF is capable of inducing growth and increased proliferation of immature multi-lineage colonies in cultures supplemented with foetal calf serum. Leary *et al* (1990) looked at the effects of LIF on IL-3 dependent colony formation by human haematopoietic progenitors (very primitive blast colony forming cells) methycellulose culture supplemented with foetal calf serum. They concluded that LIF was effective in enhancing colony formation. This correlates well with the work of Metcalf *et al* (1989). They demonstrated that, when present at high concentrations, LIF causes an increase in the number of immature blasts present in murine haematopoietic organs. The

blast-CFC cell population is thought to be closely related in the haematopoietic developmental hierarchy to HPP-CFC. This would suggest that whilst LIF can be active on primitive blast cells, as the HPP 1 and HPP 2 populations tested are insensitive to it they are thus somehow distinct from the BL-CFC. It should be noted here however that although a murine BL-CFC has been assayed Leary *et al* worked on human BL-CFC. The HPP populations tested were murine in origin although they do have an *in vitro* human counterpart. It is perhaps also worth noting that the LIF used in Leary's work came from a different source to that used in the work contained in this thesis.

At this point it seems prudent to consider the HPP-mCFU-MK population of Han (1994). This HPP population is argued to be HPP1 expressing a latent potential for megakaryocytic development in the presence of relevant concentrations of IL-6 and IL-3, or unknown factors from a conditioned medium. In the development of megakaryocytes, IL-6 and LIF have similar roles to play with a probable degree of functional overlap at certain points. (Review Burnstein, 1994; Long, 1993). Given LIF's noted ability to enhance megakaryocytopoiesis, it seems reasonable to suggest that LIF, at appropriate levels, may have an action like IL-6 in stimulating HPP-mCFU-MK. In Han's work there is a very slight increase in overall colony numbers in assays for HPP-mCFU-MK as compared to assays for HPP1. They identify the HPP-mCFU-MK on a morphological basis. It may be that in these experiments LIF has stimulated the formation of HPP-mCFU-MK without a significant rise in the overall colony numbers. As a consequence the nature of its

action in stimulating differential HPP populations has not been evident as a morphological assay for this population was not undertaken. Equally it may be that LIF was not used at a concentration likely to promote formation of this population although its use at these concentrations is justified by other work, as discussed in the methods. The development of HPPmCFU-MK may be completely independent of LIF. If HPP (and at least three populations of different maturity have been tested here) are insensitive to LIF it would suggest that BL-CFC are in this respect a distinct population. A sensitivity to LIF poses the question "is this important in megakaryocytopoiesis ?". The differentiation of a primitive stem cell into megakaryocyte progenitors is poorly understood. Is there a role for BL-CFC and is it related to Han's HPP population ? Interestingly IL-6, an important ingredient in the development of HPPmCFU-MK, was shown to act in a similar but more rapid manner to LIF in promoting the IL-3 dependent blast cell colony formation.

Finally there is a question of experimental protocol. In both the work by Leary *et al* (1990) and the work of Verfaillie and McGlave (1991) foetal calf serum is used in assays. In all of the work in this thesis horse serum was used. Does horse serum represent a possible environment in which certain of LIF's actions are lost ? It is worth noting that colony stimulation seen with LIF alone in the presence of foetal calf serum is not seen when serum is withdrawn. (Verfaillie and McGlave, 1991) This strongly suggests that LIF interacts with some component of foetal calf serum. Schaafsma *et al* (1992) report work in which

LIF does not affect the proliferation or differentiation of human haematopoietic progenitor cells in LTBMC. In noting that these experiments produce contrasting results to Verfaillie and Mcglave (1991), Schaafsma *et al* point out that the source of LIF was different. The LIF used by Verfaillie and Mcglave was derived from a different cell line to that used by Schaafsma *et al*. The implication is that other factors produced by cell lines may interact with LIF. This could explain apparent contradictions in reports of LIF's actions. Given that Verfaillie seems to have controlled for this possibility it is perhaps worth considering that in the LTBMC studied by Schaafsma *et al* they do not seem to have controlled for endogenously produced LIF (membrane bound). Thus this particular assay system may already be saturated with LIF. Notably, however, Schaffsma does not use foetal calf serum in his assays. Could the inability of LIF to stimulate proliferation be related to the absence of this serum ?

In the assay system used in the work contained in this thesis LIF is unable to support HPP-CFC proliferation and has no effect on the level of colony formation by populations of HPP-CFC.

3D2: LIF and DNA synthesis.

In all cases LIF has had no statistically significant effect on the proportion of HPP-CFC or GM-CFC in S-phase. Robinson *et al* (1993) demonstrated the importance of the -SDK- sequence in the biology of the pentapeptide AcSDKP. It was shown to be

that part of the molecule which blocked the action of stimulator on a population of HPP-CFC. Murine LIF contains the -SDK- sequence. If the sequence were responsible for a similar activity in LIF then this result would be unsurprising as LIF would be expected to have a mode of action similar to the tetrapeptide AcSDKP. However set against work by Fletcher *et al* (1990) these results (specifically for the HPP sub population) initially seem puzzling. Their work was designed to test for the effects of LIF on the recovery and retroviral infection of murine CFUS13 *in vitro*. They conclude that LIF enhances the recovery and retroviral-vector infection efficiency of normal haematopoietic progenitors. This would suggest that LIF may well stimulate HPP-CFC proliferation. This would be in direct contrast to the work of Robinson *et al* (1993). Interpretation of these results requires care. Firstly the enhanced recovery of CFUS13 brought about by LIF is open to a variety of explanations. It may be that LIF, in cultures of whole bone marrow, acts to stimulate proliferation of CFUS13. The main evidence for this would be the increased numbers of CFUS13 as compared to controls, and the high infection rate in these colonies. Several points weigh against this possibility. Most importantly the populations of CFUS13 from LIF treated cultures show no loss in differentiative capacity as assayed by morphological study of spleen colonies. This would imply that LIF had induced proliferation without differentiation. This is of course a documented function of LIF in ES cell cultures (Smith *et al*, 1990). So initially this would seem an inviting prospect. Fletcher *et al* however point out that many of the CFUS13

derived colonies have identical pro-viral insertion patterns. This could only occur if a CFUS13 precursor had been infected, subsequently divided, and given rise to two identical daughter cells from which these colonies arose. This suggests that LIF acts on an earlier precursor. This earlier precursor may not be equivalent to the HPP populations assayed by Robinson *et al* (1993). This explanation relies on proliferative status alteration of CFUS13 or a precursor cell population by LIF.

A second explanation may be that LIF does not increase the size of the CFUS population but increases the survival of that population or indeed a precursor population which gives rise to CFUS13. Cultures lasted 72 hours, certainly a long enough period to allow cell death. The assumption with retro-viral labelling is that proliferation and labelling are directly proportional. Although Fletcher *et al* highlight significant evidence for this possibility they point out that infection may be unrelated to replication status. There is evidence that LIF may play a role in the maintenance of haematopoietic stem cell numbers (Escary *et al*, 1993). It has also been documented (Pesce *et al*, 1993) that LIF (in common with the cytokine SCF) reduced the occurrence of apoptosis, thus increasing cell survival, in Primordial germ cells during the first hours in culture. This ability certainly did not extend to a 72 hour time period, however, as Pesce points out the PGC's were cultured with the soluble form of LIF. In Fletcher's work bone marrow aspirates were in contact with stroma over the incubation period and thus the fibroblasts could have been induced to express membrane bound LIF or other cytokines. Work by

Verfaillie (1993) seems to corroborate this. In a delicate manipulation of LTBMC she cultures primitive human cells either in the absence of a stromal layer (stroma free) or separated from a stroma layer by a microporous membrane (Stroma non-contact). She concludes that although both systems were supplemented with growth factors (including LIF) clonogenic cell expansion in stroma non-contact cultures was greater. Equally conservation of LTBMC-IC was greater in stroma cultures. This suggests that factors such as LIF are important for early stem cell survival but may function by inducing stroma to produce soluble factors. This is a subtle dynamic and may relate to the findings of Schaffsma *et al* 1992 (although they have not examined the LTBMC-IC survival.) Relating this work to the findings contained in this thesis for GM-CFC and HPP-CFC populations, raises several points. The suicide assay technique results for GM-CFC cell populations are in agreement with the findings of Schaffsma *et al* (1992); Verfaillie and McGlave (1991); Metcalf *et al* (1989) The tritiated thymidine work confirms these findings. LIF alone is not able to enhance proliferation as compared to controls within the normal bone marrow cell population. Equally where assays have been stimulated with GM-CSF which should induce proliferative activity within GM-CFC, LIF does not enhance the level of proliferation as compared to controls. The apparent insensitivity of HPP-CFC to LIF could be a consequence of the HPP sub-population studied. HPP studied were stimulated with WEHI/L929 essentially IL-3/M-CSF (review methods). As noted in earlier discussions on HPP this would relate to a

relatively mature HPP population. The CFUS-13 or indeed the precursor which appears sensitive in Fletcher's work may well be less mature than this population. The HPPmCFU-MK and BL-CFC populations may indeed overlap with Fletcher's population and thus it could be that LIF has a window of sensitivity which is being missed in work contained in this thesis. LIF is certainly not stimulatory or directly inhibitory for DNA synthesis in this particular HPP sub-population. The question of LIF acting as a survival agent should be considered. This could be manifested as proliferation without differentiation ie expansion of the cell pool. This seems unlikely however as there is no increased killing effect in LIF stimulated cultures. Secondly it could be maintenance in G0 without cell death. Again this seems unlikely as there was no drop in colony numbers in HPP from non LIF treated controls. Incubation periods used here (3 hours stimulation/5 hours inhibition) were not sufficient to allow induction of secondary cytokines which may act as outlined by Verfaillie (1993.) If LIF could induce secondary factors which could alter the level of DNA synthesis in populations of haematopoietic cells this would only reinforce the finding that LIF does not directly alter the proliferative status or survival of HPP stimulated to colony formation with WEHI/L929.

3D3: The interaction of LIF with a stem cell stimulator

Robinson *et al* (1993) demonstrated that -SDK- was the functionally important part of the AcSDKP molecule with respect to inhibition of stimulatory activity. Thus this work examined whether LIF, which contained the -SDK- sequence, would have a similar role to play in blocking the action of a stem cell stimulator. LIF is not unique in containing this sequence, perhaps most notably murine and human TNF-α also contain the sequence of the peptide. (L'enfant *et al*, 1989). This is a known inhibitor of progenitors and primitive stem cells. However as reported by Bonnet *et al* (1995) the likelihood is that TNF-α does not exert its inhibitory activity via the -SDK- sequence; although there appears to be no work which directly assays the effects of TNF-α on a stem cell stimulator. In the work presented here LIF (murine) has failed to block the action of a stem cell stimulator. The proportion of the *in vitro* HPP-CFC in S-phase in normal femoral marrow was 10.6%±2.3. In the presence of medium conditioned by regenerating femoral marrow it was 34.5%±9.2(a significant increase $p<0.025$). This increase was not significantly altered by the addition of 1000U/ml LIF, 29.9%±10.12, $p>0.1$. Immediately this poses the question: "what is the significance of this sequence ?" In order to fully address this question it would be necessary to fully understand two points of the stimulator/stem cell interaction. Firstly the nature of the stimulator itself; as yet its identity is unknown. Secondly the precise manner in which the -SDK-

sequence and stimulator interact. Do the two interact directly ? Does -SDK- block the stimulator receptor ? Does it bind to cells directly (Bonnet *et al*, 1993) and interfere with an intracellular pathway or down-regulate stimulator receptors as has been seen with TGF-β and MIP-1-α ? (Maltman *et al*, 1993) Only with an accurate understanding of this interaction can an explanation for murine LIF's inability to mimic this response be offered. Considering the work on TNF-α it is obvious that simply possessing the sequence is not sufficient to guarantee functional equivalence to the tetrapeptide. The assumption must be that its potential inhibitory function is modified by the sequences which surround it.

Although the -SDK- sequence is known to block the action of an already manufactured stem cell stimulator it was noted that monocytes and macrophages have high numbers of LIF receptors where earlier cells have few (Metcalf *et al*, 1989; Fletcher *et al*, 1990). Cells of the monocyte/macrophage lineage were the cells shown to produce stimulator and indeed inhibitor (review intro). It had been demonstrated the LIF did not affect in vitro differentiation of GM-CFC (Metcalf *et al*, 1989) although it may play some role in the survival of spleen derived GM-CFC and to a lesser extent of NBM GM-CFC (Escary *et al*, 1993). It seemed possible that if LIF did not play a significant role in the regulation of these cells yet receptors were present in monocytes and macrophages then it may be that LIF modulated some end function not directly concerned with cell differentiation. Could this possibly be the production of stimulator ? In all cases LIF has failed to inhibit the

production of a stem cell stimulator by populations of aspirated bone marrow. Presuming LIF bound to receptors on moncyte/macrophage cells this would suggest that LIF's function here is not concerned with the manufacture of stimulator, at least as an inhibitory activity.

3D4: LIF and a murine myeloid Leukaemic cell line

LIF was initially characterised on the basis of its ability to induce macrophage differentiation in a murine myeloid Leukaemic cell line M1. SA2JMB1 was an X-irradiation induced murine myeloid leukaemia.

In all cases LIF has had no effect on the clonogenicity of SA2JMB1 cells in semi-solid agar culture either alone or in combination with GM-CSF. At all concentrations the level of colony formation is not statistically different to that seen in the control ($p>0.05$ in all cases). SA2JMB1 colonies were distinct and easy to distinguish from normal GM-CFC colonies. LIF had no effect on the appearance of SA2JMB1 colonies. Where SA2JMB1 cells were pre-incubated with LIF the picture is more complicated. In preincubation cultures supplemented with 100 or 500 U/ml LIF there has been a significant decrease in the level of colony formation as compared to controls. This difference is not apparent at higher concentrations and has not been accompanied by a change in size or appearance of the remaining colonies. In pre-incubation cultures stimulated with GM-CSF there is a statistically significant increase in colony

formation with 100U/ml LIF. In the presence of 500 and 1000 U/ml LIF this increase is not present. This pattern suggests two distinct effects of LIF on SA2JMB1 colony formation. In the former case it is able to reduce it whereas in the latter (when incubated with GM-CSF) it is able to increase it. As in earlier discussions on the effect of LIF on colony formation the first striking point about these results is the magnitude of the difference. Where LIF has decreased clonogenicity the difference is slight (Control 100%; 100U/ml LIF 94.83%±1.83; 500U/ml LIF 96.9%±1.05U/ml). This change is so small, seemingly decreasing as the LIF concentration increases, that its relevance must be questioned. It is possible that LIF has such a small effect when used alone in pre-incubation cultures that it is barely apparent. Weighing against this is the fact that in cultures where GM-CSF was also present the reduction in clonogenicity is not seen at the appropriate concentrations and in fact at 100U/ml LIF is replaced with an increase. This would mean that this particular factor combination was able to increase the fraction of the population forming colonies presumably by increasing the numbers in cell cycle. Thus as a transient agent alone LIF did not alter clonogenicity whereas in association with GM-CSF it increased it. The effects of LIF on Leukaemic cells are often very different. In the case of M1 cells LIF is able to induce significant differentiation within cell populations in agar culture. (80-100% at 400U/ml after 7 days in culture) (Metcalf, 1989). Interestingly molecules which had no or a very slight effect on M1 cell differentiation when used in conjunction with LIF or IL-6 significantly enhanced the level

of differentiation. If LIF were having only a slight effect on SA2 cells it may be that this could be accentuated in conjunction with appropriate growth factors. The work of Maekawa *et al* 1990 on the growth of human myeloid Leukaemic cell lines HL60 and U937 concludes that LIF in combination with GM-CSF can reduce HL60 colony numbers where LIF alone cannot. Similarly in U937 cells LIF in a synergistic interaction with IL-6 and GM-CSF can significantly decrease colony numbers, alone it has no such action. Takanashi *et al* (1993) presented work on the growth of human acute myeloid leukaemias in an assay system which eliminates the need for foetal calf serum. They studied the effects of LIF, IL-6, IL-3 and GM-CSF on various parameters of Leukaemic cell growth. In AML cells from 11 patients LIF was shown to have a variety of actions. In 2 cases it reduced colony formation, in 7 it had no effect and in a further 2 it increased colony formation. In the cases where there was no stimulation of colony formation combination of other cytokines IL-6 showed a colony stimulating activity. In combination with GM-CSF LIF was able to significantly increase the number of colonies seen when compared to those produced by GM-CSF alone. It may be that LIF is acting here to increase the number of blasts able to be stimulated by GM-CSF. If this is the case and a comparable stimulation is being observed in this work then why is it only seen in the case where LIF is present as a transient agent ? In the work of Takanashi *et al* cytokines are available throughout the incubation period (7 days). Differences exist in the protocol used by Takanashi and that

used in these experiments. Specifically these relate to factor concentrations, culture media, inclusion of serum, use of a cloned cell line. Nevertheless Takanashi's work reflects a general pattern for the interaction of LIF with Leukaemic cell populations. It may stimulate, inhibit or have no effect on colony formation. Further confirmation of this comes from Wang *et al* (1990). They note that LIF could stimulate or inhibit human Leukaemic blast cell colony formation. In using a cloned cell line it had been hoped to overcome most of these problems however results suggest that LIF has opposing effects on a Leukaemic cell line dependent on the incubation conditions. These results are certainly significant but how relevant are they ? Would a larger pool of data accentuate or diminish them ? It should be noted that it is only where LIF is present as a pre-incubation agent that any effect is detected. This suggests that this four hour incubation period is where LIF at certain concentrations may be interacting with the Leukaemic cell population.

In the light of previous reports on the effects of LIF on murine or human Leukaemic cells, it is reasonable to conclude that a variation in response is observed.

The effect of LIF on the proliferation of SA2JMB1 cells

LIF at concentrations of 100 or 1000U/ml has had no statistically significant effect on the level of DNA synthesis as compared to the control. ($P>0.05$ in both cases) tritiated thymidine studies confirm this work and extend it. Over three days in culture LIF fails to significantly alter the level of thymidine uptake as compared to controls. These results indicate that LIF does not interact with the Leukaemic cell cycle. If this is the case then it suggests that in the previous experiments LIF's ability to alter colony numbers is not brought about by LIF altering the level of the population in S-phase. It has been suggested that LIF is capable of inducing the G0-G1 transition in normal blast cells (Leary *et al* (1990); Fletcher *et al*, 1990; Ikebuchi *et al*, 1987). Equally Wang *et al* (1990) report that LIF can prolong the doubling time of newly established human acute myeloblastic cell lines in liquid culture. A combination of effects such as this might appear to have excellent value in the therapeutic treatment of leukaemia. However as Wang *et al* point out although the generation of Leukaemic blasts is slowed down consequently the effect of an S-phase specific killing agent is reduced. This coupled with LIF's extreme plieotrophic actions appears to discount its possible use as a chemotherapeutic agent. Again this work highlights the disparate nature of LIF's actions on Leukaemic populations.

3D5: The effects of LIF on HPP-CFC derived from foetal liver

The basis for testing LIF on primitive haematopoietic cells derived from foetal liver was the observation that LIF differentially regulated the maintenance of pluripotent stem cells (CFU-S) and progenitor cells (BFU-E and GM-CFC) between spleen and bone marrow. (Escary *et al*, 1993; Dumenil *et al*, 1993). With respect to colony formation the effects of LIF are varied depending on the concentration used and the factor combinations used to stimulate colony forming cells. In the case of HPP-CFC stimulated by GM-CSF + M-CSF or IL-3+M-CSF LIF has had no effect on colony formation as compared to controls. Where colonies have been stimulated by L929 +WEHI cm the inclusion of 100U/ml LIF in cultures has significantly reduced the level of colony formation as compared to the control (83.77%±7.33 against 100% in the control). This reduction is not observed at 500U/ml or 1000U/ml LIF. This finding is curious in that it is not observed in cultures stimulated with IL-3+M-CSF the recombinant factor combination thought to be responsible for stimulation in WEHI/L929 cm. With 100U/ml added to cultures stimulated by IL-3+M-CSF there is a comparable decrease in the level of colony formation however in this instance it is not significant. (84.5%±15.5 against 100% $P>0.05$). The basis for this inhibition of colony formation is difficult to define. It is apparent from other studies that LIF is capable of enhancing the activity of IL-3 with respect to colony formation. (Leary *et al*, 1990; Debilli *et al*, 1993) and

Leukaemic cell growth (Takanashi *et al*, 1993). Since the inhibition of colony formation is not seen in the recombinant IL-3+M-CSF cultures it seems likely that LIF is interacting with some component of conditioned medium. This observation would gain validity from the observations of Verfaillie and McGlave (1991) that LIF can interact with components of serum to induce proliferative changes which otherwise it would not. Similarly the proposal of Schaffsma *et al* that LIF could be interacting with components of conditioned medium to give alterations in the proliferative status of haematopoietic cells is worth noting here. In the case of HPP stimulated with IL-1+IL-3+M-CSF it seems that LIF at 500U/ml and 1000U/ml is able to significantly reduce the level of colony formation as compared to the control. Thus the effect of LIF has been to reduce the size of the cell population on which IL-1+IL-3+M-CSF can have its colony stimulating activity. If, as in bone marrow, this combination of growth factors is stimulating a primitive stem cell compartment this would mean that LIF was protecting/removing part of the compartment, from the effects of differentiation cues thus preventing terminal differentiation. The purpose of this is not clear. Is exogenously added LIF acting as a survival agent ?

Foetal liver represents a tissue in which HPP-CFC are already in cycle. This is an excellent opportunity to study an innately cycling population i.e. one that has not been induced to cycle by exceptional non-physiological parameters. LIF is unable to block the cycling associated with this cell population. This

indicates that LIF does not appear to play a role in the proliferation regulation of foetal liver HPP-CFC.

CONCLUSIONS: LIF and Haematopoiesis

LIF appears to have a role in murine and non human primate megakaryocytopoiesis/development and function of platelets. (Metcalf *et al*, 1990; Metcalf *et al*, 1991; Debilli *et al*, 1993; Waring, 1993). It may have a role in the proliferation of early human haematopoietic stem cells or their precursors (Leary *et al*, 1990) and multipotential human haematopoietic progenitors(Verfaillie and McGlave, 1991). It is necessary for maintenance of normal haematopoietis in mice and thus may have a role as a possible survival factor (Escary *et al*, 1993; Dumenil *et al*, 1993) such as that seen for LIF (In common with stem cell factor) in PGC's (Williams *et al*, 1989; Pesce *et al*, 1993). LIF is thought to manage this by preventing apoptosis. It is perhaps worth considering that terminal differentiation can itself be seen as cell death.

It is thus possible to envisage a situation in which LIF may have actions at several points in haematopoiesis (Review diagram 8).

It is clear from the work contained in this thesis that LIF is not active in altering colony formation by the majority of HPP-CFC or GM-CFC populations in semi-solid agar culture. Where it is its actions are not straight-forward and there is the possibility that it may be acting as a survival agent. Furthermore the possibility that LIF induces morpholgical changes within

colonies derived from HPP cells is not discounted. With respect to proliferation regulation of an HPP-CFC sub-population LIF was unable to alter the level of proliferation either directly or indirectly. This finding suggests that although LIF contains the -SDK- amino acid motif, thought to be the important part of the AcSDKP molecule with respect to inhibition of stimulator activity, it is unable to mimic the inhibitory action of AcSDKP. This may be unsurprising due to the fact that the sequence is not conserved between species and there is little overlap between the functions of LIF and AcSDKP (Review Bonnet *et al*, 1995; Hilton *et al*, 1992). On a murine myeloid Leukaemic cell population LIF appears to exert +ve and -ve effects on *in vitro* colony formation but is unable to alter the level of proliferation. In some populations of HPP-CFC derived from foetal liver certain concentrations of LIF are able to inhibit colony formation. This may represent suppression of terminal differentiation and thus maintenance of an early stem cell population.

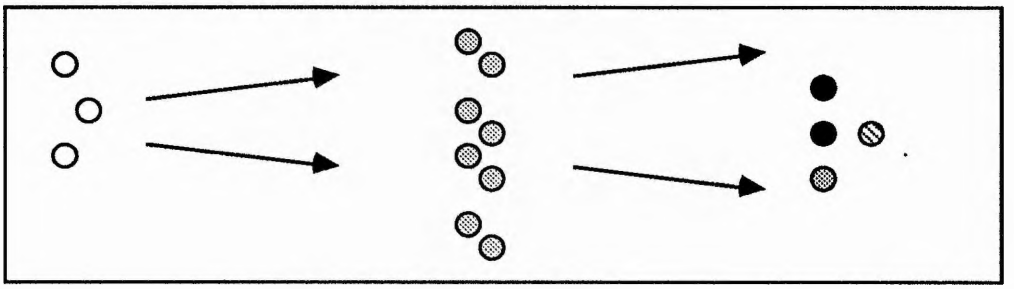
CHAPTER 4: SA2JMB1 CONDITIONED MEDIUM

The term leukaemia is used to categorise a group of haematopoeitic proliferative disorders which are malignant in nature. Leukaemic patients present with symptoms associated with the suppression of normal haematopoiesis: anaemia; thrombocytopenia and infection. This reflects the nature of the disease it is a perturbation of the dynamic balancing the level of primitive cell proliferation with the level of end cell output. This can be construed as a spectrum of leukaemias differing in the particular cell lineage in which neoplastic proliferation is noted and the level of differentiation associated with the leukaemia. A leukaemia can be acute with poorly differentiated cells or chronic in which cells are more differentiated. In both the result is abnormal white blood cell production. (Diagram 19) These abnormal cells infiltrate the haematopoietic system, liver and spleen amongst other tissues Perhaps the most significant aspect of leukaemia is the suppression of normal haematopoiesis. It seems likely that this phenomenon is due to the production by Leukaemic cells of leukaemia associated factors. These factors are able to alter the proliferative status of normal cells whilst Leukaemic cells remain insensitive to them. (Bognacki *et al*, 1981; Riches and Robinson, 1993). Identification of such factors is thus of fundamental importance in the treatmentt of leukaemias.

In this chapter SA2JMB1 conditioned medium (SA2cm) was assessed *in vitro* as an agent affecting certain aspects of haematopoeisis. Attempts were made to identify activities interacting with aspects of haematopoiesis .

HAEMATOPOIESIS IN LEUKAEMIA

TAKES PLACE WITHIN BONE MARROW



STEM CELLS	PROGENITOR CELLS	FUNCTIONAL END CELLS
-A leukemic lesion may develop in one stem cell .	- As cells mature leukemic lesions may accumulate resulting in the development of large numbers of leukemic progenitor cells.	- Do NOT get increased numbers of end cells in line with increased numbers of progenitors actually see a decrease in numbers.

DIAGRAM 19 SCHEMATIC APPRECIATION OF BLOOD CELL FORMATION IN LEUKEMIC INDIVIDUALS

4.1: FEMORAL MARROW

Again as in work investigating LIF, this work falls into two broad categories: **direct** action and **indirect** action. SA2JMB1 were grown (according to the protocol laid out in 2(1.3.3)) in Fischer's 10%. In all experiments SA2cm was routinely used at a range of concentrations not exceeding 20%(v/v). (Diagram 20)

All results for individual experiments were normalised as a percentage of the control. This validated the comparison of data between individual experiments and also meant that data could be pooled as a mean.

An unpaired two-tailed t-test was used to analyse data. (See 2(3.1.1)) p values are shown beneath the mean for each condition.

Means of data for individual experiments are shown beneath p values.

METHOD

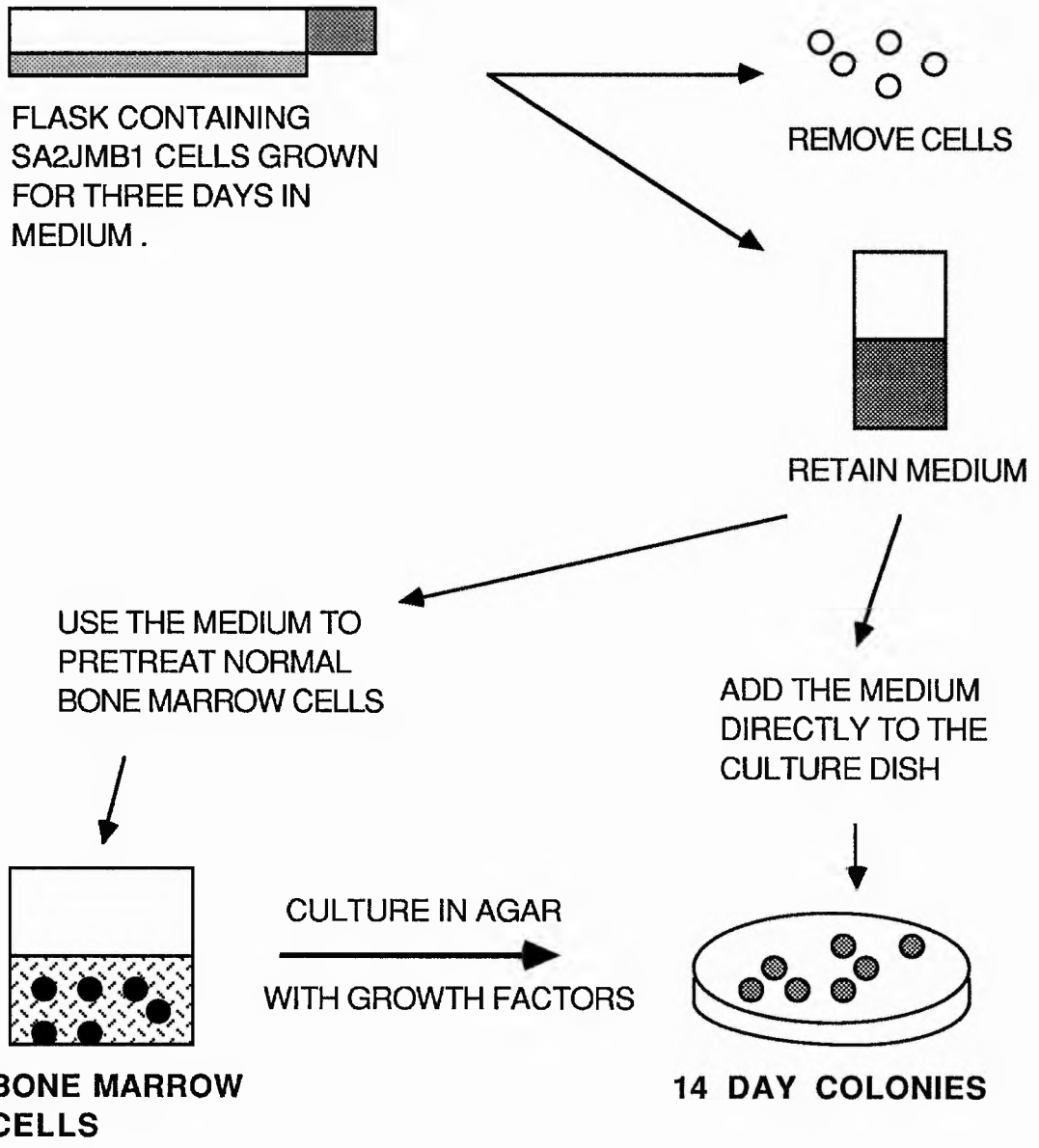


DIAGRAM 20 SUMMARY OF METHODS TO STUDY SA2CM ACTION

DIRECT ACTION

4(1.1)The effect of SA2JMB1 conditioned medium (SA2 cm) on the colony forming potential of GM-CFC in semi-solid agar culture

It was decided to test a range of concentrations of SA2cm *in vitro* for colony stimulating activity on 7 day semi-solid agar cultutres. SA2cm was tested as an agent acting alone and as a synergistic agent with GM-CSF, IL-3, WEHIcm and L929cm.

4(1.1.1) PROCEDURE

a) GM-CFC stimulated with SA2cm alone.

SA2cm was assayed at 20%(v/v), 10%(v/v), 5%(v/v), 2%(v/v) and 1%(v/v)

The protocol was a modification of the standard GM-CFC assay (2(1.3.1)). 30ml universals were supplemented with appropriate volumes of SA2cm. (E.G 2mlSA2cm in 10ml prep for 20%v/v; 1ml in 10ml prep for 10%v/v etc). A control universal was prepared which was not supplemented with SA2cm. A standard GM-CFC assay was then performed for each condition.

b) GM-CFC stimulated with SA2cm and other factors.

The above protocol was repeated but with universals supplemented with various other growth factors, final volumes were altered appropriately.

The table below summarises the factors tested:

	FACTOR			
ALL CONC SA2CM	GM-CSF (40U/ml)	IL-3 (80U/ml)	WEHI CM 10% (v/v)	L929 CM 10%(v/v)

TABLE 4.1 *summary of factors used to stimulate 7-day cultures*

Four replicate experiments were performed.

(4.1.1.2) RESULTS

Table 4R.1a, 4R.1b and Figures G4.1a, G4.1b show the results for 7 day colony formation in the presence or absence of varying concentrations of SA2cm.

In the case of cultures supplemented with SA2cm only, table 4R.1a shows that a complicated pattern emerged with no consistent finding throughout the four individual experiments. In cultures supplemented with 1% V/V SA2cm there is no significant increase in the level of colony formation as compared to the control in any of the individual experiments. At 2%SA2cmV/V a significant increase is observed in 2 of the experiments ($P<0.05$). At 3% SA2cm V/V there is a statistically significant increase in the level of colony formation in three of the experiments. At 10 and 20% SA2cm V/V a similar pattern emerges with three of the four experiments showing a significant increase in the level of colony formation. In all instances the level of colony formation is not high. This is illustrated in figure G4.1a.

In 7 day culture of femoral marrow SA2cm appears able to support limited colony formation.

Table 4R.1b show the effect of SA2cm on the level of colony formation induced by other growth factors. In the case of IL-3 stimulated cultures supplemented with varying concentrations of SA2cm there is no statistically significant difference in colony formation with 1 ,2, 5, and 10 %v/v SA2cm. ($P.>0.05$) in all cases. In the case of IL-3 stimulated cultures supplemented with 20% SA2cm there is a significant increase in the level of colony formation as compared to the control. ($P<0.05$)

In cultures supplemented with GM-CSF there is a statistically significant increase in the level of colony formation at all concentrations of SA2cm ($P<0.05$) except 1%v/v ($P>0.05$). The same picture emerges for L929 stimulated cultures.

In the case of cultures stimulated with WEHIcm there is no statistically significant increase in the level of colony formation at any concentration of SA2cm ($p>0.05$).

Thus with respect to influencing colony formation in 7-day semi-solid agar culture SA2cm shows a differential pattern of activity.

TABLE 4R.1a: The effect of varying concentrations of SA2cm on the colony forming potential of GM-CFC from NBM in 7-day semi-solid agar cultures .Data for four individual experiments is shown.

CONC SA2CM (%V/V)	0	1	2	5	10	20
	COLONY	FORMAT	ION	(PER	$5x10^4$	cells)
7 DAY ASSAY WITH SA2CM ALONE	0.0±0.0	0.0±0.0	0.0±0.0	1.5±0.5	2.0±0.0	12.0±1.0
				.025<P<.05	P<.0005	P<.0005
	0.0±0.0	0.0±0.0	2.0±0.0	2.0±1.0	10.5±5.5	18.5±1.5
			P<.0005	.005<P<0.1	.05<P<.1	P<.005
	0.0±0.0	0.5±0.5	3.0±0.0	3.5±0.5	9.5±2.5	7.5±4.5
		.1<P<.375	P<.0005	P<0.01	P<0.05	.1<P<.375
	0.0±0.0	0.0±0.0	0.0±0.0	3.0±1.0	4.0±0.0	10.5±2.5
				P<0.05	P<.0005	P<.05

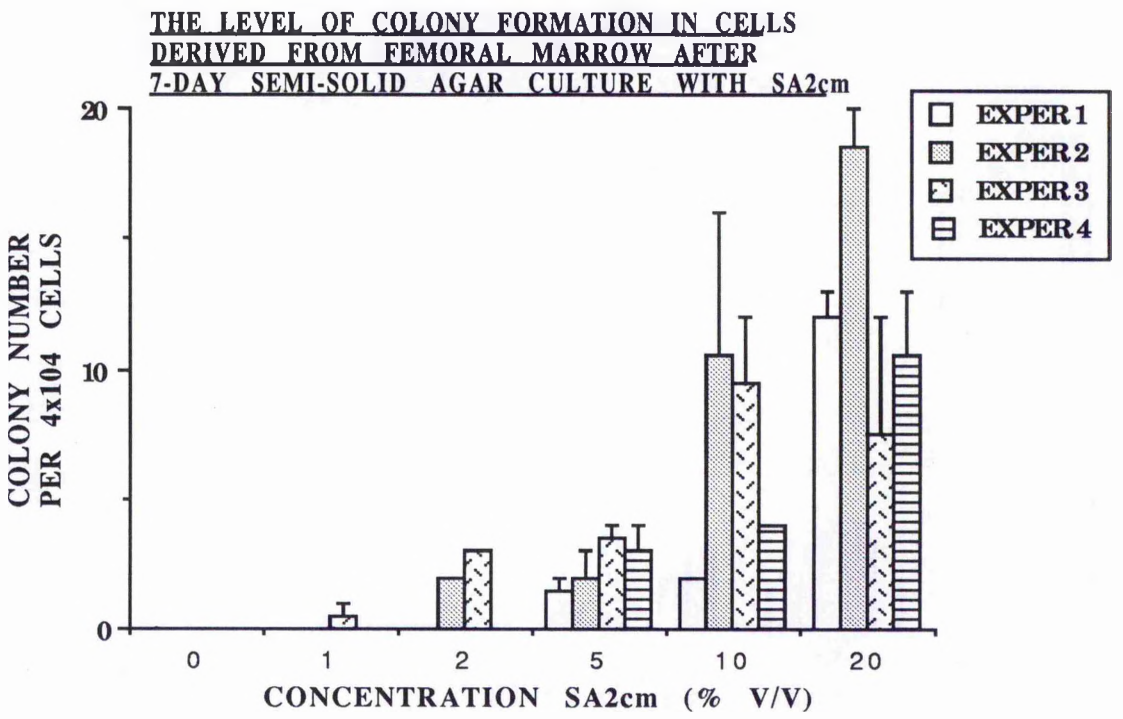


FIGURE G4.1a: Colony formation(expressed as colony number per 5x 10^4 cells plated) in 7-day semi-solid agar cultures of NBM in the presence or absence of varying levels of SA2cm. Data for four individual experiments is illustrated.

TABLE 4R.1b: The effect of varying concentrations of SA2cm on the colony forming potential of a variety of GM-CFC in 7-day semi-solid agar cultures .

CONC. SA2CM (%V/V)	0	1	2	5	10	20
GM-CFC ASSAY	COLONY FORMATI ON (%/C ONTROL)					
IL-3	100.0	101.7± 6	99.7±14	121.3±11	120±10.5	138±9.6
		.375<P≤.4	P>0.4	0.05<P≤.1	0.05<P≤.1	P<0.005
	15.0±1.0	15.5±0.5	19.0±1.5	20.5±2.5	21.0±1.0	24.5±2.5
	14.0±2.0	14.5±0.5	16.5±1.5	20.0±3.0	19.0±1.0	20.5±0.5
	24.5±2.5	28.0±3.0	22.5±1.7	28.5±3.5	25.5±4.2	31.5±4.5
	23.0±9.0	20.0±2.3	14.5±1.5	21.5±3.5	23.0±2.3	27.5±0.5
GM-CSF	100.0	107±7.8	134±8.8	185±36.8	213±35	220.7±41
		.1<P≤.375	P<0.005	P<.005	P<0.01	.01<P≤.03
	15.0±4.0	16.0±3.0	23.0±3.0	33.0±2.0	40.5±2.5	39.0±1.0
	11.0±1.0	11.0±1.0	16.0±8.0	30.0±0.0	31.0±1.0	34.5±3.5
	22.0±4.0	20.2±2.5	26.0±1.0	26.5±4.5	31.0±5.0	31.0±3.0
	21.0±2.0	27.0±1.0	25.0±3.0	27.5+10	35.0±1.0	35.0±1.5
L929	100.0	84.5±39	141.2±20	168.7±22	215±9	251±27.6
		.1<P≤.375	.03<P≤.05	.01<P≤.03	P<0.0005	P<0.005
	14.5±4.5	18.0±0.0	20.0±1.0	30.5±7.5	35.0±1.0	37.5±3.5
	20.0±3.0	9.0±1.0	28.0±3.0	28.0±5.0	40.0±2.0	46.5±5.5
	17.0±1.0	17.0±4.0	26.0±2.0	30.0±2.0	36.5±2.5	39.0±0.0
	15.0±1.0	19.0±1.0	18.0±1.0	21.5±0.5	31.0±1.0	40.5±3.5

WEHI	100.0	90.5±27.5	101.3±8.5	89.3±12.2	116.5±25	106.3±12
		.1<P≤.375	1<P≤.375	.1<P≤.375	.1<P≤.375	.1<P≤.375
	11.0±3.0	13.0±0.0	14.0±3.7	13.5±3.5	20.5±5	15.0±2.0
	18.5±5.0	11.5±0.5	14.5±0.5	11.0±1.5	13.0±5.0	15.0±2.0
	20.5±1.5	22.0±1.0	22.0±1.0	17.5±0.5	22.5±1.5	20.0±3.0
	21.5±2.5	21.5±1.5	21.5±11	20.5±2.5	21.5±2.5	23.0±1.0

In all cases N = 4

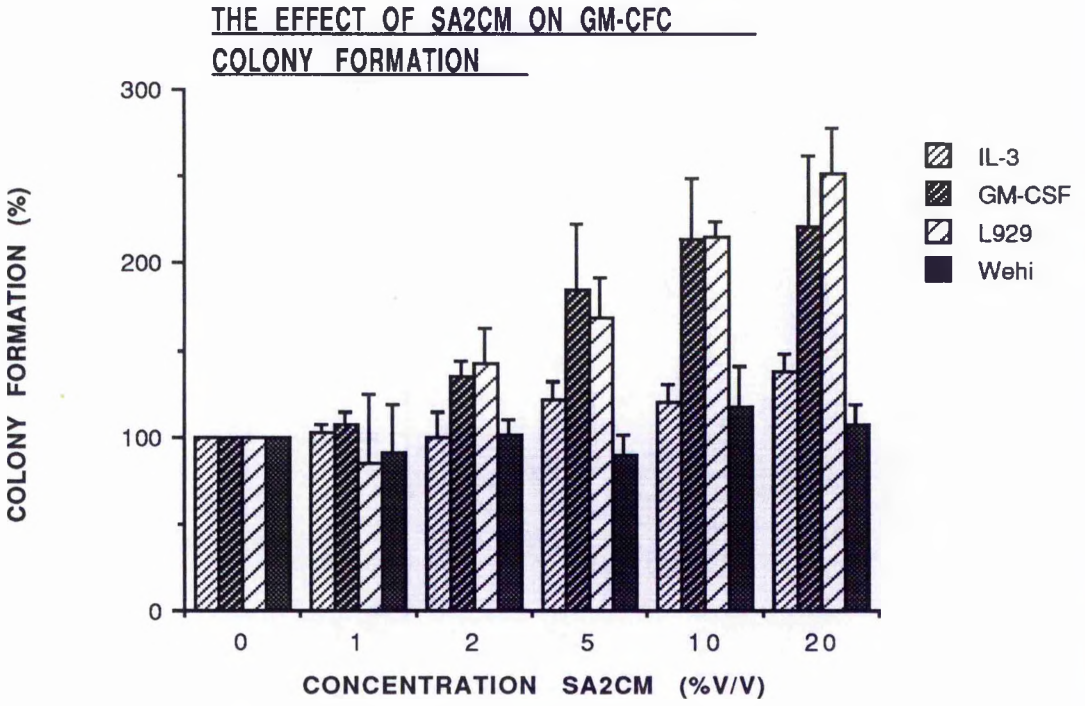


FIGURE G4.1b: Colony formation(expressed as a percentage of the control 0%V/V SA2cm) in 7-day semi-solid agar cultures of NBM stimulated with varyious growth factors in the presence or absence of varying levels of SA2cm

4 (1.2)The effect of SA2cm on the colony forming potential of HPP-CFC in semi-solid agar culture

In an attempt to define a role for SA2cm in interacting with primitive haematopoietic cell populations SA2cm was assayed *in vitro* for colony stimulating activity on HPP-CFC. It was assayed both as an agent able to stimulate colony formation on its own and as a synergistic agent in cultures stimulated with :

- 10% WEHI cm + 10% L929 cm;
- GM-CSF + M-CSF (HPP 3);
- IL-3 + M-CSF (HPP 2);
- IL-1α + IL-3 + M-CSF (HPP 1);
- IL-1α + IL-3;
- IL-1α + M-CSF;

4(1.2.1) PROCEDURE

The protocol used was a modification of the standard HPP-CFC assay 2(2.1.3).

a) HPP-CFC stimulated with SA2cm alone

FEEDER LAYERS

SA2cm was assayed at 20%(v/v), 10%(v/v) and 5%(v/v) feeder layers were prepared containing SA2cm at these final concentrations. A control was prepared in which feeder layers contained no SA2cm (i.e. 9ml Dulbecco's 20% and just prior to plating 1ml 5% agar).

b) HPP-CFC stimulated with SA2CM and other growth factors.

The above protocol was repeated but with universals supplemented with various other growth factors, final volumes altered apporopriately.
The table below summarises the conditions tested:

			FACTOR			
ALL CONC. SA2CM	WEHI CM (10% v/v) + L929 CM (10% v/v)	GM-CSF (40U/ml) + M-CSF (50U/ml)	IL-3 (80U/ml) + M-CSF (50U/ml)	IL-1α (10U/ml) + IL-3 (80U/ml) + M-CSF (50U/ml)	IL-1α (10U/ml) + IL-3 (80U/ml)	IL-1α (10U/ml) + M-CSF (50U/ml)

TABLE 4.2: *Summary of factors used to stimulate HPP-CFC in 14-day semi-solid agar cultures.*

Standard HPP-CFC assays were performed for each condition Three replicate experiments were performed.

4(1.2.2) RESULTS

Table 4R.2a, 4R.2b and Figures G4.2a, G4.2b show the results for the effect of SA2cm on the level of colony formation in 14 day semi-solid agar cultures .

Table 4R.2b shows that in three individual experiments where cultures were supplemented with SA2cm an inconsistent

pattern of colony formation emerges. With 5 or 20%V/V SA2cm a significant increase in the level of colony formation emerges in two of the three experiments. At 10%SA2cmV/V a significant increase in colony formation is seen in only one experiment. Thus SA2cm does appear able to support limited colony formation in 14 day culture. Notably the level of colony formation is far less than that seen in 7day cultures. There is also no sustantial increase in the level of clonony for mation seen with 20%SA2cm V/V as compared to that seen with 5%SA2cm V/V. This is illustrated in figure 4G.2a. This would suggest that SA2cm contains very low levels of factors able to stimulate the production of HPP-CFC.

Table 4R.2b shows that in cultures stimulated with WEHI/L929 there is no significant difference in the level of colony formation seen in the presence of any concentration of SA2cm as compared to that seen in the control. ($P>0.05$ in all cases)

In cultures stimulated with GM-CSF/M-CSF there is a statistically significant **increase** in the level of colony formation at 5 and 10%v/v SA2cm as compared to the control($P<0.05$). This is not observed at 20%v/v SA2cm where $P>0.05$.

In the case of cultures stimulated with IL-3/M-CSF there is no significant difference in the level of colony formation at concentrations of 5 or 10 %v/v SA2cm ($P>0.05$). With 20%v/v SA2cm there is a significant increase in the level of colony formation ($P<0.05$).

For cultures stimulated with IL-1/Il-3/M-CSF or IL-1/M-CSF a similar picture emerges. At concentrations of 5 or 20 %v/v

SA2cm there is a significant increase in colony formation ($P<0.05$). At 10%v/v SA2cm no such increase is observed ($P>0.05$).

The actual increase in the level of colony formation is much greater in cultures supplemted with IL-1+ M-CSF than those supplemented with IL-1+IL-3+M-CSF.

Thus SA2cm has a variety of effects on the level of colony formation seen in 14 day agar culture. These effects appear to be related to the growth factor combinatioins with which cultures were supplemented.

TABLE 4R.2a: The effect of SA2CM on the colony forming potential of HPP-CFC from NBM in semi-solid agar culture. Data from three individual experiments is shown.

CONC SA2CM (%V/V)	0	5	10	20
		COLONY	FORMATION	(%/control)
14 DAY ASSAY WITH SA2CM ALONE	**0.0±0.0**	**1.0±0.4**	**0.5±0.3**	**1.0±0.7**
		.01<P<.025	.05<P<.01	.1<P<.375
	0.0±0.0	**0.5±0.5**	**2.0±2.0**	**1.0±0.0**
		.1<P<.375	.1<P<.375	P<.0005
	0.0±0.0	**1.0±0.0**	**3.0±0.0**	**1.5±0.5**
		P<.0005	P<.0005	.025<P<.05

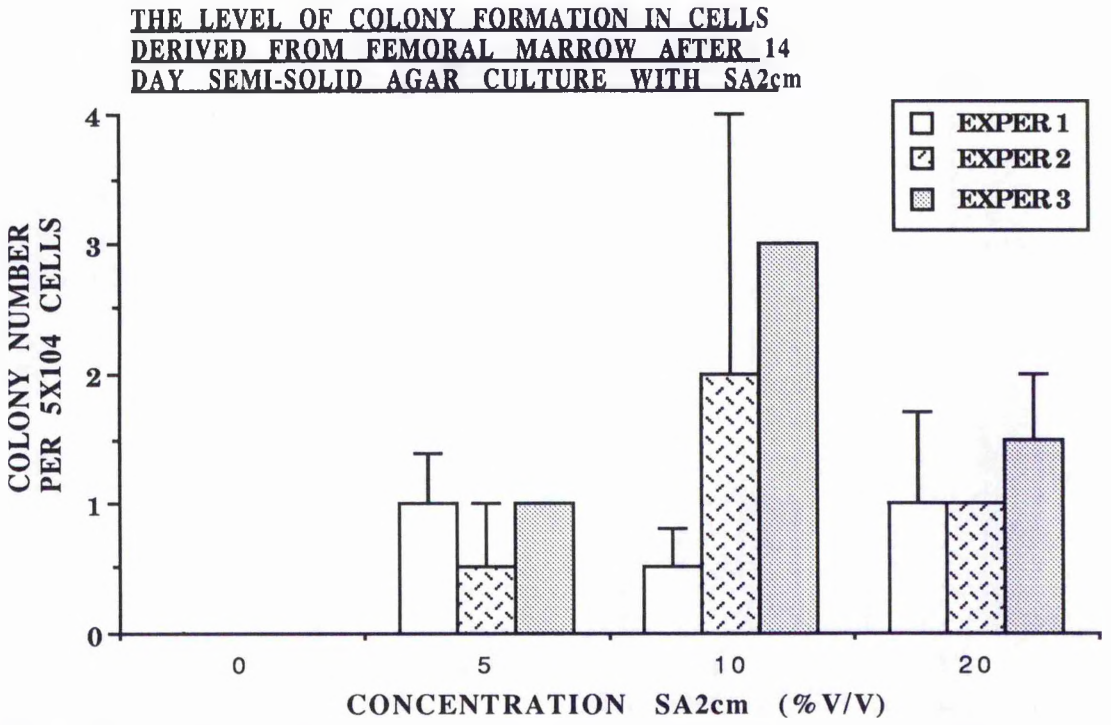


FIGURE 4G.2a: Colony formation (expressed as colony number per $4x10^4$ cells plated) in 14 day semi-solid agar cultures of NBM in the presence or absence of varying concentrations of SA2cm Data from three individual experiments is illustrated.

TABLE 4R.2b: The effect of SA2CM on the colony forming potential of HPP-CFC from NBM in semi-solid agar culture.

CONC SA2CM (%V/V)	**0**	**5**	**10**	**20**
HPP-CFC ASSAY		**COLONY**	**FORMATION**	**(%/CONTROL)**
WEHI/L929	**100.0**	**103.7±3.7**	**98.7±7**	**112.3±9.1**
		0.1<P≤.375	P>0.4	0.1<P≤.375
	29.0±3.0	29.5±1.5	25.5±3.5	29.5±2.5
	18.0±0.0	29.5±2.4	28.5±1.5	31.0±2.0
	29.5±7.0	20.5±1.5	22.0±2.0	23.5±0.5
GM-CSF + M-CSF	**100.0**	**154.3±24**	**181.9±26**	**178±63**
		.025<P≤0.05	0.01<P≤.025	0.1<P≤.375
	11.0±1.0	11.0±3.0	20.0±3.0	10.0±1.0
	5.5±3.5	16.0±1.0	12.5±0.5	19.5±1.5
	13.5±3.5	16.0±4.0	18.5±2.5	15.0±0.5
IL-3 + M-CSF	**100.0**	**92.3±5.0**	**102.7±6.2**	**104.3±1.3**
		0.05<P≤0.1	0.1<P≤0.375	0.01<P≤.025
	31.5±2.5	26.5±2.5	30.5±3.5	32.5±1.5
	38.5±4.5	27±1.5	26.5±2.5	29.5±0.5
	27.5±1.5	25±3	31±3	28±2
IL-1 + IL-3 + M-CSF	**100.0**	**106.0±2.5**	**116.0±9.2**	**119.0±7.9**
		0.025<P≤.05	0.05<P≤0.1	0.025<P≤.05
	40.0±5.0	41.5±3.5	46.0±4.0	46.5±2.5
	31.0±7.0	34.5±2.5	41.5±0.5	41.0±3.0
	38.5±0.5	40.0±1.0	38.0±6.0	41.5±1.5

IL-1+ M-CSF	**100.0**	**150.7±12.0**	**157.3±37.0**	**213.3±33.0**
		0.05<P≤0.1	0.1<P≤.375	0.01<P≤.025
	20.5±4.5	26.0±1.0	10.0±3.0	33.5±3.5
	8.5±0.5	8.5±0.5	10.5±0.5	16.5±2.5
	4.5±0.5	4.5±0.5	25.0±0.0	12.5±0.5

In all cases N=3

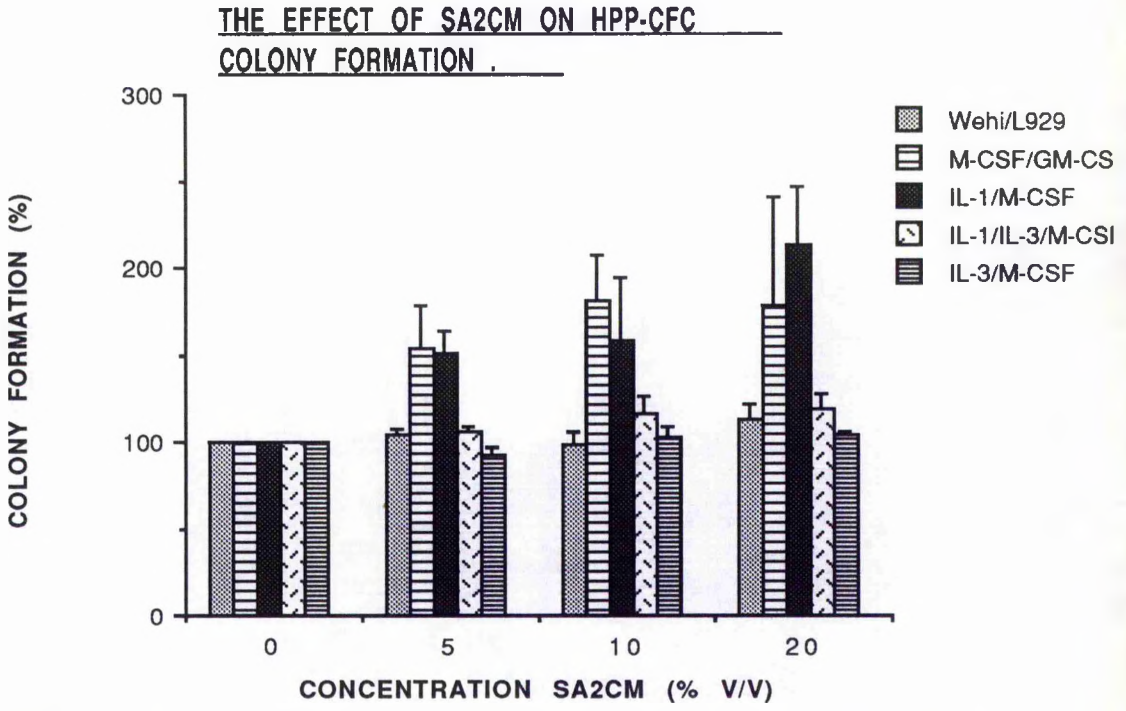


FIGURE 4G.2 b: Colony formation (expressed as a percentage of the control) in 14 day semi-solid agar cultures of NBM stimulated with varying growth factor combinations in the presence or absence of varying concentrations of SA2cm.

4 (1.3) The effect of SA2cm *in vitro* on the proportion of GM-CFC and HPP-CFC in DNA synthesis.

The principle here was identical to that employed when testing the effect of LIF on the proportion of a cell population in S-

phase. It is possible that Leukaemic cells may gain a proliferative advantage by actively altering the proliferative status of normal haematopoietic cells. Thus normal cells cannot respond to the decreased end cell production associated with leukaemia.

In this experiment SA2cm was assessed as an agent able to alter the proportion of haematopoietic cell populations in S-phase.

4(1.3.1) PROCEDURE

As a potential stimulator of DNA synthesis

In normal femoral marrow the HPP-CFC population is largely quiescent with 10-15% in S-phase. Thus it is possible to assay SA2cm as a stimulator of DNA synthesis in populations of HPP-CFC derived from femoral marrow.

The protocol was a modification of the standard stimulator-S-phase suicide assay. Four red-topped conical bottomed centrifuge tubes recieved 1ml aliquots of cell dilution. The tubes were then set out in two pairs. 200ul of SA2cm was aliquoted into both tubes of the first pair. This gave two identical tubes containing 1ml $5x10^6$ cells, 20%(v/v) SA2cm. The second pair, the control tubes, received aliquots of 200ul Fischer's 10% .A standard stimulator S-phase suicide assay with ARA-C was then performed.

Assays for HPP-CFC stimulated with WEHI/L929 conditioned media were performed for each tube

As a potential inhibitor of DNA synthesis

A high proportion of the GM-CFC population in normal femoral marrow is in S-phase. It is thus possible to assay SA2cm as an agent able to reduce levels of DNA synthesis within this cell population.

GM-CFC

The method used is exactly as that outlined to test SA2cm as a stimulator with the exception that cells are incubated for three hours prior to the one hour ARA-C incubation. Assays for GM-CFC stimulated with WEHI were performed.

HPP-CFC

As in the earlier studies involving LIF, regenerating bone marrow was used as a source of cycling HPP-CFC. The protocol was identical to that used to test SA2cm as a stimulator with the exception that the cells were incubated for four hours prior to the one hour ARA-C incubation.

In all cases four replicate experiments were performed.

4(1.3.2) RESULTS

Table 4R.3.1 and Figure G4.3 show the effect of SA2cm on DNA synthesis in a population of HPP-CFC. There is no statistically significant difference between the level of cycling seen in the control and that seen in the population treated with 20%v/v SA2cm (control13.9%±1.8 / 20%V/V SA2cm 13.6±1.4 where $0.1<p\leq0.375$).

Table 4R.3.2 and Figure G4.4 show the effect of SA2cm on DNA synthesis in a population of GM-CFC. The addition of SA2cm to

cultures failed to alter the proportion of the cell population in DNA synthesis as compared to the control (control 26.7%±2.43 / 20% SA2cm 25.0±2.7 where P > 0.4).

The addition of Table 4R.3.3 and fugure G4.5 show the effect of SA2cm on DNA synthesis in HPP-CFC derived from regenerating femoral tissue. In this tissue a relatively high proportion of the population is in DNA synthesis (Control 28.0% ±4.4) The addition of SA2cm fails to alter the level of DNA synthesis seen in this population (20% SA2cm 21.4%±2.6 where P>0.05)

Thus SA2cm at this test concentration does not alter the level of DNA synthesis in any of the colony forming cell populations tested.

TABLE 4R.3.1: HPP-CFC

CONDITION	NBM	N B M + SA2CM(20%V/V)
	PROPORTION	*S-PHASE (%)*
FACTORS (HPP-CFC)		
L929/WEHI	**13.9±1.8**	**13.5±1.4**
		0.1<P≤0.375
	13.24	13.50
	10.81	10.71
	12.39	12.64
	18.97	17.27

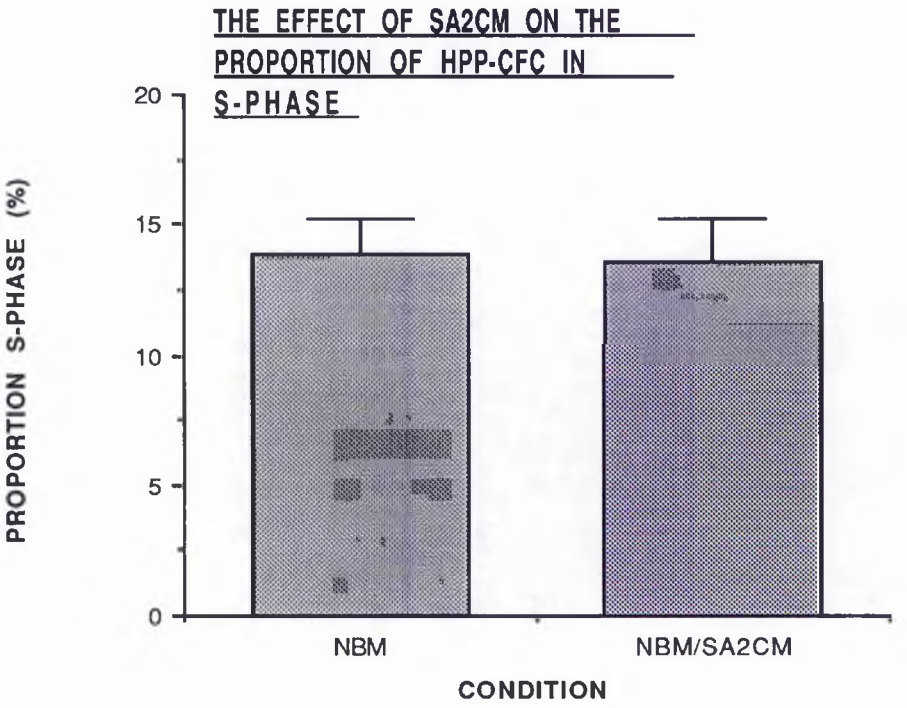


FIGURE G4.3: The proportion of HPP-CFC(WEHI/L929) from NBM in D.N.A synthesis(%) following incubation with varying concentrations of LIF.

AS A POTENTIAL INHIBITOR OF D.N.A SYNTHESIS

TABLE 4R.3.2: GM-CFC

CONDITION	NBM	N B M + SA2CM(20%V/V)
	PROPORTION	*S-PHASE (%)*
FACTORS (GM-CFC)		
WEHI	**26.8±2.4**	**25.0±2.7**
		P>0.4
	28.03	20.21
	22.00	31.05
	24.56	24.00
	33.82	27.00

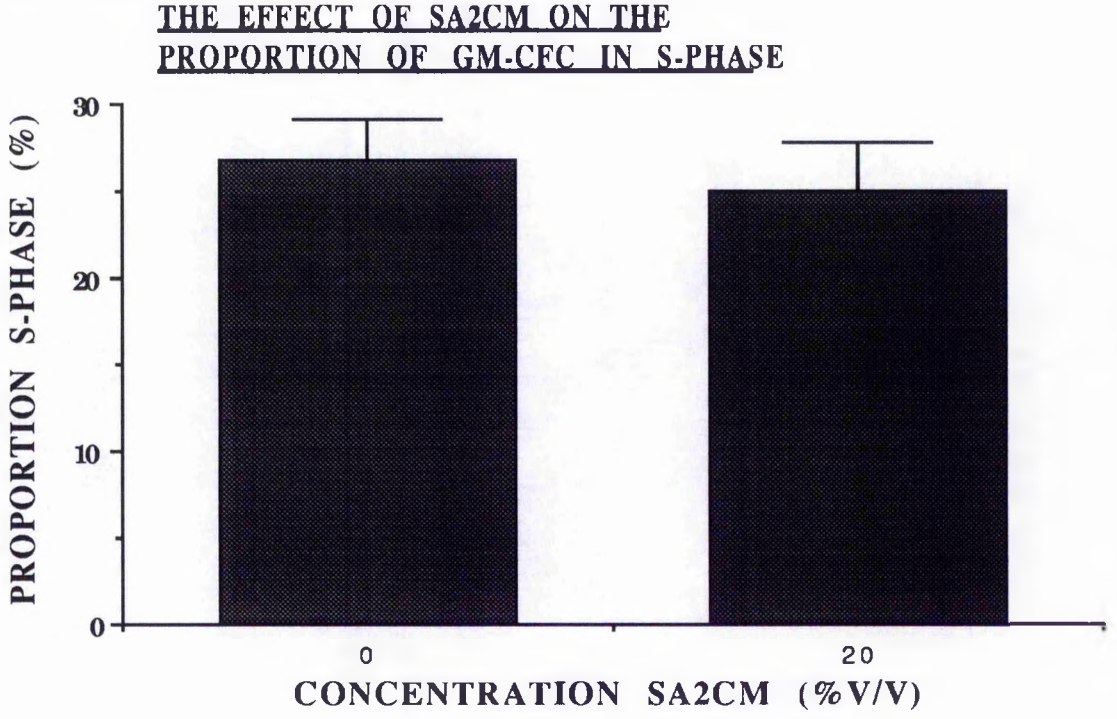


FIGURE4.4: The proportion of GM-CFC (WEHI) from NBM in S-phase (%) following incubation with varying concentrations of LIF

TABLE 4R.3.3: HPP-CFC

CONDITION	RBM	R B M + SA2CM(20%V/V)
	PROPORTION	*S-PHASE (%)*
FACTORS (HPP-CFC)		
L929/WEHI	**28.0±4.4**	**21.4±2.6**
		0.1<P≤0.375
	38.46	25.00
	31.94	26.50
	22.09	15.29
	19.47	18.86

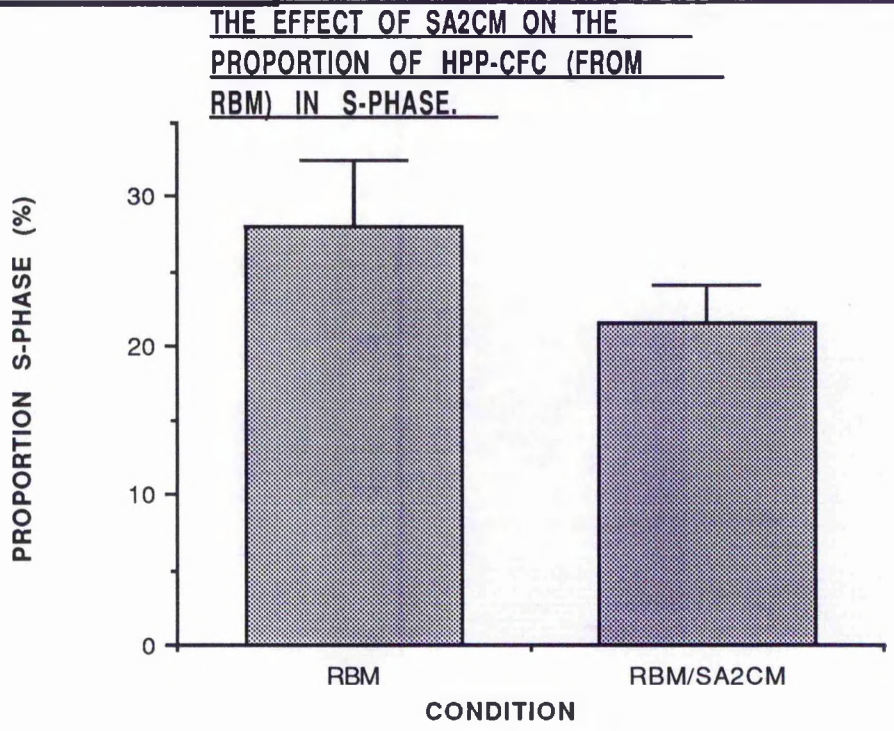


FIGURE G4.5: HPP-CFC (WEHI-L929) from regenerating bone marrow in S-phase(%) following incubation with varying concentrations of SA2cm

INDIRECT ACTION

A Leukaemic cell population could gain a proliferative advantage over normal haematopoietic cell populations in a number of ways. Of these interference with the level of DNA synthesis in cell populations has been excluded in the above work. It is possible that Leukaemic associated factors could act to interfere with those activities responsible for the initiation of DNA synthesis in normal haematopoietic cell populations. This work attempted to define a role for SA2cm in interacting with a stem cell stimulator.

4 (1.4) Does SA2cm alter the action of a haematopoietic stimulator on a defined HPP-CFC subpopulation?

A stem cell stimulator has been shown to be produced reproducibly by femoral marrow recovering from irradiation. It is reasonable to assume that such a stimulator may be produced *in vivo* in response to haematopoietic stress such as that associated with the development of a leukaemia. This experiment was designed to assay the ability of SA2cm to interfere with the production of this stimulator.

4(1.4.1) PROCEDURE

The protocol was a modification of the standard stimulator block assay.

A single cell suspension was prepared and cellularity adjusted to 5 x 10^6 cells/ml. 8 ml of this dilution was prepared. 1ml

aliquots of this cell dilution were pipetted into each of six red-topped conical bottomed centrifuge tubes. The tubes were then set out in three pairs. 1ml of 'stimulator' was added to four of the tubes. 400ul SA2cm was aliquoted into two of these tubes. 400ul Dulbecco's 20% into the other two. 1400ul of Dulbecco's 20% was added to each of the two remaining tubes, the control tubes. A standard stim-block assay was performed.

Assays for HPP-CFC stimulated with WEHI/L929 conditioned media were performed for each tube.

Five replicate experiments were performed.

4(1.4.2) RESULTS

Table 4R.4 and figure G4.6 show the effect of SA2cm on the action of a stem cell stimulator.

In Normal bone marrow a small proportion of this HPP-CFC sub-population are involved in DNA synthesis (17.08%±1.16). In the presence of a stem cell stimulator there is a statistically significant increase in the proportion of the population in DNA synthesis. (30.31%±3.42 where $0.0005<P\leq0.005$). In the presence of 20%v/v SA2cm this increase in the level of DNA synthesis is lost. (18.09% ±2.70 where $0.1<p\leq0.375$).

Thus SA2cm has blocked the action of a stem cell stimulator on a population of HPP-CFC.

TABLE 4R.4: The effect of LIF on the action of a stem cell stimulator.

CONDITION	NBM	NBM+STIM	NBM + STIM + SA2CM(20%V/V)
FACTORS (HPP-CFC)	*PROPORTION S-PHASE (%)*		
WEHI/L929	**17.08±1.16**	**30.31±3.42**	**18.09±2.70**
		0.0005<P≤0.005	0.1<P≤0.375
	17.20	36.84	16.07
	12.87	34.78	21.65
	20.00	35.71	26.63
	17.86	24.04	13.27
	17.48	20.18	12.87

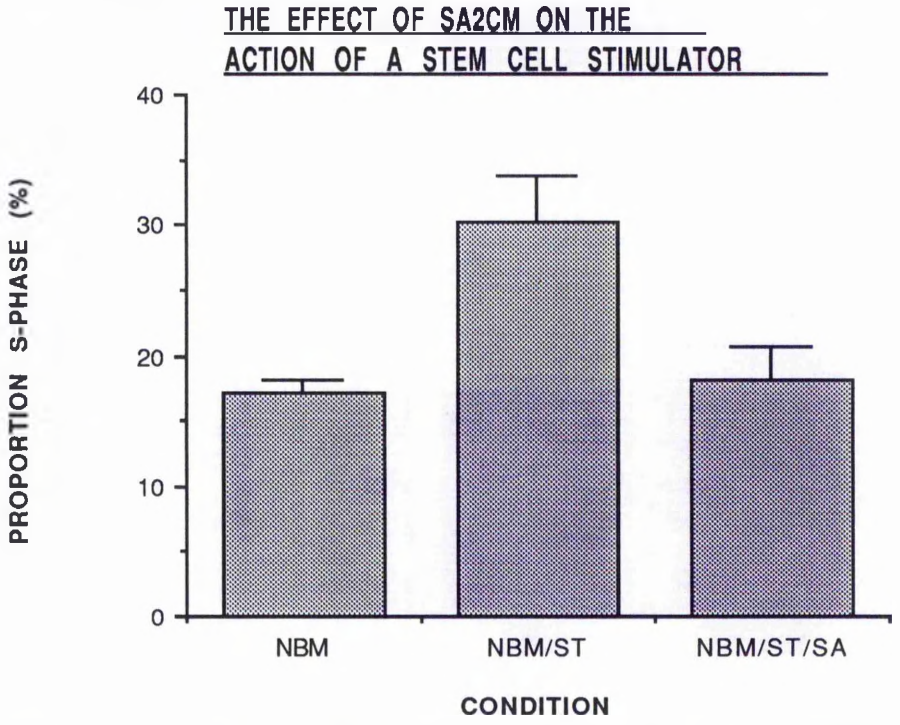


FIGURE G4.6: The proportion of HPP-CFC(WEHI-L929) from NBM in S-phase(%) after treatmentt with a stem cell stimulator in the presence or absence of SA2cm.

4.2: IDENTIFICATION OF THE ACTIVITIES WITHIN SA2cm

SECTION1: COLONY PROMOTING ACTIVITY

The pattern of colony promoting activity emerging for SA2cm suggested that it may contain IL-3. (See results and discussion). This is not to suggest that SA2cm was thought to be a source of IL-3 alone, but that its actions in promoting colony formation in both HPP-CFC and GM-CFC cultures suggested that it may contain IL-3 as one of its exponents.
A series of experiments were undertaken to confirm this.

4(2.1.1) The effect of SA2cm on the proliferation of IL-3 factor dependent cell lines

Cell lines were available (A4 and C2GM) whose proliferation was dependent on the presence of IL-3. It had been shown that SA2cm supported the proliferation of these factor dependent cell lines. This work set out to determine the molecular weight band in which the signal was contained .

4(2.1.1.1) PROCEDURE

Concentration and filtration of SA2cm conditioned media

It was necessary initially to filter and concentrate the SA2conditioned medium. Centricon microconcentrators with a 10KD molecular weight cut off were used 2(1.4.1.1).

FDCP-A4 cells (See 2) were cultured in limited volume liquid suspension culture in the presence or absence of fractions of SA2cm, F10% or WEHI cm at a range of concentrations. A4 cellularity was determined as in 2.(1.3.4) and adjusted to 5 x 10^6cells/ml in F10%. A 96 well microtitre plate was prepared and in a standard thymidine uptake assay for A4 cells 2() both fractions of SA2cm were tested at concentrations of 50%v/v, 25%v/v, 12.5%v/v, 6.25%v/v and 3.125%v/v. F10% was tested at identical concentrations. WEHIcm was tested at 10%v/v, 5%v/v, 2.5%v/v, 1.25%v/v and 0,75%v/v. Plates were then placed in the incubator at 37^0C with 5% CO_2 in air and a fully humidified atmosphere for forty eight hours.

Protocol as outlined in 2 (2.1.5.3)-2(2.1.5.5.)

Two replicate experiments were performed.

4(2.1.1.2) RESULTS

Table 4R.5.1, Table 4R.5.2, Table 4R.5.3 and figures G4.7, G4.8 show the results for the effect of SA2cm, F10% and WEHIcm on the uptake of tritiated thymidine by FDCP-A4 cells.

Table 4R.5.1 shows that there is a significant increase in the mean thymidine uptake in wells supplemented with 3.125 or 6.25 %v/v SA2cm<10KD; $P<0.05$ in both cases .At concentrations of 12.5, 25 or 50%v/v no comparable increase in thymidine uptake is observed; $P>0.05$.

For the fraction of SA2cm>10KD, there is a significant increase in the mean thymidine uptake in wells supplemented with all concentrations of SA2cm ($P<0.05$) except those wells supplemented with 50%v/v SA2cm> 10KD ($P>0.05$). The

magnitude of the increase in thymidine uptake is noteable. At a concentration of 25%V/V SA2cm>10KD the uptake was over three hundred times that which was seen in the control.

Table 4R.5.2 shows that there is a significant increase in the mean thymidine uptake in wells supplemented with 3.125, 6.25 and 50%v/v F10%V/V ($P<0.05$ in all cases) .At concentrations of 12.5 or 25%v/v no comparable increase is observed $P>0.05$. Importantly the change in the level of thymidine uptake is not large and, alone, could not explain the substantially larger increase seen in Table 4R.5.1 for SA2cm>10KD.

Thus SA2cm >10KD significantly increased the level of thymidine uptake by A4 cells in a way that F10% or SA2cm<10KD did not. This is illustrated in Figure G4.7.

Table 4R.5.3 and figure G4.8 show that WEHIcm increases the level of thymidine uptake in a titratable manner. There is a statistically significant increase in thymidine uptake with all concentrations of WEHIcm as compared to the control. ($P<0.05$ in all cases). The level of thymidine uptake is far greater in the presence of WEHIcm than in the presence of SA2cm>10KD. (25%V/V SA2cm>10KD mean thymidine uptake 4855.0±293.8 cpm / 10% WEHIcm mean thymidine uptake 10479.3±211.7cpm). Thus WEHIcm was a richer source of a factor able to induce A4 cell proliferation than SA2cm >10KD.

TABLE 4R.5.1: The effect of Fractions of SA2cm on the proliferation of FDCP-A4 cells.

CONCENTRATION SA2CM (%V/V)	LARGE FRACTION (>10KD)	SMALL FRACTION (<10KD)
	MEAN UPTAKE (CPM) THYMIDINE	
50	**968.1±642.5** 0.1<P≤0.375 1610.63 325.54	**40.2±10.5** 0.05P≤0.1 29.71 50.77
25	**4855.0±293.8** 0.0025<P≤0.005 5148.87 4561.18	**43.5±15.2** 0.1<P≤0.375 28.23 58.70
12.5	**4929.5±86.4** P≤0.0005 5015.81 4843.06	**43.2±15.5** 0.1<P≤0.375 27.78 58.72
6.25	**59.7±8.4** 0.01<P≤0.025 68.03 51.32	**57.6±9.7** 0.025<P≤0.05 47.90 67.37
3.125	**58.3±5.5** 0.01<P≤0.025 63.89 52.80	**45.7±2.1** P≤0.005 47.84 43.59
0	**16.2±3.7** 12.48 19.92	**16.8±0.7** 17.51 16.15

TABLE 4R.5.2: The effect of F10% on the proliferation of FDCP-A4 cells.

CONCENTRATION F10% (% V/V)	MEAN THYMIDINE UPTAKE (CPM)
50	**53.3±6.3** 0.005<P≤0.01 59.59 46.93
25	**43.5±15.5** 0.05<P≤0.1 28.23 58.70
12.5	**43.2±15.5** 0.05<P≤0.1 27.78 58.72
6.25	**57.6±9.7** 0.01<P≤0.025 47.90 67.37
3.125	**45.7±2.1** 0.0005<P≤0.005 47.80 43.59
0	**100** 0.0 0.0

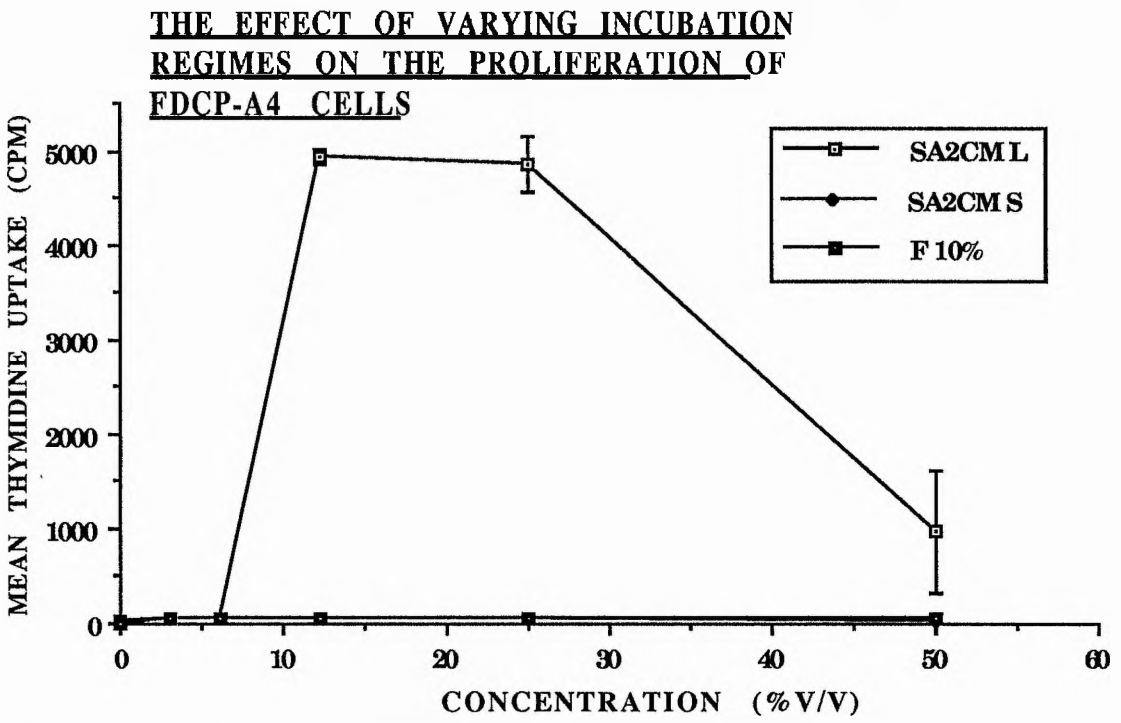


FIGURE G4.7: Mean thymidine uptake (CPM) by FDCP-A4 cells in the presence of varying concentrations of SA2cm fractions.

TABLE 4R.5.3: The effect of WEHIcm on the proliferation of FDCP-A4 cells.

CONCENTRATION WEHICM (% V/V)	MEAN THYMIDINE UPTAKE (CPM)
10	**10479.3±211.7** P≤0.0005 10691.04 10267.61
5	**8745.5±521.4** P≤0.005 9266.84 8224.06
2.5	**6683.6±137.5** P≤0.0005 6546.14 6821.04
1.25	**5997.8±1421.3** 0.01<P≤0.025 7419.10 4576.50
0.75	**3671.3±152.1** P≤0.0005 3823.47 3519.18
0	**16.8±0.5** 17.33 16.33

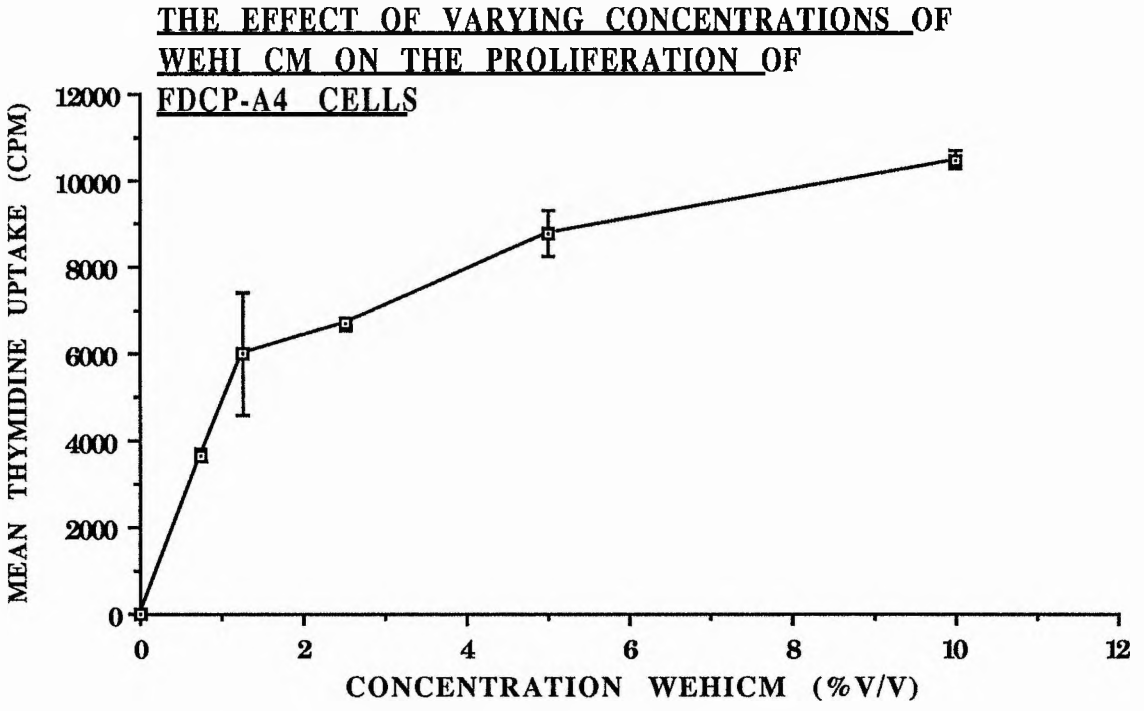


FIGURE G4.8: Mean thymidine uptake (CPM) in the presence of varying concentrations of WEHIcm.

4(2.3.)The effect of anti-murine IL-3 neutralising antibody on the colony promoting activity of SA2cm

Work was undertaken to determine whether the IL-3 like activity responsible for proliferation within the IL-3 dependent cell line was also the activity responsible for the altered pattern of colony formation seen in colony assays. This was accomplished by pre-incubating SA2cm with anti-murine IL-3 antibodies. It could then be assayed for its colony enhancing ability in GM-CFC and HPP-CFC cultures.

4(2.1.3.1)PROCEDURE

Initially SA2cm was pre-treated with Goat anti-Murine IL-3 as outlined in .

Assays for HPP-CFC stimulated with GM-CSF+L929cm(a source of M-CSF) and HPP-CFC stimulated with IL-1α + L929cm ,were carried out. For each condition three replicate sets of plates were prepared one set of which was supplemented with pre-treated SA2cm, another with normal SA2cm and a control withF10% only.

Four replicate experiments were performed .

4(2.3.2) RESULTS

Table 4R.6 and figures 4G.9a and 4G.9b show the effect of pretreating SA2cm with antiIL3 on the colony enhancing activity of SA2cm in HPP-CFC cultures.

In the case of HPP-CFC populations stimulated with L929cm+GM-CSF or IL-1a +L929cm there was a significant

increase in the level of colony formation when cultures were supplemented with 10%v/v SA2cm. ($P<0.05$ in both cases).

No comparable increase in colony formation is observed in L929+GM-CSF cultures supplemted with 10%v/v SA2cm which had been pre-treated with anti-IL-3 (Control 100 % / 10% V/V SA2cm pre-treated with anti-IL-3 110.0%±14.1 where $P>0.05$)

In the case of IL-1-a + L929cm cultures supplemented with SA2cm pre-treated with anti-IL-3 there is an increase in the level of colony fomation (Control 100% / 10% SA2cm pre-treated with anti-body 112.0% ±5.80 where $0.025<p\leq0.05$). This increase is however much less than that seen in culture supplemented with SA2cm not pre-treated with antibody. (10% V/V SA2cm not pre-treated 217.5 ±4.9 / 10% V/V SA2cm pre-treated 112.0±5.8).

Thus in one instance the pre-treatmentt of SA2cm with anti-body blocks the colony enhancing activity of SA2cm. In the second instance it significantly reduces it. Photographs of colonies formed in the presence of normal SA2cm or SA2cm pre-treated with anti-IL-3 are shown in figure PH2.

TABLE 4R.6: The effect of IL-3 antibodies on the synergistic activity of SA2cm on 14 day HPP-CFC NBM semi-solid agar cultures.

CONDITION	CONTROL	SA2CM (10%V/V)	SA2pretreated with antibody (10%v/v)
FACTORS (HPP-CFC)	**COLONY**	**FORMATION**	**(%/control)**
L929 + GM-CFC	**100.0**	**219.3±27.6**	**110.0±14.1**
		0.005<p≤0.005	0.1<p≤0.375
	11.75±1.00	23.50±1.80	13.50±1.55
	10.25±1.00	16.75±2.50	13.25±1.25
	9.75±1.25	21.25±1.00	12.50±1.26
	9.00±1.58	26.25±3.25	6.25±1.38
IL-1α + L929	**100.0**	**217.5±4.9**	**112.0±5.8**
		p≤0.0005	0.025<p≤0.05
	9.00±1.96	19.75±1.25	11.25±2.17
	8.25±0.5	18.25±2.25	9.50±3.00
	8.75±1.31	19.50±2.10	8.50±1.00
	9.00±1	18.75±1.00	10.00±1.00

N=4 in all cases

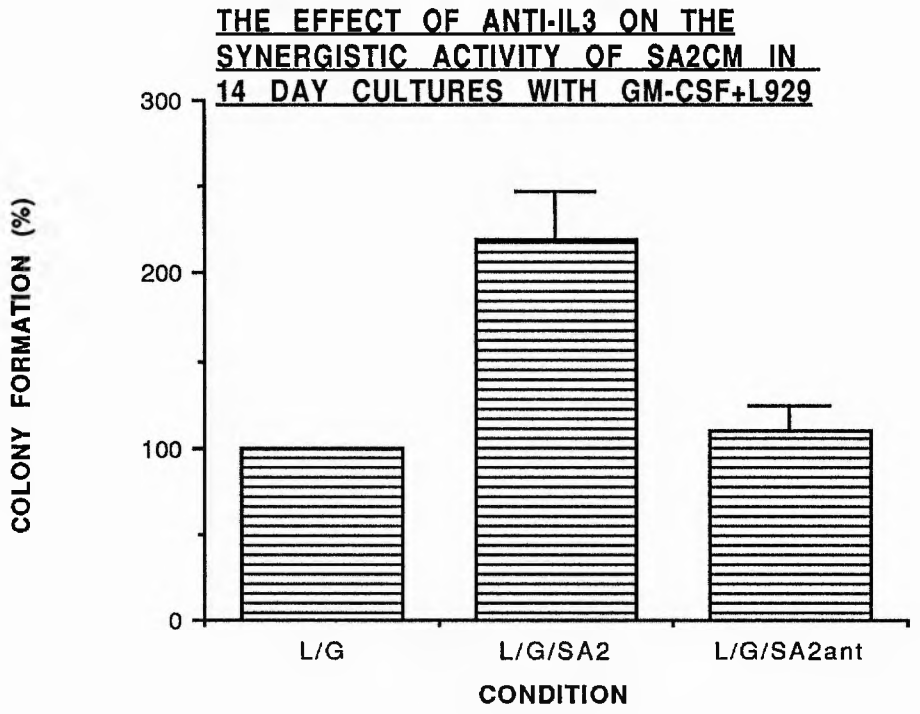


FIGURE G4.9a: Colony formation (expressed as a percentage of the control 0% V/V SA2cm) in 14 day semi-solid agar cultures of NBM stimulated with GM-CSF+L929 in the presence or absence of SA2cm ± anti-IL-3 antibodies.

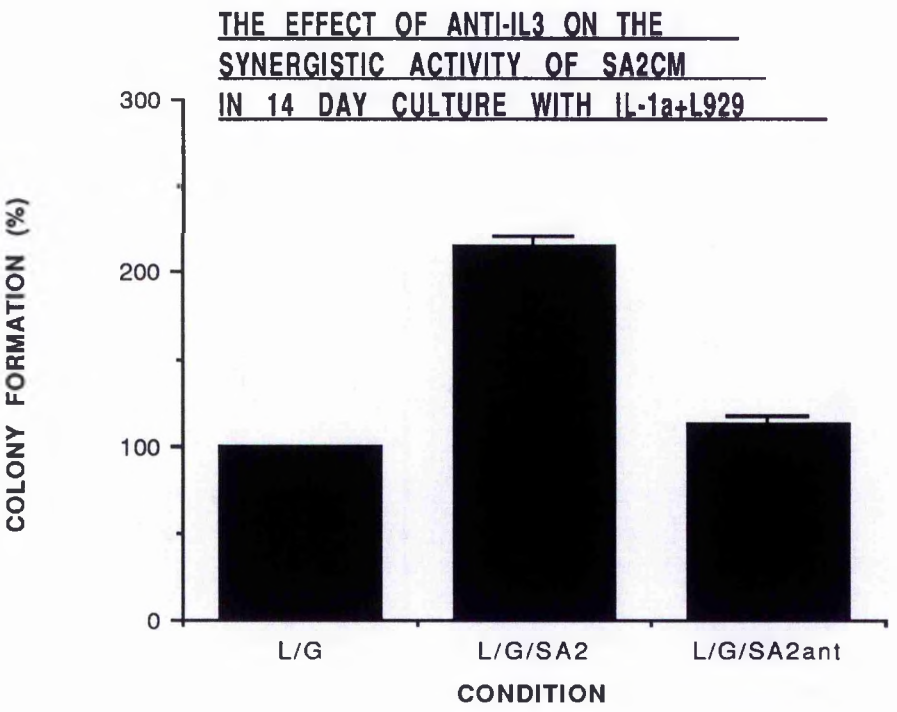


FIGURE G4.9b: Colony formation (expressed as apercentage of the control 0% V/V SA2cm) in 14 day semi-solid agar cultures of NBm stimulated with IL-1a+L929 in the presence or absence of SA2cm ± anti-IL-3 antibodies.

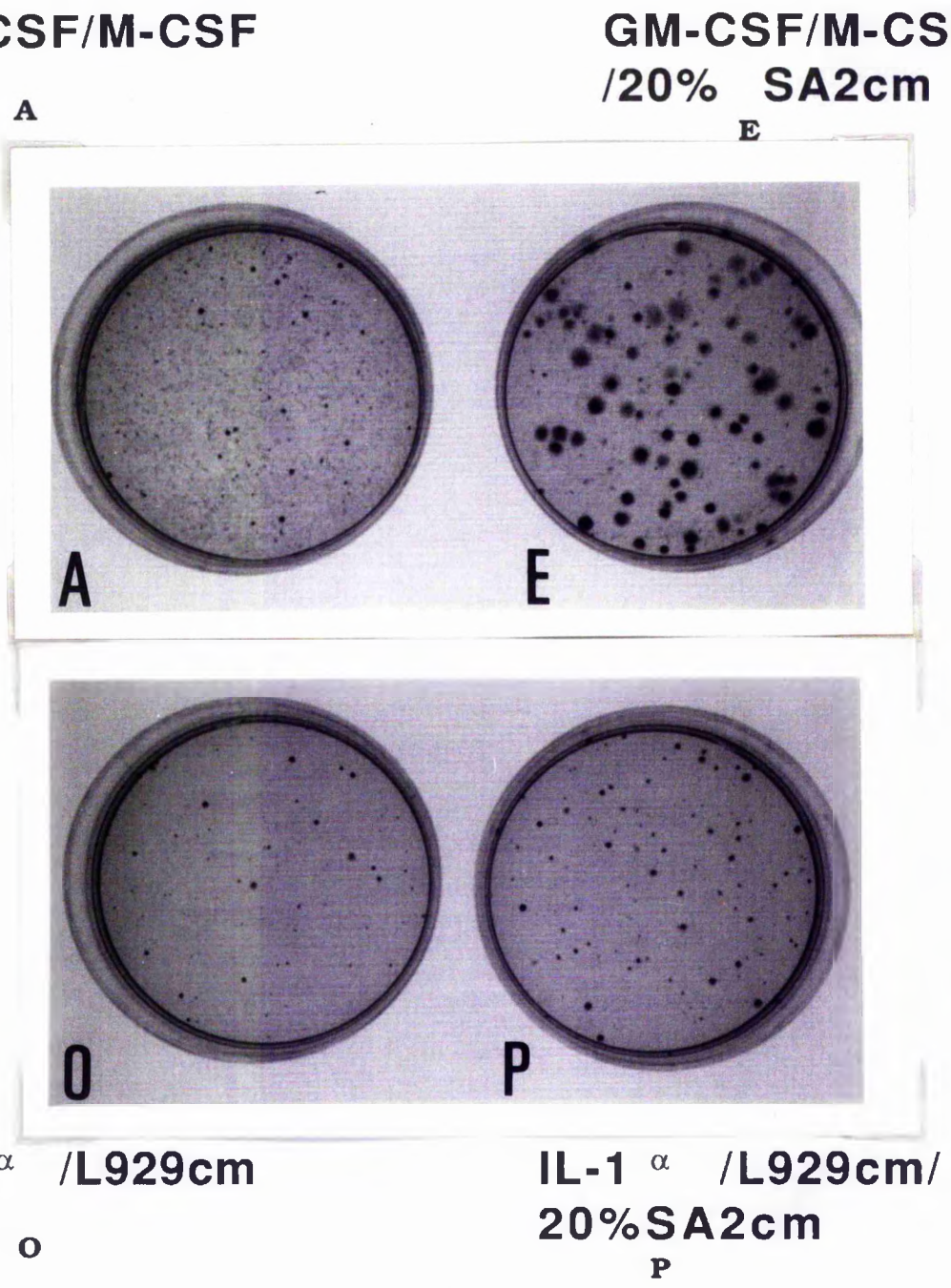


FIGURE PH2 : The effect of SA2cm on the colony formation seen in assays stimulated with varying growth factor regimes

4.2.4 ACTIVITY INTERFERING WITH THE ACTION OF STIMULATOR

It had been demonstrated that SA2cm blocked the action of a stem cell stimulator. However the identity of this activity was unknown.

A series of experiments was carried out in which fractions of SA2cm or other factors which could be the active component of SA2cm, were assayed in attempts to match the action of whole SA2cm in blocking stimulator.

4(2.4.1) PROCEDURE

In all cases these experiments were modifications of the protocol laid out in 4(1.4). The HPP-CFC population examined was always that stimulated to colony formation by WEHI/L929. The test conditions used are outlined in the table below:

EXPERIMENT	CONDITION
	N (Normal)
1	**NS** (Normal +Stimulator)
	NSSa(Normal + stimulator +20%v/v SA2cm)
	NSIL-3 (Normal + stimulator + 80U/ml IL-3)
	NSW(Normal + stimulator +10%V/V WEHI cm)
	NSSas(Normal + Stimulator +SA2cm fraction < 10KD 20%v/v)
	NSSal(Normal +Stimulator +SA2cm fraction >10KD 20%v/v)
	N (As above)
	NS(As above)
	NSSa(As above)
2	**NSSas**(Normal +Stimulator +SA2cm fraction <50KD 20%v/v)
	NSSal(Normal +Stimulator +SA2cm fraction >50KD 20%v/v)
	NSSaH (Normal +Stimulator +heat treated (+80^{O}C) SA2cm 20%v/v)
	NSM (Normal + stimulator +MIP-1-α 300 ng/ml)

TABLE 4.3: *Summary of the conditions used to assay action of factors on a stem cell stimulator.*

For group 1 factors three replicate experiments were carried out. For group two factors six replicate experiments were carried out.

4(2.4.2) RESULTS

Table 4R.6.1 and figure G4.10 show the effect of exogenous factors in group 1 on the action of a stem cell stimulator.

There is a significant increase in the proportion of HPP-CFC(WEHI/L929) in S-phase in the presence of a stem cell stimulator as compared to the control. (Control 7.37%±4.57 / In the presence of stimulator 22.36% ± 2.26 where $P<0.05$) .

In the presence of 20%V/V SA2cm stimulator fails to induce a significant increase in the level of HPP-CFC DNA synthesis as compared to the control. (20% V/V SA2 5.82% ±6.52 where $P>0.4$). Thus SA2cm at this concentration prevents the action of stimulator on this cell population.

Similarly addition of stimulator to cells in the presence of the fraction of SA2cm larger than 10KD fails to increase the level of DNA synthesis as compared to controls. (8.34%± 8.08 where $P>0.4$).

All other test factors fail to inhibit the action of a stem cell stimulator with the proportion of the cell population in DNA synthesis raised in all instances. There is thus a significant increase in the level of DNA synthesis as compared to the control .($P<0.05$ in all cases).

In this first set of test conditions only 20%V/V SA2cm or 20%V/V SA2cm > 10KD were able to alter the action of a stem cell stimulator.

Table 4R.6.2 and figure G4.11 show the effect of exogenous factors on the action of a stem cell stimulator. There is a significant increase in the proportion of HPP-CFC(WEHI/L929) in S-phase in the presence of a stem cell stimulator. (Control 13.08 %±4.13 / In the presence of stimulator 24.77%±4.10 where $P<0.05$) .The addition of stimulator in the presence of 20%v/v SA2cm failed to increase the level of DNA synthesis as compared to the control (7.51% ±3.31 where $P>0.05$). Thus 20%V/V SA2cm is able to inhibit the action of a stem cell stimulator on this population.

The addition of MIP-1-a, 20%V/V heat treated SA2cm or the fraction of SA2cm smaller than 50KD cannot mimic this response. There is a statistically significant increase in the level of DNA synthesis as compared to the control in all cases. ($P<0.05$)

Treatment of cultures with stimulator in the presence of the fraction of SA2cm greater than 50KD failed to increase the level of DNA synthesis seen as compared to the control. (9.49%±4.05 where $P>0.05$).

Thus whole SA2cm and a fraction of SA2cm larger than 50KD at concentrations of 20%V/V are able to inhibit the action of a stem cell stimulator on this cell population. The other activities assayed fail to mimic this response.

TABLE 4R.6.1: The effect of a variety of factors on the action of a stem cell stimulator.

CONDITION	PROPORTION HPP-CFC IN S-PHASE(%) ±STANDARD ERROR
N (Normal)	**7.37±4.57** 14.52 -1.14 8.73
NS (Normal +Stimulator)	**22.36±2.26** 0.01<p≤0.05 25.00 17.86 24.22
NSSa(Normal + stimulator +20%v/v SA2cm)	**5.82±6.52** P>0.4 13.87 -7.08 10.67
NSIL-3 (Normal + stimulator + 80U/ml IL-3)	**18.25±6.81** 0.1<P≤0.375 31.87 11.76 11.11
NSW(Normal + stimulator +10%V/V WEHI cm)	**24.01±8.75** 0.05<P≤0.1 29.55 6.86 35.63
NSSas(Normal + Stimulator +SA2cm fraction < 10KD 20%v/v)	**26.61±4.87** 0.01<P≤0.025 23.97 19.82 36.05
NSSal(Normal +Stimulator +SA2cm fraction >10KD 20%v/v)	**8.34±8.08** P>0.4 24.32 -1.67 2.36

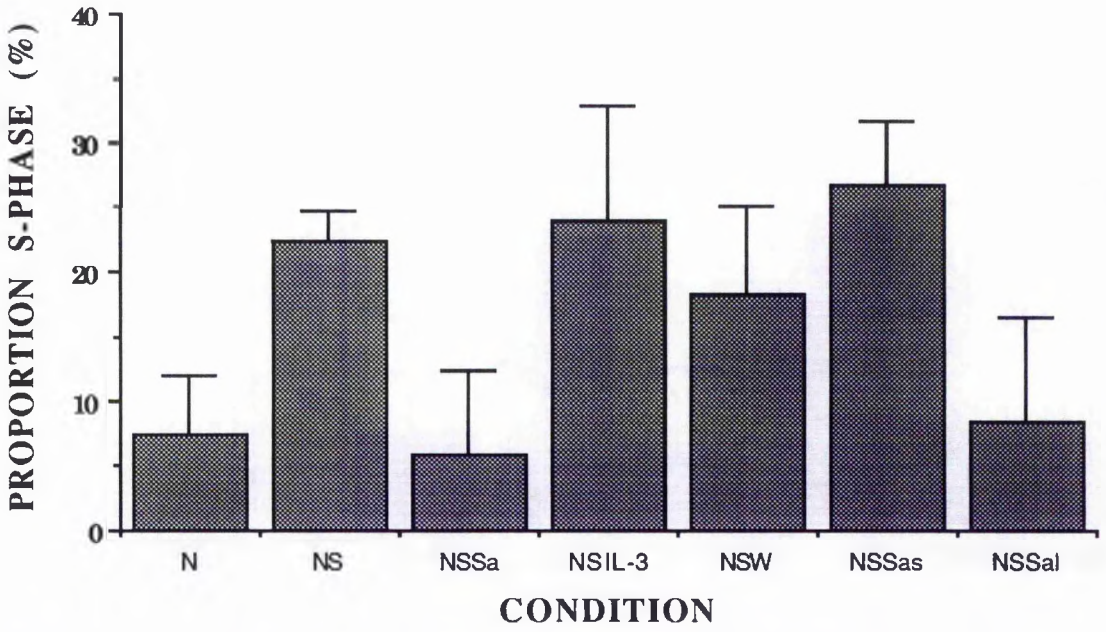


FIGURE G4.10: The proportion HPP-CFC(WEHI/L929) from NBM in S-phase(%) after pretreatmentt with a stem cell stimulator in the presence or absence of a variety of factors

TABLE 4R.6.2: The effect of SA2cm or SA2cm fractions(<or >50KD) or heat treated SA2cm or MIP-1-α on the action of a stem cell stimulator.

CONDITION	PROPORTION HPP-CFC S-PHASE(%) ± STANDARD ERROR
N (As above)	**13.08±4.13** 16.33 14.49 26.61 13.08 -4.72 12.72
NS(As above)	**24.77±4.10** 0.025<P≤0.05 33 29.92 26.43 15.97 12.15 37.16
NSSa(As above)	**7.51±3.13** 0.1<P≤0.375 11.34 7.59 20.18 5.74 -1.69 1.92
NSSas(Normal +Stimulator +SA2cm fraction <50KD 20%v/v)	**18.91±4.48** 0.005<P≤0.01 30.21 12.33 29.51 22.58 1.52 17.33

NSSaI(Normal +Stimulator +SA2cm fraction >50KD 20%v/v)	**9.49±4.05** 0.1<P≤0.375 13.64 16.54 22.11 5.43 -5.56 4.76
NSSaH (Normal +Stimulator +heat treated (+80^{o}C) SA2cm 20%v/v)	**28.03±3.00** 0.1<P≤0.375 35.04 28.57 29.85 19.00 19.59 36.11
NSM (Normal + stimulator +MIP-1-α 300 ng/ml)	**18.79±3.13** 0.1<P≤0.375 27.76 25.61 21.21 20.62 -6.06 23.60

In all cases N=6

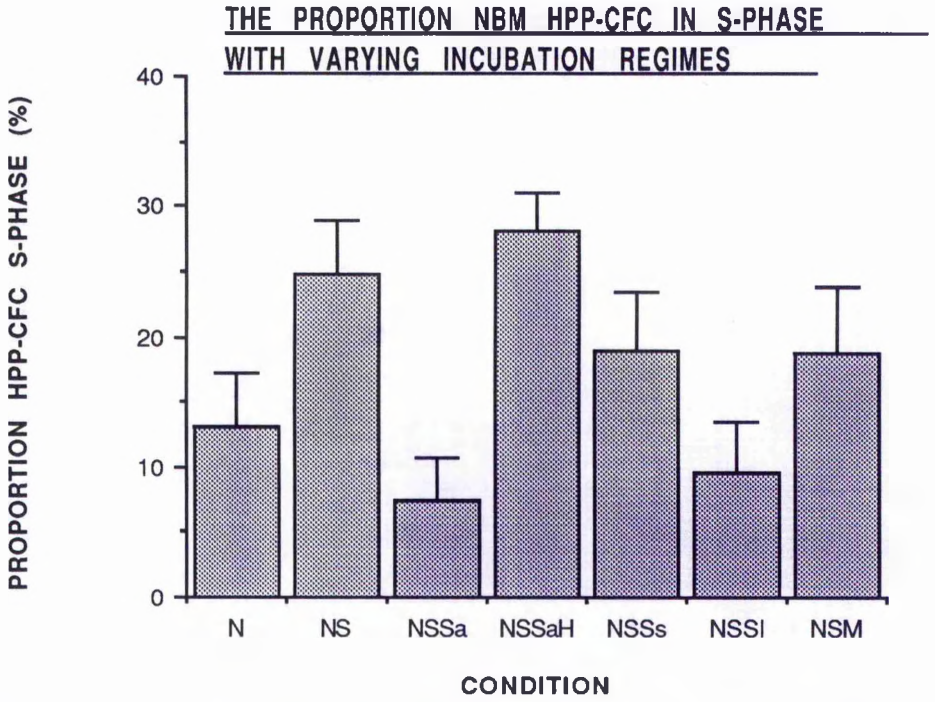


FIGURE G4.11: The proportion HPP-CFC(WEHI/L929) from NBM in S-phase(%) after pretreatmentt with a stem cell stimulator in the presence or absence of various factors.

4D: DISCUSSION

4D1: The effect of SA2cm on colony formation in semi-solid agar culture by a variety of NBM cell populations

The pattern of colony formation resulting from the addition of varying concentrations of SA2cm to LPP-CFC populations is complex. Alone it has limited colony stimulating ability in 7 day cultures. At concentrations of 20%v/v it is able to support proliferation of progenitor cells. In the case of GM-CFC stimulated with GM-CSF or L929cm there is a statistically significant increase in the level of colony formation with 2, 5, 10 and 20% SA2cm. The first striking point about this increase is that it is large and apparently titratable (For GM-CSF, control 100%, against 2% SA2cm 134%± 8.8 and 20% SA2cm 220.7±41). A similar increase is seen with L929cm (M-CSF). Thus SA2cm contains an activity which is significantly enhancing colony formation, presumably by synergistic interaction, with these factors. Interestingly in the case of WEHIcm (IL-3) stimulated colony formation, there is no statistically significant increase in colony formation as compared to controls. Where recombinant IL-3 has been used there is a statistically significant increase only with 20% SA2cm (Control 100%, against 138%±9.6). Also this increase in the level of colony formation is not as large as that seen for GM-CSF or L929 cm. Thus in this case SA2cm contains an activity which has been only slightly effective in increasing the level of colony formation as compared to controls. As both of these particular assays were stimulated

with IL-3 then the assumption was that SA2cm may itself contain IL-3, or an IL-3 like activity, or an activity which did not synergise with IL-3. The result in which 20% SA2cm enhanced the level of IL-3 stimulated colony formation was confusing. It could be explained if the activity did not synergise with IL-3 or if the recombinant IL-3 used to stimulate assays was in fact at sub-optimal levels. Thus at 20%V/V, the SA2cm supplied sufficient IL-3 to optimise levels. The fact that recombinant IL-3 had been titrated prior to assay and 80U shown to be maximally stimulatory, should discount this possibility. If this particular activity were actually some other factor present at low levels but nevertheless at sufficient concentration in 20%V/V to be synergistic with IL-3 then this may explain the result. This is not an unlikely scenario, consider the multi-factorial nature of conditioned medium such as WEHIcm. Although largely a source of IL-3 it undoubtedly contains other factors which "improve " the colony formation seen as compared to that seen with IL-3 alone. SA2cm could in addition to IL-3 or an IL-3 like activity also contain an activity which improves colony formation in conjunction with IL-3. Interestingly there is no increase in the level of colony formation with WEHI cm. Is such a secondary activity present in this SA2cm. ? The fact that GM-CSF and L929 are both synergistic with low levels of this cm would seem to suggest that if it is, it is likely to be independent of the IL-3 like activity.

HPP-CFC

The pattern of colony formation resulting from these cultures appears to support the assumptions made above. In cultures stimulated with GM-CSF+M-CSF or IL-1+M-CSF there is a statistically significant increase in the level of colony formation in the presence of SA2cm as compared to controls. Notably this is a large increase. With 20% SA2cm it is almost double the level seen in the control. (For IL-1+M-CSF control: 100%, against 20%SA2cm: 213%±33.58). Where IL-3 is present in cultures (IL-3+M-CSF or IL-1+IL-3+M-CSF) in some cases there is an increase in the level of colony formation, however, it is not large. (For IL-3+M-CSF control :100%, against 20%SA2cm: 104.33%±1.33; For IL-1+IL-3+M-CSF control :100%, against 20% SA2cm: 119%±7.94). The arguments used to explain the pattern of colony formation seen in GM-CFC cultures would thus also seem to apply here. The cultures which lack IL-3 may be receiving it from SA2cm and as a consequence actually stimulating more primitive HPP-CFC populations to colony formation. Those cultures already stimulated with IL-3 may be receiving some secondary factor from SA2cm. Notably where cultures were stimulated with WEHIcm+L929cm (essentially IL-3 +M-CSF +unknown factors) there is no alteration in the level of colony formation as compared to controls.

4D2: The effect of SA2cm on DNA synthesis in various NBM derived cell populations.

This assay was essentially an assay of the manner in which a Leukaemic conditioned medium might perturb normal haematopoiesis. Could factors associated with this particular leukaemia directly interact with cells to alter their cell cycle status ?

SA2cm has not significantly altered the level of cycling in normal GM-CFC (WEHIcm), HPP-CFC (WEHIcm+L929cm) or cycling HPP-CFC (WEHIcm+L929cm). It has thus not acted as a direct inhibitor or stimulator of DNA synthesis in these populations. In addressing the significance of this finding a number of points are worth considering. Firstly two major bodies of work on inhibitory activities associated with leukaemias.

The first of these " leukaemia associated inhibitory activity" (LIA) was identified as an S-phase specific inhibitory activity against normal GM-CFC (Broxmeyer *et al*, 1978; Bognacki *et al*, 1981; Broxmeyer *et al* 1981). It was produced by bone marrow spleen and blood cells from patients with acute and chronic myeloid leukaemia. Importantly this activity was not detected in normal patients and at significantly reduced levels in remission cases. (Broxmeyer *et al*, 1979). With molecular weight originally localised to 55KD. It has subsequently been reported to be monocyte-macrophage derived acidic isoferritin (Broxmeyer *et al*, 1981; Broxmeyer *et al*, 1982). Ferritin is a large molecule in the molecular weight range of 55KD which can be separated into isoforms differing in charge and size. Acidic isoferratins are thought to be composed mainly of so called H sub-units. Having identified LIA as acidic isoferratin it

was shown that it could be detected in normal bone marrow and blood cells belonging only to the mononucleur phagocytic lineage. This suggests that they may have a role to play in normal GM-CFC proliferation regulation. (Broxmeyer *et al*, 1982; Broxmeyer *et al*, 1983) LTBMC cultures were also shown to endogenously produce acidic isoferritin (Oblonet *et al*, 1983). Lilu *et al* (1983) also implicate acidic isoferratin in the negative proliferation of BFU-E and GEMM-CFC interestingly noting that purified acidic isoferratin is active where LIA is not . Does this suggest that in some way LIA is a slightly modified acidic isoferratin ? Sala *et al* (1986) could not demonstrate a role for acidic isoferritins as a regulator of GM-CFC *in vitro* in mice. Broxmeyer *et al* (1989) demonstrated that in vivo in mice recombinant human H-subunit isoferratin decreased cycling rates and absolute numbers of haematopoietic progenitors (GM-CFC, BFU-E and GEMM-CFC).

A second inhibitory activity " leukaemia associated inhibitor" (LAI) has also been shown to suppress normal granulopoiesis *in vitro*. (Olofsson and Olsson, 1980a; Olofsson and Olsson, 1980 b; Olofsson and Olsson 1980 c; Olofsson and Sallerfors, 1987). It is produced by human acute or chronic myeloid Leukaemic cells and the human promyelocytic cell line HL60 (Oloffson and Olsson, 1980a). Normal cells failed to produce detectable levels. It has been identified as a heat stable glycoprotien with apparent molecular weight of 500KD but with biological activity residing in a sub-unit of 150-170KD. It reversibly inhibits the normal cycling of human GM-CFC stimulated to colony formation with 10% human placenta conditioned

medium as a source of growth factor. Leukaemic cells are insensitive to this regulation. (Olofsson and Olsson, 1980b). As with LIA, it has seemed likely that LAI may in fact be produced in normal haematopoietic tissue with a potential role in the regulation of normal granulopoiesis. (Olofsson *et al*, 1984). This was largely due to the fact that LAI producer cells -characterised as non-phagocytic, non-adherent, non T, non B, Fc receptor +ve cells were found within the non-phagocytic Fc+ve compartment of normal cells. Importantly they are different cells to those producing LIA (Olofsson *et al*, 1984).

It is conceivably that both of these inhibitory activities represent normal inhibitors, possibly with subtly different roles, locally active and subject to degradation out with that environment. In leukaemia they may be over produced, perhaps in some slightly modified manner.

In this work no granulopoiesis inhibiting effect is observed. There are potentially a number of explanations for this. Firstly there are differences between the protocols used here and those used in the work of the groups discussed above. Specifically these relate to the killing agent and the factors used to stimulate colony formation. The former of these should certainly be discountable as ARA-C is a routinely used killing agent. The possibility that the populations being assayed are slightly different should certainly not be dismissed. However the ability of LIA to inhibit a broad spectrum of progenitor cell populations suggests that such an activity should be seen in these cultures. It may be that although these factors represent one manner in which a Leukaemic cell population may

manifest its proliferative advantage there may well be others. The fact that SA2cm can actually enhance colony formation within certain GM-CFC cell cultures is strongly supportive of this notion.

LAI and LIA remain untested on the more primitive HPP populations. However SA2cm has no direct effect on the level of cell cycling in a population of HPP cells. On this basis it can be assumed that SA2cm does not manifest its inhibitory activity by production of some of the known inhibitors discussed in the introduction. For example MIP-1-α, which is known to be active in the inhibition of CFU-A cycling. As discussed HPP and CFU-A are thought to closely related and thus it would seem that MIP-1-α is not the relevant activity unless it is complexed and thus inactive *in vitro*. TGF-β, a highly conserved plieotrophic protein known to promote a three to five fold increase in the number and size of GM-CFC colonies, initially seems a potential candidate. (Keller *et al*, 1990). However TGF-β is known to be a direct inhibitor of primitive haematopoietic cells similar in action to MIP-1-α. (Maltman *et al*, 1993; Bradley *et al*, 1991; Hampson *et al*, 1990; Migdalska *et al*, 1990). The pentapeptide pEEDCK is another haematopietic inhibitory activity. Oxidation of the cysteine thiol groups within pEEDCK leads to the formation of a disulphide bridged homodimer (pEEDCK2) which is a stimulator of *in vitro* and *in vivo* haematopoiesis (Paukovits *et al* 1991). pEEDCK has been shown to prevent the post chemotherapy proliferation of haematopoiesis stem cells CFU-S (Paukovits *et al* 1990) and thus to prevent the recruitment of CFU-S. This may seem a

good candidate for a potential inhibitory activity however it is noted not only to be an active inhibitor of CFU-S but also GM-CFC. This would seem to rule out this particular factor. As outlined in discussions on LIF the assay system used in this thesis is limited in that only 1 population is studied. As discussed for LIA and LAI it is conceivably that inhibitors may act to regulate subtly different cell populations. It may be that any inhibitory activity is missed in this assay system.
This Leukaemic cell conditioned medium was not acting via known inhibitory molecules which have a direct action on the cycling of stem or progenitor cells. Could it work like AcSDKP? Could it be AcSDKP?

4D3: The interaction of SA2cm with a stem cell stimulator

20%V/V SA2cm blocks the action of a stem cell stimulator on a population of HPP-CFC. This is an extremely important finding for a number of reasons. Firstly it confirms the finding that SA2cm does not manifest Leukaemic suppression via any of the agents discussed above. Secondly it initially opens up the possibility that the inhibitory activity may be AcSDKP. This seems unlikely however as AcSDKP is noted to inhibit the *in vitro* growth of human progenitor cells (GM-CFC/BFU-E and CFU-E (Anagnostou *et al* 1991). SA2cm is clearly not inhibitory for GM-CFC. This finding also indicates that the conditioned medium contains both an inhibitory and a stimulatory activity. The question which immediately springs to mind is "are they

the same activity?". This is certainly possible. Consider pEEDCK and the action of IL-1b on human LTBMC (Marley *et al* 1992). Only with identification of these activities will it be possible to answer this question. Similarly the precise action of the inhibitory activity is unknown. Does it block receptors ? Does it degrade stimulator ? Does it interfere with gene transcription following stimulator activation of cells ? Identification of the activity/ies would help to resolve these questions.

The fact that this activity appears to be different from any of the other inhibitory activities outlined suggests that it is either a new activity or an activity such as AcSDKP expressing only part of its inhibitory potential.

4D4: SA2cm as a source of IL-3

SA2cm appears to have a colony stimulating activity very like that of IL-3. The above studies provide indirect evidence in support of this. Initially the observation that IL-3 could support the proliferation of an IL-3 dependent cell line seemed strongly indicative of the presence of IL-3 in the medium. By filtering the medium such that small molecules could be removed from it (or substantially diluted) it seems clear that this activity is localised to a fraction > 10KD. (25% SA2cm >10KD: 32073cpm±9167, against, 25%SA2cm < 10KD: 262cpm±101). The molecular weight of IL-3 is 28KD with possible variation due to the level of glycosylation (Farrar *et al* 1989). It can stimulate the proliferation and differentiation of pluripotent haematopoietic stem cells as well as progenitor

cells (Miyjima *et al* 1993). Coupled with the antibody studies it seems likely that IL-3 is present in the SA2cm and more-over is the activity responsible for the stimulation of colony formation seen in these cultures. Could IL-3 be the factor responsible for Inhibition ? There appears to be no precedent for such a possibility given that the inhibition is not attributable to toxicity.

4D5: SA2cm as a source of a stem cell inhibitor.

In the first series of these experiments it is clear that whole SA2cm blocks the action of a stem cell stimulator. The fraction of SA2cm larger than 10KD is similarly inhibitory whilst the fraction lower than 10KD fails to block the initiation of DNA synthesis. This suggests that the inhibitory activity is larger than 10KD or a smaller molecule at such a high concentration that even following significant dilution it is still a potent inhibitory molecule. The results for IL-3 and WEHIcm are less conclusive. Clearly they do not inhibit to the same extent as either whole SA2cm or SA2cm > 10KD. The results however do show that they significantly alter the action of the stimulator. The explanation for this finding is elusive. Firstly the degree of inhibition seen for WEHIcm and IL-3, different to that seen for SA2cm, could reflect the action of a molecule present at lower concentrations in WEHIcm. However where WEHIcm could possibly contain some inhibitory component it seems unlikely that recombinant IL-3 is a stem cell inhibitor. Not least because colony assay studies above show that IL-3 is at lower

concentrations in SA2cm. Conclusive proof implicating IL-3 as an inhibitory activity in this assay system would come if SA2cm were pre-treated with IL-3 antibodies and this medium then subsequently used to block the action of a stimulator.

The second test block in which the inhibitory activity is clearly demonstrated not to be heat stable supports the notion that the activity is attributable to a protein. The finding that the activity appears to reside in a fraction >50KD however suggests that it is unlikely to be IL-3 (Although this is a crude estimate of molecular weight and it is worth noting that in the early stages of molecular identification molecular weight is over-estimated). This later work confirms that the activity is not MIP-1-a which failed to block the action of a stem cell stimulator in these assays.

To resolve these problems a comprehensive identification strategy would have to be undertaken.

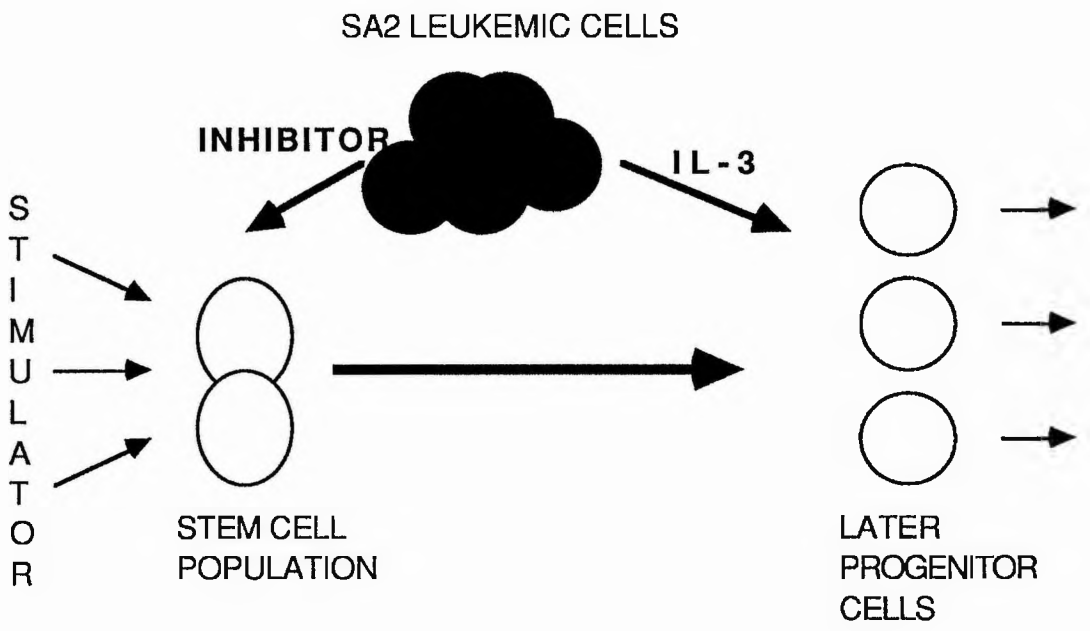
CONCLUSIONS: SA2JMB1 CM

The murine myeloid Leukaemic cell line SA2JMB1 can be used to generate a conditioned medium which has various properties. Firstly this medium has limited colony stimulating abilities in 7 day semi-solid agar culture of NBM derived haematopoietic cells. In association with other factors it has a significant synergistic action with respect to the promotion of colony formation. Notably where IL-3 is present in cultures this synergistic activity is significantly reduced. Studies to determine the nature of this activity strongly suggest that it may be IL-3 as the activity was lost in cultures pre-treated with IL-3 antibodies. It is thus reasonable to conclude that SA2cm contains an activity which has functional similarities to IL-3. At least one of these functions can be inhibited by antibodies to IL-3.

A second activity blocks the action of a stem cell stimulator on a population of HPP-CFC. The nature of this activity has not been determined but it seems susceptible to heat treatmentt, larger than 50KD and thus probably independent of the activity synergising to enhance the level of colony formation. Several of the known inhibitory activities have been compared to this activity and it seems reasonable to conclude that this is a new activity .Identification of this activity would require a comprehensive identification protocol. Such a strategy could entail:

lysis of Leukaemic cells, concentration of lysate and production of fractions using either centricon microconcentrators or sephadex beads;
assay of concentrated fractions for stimulator blocking activity ; active fractions assayed for affinity for Concanavilin A sepharose, susceptibility to periodate treatmentt, protease digestion and resistance to heating ;
active fractions run on SDS-polyacrylamide gel electrophoresis., subsequent quantification of protein levels present in bands, and assay for the presence of lysate.

From the work in this thesis it is possible to speculate as to how this particular leukaemia may interact with normal haematopoiesis.
The leukaemia could actually be promoting the proliferation of white blood cells through the IL-3 like activity. However as the proliferation of more primitive cells is signalled to reconstitute decreased levels of more mature cells, the inhibitory activity prevents their proliferation. The balance of haematopoiesis is perturbed. Meanwhile the Leukaemic cells gain a proliferative advantage proliferating unchecked, filling the marrow with useless blasts .(Diagram 21)



SA2 LEUKEMIC CELLS GAIN PROLIFERATIVE ADVANTAGE NUMBERS INCREASE

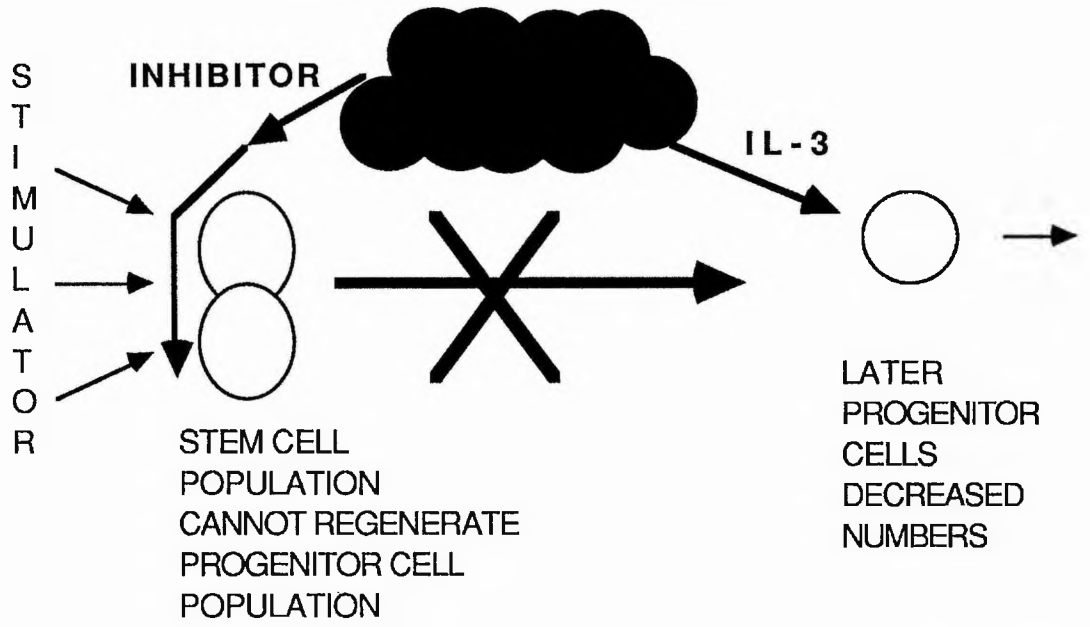


DIAGRAM 21: SCHEMATIC OF THE PROPOSED MANNER IN WHICH SA2 LEUKEMIA MAY DISRUPT NORMAL HAEMATOPOIESIS .

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