PROLIFERATION REGULATION OF HAEMATOPOIETIC STEM CELLS IN NORMAL AND LEUKAEMIC HAEMATOPOIESIS

Simon N. Robinson

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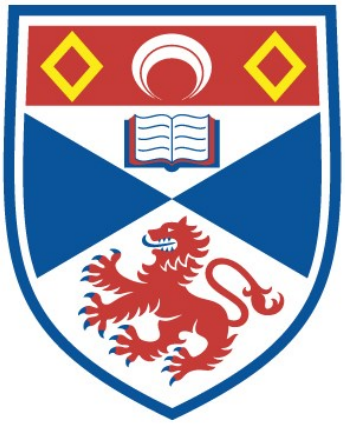
University of St Andrews



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Proliferation regulation of haematopoietic stem cells in normal and leukaemic haematopoiesis

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A research thesis submitted for the Degree of

Doctor of Philosophy (Ph.D.)

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Declaration For The Degree of Doctor of Philosophy (Ph.D.)

I 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 fulfillment, of any other degree or professional qualification.

Simon N. Robinson

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Doctor of Philosophy (Ph.D.), Faculty of Science, University of St.Andrews.

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Copyright Declaration

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Computer Software.

Text Processor - Microsoft Word, Microsoft Corporation.

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Dedication.

I respectfully dedicate this research thesis to my mother Jean, without whose constant love, support and encouragement this thesis would not have been possible, my sister Caroline, my Grandfather Victor Lockett and my Great Aunts, Dolly Sargeant and Florence Cartwright.

"Each one of us adds
a little to our
understanding of nature,
and
from all the facts assembled
arises a certain grandeur."

Aristotle (384-322 B.C.)

Abstract

The cellular integrity of the blood is maintained by the cellular output of the *haematopoietic stem cell* population which produces the specialized precursors and differentiated cells which constitute the blood. The investigation of haematopoietic stem cell behaviour and regulation has been hampered by both the difficulty in their identification and the development of relevant assay systems. The purpose of this investigation was to study the behaviour and regulation of the haematopoietic stem cell population in normal and leukaemic haematopoiesis using an *in vitro* assay of a primitive haematopoietic precursor.

The use of a combination of haematopoietic colony-stimulating factors [*interleukin 3 (IL3)/multi-CSF* and *macrophage colony-stimulating factor (M-CSF/CSF-1)*] in semi-solid agar culture of murine haematopoietic tissue, stimulated the proliferation of a haematopoietic colony-forming cell, defined as the "HPP-$CFC_{IL3+CSF-1}$" population, which was characterized by a high proliferative potential, a multipotency and behavioural and regulatory properties consistent with its being a primitive haematopoietic precursor and possibly a component of the haematopoietic stem cell population.

The proportion of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population in S-phase in normal murine marrow, was determined to be relatively low at approximately 10%, increasing to approximately 40% in sublethally X-irradiated, regenerating murine marrow and the respective presence of the haematopoietic stem cell proliferation *inhibitor* and *stimulator* was demonstrable by the induction of appropriate kinetic changes in the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population.

In leukaemic haematopoiesis, leukaemic proliferation often occurs at the expense of apparently suppressed normal haematopoiesis. *In vitro* HPP-$CFC_{IL3+CSF-1}$ assay of the haematopoietic stem cell proliferation regulators in a number of murine, myeloid leukaemic cell lines, failed to

demonstrate either increased levels of the haematopoietic stem cell proliferation *inhibitor*, or evidence of a direct-acting, leukaemia-associated proliferation inhibitor, however, evidence of a leukaemia-associated impairment of *inhibitor* and *stimulator* production was observed and this may be a possible mechanism by which the leukaemic population develops a proliferative advantage over normal haematopoietic tissue. The identification of a possible mechanism of leukaemic progression and suppression of normal haematopoiesis may subsequently allow the development of potentially more effective disease treatment and management regimes.

The endogenous haemoregulatory tetrapeptide: Acetyl-N-Ser-Asp-Lys-Pro [AcSDKP, Mr=487 amu] is reported to prevent the G_0-G_1 transition of haematopoietic stem cells into S-phase. The mechanism of action of AcSDKP and a number of related peptides, was investigated in relation to the stem cell proliferation *stimulator* and *inhibitor*. AcSDKP demonstrated no direct haemoregulatory role against the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population, which is consistent with reports that AcSDKP is not active against cells already in late G_1, or S-phase, rather it appeared to act indirectly by impairing the capacity of the haematopoietic stem cell proliferation *stimulator* to increase the proportion of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population in S-phase. An apparent impairment of *stimulator* action may explain the reported AcSDKP-associated 'block' of haematopoietic stem cell recruitment. A putative endogenous AcSDKP precursor and synthetic and degradative enzyme systems have been reported and the possible physiopathological role of AcSDKP in a number of myeloproliferative disorders has been implicated. The potential application of AcSDKP as a 'haemoprotective' agent administered prior to the use of S-phase-specific chemotherapy may be of clinical significance.

The *in vitro* HPP-$CFC_{IL3+CSF-1}$ assay of a primitive haematopoietic precursor cell population, which may be a component of the haematopoietic stem cell population, should play a significant role in the investigation of haematopoietic stem cell behaviour and regulation in both normal and aberrant haematopoiesis. With the characterization of the mechanism(s) of action of the haematopoietic stem cell proliferation *inhibitor* and *stimulator* and the haemoregulatory tetrapeptide AcSDKP, the manipulation of the haematopoietic system to clinical advantage can be envisaged, while the identification of the aberrant regulatory mechanism(s) in haematopoietic dysfunction may allow the development of more effective disease treatment and management regimes.

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Chapter 1:

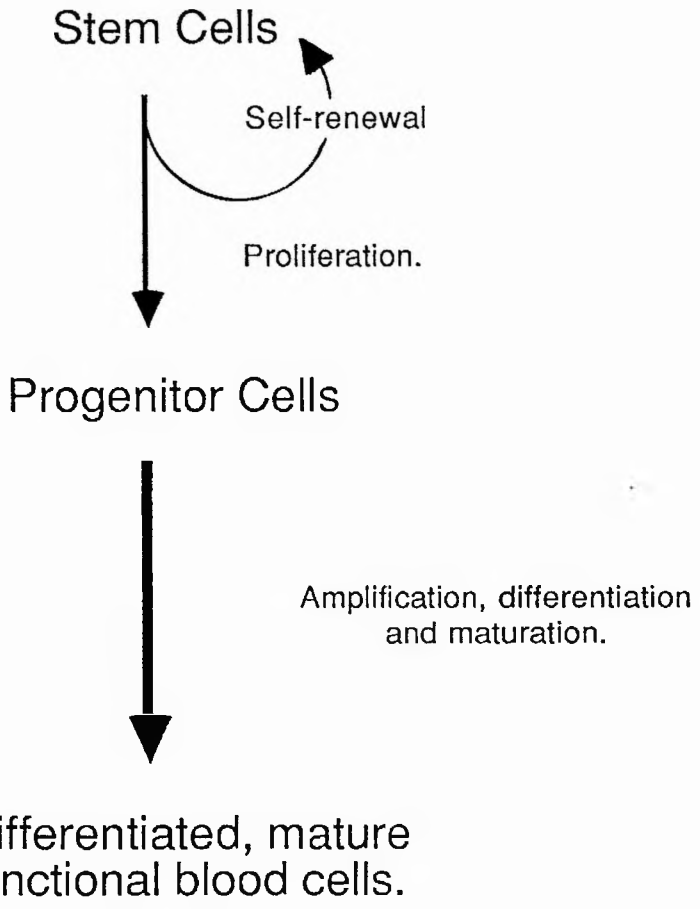
Introduction.

1.1 Introduction.

All the cells of the body are descended from a small population of specialized precursors known as "*stem cells*". Stem cells are capable of multiplying rapidly, under the control of subtle regulatory cues, to give rise to a large number of terminally differentiated, non-proliferating progeny. This cellular amplification occurs through a population of 'transit' progenitor cells which, with maturation, develop a reduced probability for proliferation and an increased probability for differentiation. In this way cell multiplication is kept in check, matching cell production to the requirements of the body - sufficient new cells are produced to replace those dead or dying, so maintaining tissue integrity, or to produce new tissue as required. While providing a continuously varying output to meet the cellular requirements of the body, stem cells must, of necessity, be capable of maintaining their own numbers, through a stem cell-specific 'self-renewal' mechanism. The processes which regulate stem cell proliferation and cellular differentiation are of considerable interest and an understanding of such processes will undoubtedly make it possible to investigate tissues where cell production is proceeding in an inappropriate manner in, for example, cancerous growth.

"*Haematopoiesis*" is the process by which the body generates new blood cells. The cellular integrity of the blood is maintained by the *haematopoietic stem cell population*, which produces the specialized precursor and differentiated cells which constitute the blood [*Figure 1*]. Investigation of haematopoietic stem cell regulation and behaviour is of considerable interest, however, difficulty in both their identification and the development of relevant assay systems has hampered such investigations.

Figure 1: Organization of the haematopoietic system.



- Erythrocytes
- Granulocytes
- Monocytes → Macrophages
- Eosinophils
- Megakaryocytes → Platelets
- Basophils / Mast Cells
- T - and B - Lymphocytes

1.2 Evidence for a haematopoietic stem cell.

Mice subjected to 8.2 Gray (Gy) whole body X-irradiation die after approximately 14 days due to haematopoietic failure. If the spleens of such mice were exteriorized and shielded prior to irradiation this haematopoietic death could be prevented [Jacobson *et al*,1949]. Left *in situ* for 1-48 hours before removal a shielded spleen would still improve haematopoietic recovery [Jacobson *et al*,1951]. Transplanted unirradiated, syngeneic spleen cells also enhanced the survival of lethally irradiated mice and similar results were obtained for syngeneic bone marrow. Initially a haematopoietic humoral factor was considered responsible, however, a cell within the donor tissue was determined to be the agent responsible for the haematopoietic recovery [Ford *et al*,1956]

To maintain adequate haematopoietic output, mice utilize all bone cavities in active haematopoiesis. If haematopoietically stressed, mice resort to *extramedullary haematopoiesis*, that is haematopoiesis outside the bone cavities, and the murine spleen becomes a focus of haematopoiesis. If lethally X-irradiated mice are given sufficient haematopoietic cells in a transplant, they survive and an enlarged haematopoietically active spleen is usually evident. However, if fewer haematopoietic cells are given in a transplant, a point is reached where insufficient cells for haematopoietic recovery are present and the mice die due to haematopoietic failure. Examination of the spleens from such mice at autopsy, revealed discrete 'patches', or 'islands', of light-coloured tissue within the otherwise degenerating spleen. These 'islands', or 'nodules', were found to contain recognisable haematopoietic cells and precursors and were foci of haematopoietic regeneration. The number of spleen 'nodules' was found to be proportional to the number of haematopoietic cells given

in the transplant [Till & McCulloch,1961]. Unique chromosome markers identified the spleen nodules as derived from donor tissue and the use of combinations of chromosomal markers demonstrated that individual nodules were derived from a single cell and so were *clonal* in nature [Becker *et al*,1963; Wu *et al*,1967; Chen & Schooley,1968]. To give rise to visible, macroscopic nodules, the single colony-forming cell responsible was demonstrating a high proliferative potential and histological analysis of individual colonies demonstrated that while some colonies consisted of cells of a single lineage, others consisted of cells of two, or three different lineages, evidence that the single colony-forming cell also had a multipotential nature [Lewis & Trobaugh,1964; Fowler *et al*,1967; Lewis *et al*,1968]. Excised single spleen colonies, if re-transplanted to lethally X-irradiated mice were demonstrated to produce more than one nodule in the recipient spleen, also giving evidence of a self-renewal capacity within the spleen colony-forming cell population [Siminovitch *et al*,1963]. To summarize, a haematopoietic colony-forming cell population of high proliferative potential, with a multipotential nature and self-renewal capacity was demonstrable in murine haematopoietic tissue. The significance of this spleen colony-forming unit (CFU-S) was realised since such characteristics would be expected of a putative haematopoietic stem cell population [*Figure 2*]. The CFU-S assay was thus adopted as a method with which to quantitatively and qualitatively investigate the behaviour and regulation of the haematopoietic stem cell population.

1.3 The murine spleen colony-forming unit (CFU-S) assay.

[Till & McCulloch,1961] Recipient mice are subjected to 8.2 Gy whole body X-irradiation, a dose which ablates host haematopoiesis

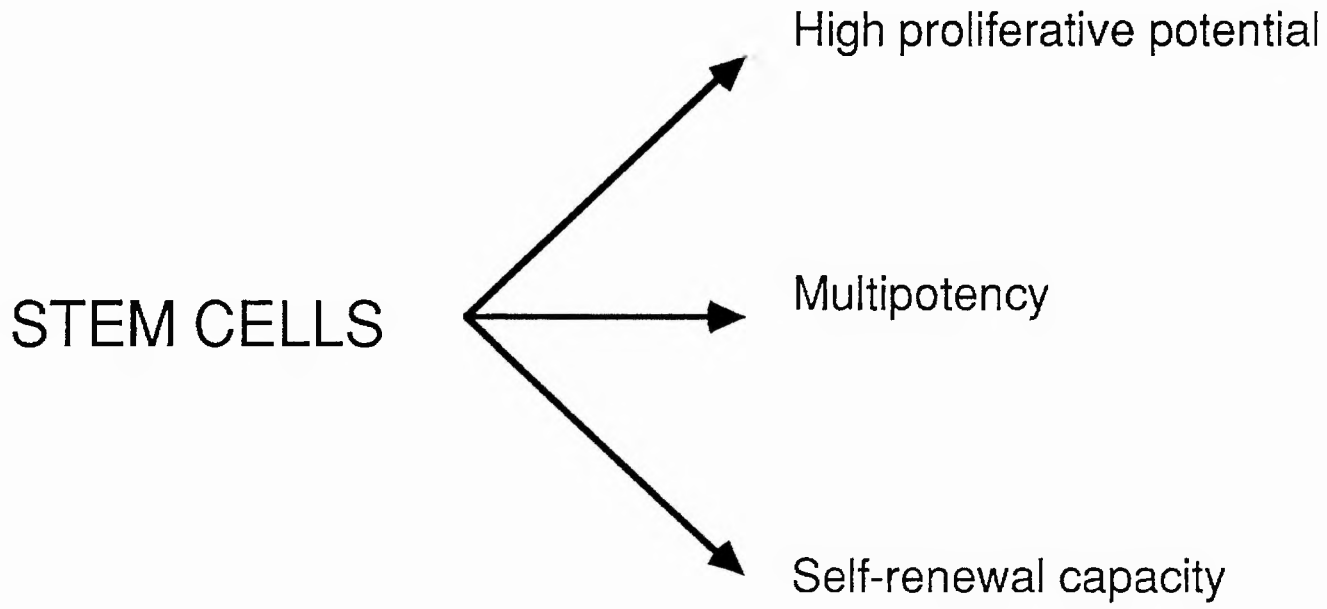


Figure 2: Characteristics of the haematopoietic stem cell population.

and will prove lethal after approximately 14 days. A single cell suspension of donor syngeneic bone marrow, spleen or haematopoietic foetal liver, is produced, its cellularity adjusted and is injected intravenously into one of the two lateral tail veins of prewarmed recipient mice.

Recipient mice will die after approximately 14 days due to haematopoietic failure since the inoculum contains a sub-survival haematopoietic dose. Spleen colonies are assayed by killing the mice after 8-12 days. The time of assay is again critical since, while the number of colonies on a spleen may be the same at day 8 or day 12, some spleen colonies are transient [Magli *et al*,1982]. Some colonies arise to be counted at day 8, disperse and are absent at day 12. Similarly, other colonies do not arise until after day 8 to be counted at day 12. Other spleen colonies appear fixed and are present at both day 8 and day 12. This has important implications in relation to an age-related hierarchy within the CFU-S population and will be discussed. At assay colonies are counted on both sides of the spleen, and a near maximal 15 colonies *per* spleen is apparent with larger numbers increasing counting errors due to colony overlap.

Only a fraction of the haematopoietic colony-forming cells in an inoculum will seed into the spleens of lethally X-irradiated mice to subsequently give rise to macroscopic colonies (CFU-S). This fraction of colony-forming cells is determined by a secondary transplantation technique [Siminovitch *et al*,1963]. Lethally X-irradiated mice receive an inoculum containing a known dilution of colony-forming cells and from the number of colonies which subsequently form on the spleen a "seeding factor", or "f-number" can be calculated [Siminovitch *et al*,1963]. The determination of a seeding factor allows an assessment of the absolute numbers of colony-forming cells in an inoculum.

Siminovitch *et al* [1963] calculated a seeding factor of 17%, while Playfair and Cole [1965] calculated a seeding factor of 8% (that is, 17% and 8% respectively, of potential colony-forming cells injected, seed into the spleen to give rise to CFU-S). A difference in experimental protocol relating to the post-irradiation collapse of the spleen was responsible for the observed discrepancy. Siminovitch *et al* [1963] allowed 2-3 hours to elapse between inoculation of the lethally X-irradiated mice and removal of the seeded spleen for retransplantation, while Playfair and Cole [1965] allowed a 24 hour period to elapse. Lord [1971] found that a 2-3 hour period was sufficient for maximal colony-forming cell accumulation in the spleen, however spleens removed at this time had yet to undergo post-irradiation collapse. With this collapse, which is complete after 24 hours, spleen cellularity reduces and a proportion of the colony-forming cells initially seeding into the spleen are expelled [Lord,1971]. Assay of the colony-forming cell content of a collapsed spleen [Playfair & Cole,1965] is thus a more representative model with which to investigate seeding.

The complications associated with the post-irradiation collapse of the spleen and the determination of a seeding factor, are reduced if mice are inoculated 24-48 hours after X-irradiation. After 2-3 hours, colony-forming cells maximally seed into the already collapsed spleen and it is removed for secondary transplantation. In such a case, a seeding factor of approximately 8% is observed.

1.4 Haematopoietic stem cell self-renewal.

The murine haematopoietic transplantation (CFU-S) assay demonstrates a cell population which fulfills many of the criteria for a putative haematopoietic stem cell population. The capacity of CFU-S

to self-renew under different conditions has revealed evidence of an age-related hierarchical organization within the CFU-S population. The self-renewal capacity of CFU-S does not differ significantly between very young and very old mice [Lajtha & Schofield,1971] implying a stem cell 'immortality'. However, serial transplantation of bone marrow from healthy to lethally irradiated mice and subsequently into other lethally irradiated mice, demonstrates a rapid and progressive decline in the regenerative capacity of the bone marrow transplant [Siminovitch *et al*,1964]. Even if long periods separated successive transplants and CFU-S numbers were standardized, the decline in the regenerative capacity remained [Lajtha & Schofield,1971; Pozzi *et al*,1973]. Investigation of serially transplanted CFU-S self-renewal showed a reduction with each transplant. Sublethal chronic [Wu & Lajtha,1975] or repeated acute [Hendry & Lajtha,1972] X-irradiation showed a similar reduction in CFU-S self-renewal.

To explain the contradictory evidence of stem cell 'immortality' and 'mortality' the '*niche hypothesis*' was developed [Schofield,1978]. A specialized microenvironmental niche was envisaged within the haematopoietic system. If a stem cell inhabits a niche, it exhibits it's immortality - it has a high self-renewal capacity. Outside the niche, it becomes subject to 'maturation' cues - it develops a reduced self-renewal capacity and an increased probability for differentiation. This hypothesis may also explain the asymmetry of fate of stem cell progeny. At division, one daughter cell remains in the niche microenvironment, the other is expelled and becomes subject to differentiation cues [*Figure 3*].

It is proposed that X-irradiation damages the haematopoietic system by subjecting the niche-inhabiting stem cells to lethal damage. Transplanted haematopoietic tissue and specifically the

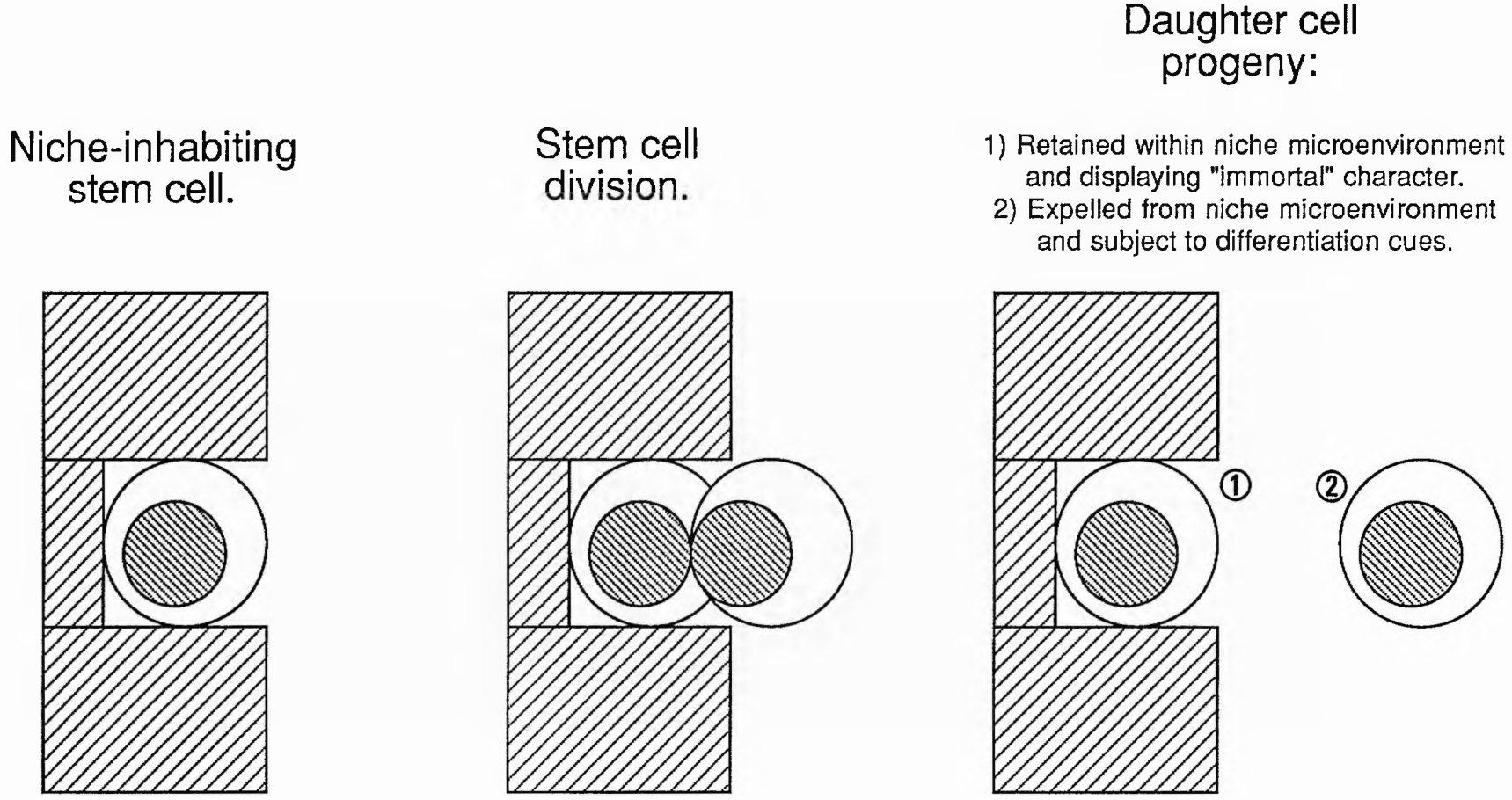


Figure 3: The "niche" hypothesis. A possible explanation of the assymetry of fate of stem cell progeny.

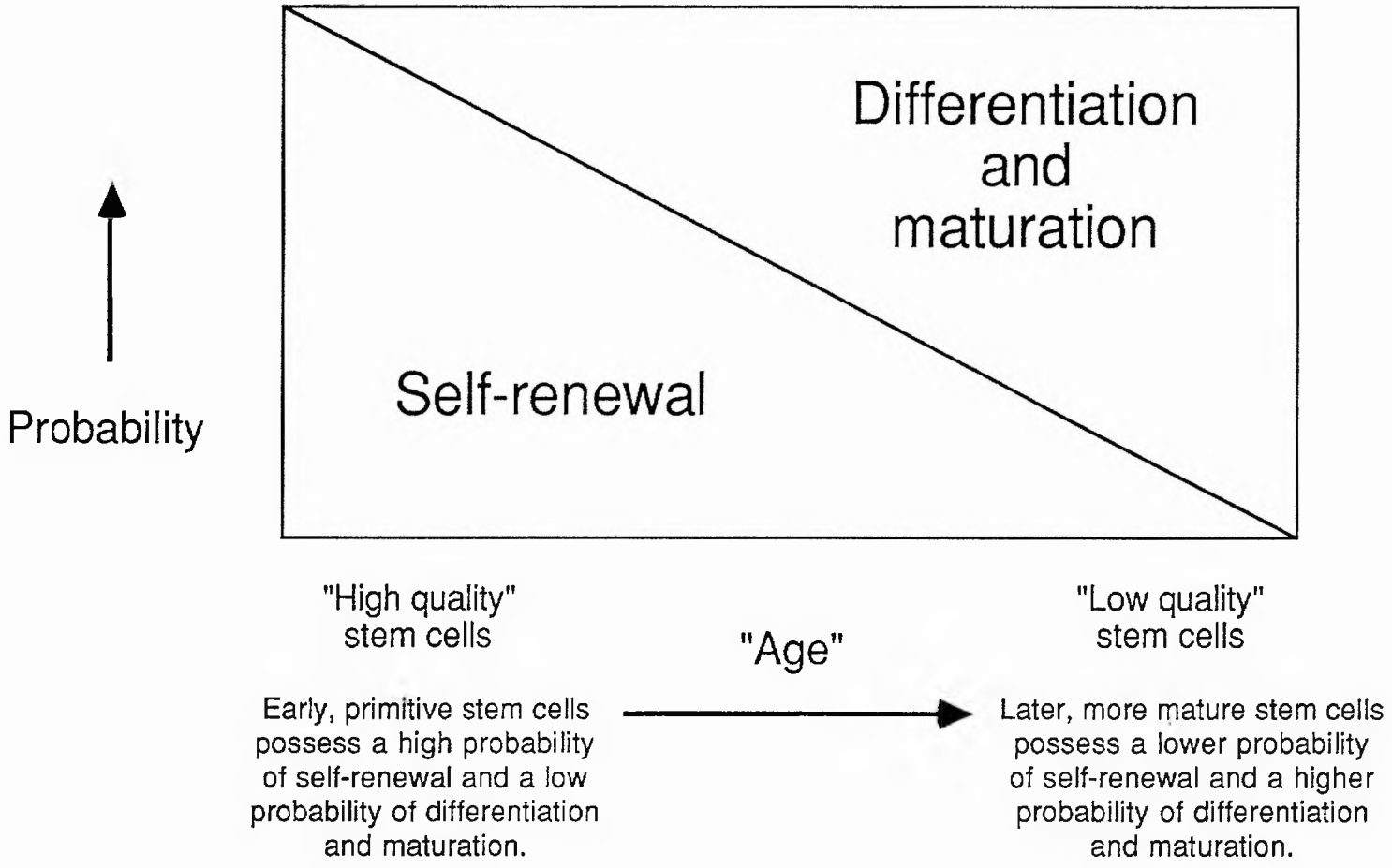
stem cells therin, can seed into the now 'empty' niches and adopt an immortal character. However, the majority of donor stem cells seeding into the niches are likely to be the more numerous, more mature haematopoietic stem cells of lower self-renewal capacity - essentially of a lower 'quality' than the original niche-inhabiting stem cell. While at the first transplantation sufficient haematopoietic output is sustained to allow haematopoietic recovery, with successive transplantation, the niche-filling stem cells are of a progressively poorer 'quality', with a still lower self-renewal capacity. By the third successive transplant insufficient self-renewal and proliferative potential exists to supply differentiated haematopoietic precursors and haematopoietic failure ensues.

Similarly, both sublethal chronic and repeated acute X-irradiations directly insult the niche-inhabiting stem cell population. 'High quality' stem cells are subsequently replaced by successively 'lower quality' stem cells giving the observed and sustained reduction in both CFU-S numbers and CFU-S self-renewal.

This evidence suggests an age-related hierarchical organization within the haematopoietic stem cell system. A component of the stem cell population is a primitive precursor with a high self-renewal capacity and proliferative potential. It possesses a high probability of self-renewal and a low probability of differentiation - essentially a 'high quality' stem cell. With maturation, a haematopoietic precursor with a lower self-renewal and proliferative potential develops. It possesses a lower probability of self-renewal and a higher probability of differentiation - essentially a 'lower quality' stem cell [*Figure 4*].

Use of chemical agents reinforces this age-related heterogeneity of the haematopoietic stem cell population. Isopropylmethane sulphate (IMS)[Schofield & Lajtha,1973] reduces CFU-S numbers dramatically, however, haematopoietic recovery is rapid and

Figure 4: Putative age-related hierarchical organization within the haematopoietic stem cell population.



complete. IMS appears to act against CFU-S of a specific age within the CFU-S maturation scheme. 'High quality', primitive and more mature 'low quality' stem cells are spared. Myleran [Morley & Blake,1974] also reduces CFU-S numbers, however, haematopoietic recovery is never complete, implying damage at the level of the more primitive 'high quality' stem cell population. Haematopoietic transplantation, replacing the damaged, primitive, 'high quality' stem cells, corrects the haematopoietic failure [Morley *et al*,1975].

While the niche hypothesis is essentially a theoretical model, considerable evidence for the existence of a specific stem cell microenironment, or 'niche', exists [Dexter *et al*,1973; Dexter & Lajtha,1974; Allen & Dexter,1976]. Use of genetic haematopoietic disorders in mice has reinforced this evidence. Briefly, the W/W^v defect is an apparent stem cell maturation defect. 'Low quality' stem cells with a low probability of self-renewal and a high probability of differentiation are produced. In Sl/Sl^d mice a microenvironmental defect exists possibly at the level of the haematopoietic stem cell niche. Neither defective systems will sustain haematopoiesis in culture, however, a combination of W/W^v stromal tissue and Sl/Sl^d haematopoietic tissue will sustain haematopoiesis in culture - implying a healthy stem cell population and a healthy stromal microenvironment are required [Dexter & Moore,1977].

Haematopoietic stem cells are not uniformly or randomly distributed throughout the marrow. Axial cores of actively haematopoietic murine femoral marrow demonstrate a considerable degree of microarchitectural organization exists. This may reinforce the evidence of a specialized microenvironmental stem cell niche. Samples of endosteal marrow have a 2-3 fold higher CFU-S content than samples of axial marrow [Lord *et al*,1975]. Conversely, samples of endosteal marrow have markedly fewer of the more mature

haematopoietic progenitors than samples of axial marrow [Frassoni *et al*,1982]. This may imply a migration of haematopoietic progenitors with maturation from the endosteal to the axial marrow. The significance of this microarchitecture may become more apparent during the discussion of the relative distribution of the stem cell proliferation regulator-producing cells.

Endosteal marrow is a particularly rich source of CFU-S [Gong *et al*,1978] and the use of a bone-seeking radionuclide: radium (^{226}Ra)[Svoboda,1975], a source of alpha radiation, demonstrates a marked reduction in CFU-S numbers with cells near the endosteal surface of the bone subject to intense radiation. Observations of haematopoietic recovery after X-irradiation also demonstrate the significance of this region with haematopoietic repopulation occuring from this endosteal tissue [Maloney & Pratt,1969; Lambersten & Weiss,1984] and while the majority of CFU-S can be removed in femoral marrow, even if the marrow cavity is vigorously washed a further CFU-S population can be released if the bone itself is ground and washed, implying a very intimate contact between a component of the CFU-S population and bone. This endosteal region may well contain certain components of the specific microenvironmental stem cell niche.

1.5 Evidence of a spleen colony-forming unit (CFU-S) age-related hierachy.

Magli *et al* [1982] described the transient nature of some spleen colonies in the murine CFU-S assay. While colony numbers may not differ between spleens assayed at day 7-8 and day 10-12, some colonies appear and are counted at day 7-8, to disperse by day 10-12, while others do not appear until after day 7-8 to be counted at day 10-

12. Other colonies appear fixed and are present both at day 7-8 and day 10-12. Analysis of day 8 CFU-S and day 12 CFU-S demonstrates a difference in character. Day 8 CFU-S have a relatively low self-renewal capacity which would be consistent with their being a component of the more mature haematopoietic stem cell population, while day 12 CFU-S have a relatively high self-renewal capacity, consistent with their being a more primitive component of the haematopoietic stem cell population [Magli *et al*,1982]. This evidence of an age-related CFU-S and so stem cell heterogeny, is reinforced by a number of drug studies.

5-fluorouracil (5FU)[Hodgson & Bradley,1979; Hodgson *et al*,1982], cyclophosphamide (CY)[Molineux *et al*,1986], hydroxyurea (HU)[Rosendaal *et al*,1979] and bromodeoxyuridine (BrdU)[Hodgson & Bradley,1984], preferentially kill more mature haematopoietic precursors. CFU-S assay of treated marrow showed a reduction in day 8 CFU-S numbers with little, or no change in day 12 CFU-S. This again implies the day 8 CFU-S is a more mature haematopoietic precursor than the day 12 CFU-S. This CFU-S age-related hierarchy becomes of greater significance when considering their respective sensitivities to the haematopoietic stem cell proliferation regulators.

1.6 Evidence of a 'pre-CFU-S' haematopoietic precursor.

The murine CFU-S assay [Till & McCulloch,1961], has been taken as the primary assay of a primitive haematopoietic precursor, however, there is evidence that it may not be an assay of the fundamental haematopoietic stem cell, with evidence of a more primitive 'pre-CFU-S' population. It is suggested that the pre-CFU-S population is responsible for both haematopoietic recovery after X-irradiation, it having a radioprotective ability (RPA) and for the

production of CFU-S. As discussed previously, the drugs 5FU, CY, HU and BrdU selectively kill the more mature haematopoietic precursors. If used in transplant, it becomes clear that the number of CFU-S in the treated tissue is not necessarily an accurate predictor of the capacity of that transplant to rescue the haematopoietically deficient recipients, rather recovery seems to be due to a 'pre-CFU-S' population.

Primitive haematopoietic precursors can be purified from haematopoietic tissue using a number of different techniques. Ploemacher and Brons [1988a; 1988b] used fluorescence activated cell sorting (FACS) of wheat germ agglutinin (WGA) - fluoroscein isothiocyanate (FITC) binding cells to purify day 12 CFU-S and 'pre-CFU-S' 50-200 fold. 50-80 of these enriched cells *per* animal (in contrast to in excess of 10^4 complete bone marrow cells) were capable of rescuing 50% of a group of lethally irradiated mice. The ability of the haematopoietic transplant to rescue it's recipients seemed closely related to it's day 12 CFU-S and 'pre-CFU-S' content rather than its more mature day 8 CFU-S content.

Bertoncello *et al* [1985], Mulder and Visser [1987] and Ploemacher and Brons [1988c; 1988d] used counter flow centrifugal elutriation (CCE) and the fluorescent supravital mitochondrial membrane stain, rhodamine-123 (Rh-123). CCE and FACS of Rh-123 treated marrow produces a Rh-123 dim fraction containing day 12 CFU-S with a high RPA/'pre-CFU-S' content and a Rh-123 bright fraction with a high day 12 CFU-S content but few RPA/'pre-CFU-S'. This suggests a possible maturation scheme of a Rh-123 dim, RPA/'pre-CFU-S' population to a Rh-123 dim, day 12 CFU-S population to a Rh-123 bright, day 12 CFU-S population and subsequently to a Rh-123 bright, day 8 CFU-S population.

Bertoncello *et al* [1989b] used antibody labelled magnetic microspheres to concentrate and resolve primitive haematopoietic precursors while Spangrude *et al* [1988], used fluorescence-labelled antibodies against cell surface antigens and FACS. Spangrude *et al* [1988] isolated a fraction of cells containing 50% of the total day 12 CFU-S and the RPA/'pre-CFU-S' population from complete marrow. (The fraction containing the remaining day 12 CFU-S had little or no RPA/'pre-CFU-S' content. This again implies a maturation of RPA/'pre-CFU-S', through a similar staining 'transition' day 12 CFU-S fraction to a differently staining day 12 CFU-S population). 30-40 of these cells *per* animal were capable of rescuing 50% of a group of lethally X-irradiated mice.

Jones *et al* [1990] have applied CCE, separating cells on the basis of their sedimentation velocities, to samples of haematopoietic tissue. Sedimentation velocity varies according to cell size and density. A clear demarcation is reported between the RPA/'pre-CFU-S' and CFU-S populations. This technique also suggests there may be a more primitive cell than the RPA/'pre-CFU-S', responsible for long-term haematopoietic repopulation. Ploemacher and Brons [1988b; 1989a] observed similar evidence.

It is proposed that for successful haematopoietic regeneration, two vital classes of haematopoietic precursors are required in transplanted tissue. Firstly, the cells capable of the initial short-term, sustained proliferation, producing the committed haematopoietic progenitors, namely the CFU-S subpopulations and secondly, the cells capable of long-term sustained haematopoiesis. It is clear that a complex hierarchy exists within the haematopoietic stem cell population and investigation into the behaviour and regulation of the various components of this very primitive compartment is limited by the assay techniques presently available. However, Sutherland *et al*

[1989] and Ploemacher *et al* [1989b] report the development of assays of such primitive haematopoietic precursors responsible for the initiation of long-term haematopoiesis, which should allow their subsequent investigation.

1.7 Determination of the proportion of haematopoietic stem cells in S-phase.

The murine *in vivo* CFU-S haematopoietic transplantation assay [Till & McCulloch,1961] has allowed qualitative and quantitative investigation of the haematopoietic stem cell compartment. One such factor investigated is the degree of haematopoietic stem cell proliferation. Cellular proliferation is intimately linked with DNA synthesis at S-phase in the cell cycle and this can be exploited by the use of S-phase specific cytotoxic agents. Proliferating cells take up the agent at S-phase and are killed. If such cells had colony-forming potential, this is not realised during any subsequent assay and by a comparison of colony numbers in the absence and presence of the cytotoxic agent, a measure of the proportion of colony-forming cells in S-phase and so proliferating, can be made. This is the basis of the '*S-phase suicide assay*'.

Two S-phase specific agents widely used are tritiated thymidine (^{3}H-TdR) and 1-ß-D-arabinofuranosyl cytosine (also known as: cytosine ß-D-arabinoside, cytosine arabinoside or cytarabine)(ARA-C). Tritiated thymidine (^{3}H-TdR) competes with native thymidine for incorporation into newly synthesizing DNA at S-phase. On incorporation, intense localized radiation induces lethal DNA lesions and the result is cell death [Becker *et al*,1965]. 1-ß-D-arabinofuranosyl cytosine (ARA-C) is a nucleoside analogue, competing with native deoxycytidine for incorporation into newly

synthesizing DNA at S-phase. Deoxycytidine kinase converts ARA-C to ARA-C triphosphate (ARA-CTP) [Wist *et al*,1976] and incorporated by the complex DNA-synthesizing enzymes into the elongating DNA molecule, it prevents subsequent nucleoside polymerization probably by stereochemically hindering its access to the 3'-OH group of the DNA macromolecule. As such it acts as a 'chain-terminator' and its incorporation is a lethal event for the cell [Millard & Okell,1975].

The kinetic behaviour of the haematopoietic stem cell population during chemotherapeutic and radiotherapeutic regimes and during haematopoietic transplantation and embryonic development is of great interest. With respect to chemotherapeutic and radiotherapeutic regimes, widely used in the treatment of malignancy, the survival and behaviour of the haematopoietic system is an important consideration and a major limiting factor in regulating the levels of use of such therapeutic agents.

1.8 The proportion of haematopoietic stem cells in S-phase.

The CFU-S assay and S-phase suicide techniques have allowed the investigation of the behaviour of the haematopoietic stem cell population under different conditions. In normal unstressed haematopoietic tissue, the proportion of haematopoietic stem cells in S-phase is relatively low at approximately 10% [Becker *et al*,1965] [*Figure 5*]. The majority of stem cells in normal unstressed haematopoietic tissue, appear to be in a reversible, non-proliferative, 'G_o-state' within the cell cycle. This G_o-state may be of particular significance, perhaps allowing a genetic 'house-keeping' mechanism to act and maintain the 'high quality' genetic integrity of the haematopoietic stem cell population. A rapidly proliferating cell

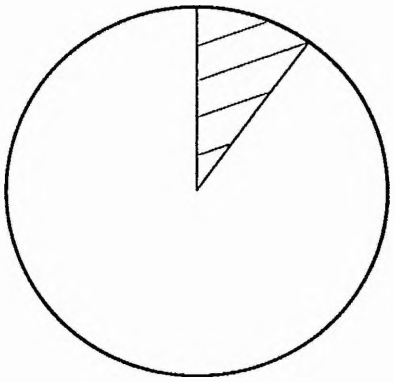
system would be forced to express any genetic damage, the defects possibly passed on to all subsequent progeny perhaps to effect aspects of their proliferation, differentiation or maturation.

The relatively large proportion of stem cells in a 'resting' G_o-state also ensures the haematopoietic tissue has a very great 'functional reserve'. As haematopoietic output requires, the proportion of stem cells in S-phase can increase to meet the demand. During sublethal whole body X-irradiation, this mobilization of the G_o-state, 'resting' stem cells can be demonstrated. 1.5 Gy, 3.0 Gy and 4.5 Gy whole body X-irradiation doses reduce CFU-S numbers to approximately 20%, 4% and less than 1% of normal levels respectively [Hendry & Lajtha,1972]. After such treatment the proportion of CFU-S in S-phase increases from approximately 10% to 40-50% [Guzman & Lajtha,1970] [*Figure 5*]. Haematopoietic stem cell proliferation is increased in an attempt to both maintain the integrity of the damaged haematopoietic system and restore CFU-S numbers to pretreatment levels, through self-renewal. Once the haematopoietic system has been reconstituted, the proportion of the haematopoietic stem cell population in S-phase reduces to pretreatment levels.

A similar effect is observed if the haematopoietic system is stressed through the use of a haemolytic agent - phenylhydrazine [Rencricca *et al*,1970]. The anaemia induced by phenylhydrazine, is compensated by an increased haematopoietic output, met inturn by an increase in the proportion of haematopoietic stem cells in S-phase. An increase from approximately 10% to 30-50% in S-phase is reported [Rencricca *et al*,1970].

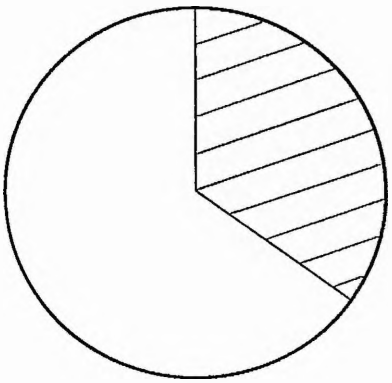
Similarly, during embryonic development a constant increase in haematopoietic output is required with growth. Haematopoiesis initiated in the murine (and human) yolk sac, develops in the foetal liver, to eventually become established in the marrow cavities

Figure 5: The proportion of haematopoietic stem cells (CFU-S) in S-phase.



Normal bone marrow.

Approximately 10% of haematopoietic stem cells (CFU-S) in S-phase.



Regenerating or foetal haematopoietic tissue.

30-40% of haematopoietic stem cells (CFU-S) in S-phase.

[Metcalf & Moore,1971]. The proportion of stem cells in S-phase in the foetal liver is relatively high at 30-40% [Thomas *et al*,1981] to meet the increasing demand for haematopoietic output.

The mechanism(s) by which the proportion of haematopoietic stem cells in S-phase is modified to meet haematopoietic demand, is of particular interest.

1.9 Evidence for the local nature of haematopoietic stem cell proliferation.

The proportion of haematopoietic stem cells in S-phase, and so proliferating, is able to change to match haematopoietic output to demand. In unstressed haematopoietic tissue the proportion of stem cells in S-phase is relatively low at approximately 10%, while in haematopoietically stressed tissue, for example after cytotoxic drug treatment, irradiation or during foetal development, the proportion of stem cells in S-phase is increased at 30-40%. The mechanism(s) by which the proportion of haematopoietic stem cells in S-phase is modified has been elucidated through the use of part-body X-irradiation.

During whole body X-irradiation, haematopoietic survival is enhanced if either the spleen is exteriorized and shielded [Jacobson *et al*,1949], or if a hind limb is shielded [Croizat *et al*,1970; Gidali & Lajtha,1972]. Initially the shielded tissue acts as a stem cell 'reservoir', exporting stem cells to the irradiated, haematopoietically deficient haematopoietic tissue [Hanks,1964]. Approximately 30% of the total hind limb stem cells, as measured by the CFU-S assay, may be exported within 6-12 hours of the initial irradiation insult. The depletion of stem cells in the shielded tissue, leads to an increase in the proportion of stem cells in S-phase, sufficient to restore stem cell

numbers to pre-export levels, through self-renewal, after which the proportion of stem cells in S-phase reduces. This is in marked contrast to the stem cells seeding into the haematopoietically-deficient, irradiated tissues. Here the proportion of stem cells in S-phase is high to develop the extensive haematopoietic regeneration required. Thus, within one animal, two distinct populations of proliferating stem cells can be demonstrated. Despite the major whole-body haematopoietic stress, the proportion of stem cells in S-phase in the shielded tissue remains low. This essentially rules out the existence of a body-wide stem cell proliferation regulation mechanism, with the haematopoietic stem cell population essentially responding to local microenvironmental cues. In shielded tissue, once the initial export depletion is corrected, the local haematopoietic microenvironment is essentially normal, and the proportion of stem cells in S-phase is correspondingly low. Conversely, in the irradiated tissue, the local haematopoietic microenvironment is depleted and the proportion of stem cells in S-phase is correspondingly high, reflecting the local need for haematopoietic regeneration.

Similar evidence of a locally active proliferation regulation mechanism, is observed with the use of the haemolytic agent phenylhydrazine [Rencricca *et al*,1970; Wright & Lord,1977]. Phenylhydrazine induces a marked haemolytic anaemia and in response, the proportion of stem cells in S-phase the bone marrow is markedly increased. Stem cells are exported to the spleen from the bone marrow, however, in marked contrast, the proportion of stem cells in S-phase in the spleen is low, despite the body-wide haematopoietic stress. Again, within one animal, two distinct populations of proliferating stem cells can be demonstrated, implying the involvement of a localized haematopoietic stem cell proliferation

regulation mechanism, the stem cells responding to local micoenvironmental cues rather than body-wide regulatory signals.

1.10 The mechanism of haematopoietic stem cell proliferation regulation.

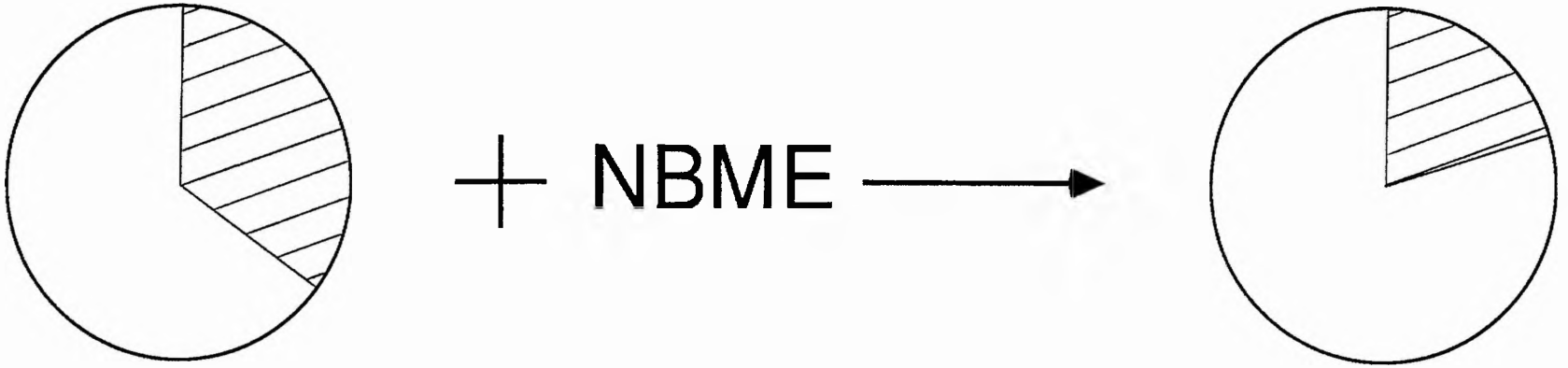
The putative, locally active regulatory mechanism(s) reponsible for modifying haematopoietic stem cell proliferation, were investigated using crude cell-free extracts of haematopoietic tissues [Lord *et al*,1976; Lord *et al*,1977; Wright & Lord,1977; Wright & Lord,1978].

Normal bone marrow extract (NBME), derived from a tissue in which the proportion of stem cells in S-phase is low [Becker *et al*,1965], has the capacity to reduce the proportion of stem cells in S-phase from haematopoietically stressed or regenerating bone marrow, or foetal liver. This is evidence of a proliferation *inhibitor* [Lord *et al*,1976; Cork *et al*,1981] [*Figure 6*].

Conversely, haematopoietically stressed or regenerating bone marrow extract (RBME), (or foetal liver extract), derived from a tissue in which the proportion of stem cells in S-phase is increased [Croizat *et al*,1970; Gidali & Lajtha,1972; Rencricca *et al*,1970; Wright & Lord,1977; Thomas *et al*,1981], has the capacity to increase the proportion of stem cells in S-phase from normal unstressed bone marrow. This is evidence of a proliferation *stimulator* [Frindel *et al*,1976a; Lord *et al*,1977; Frindel *et al*,1978; Thomas *et al*,1981; Dawood *et al*,1990] [*Figure 7*].

The reduced proportion of stem cells in S-phase in normal bone marrow is thus maintained by the presence of an endogenous proliferation *inhibitor*, while the increased proportion of stem cells in S-phase in haematopoietically stressed or regenerating bone

Figure 6: Evidence for a stem cell proliferation inhibitor.

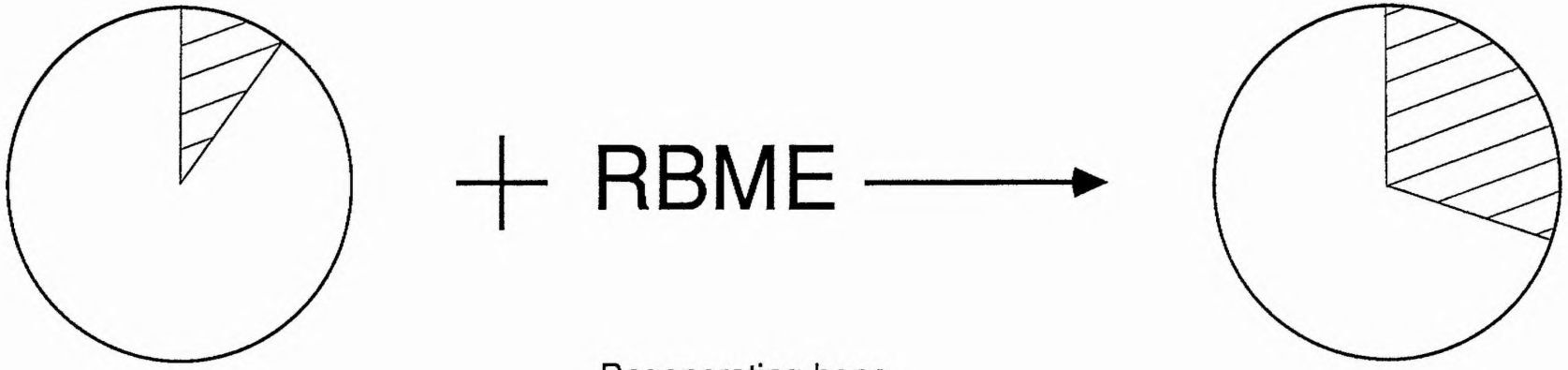


Regenerating or foetal haematopoietic tissue Approximately 30-40% haematopoietic stem cells (CFU-S) in S-phase.

Normal bone marrow extract. Derived from a tissue in which the proportion of stem cells (CFU-S) in S-phase is low (approximately 10%).

Proportion of stem cells (CFU-S) in S-phase reduced.

Figure 7: Evidence for a stem cell proliferation stimulator.



Normal bone marrow. Approximately 10% haematopoietic stem cells (CFU-S) in S-phase.

Regenerating bone marrow (or foetal haematopoietic tissue) extract. Derived from a tissue in which the proportion of stem cells (CFU-S) in S-phase is high (approximately 30-40%).

Proportion of stem cells (CFU-S) in S-phase increased.

marrow, or foetal liver, is maintained by the presence of an endogenous proliferation *stimulator*.

Significantly, the slowly proliferating stem cells of normal, unstressed haematopoietic tissues, remain slowly cycling, even if the endogenous *inhibitor* is washed away. Similarly, rapidly cycling stem cells from haematopoietically stressed, regenerating or foetal tissues, remain rapidly cycling, even if the endogenous *stimulator* is washed away [Lord *et al*,1979]. Haematopoietic stem cells thus remain either slowly, or rapidly cycling, unless acted upon by the opposing proliferation regulator. Removal of the existing regulatory activity is not sufficient to change the proliferative status of haematopoietic stem cells.

As previously discussed, the local nature of stem cell proliferation regulation is demonstrated by either part body X-irradiation, or the use of the haemolytic agent phenylhydrazine. The existence of two distinct populations of cycling stem cells within the one animal can be explained in part by examination of the respective local levels of proliferation *inhibitor* and *stimulator*. Unshielded, X-irradiated tissue contains rapidly cycling haematopoietic stem cells and this is consistent with the local presence of *stimulator*. In shielded tissue, containing slowly cycling haematopoietic stem cells, *inhibitor* is present. Similarly, in phenylhydrazine-treated marrow, a source of rapidly proliferating stem cells, *stimulator* is demonstrable, while in phenylhydrazine-treated spleen, containing slowly cycling stem cells, *inhibitor* is demonstrable [Lord *et al*,1979; Wright & Lord,1977; Wright & Lord,1978].

Use of Amicon Diaflo Ultrafiltration, a technique separating factors by molecular weight, has isolated the *inhibitor* activity to a 50,000-100,000 dalton fraction of normal bone marrow extract, termed *NBME-IV* [*Figure 8*]. *Stimulator* activity has been similarly isolated to

a 30,000-50,000 dalton fraction of regenerating bone marrow extract (and also of haematopoietically stressed marrow and foetal liver extracts), termed *RBME-III* [*Figure 8*] [Lord *et al*,1977]. (1 dalton = 1 atomic mass unit (amu) = 10^{-24}g)

No significant *inhibitor* activity is detected in the 50,000-100,000 dalton fraction of RBME, or *stimulator* activity detected in the 30,000-50,000 dalton fraction of NBME, implying that the two endogenous factors are not present simultaneously but act antagonistically to modify the proportion of stem cells in S-phase and tailor stem cell proliferation and so haematopoietic output, to haematopoietic demand. *Stimulator* and *inhibitor* are not detected in non-haematopoietic tissues, demonstrating their role is confined to the regulation of haematopoietic tissues [Riches *et al*,1981a; 1981b].

1.11 Cellular sources of the haematopoietic stem cell proliferation regulators.

In producing cell-free extracts of normal (NBME) and regenerating, haematopoietically stressed or foetal liver tissue (RBME), the haematopoietic cells are effectively washed and the proliferation regulators removed. As previously stated, this does not affect inherent stem cell proliferation [Lord *et al*,1979] - slowly cycling cells remain slowly cycling and rapidly cycling cells remain rapidly cycling. If the washed cells are incubated after washing, resynthesis of the appropriate regulatory factor occurs. Over a 5 hour period, incubated at 37°C, washed, normal bone marrow will resynthesize *inhibitor*, [Wright & Lord,1978] and over a 3 hour period, incubated at 37°C, washed, regenerating or haematopoietically stressed bone marrow, or foetal liver will resynthesize *stimulator* [Wright & Lord,1978]. Resynthesis of the proliferation regulators can be

Figure 8: Stem cell proliferation inhibitor and stimulator

Inhibitor:	Isolated to a 50,000-100,000 dalton Amicon ultrafiltration fraction of normal bone marrow conditioned medium.	NBME-IV
Stimulator:	Isolated to a 30,000-50,000 dalton Amicon ultrafiltration fraction of regenerating bone marrow (or foetal haematopoietic tissue) conditioned medium.	RBME-III

blocked by the action of *cyclohexamide*, an inhibitor of protein synthesis, demonstrating the production of the proliferation regulators requires active protein synthesis [Wright *et al*,1980b; 1982].

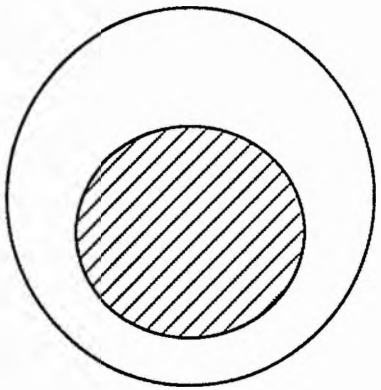
Cellular fractionation studies of haematopoietic tissue demonstrate that the cells responsible for the production of the proliferation regulators are a non-stem cell population. Fractionation of normal murine bone marrow, demonstrates a cell population of 1.052-1.062 g/ml density which are adherent, phagocytic, relatively radio-resistant, Thy 1.2^-, Fc^+, phosphodiesterase positive, is responsible for *inhibitor* production [Wright & Lord,1979; Wright *et al*,1980b] [*Figure 9*]. Similar fractionation of regenerating murine marrow, demonstrates a cell population of 1.064-1.072 g/ml density which are adherent, phagocytic, relatively radio-resistant, Thy 1.2^-, Fc^+, is responsible for *stimulator* production [Wright & Lord,1979; Wright *et al*,1982] [*Figure 9*].

Microscopic examination of cells at these densities shows a wide variety of cell types present, and while two distinct cell densities exist for the *inhibitor*- and *stimulator*-producing cell populations, it may be that a single cell-type is capable of producing either factor with it's density varying accordingly. It is clear however, that the factor-producing cells are not stem cells. Virtually no stem cells are found at either density and similarly, no factor-producing cells are apparent at the density where stem cells are concentrated [Wright & Lord,1979; Wright *et al*,1980b; Wright *et al*,1982].

It is likely that the proliferation regulators are produced by cells of the monocyte-macrophage lineage within the haematopoietic tissue. Macrophages make up a large component of the haematopoietic stroma and are already demonstrated to play a major role in the regulation of granulopoiesis, erythropoiesis, megakaropoiesis and

Figure 9: Summary of inhibitor- and stimulator-producing cell populations.

Both inhibitor- and stimulator-producing cell populations are characterized as:



Adherent
Phagocytic
Relatively radioresistant
Thy 1.2 -ve
Fc +ve
Phosphodiesterase +ve
F4/80 +ve

Probably cells of the monocyte-macrophage lineage.

Inhibitor-producing cells 1.052 - 1.062 g/ml density.

Stimulator-producing cells 1.064 - 1.072 g/ml density.

lymphopoiesis. A monoclonal antibody F4/80, which is specific for murine macrophages has been used in association with a density-cut procedure and fluorescence activated cell sorting to select for F4/80 positive and negative populations from murine marrow. An F4/80 positive fraction contained both *inhibitor*- and *stimulator*-producing cells and cells displayed the histochemical characteristics of macrophages. These purified macrophage populations can be cultured over an extended period and still synthesize *inhibitor* and *stimulator* [Simmons & Lord,1985; Wright & Lorimore,1987; Pojda *et al*,1988].

1.12 Identification and characterization of the haematopoietic stem cell proliferation *inhibitor*.

Since cells of the monocyte-macrophage lineage are probably responsible for the production of the stem cell proliferation regulators, a number of macrophage cell lines were screened for the production of either *inhibitor* or *stimulator*.

Crude conditioned medium from a murine macrophage cell line J774.2 was demonstrated to possess a 50,000-100,000 dalton activity which was capable of reversibly inhibiting stem cell proliferation [Graham *et al*,1990]. The activity was termed '*stem cell inhibitor*' (SCI). A 50,000 fold purification of the activity from crude J774.2 conditioned medium was performed and a protein doublet of 8000 daltons isolated [Graham *et al*,1990]. N-terminal analysis resolved amino acid sequences corresponding to a previously described superfamily of cytokines, the *macrophage inflammatory proteins* (MIPs) [Wolpe & Cerami,1989]. One component of the isolated protein doublet, a 69 amino acid polypeptide, was found to correspond to the previously described cytokine, *macrophage*

inflammatory protein - 1 alpha (MIP-1 alpha), and the second component of the protein doublet, was found to correspond to the previously described cytokine, *macrophage inflammatory protein - 1 beta* (MIP-1ß). Antibodies directed against MIP-1 alpha were able to remove inhibitory activity from the purified stem cell inhibitor (SCI), the crude J774.2 conditioned medium and significantly from normal bone marrow extract (NBME), indicating that the stem cell inhibitor, as purified SCI, crude J774.2 conditioned medium or NBME, and MIP-1 alpha are antigenically and functionally identical. The complete genomic sequence of the murine haematopoietic stem cell inhibitor/macrophage inflammatory protein 1 alpha has subsequently been determined [Grove *et al*,1990].

Use of the polymerase chain reaction and subsequent generation of an expression vector in COS cells has allowed the production of recombinant MIP-1 alpha and MIP-1ß. rMIP-1 alpha has native SCI activity while rMIP-1ß, although possessing a 67% amino acid homology with rMIP-1 alpha, shows no SCI activity, demonstrating a considerable degree of MIP-1 alpha specificity. Molecular probes have isolated the SCI/MIP-1 alpha gene to murine chromosome 11. This is significant since the genes for interleukins 3-5 and granulocyte-macrophage and granulocyte colony-stimulating factors as well as the macrophage colony-stimulating factor-receptor lie within close genomic proximity. In humans molecular probes have isolated the SCI/MIP-1 alpha gene to chromosome 17 [Irving *et al*,1990].

Similar studies are likely to isolate and characterize the haematopoietic stem cell proliferation *stimulator.*

1.13 Demonstration of the haematopoietic stem cell proliferation regulators in *in vitro* long-term bone marrow culture.

The modification of haematopoietic stem cell proliferation by the proliferation regulators can be simulated using *in vitro* long-term bone marrow culture systems. The maintainance of long-term bone marrow culture is dependent on the formation of a healthy, adherent, 'stromal' layer containing a variety of cell types including fibroblast and epithelial-like cells, fat cells and phagocytic, mononuclear macrophages. This adherent stromal layer provides a unique haematopoietic microenvironment, which allows the seeding and proliferation of haematopoietic stem cells and the production of haematopoietic progenitor cells [Dexter *et al*,1977a; 1977b; Spooncer & Dexter,1984; Dorshkind,1990]. To maintain long-term marrow cultures *in vitro*, a periodic removal of old medium and replacement with fresh medium is required. Cells released into the medium by the actively haematopoietic layer, are removed with the old medium to be replaced by fresh, cell-free medium. This cellular depletion leads to a cyclical increase in the proportion of the haematopoietic stem cell population in S-phase, however, within a few days of the depletion, the cellularity of the medium has increased sufficiently for haematopoietic output to return to normal levels, with a subsequent reduction in the proportion of stem cells proliferating [Dexter *et al*,1977a; 1977b; Toksoz,1980; Cashman *et al*,1985].

The increased stem cell proliferation in response to the cellular depletion of the culture, is induced by the endogenous production of *stimulator* by the stromal layer. As cellular repopulation occurs after 5-7 days, levels of endogenous *stimulator* fall and levels of

endogenous *inhibitor* increase, with a resultant reduction in the proportion of stem cells in S-phase. It thus appears that this artificial *in vitro* model of haematopoiesis demonstrates the same subtle stem cell proliferation regulation mechanism as is observed *in vivo*.

The degree of stem cell proliferation in long-term bone marrow cultures can be artificially manipulated by the exogenous addition of either *inhibitor* or *stimulator*. A number of chemotherapeutic drugs are S-phase specific, cytotoxic agents and, in use, damage cycling, normal tissues as well as the target cancerous tissues. Haematopoietic tissue is especially sensitive to such agents and the potential for the use of the proliferation *inhibitor* to reduce the proportion of haematopoietic stem cells in S-phase, and so sensitive to the specific action of the cytotoxic agent, is significant. Such potential can be demonstrated using the *in vitro*, long-term bone marrow culture system. Treatment of long-term bone marrow cultures with *cytosine arabinoside* (ARA-C), an S-phase specific cytotoxic agent, leads to a long term depression of haematopoietic output. In contrast, cultures pretreated with exogenous proliferation *inhibitor*, prior to treatment with ARA-C, perform at least as well as control cultures (no ARA-C) [Lord *et al*,1987].

These *in vitro* results mimic results observed *in vivo* [Lord & Wright,1980]. Exogenous *inhibitor* was observed to 'protect' haematopoietic stem cells *in vivo* from the S-phase specific cytotoxic effects of tritiated thymidine (^{3}H-TdR), hydroxyurea and ARA-C.

The use of long-term bone marrow culture systems reveals a subtle permissive stromal microenvironment is vital in the maintainance and regulation of haematopoiesis. Such a specialized microenvironment and evidence of very local haematopoietic stem cell-stromal cell interactions [Lambertsen,1984; Zipori,1989] is

somewhat reminiscent of the stem cell 'niche' proposed by Schofield [1978].

1.14 Regulation of the production of the haematopoietic stem cell proliferation *inhibitor* and *stimulator*.

The mechanism of action of the proliferation regulators has been investigated. The proportion of stem cells in S-phase in normal unstressed haematopoietic tissue is relatively low and maintained by endogenous *inhibitor* activity. In the absence of *inhibitor*, when cells are washed, the proportion of stem cells in S-phase remains low and washed, incubated cells resynthesize *inhibitor*. In regenerating, haematopoietically stressed or foetal haematopoietic tissues, the proportion of stem cells in S-phase is increased, maintained by endogenous *stimulator* production. In the absence of *stimulator*, when the cells are washed, the proportion of stem cells in S-phase remains increased and washed reincubated cells resynthesize *stimulator*. Thus, change in the proportion of stem cells in S-phase is not simply brought about by a removal of the existing regulatory factor. Changes in the proportion of stem cells in S-phase can only be induced by the presence of the opposing factor.

Distinct *stimulator* and *inhibitor* producing cells can be identified in all haematopoietic tissues. Both cell subpopulations are present regardless of the proliferative state of the haematopoietic tissue, with the potential to produce one or other factor, or both, however, only one proliferation regulator predominates at any one time. This, together with evidence that *stimulator* and *inhibitor* are relatively stable activities (with no loss of potency after a week at 37°C) [Toksoz *et al*,1980] and do not directly interact, the one not inactivating the other [Lord & Wright,1982] implies a distinct

regulatory mechanism must exist to coordinate *inhibitor* and *stimulator* production and breakdown, to ensure the appropriate degree of haematopoietic stem cell proliferation occurs.

Changes in *inhibitor* and *stimulator* production, and so changes in the degree of haematopoietic stem cell proliferation, are brought about by the action of the one factor on the cell producing the other. Isolated *inhibitor*-producing cells will resynthesize *inhibitor* if washed. However, in the presence of exogenous *stimulator*, no *inhibitor* synthesis is observed [*Figure 10*]. Similarly, isolated *stimulator*-producing cells will synthesize *stimulator* if washed. However, in the presence of exogenous *inhibitor*, no *stimulator* synthesis is observed [Lord & Wright,1982; Riches & Cork,1987] [*Figure 11*].

This elegant interaction of factor with opposing factor-producing cell populations, however, does not allow for the proliferative flexibility demonstrated by the haematopoietic system. Regulation by this mechanism alone would see haematopoietic tissue effectively 'locked' into producing one factor at the expense of the opposing factor. Questions arise: When damaged, how does haematopoietic tissue and specifically the factor-producing cell population therin, 'know' to preferentially produce *stimulator* and how is the subsequent change from *inhibitor* production brought about ? Also, once regenerated, how does the haematopoietic tissue and specifically the factor-producing cell population therin, 'know' to cease *stimulator* production and initiate *inhibitor* production and how is this change brought about ? In effect how is haematopoietic stem cell proliferation coordinated ? The answer appears to lie in a second regulatory mechanism intimately related to the haematopoietic stem cell population itself.

Figure 10: The regulation of inhibitor production.

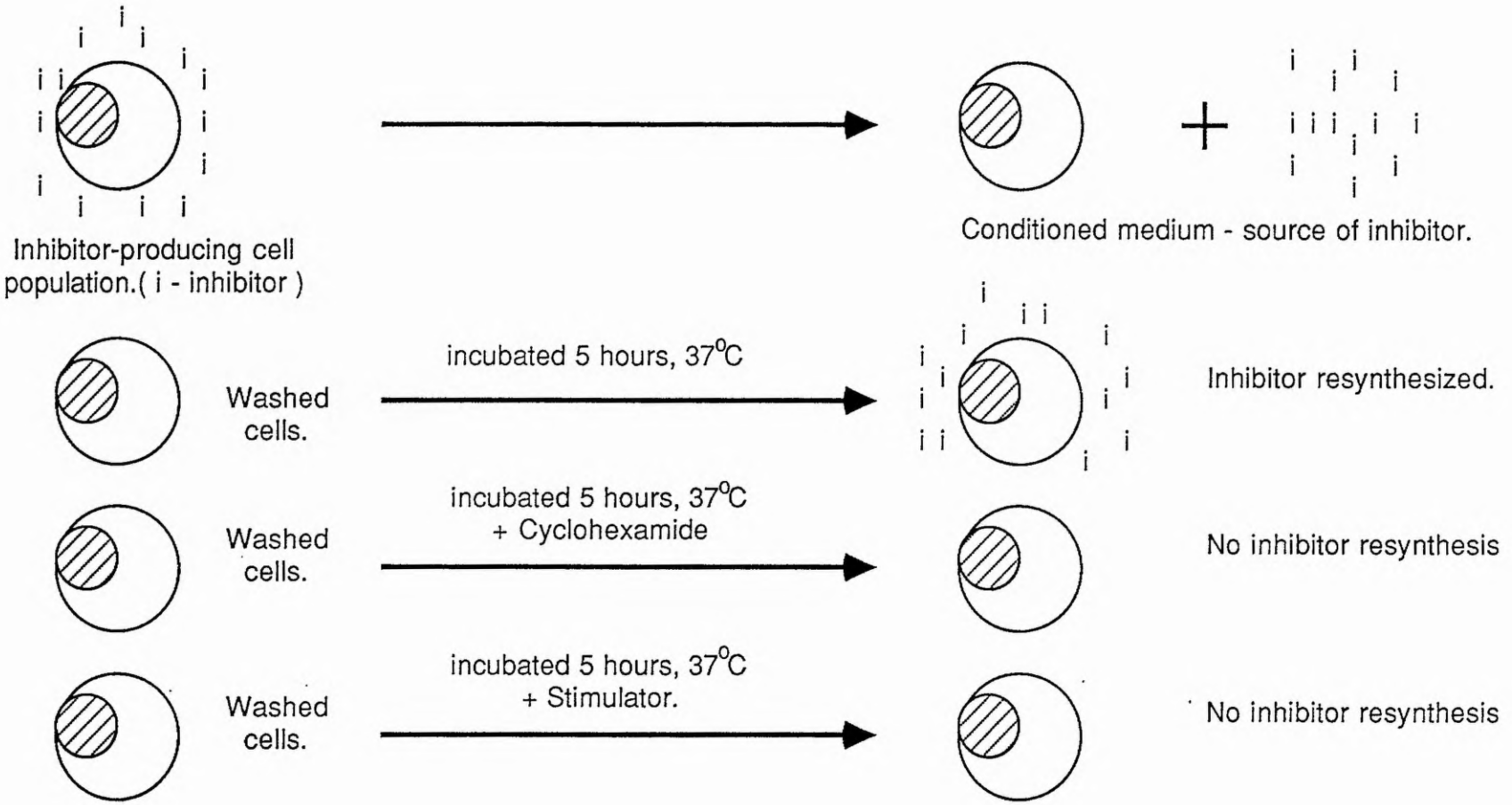
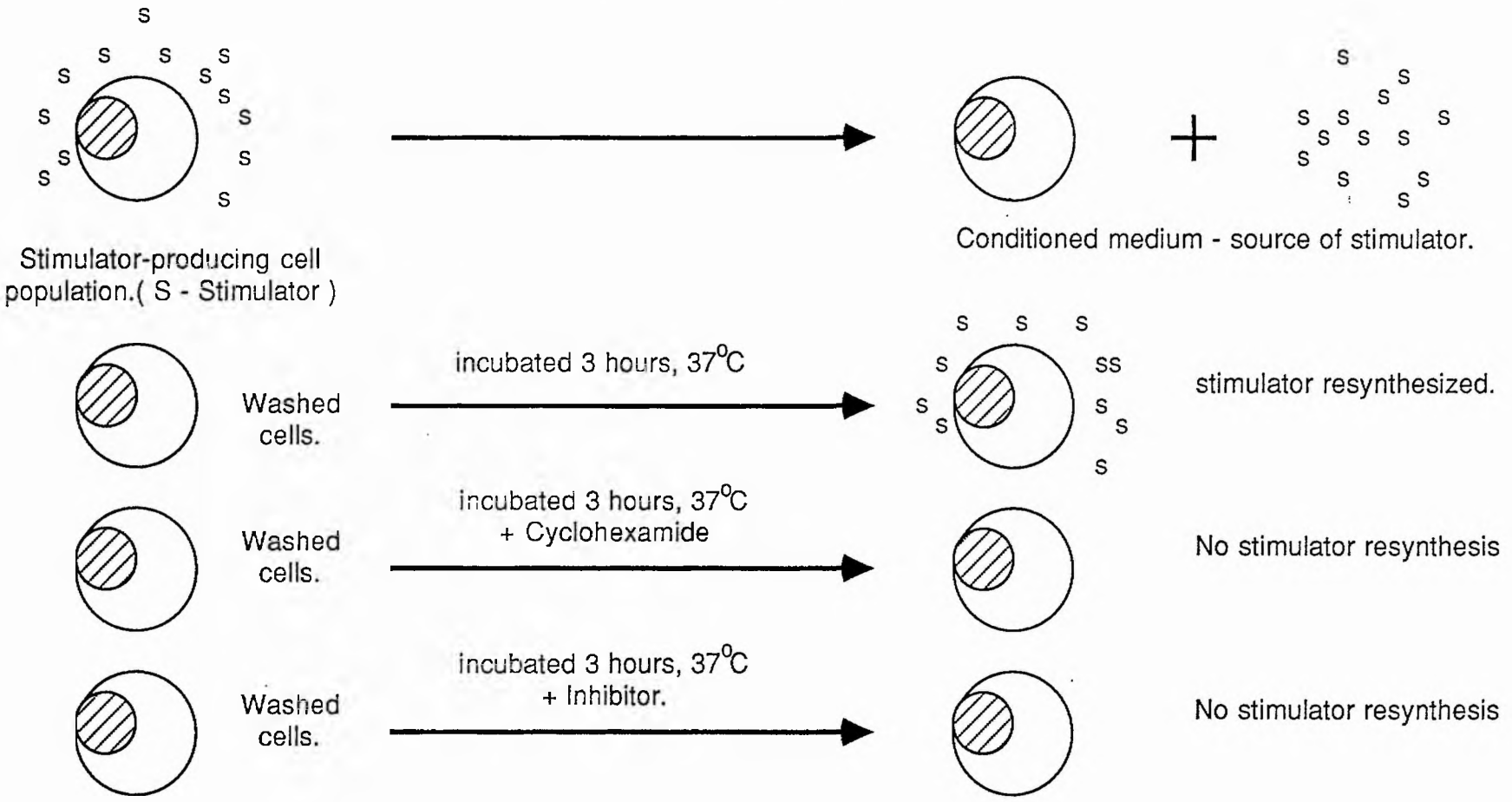


Figure 11: The regulation of stimulator production.



1.15 Evidence for a haematopoietic stem cell 'feedback factor' coordinating *stimulator* and *inhibitor* production.

A crude model for the existence of a putative stem cell 'feedback factor' can be proposed: In normal unstressed haematopoietic tissue, stem cell numbers are 'sufficient' and interact with the factor-producing cell populations to initiate *inhibitor* production and/or suppress *stimulator* production. Conversely, in haematopoietically stressed tissue, where stem cell numbers are insufficient, or depleted, the interaction with the factor-producing cells initiates *stimulator* production and/or suppresses *inhibitor* production. With increased stem cell proliferation and self-renewal, stem cell numbers increase, become 'sufficient' and interaction with the factor-producing cells once again initiates *inhibitor* production and/or supresses *stimulator* production. In this way, a secondary regulatory mechanism can be envisaged to directly initiate, or suppress, production of one of the factors and so coordinate the proliferative state of the haematopoietic tissue.

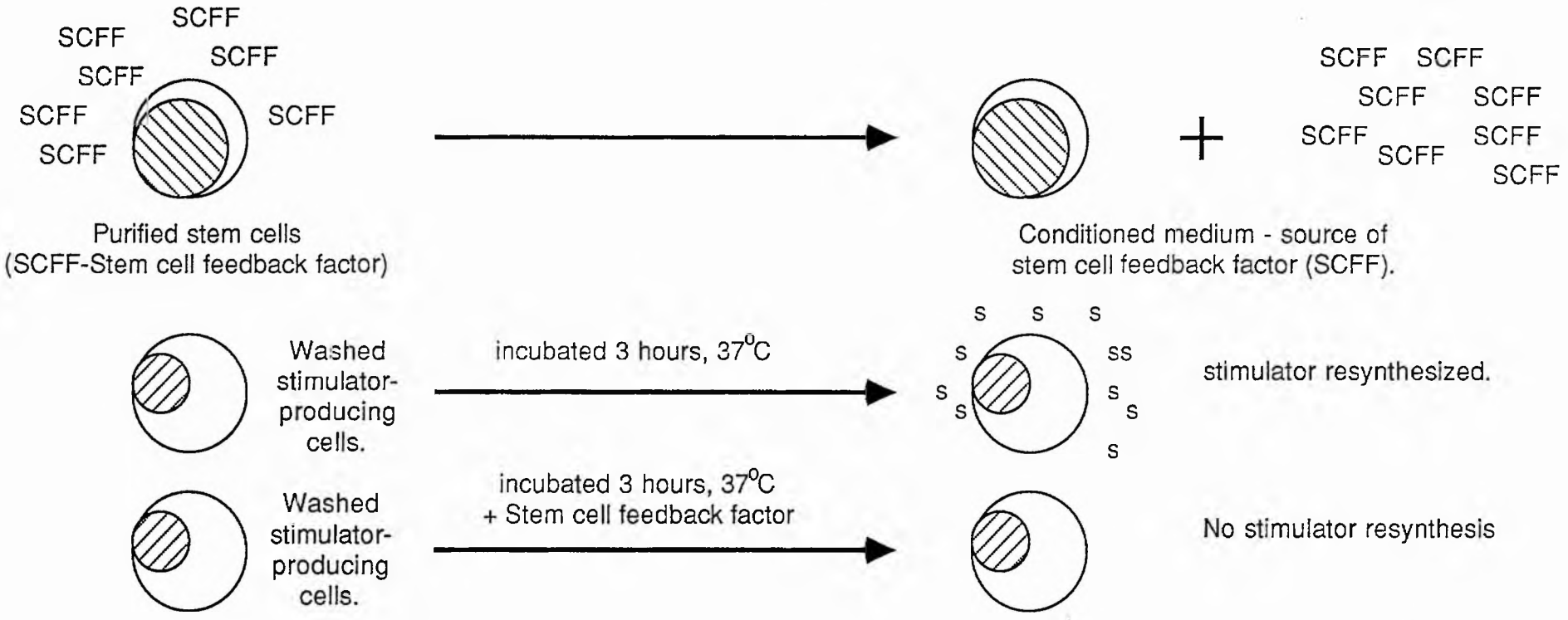
Experimental evidence exists for such a secondary regulatory mechanism [Lord,1986]. A '*stem cell feedback factor*' has been identified and extracted from stem cell conditioned medium as a 30,000-50,000 dalton moiety. If added to phenylhydrazine-treated, and so rapidly cycling, bone marrow, stem cell feedback factor has the capacity to reduce the proportion of stem cells in S-phase although not itself being a proliferation inhibitor. A reduction in *stimulator* levels and increase in *inhibitor* levels is observed. Whether the stem cell feedback factor is actively preventing *stimulator* production and subsequently allowing *inhibitor* production, or else is activating *inhibitor* production, which will inturn prevent *stimulator* production by the *stimulator*-producing cells, can be determined. Removal of the

inhibitor-producing cell subpopulation from the phenyhydrazine-treated marrow, prior to the addition of stem cell feedback factor, demonstrates that *stimulator* production is inhibited rather than *inhibitor* production initiated. The action of the stem cell feedback factor thus appears to actively suppress *stimulator* production by *stimulator*-producing cells thereby allowing *inhibitor* production [*Figure 12*].

Stimulator production in regenerating haematopoietic tissue can be explained in terms of the putative stem cell feedback factor. In sublethally X-irradiated haematopoietic tissue, stem cell numbers are depleted. With stem cell depletion, levels of stem cell feedback factor will fall. The inhibition of *stimulator* production by the *stimulator*-producing cells is removed allowing the production of *stimulator*. With the increase in endogenous *stimulator*, the proportion of stem cells in S-phase increases and stem cell numbers will be restored through self-renewal. With the restoration of stem cell numbers to pre-irradiation levels, levels of stem cell feedback factor rise to actively suppress *stimulator* production by the *stimulator*-producing cells, allowing production of the proliferation *inhibitor*, with a subsequent reduction in the proportion of stem cells in S-phase. For such a feedback factor to operate, the levels of the factor must be proportional to the size of the stem cell population. In this manner, one can envisage an elegant feedback loop coordinating the production of proliferation regulators, and so the proportion of haematopoietic stem cells in S-phase, to the required haematopoietic output.

However, high stem cell numbers, with a supposed high concentration of putative stem cell feedback factor, are not always consistent with slowly proliferating haematopoietic tissue. Similarly, low numbers of stem cells, with a low concentration of putative stem

Figure 12: The role of stem cell feedback factor.



cell feedback factor, are not always associated with rapidly proliferating haematopoietic tissue. Consider phenylhydrazine-treated bone marrow. Stem cell numbers are not significantly reduced and a high putative stem cell feedback factor concentration should exist inducing low stem cell proliferation. However, this is not the case. Where stem cell numbers are high so too is the degree of stem cell proliferation. It is also difficult to explain the increased stem cell proliferation observed in foetal haematopoietic tissues where again stem cell numbers are relatively high. Stem cell numbers must not be the sole determining factor in this feedback mechanism. Lord and Wright [1982], suggest the localized ratio of stem cell numbers to factor-producing cells may be a more significant determinant.

1.16 The distribution of *stimulator*- and *inhibitor*-producing cells in the bone marrow.

As previously discussed, stem cells are not uniformly, or randomly, distributed throughout normal bone marrow [Lord & Hendry,1972; Lord *et al*,1975], rather a considerable degree of haematopoietic microarchitecture exists. Similarly, the distribution of *inhibitor*- and *stimulator*-producing cells is not uniform, or random [Lord & Wright,1984]. Varying the size of axial cores of murine femoral marrow as axial, central and marginal regions, demonstrates that a well defined micro-organization of various cell populations exists.

Inhibitor-producing cells are most abundant in the axial core of the femoral marrow, the numbers of such cells reducing as one approaches the endosteal surface of the bone. Conversely, *stimulator*-producing cells are most abundant at the endosteal surface of the bone, in the marginal region of the marrow. The

presence of increased numbers of *stimulator*-producing cells in this subendosteal region probably accounts for the high degree of stem cell proliferation observed in this region. Axial stem cell proliferation is relatively low in comparison, consistent with the presence of increased numbers of *inhibitor*-producing cells.

The slowly cycling stem cells derived from the axial marrow are of a 'higher quality' than those found in the subendosteal marrow. Such 'high quality' stem cells possess a high self-renewal capacity and are better able to establish haematopoietic regeneration in haematopoietically-deficient mice, in comparison to the subendosteal stem cells. This implies that the stem cell population is a heterogenous population, as previously discussed, and that axial stem cells are more primitive haematopoietic precursors than those in the subendosteal marrow. It has been proposed that an age-related migration of stem cells from the primitive, axial stem cells to a more mature, subendosteal stem cell population may occur. With this putative migration, the presence of decreasing numbers of *inhibitor*-producing cells and increasing numbers of *stimulator*-producing cells will alter the subtle microenvironmental cues to which the cells are exposed.

1.17 The stem cell-specificity of *stimulator* and *inhibitor* and variation in the sensitivity to the proliferation regulators with stem cell maturation.

The relative sensitivities of a number a haematopoietic precursors to the proliferation *inhibitor* and *stimulator* have been investigated [Tejero *et al*,1984]. A comparison of day 9 CFU-S, mixed colony-forming cells (CFC-mix) (an *in vitro* cell population shown to possess characteristics which imply a close relationship to the *in vivo*

CFU-S population) [Metcalf *et al*,1978; Johnson,1980] and more mature, committed haematopoietic progenitors: granulocyte-macrophage colony-forming cells (GM-CFC) and erythroid burst-forming units (BFU-E), has demonstrated that *stimulator* and *inhibitor* are only effective against the more primitive haematopoietic precursors, the day 9 CFU-S and *in vitro* CFC-mix. GM-CFC and BFU-E, show no *stimulator* or *inhibitor* sensitivity, implying that *stimulator* and *inhibitor* are stem cell-specific in nature, regulating the proliferation of the more primitive components of the haematopoietic system. The more mature GM-CFC and BFU-E haematopoietic progenitor cell populations possess their own specific regulation mechanism, related to end-cell feedback [Lord *et al*,1974a; 1974b]. Significantly, the haematopoietic stem cell population proves insensitive to these more mature haematopoietic progenitor cell proliferation cues.

Within the heterogenous stem cell population there is a variation in the sensitivity to *inhibitor* and *stimulator* [Tejero *et al*,1984; Wright *et al*,1985; Lorimore *et al* 1990] and this contributes to the evidence of an age-related organization of the stem cell population. Day 12 CFU-S are believed to be a more primitive stem cell population than day 7 CFU-S, and possess an increased sensitivity to the haematopoietic stem cell proliferation *inhibitor* and reduced sensitivity to the proliferation *stimulator*. As the day 12 CFU-S mature to day 7 CFU-S and subsequently to the *in vitro* CFC-mix, there is a progressive reduction in *inhibitor* sensitivity and a progressive increase in *stimulator* sensitivity [Tejero *et al*,1984; Wright *et al*,1985; Lorimore *et al*,1990] [*Figure 13*]. In effect, the more primitive haematopoietic stem cells prove a more difficult population to induce into S-phase than do the more mature CFU-S and *in vitro* CFC-mix.

Figure 13: The variation in stem cell sensitivity to inhibitor and stimulator with maturation.

1) More primitive components of the haematopoietic stem cell population (day 12 CFU-S) display
INCREASED inhibitor
and
REDUCED stimulator
sensitivity.

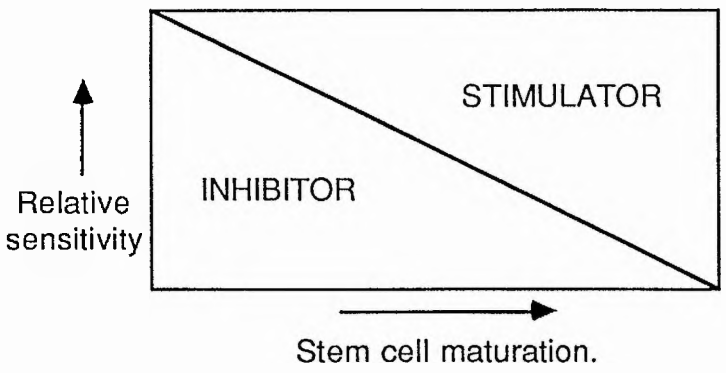
2) More mature components of the haematopoietic stem cell population (day 7 CFU-S and CFC-mix) display
REDUCED inhibitor
and
INCREASED stimulator
sensitivity.

3) Mature, committed haematopoietic progenitor populations (GM-CFC and BFU-E) display
NO sensitivity to either inhibitor or stimulator.

THEREFORE:

1) With stem cell maturation, sensitivity to inhibitor REDUCES and sensitivity to stimulator INCREASES.

2) Inhibitor and Stimulator are stem cell specific proliferation regulators.



A diagramatic representation of the variation in inhibitor and stimulator sensitivity with maturation.

In the light of this variation in proliferation regulator sensitivity with maturation, as the stem cells undergo the putative migration from the axial marrow to the subendosteal marrow, is it the subtle changes in the proliferation regulator levels which are actually responsible for the induction of 'ageing' ? In effect, is the haematopoietic microenvironment 'maturing' the stem cells as they undergo their migration, developing a stem cell of limited self-renewal and increased capacity for differentiation, with the reduction in endogenous *inhibitor* levels and an increase in *stimulator* levels ? Alternatively, is it the natural maturation of the haematopoietic stem cell population with it's reduction in *inhibitor* sensitivity and increase in *stimulator* sensitivity, which attracts it towards the *stimulator*-rich, subendosteal microenvironment ? At present this is unclear.

1.18 The non-species specificity of the stem cell-specific proliferation regulators.

While the proliferation *stimulator* and *inhibitor* are stem cell-specific in nature, there is evidence that they are non-species specific. 50,000-100-000 dalton extracts of normal human [Wright *et al*,1980a], rat [Riches *et al*,1981a; Cork *et al*,1981], guinea pig [Riches *et al*,1981b] and pig [Riches *et al*,1981b] bone marrow, have the capacity to reduce the proportion of stem cells in S-phase in regenerating murine marrow. Similarly, a 30,000-50,000 dalton extract of human foetal liver [Riches *et al*,1981b; Cork *et al*,1982; Cork *et al*,1986] has the capacity to increase the proportion of stem cells in S-phase in normal murine marrow. Significantly, extracts of human foetal thymus, a non-haematopoietic tissue, show no proliferation regulator activity implying the production of *stimulator* and *inhibitor* is restricted to haematopoietic tissues [Riches *et al*,1981a; 1981b].

Such evidence of species cross-reactivity, suggests a highly conserved haematopoietic stem cell regulatory system exists. The fact that human haematopoietic stem cell proliferation regulators can be assayed in a murine system is of significance since there is no equivalent to the murine CFU-S assay with which to directly assay the human haematopoietic stem cell. Using the murine system, the relative levels of the endogenous proliferation regulators in human haematopoietic tissues can be assessed and the kinetic state of human haematopoietic stem cells infered.

1.19 Other haemoregulatory activities.

While discussion thus far has been restricted to the normal bone marrow-derived 50,000-100,000 dalton, stem cell proliferation *inhibitor*, and regenerating or haematopoietically stressed bone marrow-, or foetal liver-derived 30,000-50,000 dalton, stem cell proliferation *stimulator*, it is acknowledged that a number of other positive and negative haemoregulatory activities have been identified. However, the effects of the majority of these factors is largely at the level of the haematopoietic progenitor cell population rather than at the level of the haematopoietic stem cell population. The target cells of most of the characterized haemoregulatory activities, appear to be the bipotential granulocyte-macrophage, early erythroid or megakaryocyte progenitors (Reviewed by Axelrad [1990] and Graham & Pragnell [1990]). However, a haematopoietic stem cell proliferation inhibitor, distinct from the 50,000-100,000 dalton normal bone marrow extract (NBME-IV), has been identified.

1.20 A low molecular weight haemoregulatory peptide.

Isolated from foetal calf bone marrow [Frindel & Guigon,1977] and from foetal calf liver [Guigon *et al*,1984] as a crude dialysable extract and as a semi-purified factor, it has the capacity to prevent haematopoietic stem cell 'recruitment' into S-phase from the quiescent G_o-state, following sublethal X-irradiation, or cytotoxic drug treatment [Guigon & Frindel,1978]. It does not act to reduce the increased proportion of stem cells in S-phase in regenerating or haematopoietically stressed bone marrow, or foetal liver, as does NBME-IV. Used *in vivo*, the foetal calf bone marrow extract has been demonstrated to 'protect' mice from potentially lethal doses of the S-phase-specific agent *cytosine arabinoside* (ARA-C), by preventing the recruitment of quiescent stem cells into a cytotoxic drug-sensitive S-phase [Guigon *et al*,1980; Guigon *et al*,1981; Guigon *et al*,1982; Wdzieczak-Bakala *et al*,1983; Guigon *et al*,1989]. Use of foetal calf bone marrow, or foetal calf liver dialysate as a crude source of the proliferation inhibitor, implied the factor was of low molecular weight. Subsequent purification isolated a tetrapeptide of 487 daltons, with no carbohydrate and of amino acid sequence: Acetyl-N-Ser-Asp-Lys-Pro-OH (AcSDKP) [Lenfant *et al*,1989a] [*Figure 14*]. The purified and synthesized molecule has native, crude extract activity and administered at 100ng *per* mouse (4ug *per* Kg body weight), shows no toxicity, but is sufficient to 'protect' mice from cytotoxic drug-induced haematopoietic damage. The tetrapeptide, isolated from foetal calf tissue is active in both murine and human haematopoietic tissues [Guigon *et al*,1990], demonstrating a non-species specificity.

The small molecular weight tetrapeptide is possibly an active fragment of a higher molecular weight molecule, degraded in the

complex purification procedure. Lenfant *et al* [1989a] using foetal calf liver as a crude source of the factor and a more gentle extraction and purification technique in an attempt to preserve any putative high molecular weight moiety, failed to find any evidence of such a molecule. This may imply the AcSDKP is active physiologically as a low molecular weight moiety.

The *SDKP* amino acid sequence has been identified in a number of existing characterized molecules [Lenfant *et al*,1989a; Pradelles *et al*,1990]. The significance of the sequence in rat liver phenylalanine hydroxylase is unclear, however, the sequence is also found in *tumour necrosis factor-alpha* (TNF-alpha), a putative haemoregulatory activity [Old,1985; Broxmeyer *et al*,1986; Munker *et al*,1987; Peetrie *et al*,1988; Slordal *et al*,1989], and *thymosin ß-4*. Thymosin ß-4 possesses a N-terminal 'AcSDKP-' sequence and proteolytic cleavage would yield an AcSDKP moiety. An endogenous enzyme *endoproteinase Asp-N* has subsequently been isolated and is demonstrated to perform such a proteolytic cleavage of thymosin ß-4 [Grillon *et al*,1990] [*Figure 15*]. This may have significance since the thymus is reported to play a role in haematopoietic stem cell proliferation regulation [Frindel *et al*,1976b; Lepault *et al*,1979; Lepault *et al*,1981; Fache *et al*,1982; Lepault *et al*,1988].

As a proliferation inhibitor, AcSDKP may act in antagonism to interleukin 3 (IL3)/multi-CSF, which has the capacity to stimulate the proliferation of multipotential haematopoietic precursors. However, AcSDKP does not inhibit IL3-dependent cell line proliferation, nor does it inhibit IL3-induced granulocyte-macrophage, or mast cell colony formation. No evidence of AcSDKP competition for IL3 receptors is observed [Lenfant *et al*,1989c]. Reported mast cell colony suppression by semipurified extracts of AcSDKP was

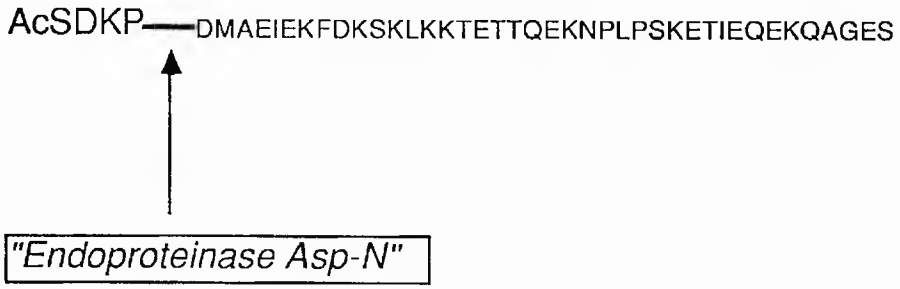
Figure: 14: The low molecular weight haematopoietic stem cell proliferation inhibitor - AcSDKP.

Acetyl - N - Ser - Asp - Lys - Pro - OH

AcSDKP

mw = 487 amu

Figure 15: Thymosin ß-4.



The possible mechanism of AcSDKP production from thymosin ß-4 by the proteolytic enzyme "Endoproteinase Asp-N".

considered due to a co-purifying activity distinct from the AcSDKP moiety [Lenfant et al,1989b].

The effects of AcSDKP on haematopoietic stem cell and stroma interactions have also been investigated. MS-1-T, a murine bone marrow stromal cell line [Itoh *et al*,1989], shows a slight reduction in proliferation in the presence of AcSDKP, however, more significant is a marked increase in stem cell adhesion to the stromal layer in the presence of AcSDKP [Lenfant *et al*,1989c]. The physiological significance of this AcSDKP-mediated increase in stem cell adhesion to stromal tissue is unclear at present.

Other cell-cell interactions are modified by the presence of AcSDKP. *In vitro*, human Jurkat lymphoma T-cells and sheep erythrocytes form 'rosettes' [Thierry *et al*,1990]. In the presence of AcSDKP, rosette formation is inhibited, a titratable effect allowing it's use as a bioassay of AcSDKP activity. Sheep erythrocytes possess a $T_{11}T_s$ membrane glycoprotein which interacts with the human CD2 T-cell membrane glycoprotein to produce the rosette. A region of the CD2 glycoprotein which modulates rosette formation contains the amino acid sequence -Ser-Asp-Lys- (-SDK-). It is thought that addition of exogenous AcSDKP competes with the CD2 -SDK- sequence for membrane recognition sites, so preventing rosette formation in a dose-dependent manner. Peptide analogues of AcSDKP, with modifications to determine the minimal active sequence of the molecule, and changed amino acids, to determine the role of chemical side chains, have been produced and both acetylated and de-acetylated peptides have been assayed for activity in the rosette formation-inhibition assay. The analogue Ser-Asp-Lys (SDK) was found to be the most potent rosette formation-inhibitor, able to inhibit rosette formation at 10^{-14}M in comparison to the native AcSDKP molecule, inhibiting at 10^{-9}M. Many of the analogues

produced showed no rosette formation-inhibition even at 10^{-3}M, demonstrating that a very specific molecular interaction may be occuring. The specific requirements for an inhibiting molecule in the rosette formation assay appear to be (1) the presence of three polar amino acid side chains and (2) the presence of a *seryl-* moiety within the peptide sequence [Thierry *et al*,1990].

The physiological role of AcSDKP in inhibiting such a T-cell interaction at the level of haematopoietic stem cell regulation is unclear. It has been proposed that T-cells may mediate the inhibitory effects of the AcSDKP molecule *in vivo*, however, nude, athymic mice, with no T-cell population, still demonstrate an AcSDKP-associated 'protection' from the cytotoxic effects of cytosine arabinoside [Monpezat & Frindel,1989]. This implies that the action of AcSDKP is T-cell independent.

The cellular specificity of AcSDKP has been investigated on a number of cell-lines *in vitro*. With specific cell lines, it is possible to synchronize cells within the cell cycle. Primary cultures of hepatocytes exist mostly in a G_0-state unless appropriately stimulated with epidermal growth factor, insulin and pyruvate. Similarly, fibroblast 3T3 cells can be arrested by serum-deprivation. Reintroduction of serum, stimulates growth. Factor-dependent cell lines (FDC) can be arrested by withdrawal of their appropriate factors and stimulated by their reintroduction. In all cases, either hepatocyte, 3T3 or FDC cells, none of the stimulatory influences were overcome by AcSDKP inhibition. Also, addition of AcSDKP to rapidly cycling cell populations, failed to reduce proliferation, although a similar addition of transforming growth factor-beta (TGF-ß), or interferon (IFN) does [Lauret *et al*,1989a].

A degree of proliferation inhibition of hepatocytes *in vivo* by AcSDKP has been reported. Hepatocytes triggered into cycle by

degradative enzyme activity, or partial hepatectomy, show a 50-70% proliferation inhibition by AcSDKP [Lombard *et al*,1990]. Since the pure primary hepatocyte culture showed no such proliferation inhibition, an explanation of this *in vivo* observation may be the presence of non-parenchymal liver cells in the heterogenous liver cell population. These cells may repond to the AcSDKP and mediate subsequent hepatocyte proliferation, a mechanism already demonstrated to exist with the production of liver-active proliferation regulators.

The degree of AcSDKP 'protection' of the haematopoietic stem cell population *in vivo* with the use of S-phase specific cytotoxic agents, is extremely sensitive to the relative times of drug and AcSDKP administration. This allows a determination of the precise point in the cell cycle at which AcSDKP exerts its proliferation 'block' after a cytotoxic drug 'recruitment trigger' [Monpezat & Frindel,1989]. AcSDKP is only active at the G_0-G_1 transition and cells progressing into early G_1 and subsequently into S-phase prove insensitive to the inhibition of AcSDKP. This would explain the observed failure of AcSDKP to inhibit an already cycling cell population. If AcSDKP is administered before the influence of the cytotoxic drug initiates stem cell proliferation, the majority of stem cells undergo the AcSDKP-associated 'recruitment block' and are spared. However, if AcSDKP is administered after the cytotoxic drug-associated 'proliferation trigger' is effective, with most of the stem cells mobilized into G_1, then it is too late for the action of the AcSDKP-associated 'recruitment block' to act. Even if the proportion of stem cells in S-phase appears relatively low at drug administration, the majority of stem cells will subsequently progress to S-phase and cytotoxic drug-induced death with the associated haematopoietic damage [Monpezat & Frindel,1989].

AcSDKP is not an immunogenic moiety, however, an amino acid extension of the tetrapeptide is immunogenic and an antiserum produced cross-reacts with AcSDKP [Lauret *et al*,1989b]. Although the antiserum is of low affinity for AcSDKP, it's use *in vivo* is reported to increase the proportion of stem cells in S-phase dramatically [Frindel & Monpezat,1989]. This demonstrates the AcSDKP moiety is produced *in vivo* and is active physiologically as an endogenous stem cell proliferation inhibitor. If the inhibition is removed by the presence of a neutralizing antibody, inhibited stem cells are allowed to enter S-phase and proliferate. Use of the neutralizing antiserum has also been demonstrated to remove the AcSDKP-associated, 'protection' of stem cells against the action of S-phase specific cytotoxic drugs. The effects of both the addition of exogenous AcSDKP and it's removal by the use of a neutralizing antiserum have thus been demonstrated and strongly imply a physiological role for the tetrapeptide.

With the development of the AcSDKP antiserum, enzyme immunoassay of AcSDKP has been developed [Pradelles *et al*,1990] and is reported to detect levels of AcSDKP down to 15 *femto* Molar ($15x10^{-15}M$). Using immunoassay the presence of AcSDKP in normal mononuclear blood cells [Pradelles *et al*,1990] and a similar molecule in human placental extract [Lopez *et al*,1991], have been demonstrated. The quantitative and qualitative immunoassay of AcSDKP should now allow the biosynthesis of the molecule to be investigated, the producer-cells to be identified and levels of endogenous AcSDKP in various haematological disorders to be determined. Interestingly, use of enzyme immunoassay implies a number of AcSDKP-like molecules may exist, perhaps suggesting evidence for a novel family of very low molecular weight, potent, haemoregulatory activities.

De novo synthesis of AcSDKP has been followed *in vivo* in mice and *in vitro* in long-term murine bone marrow cultures [Wdzieczak-Bakala *et al*,1990] using tritiated proline ([^{3}H]-proline). [^{3}H]-proline competes with native proline for incorporation into newly synthesized AcSDKP. Newly synthesized AcSDK-[^{3}H]P was detectable, secreted by bone marrow stroma and [^{3}H]-proline pulse labelling gives some evidence that AcSDKP may be derived from a larger precursor and undergoes enzymatic maturation to form biologically active AcSDKP [Wdzieczak-Bakala *et al*,1990].

Cyclical changes are reported in the proliferative state of the haematopoietic stem cell population in long-term bone marrow cultures with refeeding [Wdzieczak-Bakala *et al*,1990]. This is considered due to the removal of endogenous AcSDKP with the old, spent medium and replacement with fresh AcSDKP-deficient medium. The removal of endogenous AcSDKP removes the inhibition of stem cell proliferation, allowing the proportion of stem cells in S-phase to increase. Proliferation levels remain relatively high until the levels of endogenous AcSDKP are restored by resynthesis and stem cell proliferation inhibition restablished. (This compares with the previously discussed observations of Dexter *et al* [1977], Toksoz [1980] and Cashman *et al* [1985], who also demonstrated such cyclical proliferation changes in long-term bone marrow cultures at refeeding. They concluded such cyclical changes were due to cellular depletion and subsequent variations in endogenous levels of NBME-IV and RBME-III. How the two distinct proliferation regulation systems relate and possibly interact will be of considerable interest.)

1.21 The haematopoietic colony-stimulating factors.

The *in vivo* CFU-S assay [Till & McCulloch,1961] has greatly improved the understanding of haematopoietic stem cell regulation and behaviour, however, the assay has considerable drawbacks amongst which is the large numbers of mice required to obtain statistically significant results. The development of an *in vitro*, clonogenic assay of a similarly primitive, high proliferative potential, self-renewing, multipotential haematopoietic precursor, would be a considerable development. A number of *in vitro* clonogenic assays already exist which utilize semi-solid agar, or methylcellulose culture techniques, to support the growth of haematopoietic colony-forming cells. A consequence of the development of such *in vitro* assays was the recognition that haematopoietic progenitor cells are unable to survive, or proliferate, unless specifically stimulated. A group of specific regulatory glycoproteins, stimulating haematopoietic progenitor cell proliferation and certain aspects of mature cell function, have been isolated as *haematopoietic colony-stimulating factors* (CSFs). The best characterized haematopoietic colony-stimulating factors are those regulating the proliferation and maturation of the granulocyte-macrophage progenitors, although similar regulatory factors exist for progenitors of the erythroid, eosinophil, megakaryocyte, mast and lymphoid lineages within the haematopoietic system.

A number of granulocyte-macrophage colony-stimulating factors have been identified in both human and murine haematopoiesis and these will be briefly discussed:

Murine *granulocyte colony-stimulating factor* (G-CSF) is synthesized by a wide variety of tissues and is a 25,000 dalton glycoprotein [Nicola *et al*,1983]. Active at 10^{-12}M, it stimulates the

proliferation of lineage-committed granulocyte progenitors within haematopoietic tissue and has a considerable capacity to induce terminal differentiation [Metcalf & Nicola,1983]. The molecule has considerable tertiary structure maintained by intra-molecular disulphide bonds, without which biological activity is lost. One copy of the G-CSF gene is present in the murine genome, isolated to chromosome 11. Murine G-CSF displays a 70% amino acid sequence homology with its human equivalent, a glycoprotein of 20,000 daltons, the gene of which has been isolated to human chromosome 17. G-CSF displays cross-species activity. [Nagata *et al*,1986; Nomura *et al*,1986; Tsuchiya *et al*,1986; Nagata,1989].

Murine *macrophage colony-stimulating factor* (M-CSF/CSF-1) is synthesized by fibroblasts and is found in embryonic yolk sac and pregnant mouse uterus extract (PMUE) [Stanley & Heard,1977; Johnson & Burgess,1978]. Purified to homogeneity from mouse L-cell conditioned medium as a 70,000 dalton, dimeric glycoprotein [Burgess *et al*,1977; Waheed & Shadduck,1982; Burgess *et al*,1985], it is active at 10^{-12}-10^{-13}M, stimulating predominantly committed cells of the monocyte-macrophage lineage to proliferate and differentiate. The intact 70,000 dalton dimer is heavily glycosylated (*The purpose of this carbohydrate moiety, present to a varying degree in all native colony-stimulating factors, but not necessary for biological activity (non-glycosylated, recombinant factors still demonstrate activity), is to confer solubility, stability and resistance to proteolytic degradation to the CSFs. As such, the CSFs are extremely resilient, resisting denaturation and enzymatic degradation.) and consists of two identical subunits, of approximately 14,000 daltons, linked by inter-molecular disulphide bonds. Mercaptoethanol disruption of the disulphide bonds yields two haematopoietically inactive subunits. One copy of the gene for murine M-CSF/CSF-1 is present and has

been isolated to chromosome 3 [Gisselbrecht *et al*,1989] and murine M-CSF/CSF-1 displays an 80% amino acid sequence homology with it's human equivalent, a dimeric glycoprotein of 70,000-90,000 daltons, which consists of two identical 21,000 dalton subunits. The gene for human M-CSF/CSF-1 has been isolated to chromosome 5 [Pettenati *et al*,1987; Ladner *et al*,1988] and M-CSF/CSF-1 displays a cross-species activity.

Murine *granulocyte-macrophage colony-stimulating factor* (GM-CSF), synthesized by a wide variety of tissues, was purified to homogeneity from mouse lung conditioned medium, as a 25,000 dalton glycoprotein [Burgess *et al*,1977]. Active at 10^{-12}M, it stimulates primarily the proliferation and differentiation of both bipotental granulocyte-macrophage progenitors and lineage-committed granulocyte and macrophage progenitors from haematopoietic tissue. The molecule has considerable tertiary structure maintained by intra-molecular disulphide bonds. Mercaptoethanol-treated GM-CSF has no biological activity [Gough *et al*,1985]. Murine GM-CSF has a single gene localized to chromosome 11 [Gough *et al*,1984] and displays a 50% amino acid sequence homology with it's human equivalent, a glycoprotein of 18,000-24,000 daltons. The gene for human GM-CSF has been localized to chromosome 5. GM-CSF is a species specific factor [Yang *et al*,1988; Miyatake *et al*,1985].

Murine *interleukin 3* (IL3)/*multipotential colony-stimulating factor* (multi-CSF), is synthesized by antigen/mitogen stimulated T-cells and is produced constitutively by the myelomonocytic leukaemic cell line WEHI-3B [Warner *et al*,1969; Metcalf *et al*,1969; Lee *et al*,1982; Bazill *et al*,1983; Clark-Lewis *et al*,1985; McNiece *et al*,1985]. Purified to homogeneity [Ihle *et al*,1982], IL3/Multi-CSF is a glycoprotein of 23,000 daltons, active at 10^{-12}-10^{-13}M. It stimulates the proliferation

and maturation of multipotential haematopoietic precursors, bipotential and lineage-committed granulocyte/macrophage progenitors and committed erythroid, eosinophil, mast cell and megakaryocte progenitors from haematopoietic tissue [Ihle *et al*,1983; Hapel *et al*,1985]. A single copy of the IL3/multi-CSF gene exists in the murine genome, localized to chromosome 11. IL3/multi-CSF has a 30% amino sequence homology with it's human equivalent, a glycoprotein of 15,000-25,000 daltons, the gene of which is isolated to chromosome 5 [Yang *et al*,1988; Yang *et al*,1989]. IL3/multi-CSF is species specific.

These granulocyte-macrophage colony-stimulating factors are complemented in haematopoietic regulation by a rapidly expanding family of haematopoietically active factors. A number of these factors will be briefly discussed:

Erythropoietin (Epo) [Stephenson *et al*,1971; Miyake *et al*,1977; Jacobs *et al*,1985] is a glycoprotein which stimulates the proliferation and differentiation of relatively mature, lineage-committed erythroid progenitor cells. Earlier erythroid progenitors are stimulated if a '*burst promoting activity*' (BPA) is added prior to Epo. Both GM-CSF and IL3/multi-CSF possess such BPA activity. The single gene for murine Epo has been isolated to chromosome 7 and the mature gene product has considerable tertiary structure, maintained by intra-molecular disulphide bonds, without which, biological activity is lost.

Interleukin 1 (IL1) exists as IL1 alpha and beta. It is primarily active in the mediation of the inflammatory response, however, it also demonstrates haematopoietic activity [Bagby,1989; Henney,1989]. IL1, initially identified as '*hemopoietin-1*' [Jubinsky & Stanley,1985; Mochizuki *et al*,1987] has no colony-stimulating activity. It's major function appears to be an induction of expression of other genes, including those for interleukin 6 (IL6) [Walther *et al*,1988], the colony-

stimulating factors [Sieff *et al*,1987; Segal *et al*,1987; Lee *et al*, 1987; Kaushansky *et al*,1988; Fibbe *et al*,1988] and their receptors. It is proposed that IL1 mediates it's effects by modifying the accumulation of mRNA of the appropriate gene. Subsequent translation of this reservoir of mRNA, rapidly yields a considerable amount of protein. While mRNA is normally very short lived, IL1 appears to increase it's stability, prolonging the life of the protein-template and allowing a considerable flexibility in both the amount and rate of protein output, as required. IL1 has also been implicated as a regulator of some leukaemic populations [Sakai *et al*,1987; Griffin *et al*,1987]. Murine IL1-alpha and IL1-ß are both non-species specific and demonstrate a 62% and 30% amino acid homology respectively, with their human equivalents.

Interleukin 4 (IL4) is '*B-cell stimulating factor-1*' (BSF-1) and stimulates the proliferation and maturation of B-lymphocytes. It induces immunoglobulin synthesis, B-lympocyte surface receptor production and the production of cytotoxic T-lymphocytes. It also stimulates the proliferation and differentiation of mast cells, although IL4 receptors are detected on a wide variety of cell types [Yokota *et al*,1986; Mosmann *et al*,1986]. The gene for human IL4 has been isolated to chromosome 5 [LeBeau *et al*,1989; van Leeuwen *et al*,1989] and IL4 is species specific [Mosmann *et al*,1987].

Interleukin 5 (IL5) is '*B-cell growth factor-III*' (BCGF-III). It is a dimeric, non-species-specific factor stimulating B-lymphocyte proliferation and maturation. It also stimulates the proliferation and maturation of lineage-committed eosinophil progenitors from haematopoietic tissue [Yokota *et al*,1987; Clutterbuck & Sanderson,1988]. Some colonies stimulated by IL5 are mixed basophil/eosinophil colonies [Yokota *et al*,1987] which suggests a bipotential progenitor may exist for these lineages. IL5 also promotes

the action of mature eosinophils [Yamaguchi *et al*,1988]. The gene for IL5 has been isolated to murine chromosome 11 and human chromosome 5 [LeBeau *et al*,1989, van Leeuwen *et al*,1989].

Interleukin 6 (IL6) or *interferon beta-2* (IFN-ß2) stimulates B-lymphocyte differentiation and stimulates the production of cytotoxic T-lymphocytes [Wong & Clark,1988]. The gene for human IL6 has been isolated to chromosome 7, and the gene product, mature IL6, is produced by a number of cell types including fibroblasts, especially in the presence of IL1 [Walther *et al*,1988]. This has both immunological and haematopoietic significance. Murine IL6 has the capacity to stimulate the limited proliferation of bipotential granulocyte-macrophage progenitors and is of considerable interest since it is also proposed to be active at the level of the primitive haematopoietic stem cell and enhances the capacity of IL3 to stimulate the proliferation of multipotential precursors [Ikebuchi *et al*,1987; Ogawa & Clark,1988].

(*A number of excellent reviews of haematopoietic growth factors exist: [Metcalf,1984; 1985; 1986; Sieff,1987; Morstyn & Burgess,1988; Nicola,1989; Platzer,1989; Golde,1990; Metcalf,1990; Whetton,1990].)

With the exception of M-CSF/CSF-1 and IL5, which are dimers, the other factors discussed, are single subunit glycoproteins. Despite their similar molecular weights, general molecular nature and overlapping biological specificities, each factor is encoded by a single unique gene, binds to a unique receptor with no cross-reactivity and shows no extensive amino acid sequence homology with any other factor. M-CSF/CSF-1, G-CSF, IL1 and IL5 are remarkably well conserved between species and are able to cross-react. GM-CSF, IL3/multi-CSF and IL4, however, differ significantly between species and do not cross react. In the mouse the GM-CSF, IL3/multi-CSF, G-CSF and IL5 and the M-CSF/CSF-1 receptor

(identified as the c-*fms* oncogene product [Sherr *et al*,1985]) genes have been mapped to chromosome 11 (*The gene for the purified haematopoietic stem cell proliferation *inhibitor*, MIP-1 alpha has significantly also been mapped to murine chromosome 11 [Graham *et al*,1990]). In humans the GM-CSF, IL3/multi-CSF, M-CSF/CSF-1, IL5 and M-CSF/CSF-1 receptor [Groffen *et al*,1983] genes have all been mapped to chromosome 5. This is significant since part of murine chromosome 11 is homologous to the long arm of human chromosome 5. Another part of murine chromosome 11 is homologous to human chromosome 17, where the gene for G-CSF has been isolated (human MIP-1 alpha has also been mapped to chromosome 17 [Irving *et al*,1990]). This probably reflects a distant evolutionary relationship.

1.22 A novel multipotential growth factor encoded by the *Sl*-locus is a ligand of a receptor encoded by the *W*-locus.

The haematological significance of the 'Steel' (*Sl*) locus of murine chromosome 10 and the 'dominant white spotting' (*W*) locus of murine chromosome 5, has long been realised. Mice bearing mutations in either loci present with considerable haematological dysfunction characterized by severe anaemia and mast cell deficiency. In the case of *Sl*-mutants, haematopoietic dysfunction is the result of a defective haematopoietic microenvironment while *W*-mutants possess inherently defective haematopoietic stem cells [Dexter & Moore,1977]. While *Sl* and *W* are distinct genetic loci they possess a related haematological function and recent studies have identified and characterized the products of both loci.

The product of the 'Steel' (*Sl*) locus of murine chromosome 10 has been identified as a 31,000 dalton glycoprotein moiety [Copeland

et al,1990; Huang *et al*,1990; Williams *et al*,1990; Witte,1990; Zsebo *et al*,1990a; 1990b]. Administered to *Sl*-mutant mice it restores normal haematopoiesis, reversing both the severe anaemia and the mast cell deficiency [Zsebo *et al*,1990b]. The glycoprotein, isolated from rat buffalo liver cell line conditioned medium [Zsebo *et al*,1990] and a murine bone marrow stromal cell line supernatant [Williams *et al*,1990], strongly synergizes with interleukins 1, 3, 6 [Zsebo *et al*,1990a; 1990b] and 7 [Martin *et al*,1990], erythropoietin [Nocka *et al*,1990], GM-CSF and G-CSF [Bernstein *et al*,1991; Bertoncello *et al*,1991; Broxmeyer *et al*,1991], increasing both the number and size of colonies in semi-solid agar culture. The glycoprotein appears to act in part, by potentiating the growth factor responsiveness of the relatively mature, lineage-committed haematopoietic progenitors, although the factor is itself a relatively potent colony-stimulating activity. The glycoprotein also appears to be a relatively potent stimulatory activity for haematopoietic colony-forming cells surviving 5-fluorouracil treatment, which are considered relatively primitive haematopoietic precursors and possibly components of the haematopoietic stem cell population [Zsebo *et al*,1990a]. The glycoprotein has subsequently been termed '*stem cell factor*' (SCF) [Zsebo *et al*,1990a] and '*mast cell growth factor*' (MGF) [Williams *et al*,1990]. Interestingly the activity of SCF/MGF is reported to be compromised by the presence of transforming growth factor-beta (TGF-ß) [McNiece *et al*,1991], a previously characterized haematopoietic inhibitor [Axelrad,1990; Graham & Pragnell,1990].

The genomic sequences of murine, rat and human SCF/MGF have been determined [Copeland *et al*,1990; Martin *et al*,1990; Zsebo *et al*,1990b; Williams *et al*,1990; Huang *et al*,1990] and recombinantly expressed. Recombinant expression in *E.Coli*., producing a non-glycosylated factor, demonstrates the glycosylation of SCF/MGF is

not essential for activity. Native SCF/MGF is heavily glycosylated and this may assist solubility and protect from enzymatic degradation. There is also some evidence that native SCF/MGF exists as a dimeric molecule [Zsebo *et al*,1990a].

The product of the 'dominant white spotting' (*W*) locus of murine chromosome 5 is a transmembrane tyrosine kinase receptor. The 145,000 dalton activity [Flanagan & Leder,1990; Williams *et al*,1990; Huang *et al*,1990] is the product of the c-*kit* proto-oncogene [Chabot *et al*,1988; Geissler *et al*,1988] and mutations of the *W*-locus usually produce a kinase-deficient inactive molety.

Significantly SCF/MGF has been identified as a ligand for the c-*kit*-derived tyrosine kinase receptor [Witte,1990; Flanagan & Leder,1990; Zsebo *et al*,1990a; 1990b; Williams *et al*,1990; Huang *et al*,1990] and SCF/MGF has thus also been termed 'c-*kit* ligand' (KL), although other c-*kit* ligands may exist.

The importance of SCF/MGF/KL and c-*kit* proto-oncogene receptor interaction in haemoregulation is becoming clear. The action of SCF/MGF/KL both alone, and in conjunction with other growth factors, may prove to be of clinical significance, perhaps stimulating haematopoietic recovery after bone marrow transplantation or assisting in the management of hypoproliferative haematopoietic disorders.

1.23 The development of an *in vitro* haematopoietic stem cell assay.

Interleukin 3 (IL3)/multi-CSF has the capacity to stimulate the proliferation and differentiation of colony-forming cells of different lineages from haematopoietic tissue. It also has the capacity to stimulate the proliferation of multipotential haematopoietic

precursors which demonstrate self-renewal and have allowed a limited *in vitro* investigation of haematopoietic stem cell behaviour [Metcalf *et al*,1978; Humphries *et al*,1979a; Humphries *et al*,1979b; Johnson,1980; Nakahata & Ogawa,1982; Koike *et al*,1986a].

Recent developments in *in vitro* techniques have however, isolated a colony-forming cell population from haematopoietic tissue which is characterized by a high proliferative potential, a self-renewal capacity and a multipotency. This *high proliferative potential colony-forming cell* (HPP-CFC) population, demonstrates many behavioural and regulatory similarities to the *in vivo* CFU-S population, previously taken as the haematopoietic stem cell assay. The potential of the HPP-CFC assay as an *in vitro* assay of the haematopoietic stem cell population will be discussed.

1.24 The *in vitro* high proliferative potential colony-forming cell (HPP-CFC) assay.

The novel aspect of the *in vitro* HPP-CFC assay is it's use of combinations of colony-stimulating factors. Bradley and Hodgson [1979] first reported the stimulation of colony-forming cells with a high proliferative potential, from murine bone marrow, using a combination of crude factors derived from pregnant mouse uterus extract (PMUE) and human spleen conditioned medium (HUSPCM). PMUE alone, stimulated the proliferation of lineage-committed haematopoietic progenitor cells with limited proliferative potential. HUSPCM alone demonstrated no colony-stimulating activity. Only on combination of the two extracts were the HPP-CFC demonstrated. This illustrates haematopoietic growth factor '*synergism*' - on combination, factors produce a more than additive effect. Bradley and Hodgson [1979] demonstrated that the HPP-CFC population

isolated by the combination of PMUE and HUSPCM was a distinct, previously undetected population of colony-forming cells, through the use of 5-fluorouracil (5FU). The PMUE-stimulated colonies proved especially sensitive to 5FU, implying a relatively rapidly cycling, colony-forming cell population was being demonstrated. This would be consistent with these cells being lineage-committed haematopoietic progenitor cells. The PMUE and HUSPCM-stimulated HPP-CFC population, however, proved relatively insensitive to 5FU, implying that the HPP-CFC population was relatively slowly cycling, consistent with its being a more primitive haematopoietic precursor population.

Characterization of PMUE has demonstrated it to be a crude source of M-CSF/CSF-1 [Das *et al*,1980], however, identification of the synergizing factor in HUSPCM has proved more problematic. Other factors found to synergize with M-CSF/CSF-1 have been obtained from rat and mouse spleen and from human placenta [Kriegler *et al*,1982] and other factors have come to light [Kriegler *et al*,1984; Stanley *et al*,1986; McNiece *et al*,1987a; Song *et al*,1985; Quesenberry *et al*,1987; McNiece *et al*,1989a; Kriegler *et al*,1990]. Subsequent analysis of these synergizing factors has demonstrated at least three distinct types of activity exist, each stimulating a discrete HPP-CFC subpopulation.

Interleukin 1 (hemopoietin-1), has been demonstrated to synergise with M-CSF/CSF-1 to stimulate an HPP-CFC population and while not completely replacing the synergism obtained from a number of crude conditioned media, it does not itself possess colony-stimulating activity, which is consistent with the findings of Hodgson and Bradley [1979] and their use of HUSPCM. Other sources of an interleukin 1-like synergistic factor, termed '*synergistic factor-1*' (SF-1), have been identified as spleen and placental

extracts, medium conditioned by bladder carcinoma cell line 5637 and medium conditioned by murine EMT-6 cells [Bradley & Hodgson,1979; Kriegler *et al*,1982; kriegler *et al*,1984, Bartelmez & Stanley,1985; Stanley *et al*,1986; McNiece *et al*,1987a].

Interleukin 3 (IL3)/multi-CSF, produced constitutively by the murine myelomonocytic leukaemic cell line WEHI-3B [Warner *et al*,1969], has been purified and characterized [Ihle *et al*,1982, Ihle *et al*,1983] and demonstrated to synergise with M-CSF/CSF-1 to stimulate HPP-CFC proliferation [Bartelmez *et al*,1985]. Interleukin 3-like factors constitute the second group of synergizing factors.

A third distinct class of non-IL1-like and non-IL3-like synergizing factors has been demonstrated and are yet to be isolated and characterized. Present in TC-1 murine bone marrow stromal cell line conditioned medium [Song *et al*,1985; Quesenberry *et al*,1987], synergism with M-CSF/CSF-1 demonstrates a third discrete HPP-CFC population.

A developmental hierarchy appears to exist within the heterogenous HPP-CFC population with the HPP-CFC stimulated by SF-1/IL1-like activity and M-CSF/CSF-1, termed *HPP-CFC-1*, considered the more primitive haematopoietic HPP-CFC. These HPP-CFC-1 are proposed to mature to IL3/multi-CSF and M-CSF/CSF-1 sensitive HPP-CFC, termed *HPP-CFC-2*, which are inturn considered to mature to the TC-1 and M-CSF/CSF-1 sensitive HPP-CFC, termed *HPP-CFC-3* [Bradley *et al*,1985; McNiece *et al*,1986; 1987b].

The development of highly purified and recombinant haematopoietic colony-stimulating factors has allowed the role of individual growth factors in synergizing interactions to be investigated. A number of haematopoietic colony-stimulating factors have been demonstrated to synergize:

G-CSF and M-CSF/CSF-1 [McNiece *et al*,1988a],

GM-CSF and M-CSF/CSF-1 [McNiece *et al*,1988b; Falk & Vogel,1988; Eckmann *et al*,1988; Pragnell *et al*,1988; Bot *et al*,1990; Lorimore *et al*,1990],

G-CSF and GM-CSF [McNiece *et al*,1988a; Bot *et al*,1990],

G-CSF and IL3/multi-CSF [McNiece *et al*,1988a],

GM-CSF and IL3/multi-CSF [McNiece *et al*,1988a; 1989b],

IL3/multi-CSF and M-CSF/CSF-1 [Chen & Clark,1986; Koike *et al*,1986b; McNiece *et al*,1987b; Williams *et al*,1987; Bartelmez *et al*,1989; Morris *et al*,1990],

IL6 and G-, M-, GM-, or IL3/multi-CSFs, or IL4 [Rennick *et al*,1989],

IL1 and G-CSF [Moore & Warren,1987]

IL1 and IL3/multi-CSF, or IL1 and IL3/multi-CSF and M-CSF/CSF-1 [Bartelmez *et al*,1989].

The relationship between these HPP-CFC subpopulations is as yet unclear, however, a number of techniques may allow a hierarchical structure within the heterogenous HPP-CFC population to be investigated.

1.25 Evidence of an age-related *in vitro* HPP-CFC hierarchy.

As previously discussed Bradley and Hodgson [1979], investigated the kinetic state of the HPP-CFC isolated by a combination of PMUE and HUSPCM using 5FU. Similar studies have been performed and reveal evidence of an age-related hierarchy within the HPP-CFC population and an HPP-CFC maturation scheme has subsequently been developed.

HPP-CFC-1 isolated by the synergism of IL1-like activity and M-CSF/CSF-1 are relatively resistant to 5FU treatment. A depletion of 50% 2 days after 5FU treatment is reported [Bradley *et al*,1985]. In

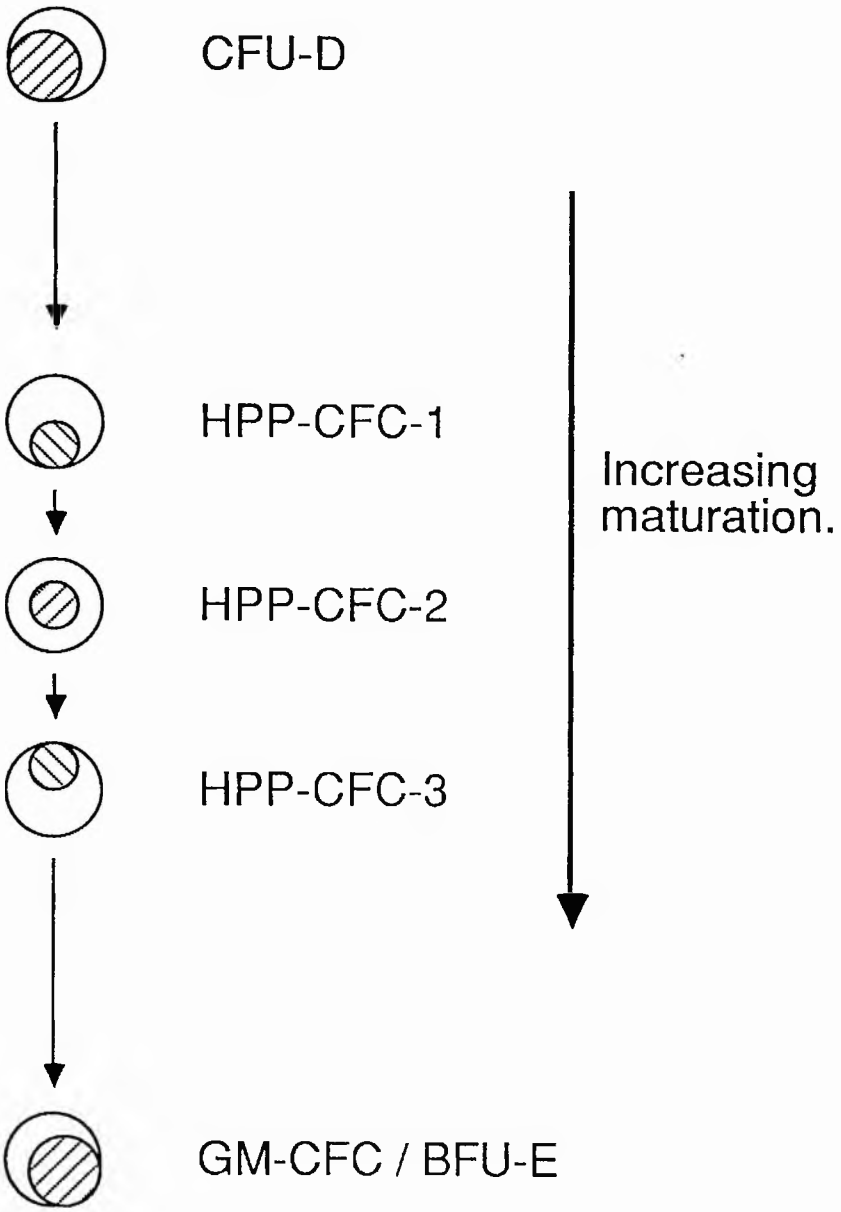
comparison HPP-CFC-2, isolated by a synergism of IL3-like activity and M-CSF/CSF-1, prove more sensitive to 5FU treatment. A depletion of 99% 2 days after 5FU treatment is reported [Bradley *et al*,1985]. This evidence implies that the proportion of HPP-CFC-1 in S-phase is lower than the proportion of HPP-CFC-2. Also, recovery after 5FU treatment by HPP-CFC-1 is rapid and complete, while HPP-CFC-2 take longer to recover [Bradley *et al*,1985]. From this the conclusion that HPP-CFC-1 is a more primitive haematopoietic precursor population, slowly proliferating and more able to self-renew than the HPP-CFC-2, has been drawn. Replating experiments have shown that HPP-CFC-1 produce HPP-CFC-2 [McNiece *et al*,1987b], implying a maturation of the one to the other, and a subsequent maturation of HPP-CFC-2 to HPP-CFC-3 is also suggested. HPP-CFC-3, prove even more sensitive to 5FU treatment than HPP-CFC-2, implying they are a more rapidly cycling population still. (*This maturation scheme would correlate particularly well with observations that primitive haematopoietic precursors have an age-related, transient sensitivity to IL3 [Koike *et al*,1986a]). A subsequent maturation of the more mature HPP-CFC-3 to the lineage-committed haematopoietic progenitor cell population could then be envisaged. Such an age related hierarchy is somewhat reminiscent of that previously discussed within the CFU-S population.

Direct and significant correlations between the *in vivo* CFU-S and *in vitro* HPP-CFC populations have been made. HPP-CFC-2 when injected into lethally X-irradiated mice give rise to day 13 CFU-S [McNiece *et al*,1987b], a CFU-S population considered a relatively primitive component of the haematopoietic stem cell compartment. This implies that the HPP-CFC-2, and the HPP-CFC-1 from which they are derived, must also be relatively primitive components of the haematopoietic stem cell compartment, likely to possess marrow,

platelet and erythroid repopulating capacity. There is evidence, however, that the HPP-CFC-1 and HPP-CFC-2, do not contain the cells responsible for long-term haematopoietic recovery, the 'pre-CFU-S', discussed previously [Bertoncello *et al*,1989a]. *In vitro* attempts to isolate such a cell population are being developed [Iscove & Yan,1990; Ploemacher *et al*,1989b] and investigations have isolated what may be a '*pre-HPP-CFC-1*' precursor, which forms colonies in *in vivo* diffusion chambers, termed 'CFU-D'. CFU-D are reported to generate *in vitro* HPP-CFC-1, HPP-CFC-2 and *in vivo day* 12 CFU-S, and possess a very high proliferative potential [Niskanen *et al*,1990] [*Figure 16*].

A number of techniques have been developed which enrich HPP-CFC from haematopoietic tissues, many of which are developments of the previously discussed techniques, used to enrich CFU-S populations. The Qa-m7 antigen is present on 90% of relatively primitive, day 13 CFU-S and HPP-CFC express relatively high levels of this antigen, allowing their enrichment using flow cytometric techniques. Specifically, use of 5FU marrow, to deplete the marrow of more mature haematopoietic progenitor cell populations [Bertoncello *et al*,1987], allows a 20% enrichment of HPP-CFC-1 and HPP-CFC-2. HPP-CFC-1 and HPP-CFC-2 also bind wheat germ agglutinin (WGA^+) and can be enriched using Rh-123. Use of Rh-123 has demonstrated that highly quiescent stem cells are Rh-123 dull [Visser & de Vries,1988; Ploemacher & Brons,1988c; 1988d], amongst these day 16 CFU-S, pre-CFU-S, and long term haematopoietic repopulating cells. More rapidly proliferating, more mature stem cells, for example day 8 CFU-S, are Rh-123 bright. HPP-CFC-1 have been resolved as a Rh-123 dull subpopulation, while HPP-CFC-2 are resolved as a Rh-123 bright subpopulation [Bertoncello *et al*,1989a], which reinforces evidence of an age-related HPP-CFC hierarchy. Use of the $Sca\text{-}1^+$,

Figure 16: Possible maturation scheme within the high proliferative potential colony-forming cell (HPP-CFC) population.



Thy-1^{lo}, lineage-negative cell 'fingerprint' by Sprangrude *et al* [1988], also appears to select HPP-CFC, specifically the more primitive HPP-CFC-1 subpopulation. Thus techniques developed primarily to enrich the *in vivo* CFU-S populations and resolve subpopulations therin, are also proving to select the *in vitro* HPP-CFC and to resolve the HPP-CFC-1 and HPP-CFC-2 subpopulations therin. This is further evidence of a very close correlation between the *in vivo* CFU-S and the *in vitro* HPP-CFC populations.

A significant development of the *in vitro* HPP-CFC assay has been the isolation of a colony-forming cell of high proliferative potential from human haematopoietic tissue [McNiece *et al*,1989]. While the *in vivo* CFU-S assay [Till & McCulloch,1961] has allowed a quantitative and qualitative analysis of haematopoietic stem cell behaviour and regulation, it is strictly a murine assay. An equivalent assay of a primitive human haematopoietic precursor does not at present exist, although as previously discussed the human stem cell proliferation regulators can be assayed in the murine system. The potential of an *in vitro* assay of a primitive human haematopoietic precursor is thus significant. The human HPP-CFC reported [McNiece *et al*,1989b] is considered equivalent to the murine HPP-CFC-2 subpopulation and should allow a direct investigation of human haematopoietic stem cell behaviour in normal, regenerating, haematopoietically stressed and foetal haematopoietic tissues and also potentially the behaviour of stem cells in haematopoietic disorders.

1.26 Refinement of the *in vitro* HPP-CFC technique 1 - The "CFU-A" assay.

While demonstrating many functional similarities to the *in vivo* CFU-S population, recent reports also demonstrate that the *in vitro*

HPP-CFC population also shares many significant behavioural and regulatory similarities to the *in vivo* CFU-S population. As previously discussed, the synergism between certain colony-stimulating factors stimulates the proliferation of a number of different HPP-CFC subpopulations. GM-CSF and M-CSF/CSF-1 have been demonstrated to synergize [McNiece *et al*,1988b; Falk & Vogel,1988; Eckmann *et al*,1988; Pragnell *et al*,1988; Bot *et al*,1990; Lorimore *et al*,1990] and this synergy and the high proliferative potential colony-forming cell stimulated by this combination of colony-stimulating factors, form the basis of the "CFU-A" assay [Eckmann *et al*,1988; Pragnell *et al*,1988; Lorimore *et al*,1990].

Eckmann *et al* [1988], Pragnell *et al* [1988], and Lorimore *et al* [1990] have demonstrated that CFU-A stimulated by a combination of GM-CSF and M-CSF/CSF-1 from murine haematopoietic tissue are multipotential precursors, giving rise to cells of the granulocytic, macrophagic, erythroid and megakaryocyte lineages and to immature, undifferentiated blast cells. CFU-A are also reported to give rise to day 12 CFU-S, considered a relatively primitive component of the haematopoietic stem cell population, when injected into lethally irradiated recipient mice. This agrees with the report of McNiece *et al* [1987b], who demonstrated the production of day 13 CFU-S on the transplantation of putative HPP-CFC-1 to lethally X-irradiated mice. As well as a considerable proliferative potential, CFU-A also exhibit a self-renewal capacity, demonstrated by replating experiments.

Perhaps of greatest significance has been the demonstration that the CFU-A population shows kinetic properties that mimic those of the *in vivo* CFU-S population in both normal and haematopoietically stressed tissues. CFU-A also show a sensitivity to the previously CFU-S-characterized stem cell proliferation *inhibitor* and *stimulator*.

In normal, unstressed bone marrow, the proportion of haematopoietic stem cells in S-phase, as measured by the *in vivo* CFU-S assay is relatively low at approximately 10%. The proportion of *in vitro* CFU-A in S-phase from such tissue is also relatively low at approximately 10%. This contrasts with the relatively high proportion of more mature, haematopoietic progenitor cells in S-phase at 30-40%. In regenerating or haematopoietically stressed haematopoietic tissue, the proportion of *in vivo* CFU-S in S-phase increases to 30-40% and a similar increase in the proportion of CFU-A in S-phase is observed.

The changes in the relative proportion of haematopoietic stem cells in S-phase are brought about by stem cell specific proliferation regulators - *inhibitor* and *stimulator*. Significantly, the *in vitro* CFU-A population shows a sensitivity to these stem cell-specific proliferation regulators with *stimulator* increasing the proportion of CFU-A in S-phase and *inhibitor* reducing the proportion of CFU-A in S-phase. (Use of the *in vitro* CFU-A assay assisted in the identification and characterization of the stem cell proliferation *inhibitor* as MIP-1 alpha [Graham *et al*,1990].)

As previously discussed, a variation in stem cell sensitivity to the proliferation regulators occurs with maturation. The more primitive haematopoietic stem cells (day 12 CFU-S) prove more sensitive to the proliferation *inhibitor* than to the proliferation *stimulator*, while more mature haematopoietic stem cells (day 8 CFU-S), prove more sensitive to the proliferation *stimulator* than the proliferation *inhibitor*. Lorimore *et al* [1990] have shown that both *inhibitor* and *stimulator* are titratable activities using the *in vitro* CFU-A assay and that the *in vitro* CFU-A population shows a greater *inhibitor* than *stimulator* sensitivity. This is somewhat analogous to the day 12 CFU-S, an example of a relatively primitive haematopoietic precursor, and

further evidence that the *in vitro* CFU-A population is indeed a relatively primitive haematopoietic precursor within the haematopoietic stem cell compartment.

Lorimore *et al* [1990] have also explored the spatial distribution of the CFU-A population within femoral marrow. The non-random, non-uniform distribution of CFU-S within femoral marrow has been discussed. Axial cores of active murine haematopoietic tissue from femora, demonstrate the majority of slowly cycling, 'high quality', relatively primitive stem cells lie axially, while the majority of more rapidly cycling, 'lower quality', more mature stem cells lie in the subendosteal marrow. Analysis of the CFU-A distribution demonstrates that the majority of CFU-A lie in a region deep to the subendosteal region, implying their being at least one step removed, and so more primitive than, the more rapidly cycling, more mature, subendosteal stem cell population. Radiation sensitivity and density separation characteristics of the *in vitro* CFU-A are also comparable to the *in vivo* CFU-S population [Lorimore *et al*,1990].

In conclusion, there is considerable evidence that the *in vitro* CFU-A HPP-CFC subpopulation is a relatively primitive haematopoietic precursor and that the *in vitro* CFU-A assay is indeed an assay of a component of the haematopoietic stem cell population. As such it should greatly assist investigation of the regulation and behaviour of the haematopoietic stem cell population.

1.27 Refinement of the *in vitro* HPP-CFC technique 2 - The "HPP-CFC$_{IL3+CSF\text{-}1}$" assay.

A high proliferative potential colony-forming cell population can be isolated *in vitro* from murine haematopoietic tissue by a synergistic interaction between interleukin 3 (IL3)/multi-CSF and M-

CSF/CSF-1 [Chen & Clark,1986; Koike *et al*,1986b; McNiece *et al*, 1987b; Williams *et al*,1987; Bartelmez *et al*,1989; Morris *et al*,1990] termed "HPP-$CFC_{IL3+CSF-1}$". A major objective of this research project will be the characterization of HPP-$CFC_{IL3+CSF-1}$ behaviour and regulation. Using a combination of WEHI-3B myelomonocytic leukaemic cell line conditioned medium, as a crude source of murine interleukin 3 (IL3)/multi-CSF [Warner *et al*,1969; Metcalf *et al*,1969; Ihle *et al*,1982; Lee *et al*,1982; Bazill *et al*,1983; Clark-Lewis *et al*,1985; McNiece *et al*,1985] and L929 fibroblast cell line conditioned medium, as a crude source of murine M-CSF/CSF-1 [Stanley & Heard,1977; Burgess *et al*,1977; Waheed & Shadduck,1982; Burgess *et al*,1985] the number, cellular composition and proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase in normal and regenerating, or haematopoietically-stressed, murine haematopoietic tissue, will be investigated and comparison made with the behaviour of the *in vivo* CFU-S [Till & McCulloch,1961] and *in vitro* CFU-A (HPP-$CFC_{GM-CSF+CSF-1}$) [McNiece *et al*,1988b; Falk & Vogel,1988; Eckmann *et al*,1988; Pragnell *et al*,1988; Bot *et al*,1990; Lorimore *et al*,1990]. Correlation between *in vivo* CFU-S, *in vitro* CFU-A and *in vitro* HPP-$CFC_{IL3+CSF-1}$ behaviour would be evidence for the primitive nature of the HPP-$CFC_{IL3+CSF-1}$ population.

HPP-$CFC_{IL3+CSF-1}$ sensitivity to the stem cell-specific proliferation regulators *inhibitor* and *stimulator* will be investigated and if demonstrable would further reinforce the evidence for the HPP-$CFC_{IL3+CSF-1}$ being a primitive haematopoietic precursor population, possibly a component of the heterogenous haematopoietic stem cell compartment. The relative sensitivity of HPP-$CFC_{IL3+CSF-1}$ to both *inhibitor* and *stimulator* may allow the position of the population within the heterogenous, age-related haematopoietic stem cell hierarchy to be determined.

If similar behavioural and regulatory characteristics are determined for the CFU-S, CFU-A and HPP-$CFC_{IL3+CSF-1}$ populations, the potential of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ assay as a technique with which to investigate haematopoietic stem cell behaviour and regulation in aberrant haematopoiesis will be developed. Specifically, the assay will be used to investigate stem cell behaviour and regulation in the neoplastic proliferative disorder of the haematopoietic system - *myeloid leukaemia*. A number of X-irradiation induced, murine, serially passaged, myeloid leukaemias have been established in this laboratory [Hepburn *et al*,1987] and will be used as models with which to investigate such aberrant haematopoiesis.

1.28 Leukaemia and leukaemia associated inhibition of normal haematopoiesis.

'*Leukaemia*', a term applied to a group of malignant haematopoietic proliferative disorders, is characterized by the abnormal production of white blood cell precursors. Leukaemias differ in both cell lineage (neoplastic proliferation is usually restricted to one cell lineage), and degree of cellular differentiation (with poorly differentiated cells usually indicative of a rapidly progressing, aggressive, '*acute*' leukaemia, while more differentiated cells usually indicate a slowly progressing, less aggressive, '*chronic*' leukaemia). The abnormal white blood cells produced enter, and circulate in the bloodstream to extensively infiltrate the haematopoietic system, liver, spleen and many other tissues. Often disease progression is associated with hepatic and splenic enlargement (hepato- and splenomegally) as the organs become diffusely infiltrated by leukaemic cells. One particularly significant aspect of most

leukaemias is a leukaemia-associated suppression of normal haematopoiesis. A consequence of this suppression of normal haematopoiesis is that death may result from anaemia, haemorrhage or infection due to a lack of functional erythrocytes, platelets and granulocyte-macrophages. This leukaemia-associated suppression of normal haematopoiesis is of particular interest and may explain the 'proliferative advantage' developed by the leukaemic cells. The selective inhibition of normal haematopoiesis by putative leukaemia-associated inhibitory activities can be envisaged. In such a system, the leukaemic cells would be insensitive to such inhibition and be able to proliferate at the expense of normal haematopoietic tissue. Evidence for such leukaemia-associated inhibitory activities has been reported.

Olofsson and Olsson [1980a; 1980b; 1980c], Olofsson *et al* [1984] and Olofsson and Sallefors [1987], report the isolation of a 500,000-600,000 dalton, heat stable glycoprotein, produced by acute and chronic myeloid leukaemic cells and the HL60 promyelocytic leukaemic cell line. The factor, "*Leukaemia Associated Inhibitor*" (LAI), is reported to inhibit normal bipotential granulocyte-macrophage progenitor cell proliferation, while leukaemic cells prove insensitive to the inhibition.

Broxmeyer *et al* [1978], Bognaki *et al* [1981], Broxmeyer *et al* [1981; 1982; 1983], Jacobs [1983], Sala *et al* [1986] and Broxmeyer *et al* [1989], report the isolation of a 550,000 dalton moiety, active at 10^{-17}-10^{-19}M and suppressing normal granulocyte-macrophage progenitor cell proliferation. Produced by leukaemic cells, which themselves prove insensitive to it's inhibitory activity, "*Leukaemia-associated Inhibitory Activity*" (LIA), has subsequently been reported to be *acidic isoferritin*. Whether LAI or LIA possess inhibitory activity at the level of haematopoietic stem cell proliferation is unclear.

1.29 Aims and objectives.

In this study, medium conditioned by the radiation-induced, serially passaged, murine, myeloid leukaemias will be investigated using the *in vitro* HPP-CFC$_{IL3+CSF-1}$ assay. The respective levels of endogenous *inhibitor* and *stimulator* will be determined and abnormalities investigated. Any abnormalities may represent a manipulation of endogenous proliferation regulator production and/or action by the developing leukaemia allowing it a proliferative advantage over, and at the expense of, normal haematopoiesis.

A possible explanation of the development of the leukaemic proliferative advantage over normal haematopoiesis may be a change in the sensitivity of the leukaemic cells to *inhibitor*. A leukaemogenic change at the level of the stem cell population may, amongst other properties, remove or reduce stem cell sensitivity to *inhibitor*. As a result, despite the relatively high endogenous levels of *inhibitor* in the normal haematopoietic tissue, leukaemic stem cell proliferation will occur. Existing haematopoietic feedback mechanisms may detect the excessive, inappropriate white blood cell production and lead to an increase in *inhibitor* levels. However, despite increased *inhibitor* levels, the *inhibitor*-insensitive leukaemic proliferation will continue at the expense of the normal, *inhibitor*-sensitive haematopoietic tissue.

Another possibility may involve the production of leukaemia-associated inhibitory activity, similar to LAI and LIA. This factor may possess inherent inhibitory activity to directly suppress haematopoietic stem cell proliferation, or it may act indirectly to block the production and/or action of the endogenous proliferation regulators. With leukaemic progression the composition of the blood becomes abnormal. Such abnormalities are normally corrected by an intricate haematopoietic feedback mechanism, adjusting

haematopoietic output to restore the composition of the blood. Part of this restorative mechanism is an increase in haematopoietic stem cell proliferation induced by *stimulator*. In effect, with leukaemic progression the haematopoietic system is 'stressed' but apparently fails to respond and the quality of the peripheral blood deteriorates. The putative leukaemia-associated activity may act to block the production and/or action of *stimulator*. The suppression of normal haematopoiesis may be a result of a failure of haematopoietic stimulation. Interestingly, if *stimulator* is produced but inactivated, it may act to prevent *inhibitor* production, so that during subsequent HPP-$CFC_{IL3+CSF\text{-}1}$ assay of the leukaemic cell conditioned medium neither factor is detectable.

Another possibility may be the production of a leukaemia-associated factor which is a stimulatory activity to which only the leukaemic cells are sensitive. Again leukaemic proliferation at the expense of normal haematopoiesis would be observed. Whether such a factor would be detectable using the *in vitro* HPP-$CFC_{IL3+CSF\text{-}1}$ assay using normal haematopoietic tissue remains to be seen.

A basic understanding of the mechanism by which leukaemic cells develop a proliferative advantage over normal haematopoietic tissue, would be a considerable step towards the development of more effective disease therapy and management. Depending on the mechanism of action of the leukaemic proliferation, it may prove possible to overcome the leukaemia-associated deterioration of the blood 'quality', by the exogenous addition of *stimulator*. If normal haematopoietic output could at least be maintained then the life threatening aspects of the leukaemia-associated anaemia, haemorrhage and infection may become less problematic and allow some degree of disease management, although not achieving a 'cure'. The potential loss of proliferative advantage by the leukaemic

system may in itself hamper disease progression and assist survival. This always assumes that exogenous *stimulator* does not promote leukaemic proliferation.

If a difference in normal and leukaemic sensitivity to *inhibitor* could be demonstrated, then this could be exploited. Chemotherapeutic and radiotherapeutic regimes are most effective against proliferating cell populations. If leukaemic proliferation were determined to be insensitive to *inhibitor*, exogenous *inhibitor* applied prior to therapy would reduce the proportion of normal haematopoietic stem cells in S-phase, while leaving a cycling leukaemic population as specific targets for such agents. This artificial manipulation of the haematopoietic system to a therapeutic advantage has great potential, but will rely on the development of an understanding of leukaemic proliferation regulation.

In addition to the investigation of leukaemia, a further objective of this research project will be an investigation of the low molecular weight stem cell proliferation inhibitor, the tetrapeptide AcSDKP. Using the *in vitro* HPP-$CFC_{IL3+CSF\text{-}1}$ assay, the mechanism of action of AcSDKP (kindly supplied by Dr. Maryse Lenfant, ICSN, CNRS, Gif-sur-Yvette, France) will be investigated to determine whether the peptide possesses inherent inhibitory activity or acts indirectly by interacting with the production and/or action of *inhibitor* and/or *stimulator*. Various peptide analogues of AcSDKP (kindly supplied by Dr. Lenfant) will also be investigated to determine the minimal active amino acid sequence of the parent molecule and to determine whether more potent haemoregulatory moieties exist.

Chapter 2:

Materials and Methods

2.1 Mice and Housing.

Experiments and procedures were performed on CBA/H strain mice bred inhouse at the University of St.Andrews Animal Facility, in accordance with the Home Office Animal (Scientific Procedures) Act 1986. Mice were exposed to a 12 hour light/dark cycle and kept at an ambient temperature of 21-23°C. Animal house feed SDS RMI(C) $^{3}/_{8}$ pellets (Special Diet Services, Essex) and chlorinated water (20mg sodium dichloroisocyanurate/l) (Presept Disinfectant Tablets, Surgikos Ltd.) were allowed *ad libitum*.

2.2 Experimental Tissue.

Haematopoietic tissue was obtained from the marrow cavities of murine femora. 9-12 week old CBA/H strain mice were killed by ether inhalation overdose and intact femora dissected and removed to a sterile plastic petridish. Tissue was transfered to a sterile laminar flow cabinet and desiccation avoided by covering tissue with sterile medium. Muscle tissue was scraped from the femora using a scalpel blade and the ball joint removed. A 1ml syringe charged with sterile medium and fitted with a 23 gauge (G) needle was inserted through the articular cartilage of the femur using a gentle 'drilling' action. Minimal trabecular bone allows a discharge of medium from the syringe to drive haematopoietic tissue from the marrow cavity as a 'plug' into sterile medium. Repeated flushing of the marrow cavity ensured maximal haematopoietic tissue was removed.
Microscopic analysis confirms that a single cell suspension was produced by a gentle repeated drawing of the marrow 'plug' through a 1ml syringe fitted with a 23G then a 25G needle. Excessive, or overvigorous, syringing should be avoided since this leads to cell

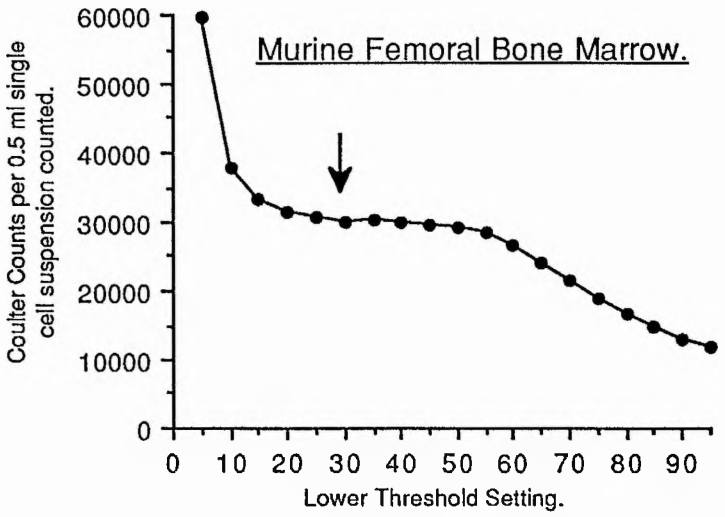
membrane damage, cell-cell adhesion and 'clumping'. Cellularity was determined using an electronic particle counter (Coulter Counter Model ZM, Coulter Electronics.) and dilutions performed in appropriate medium.

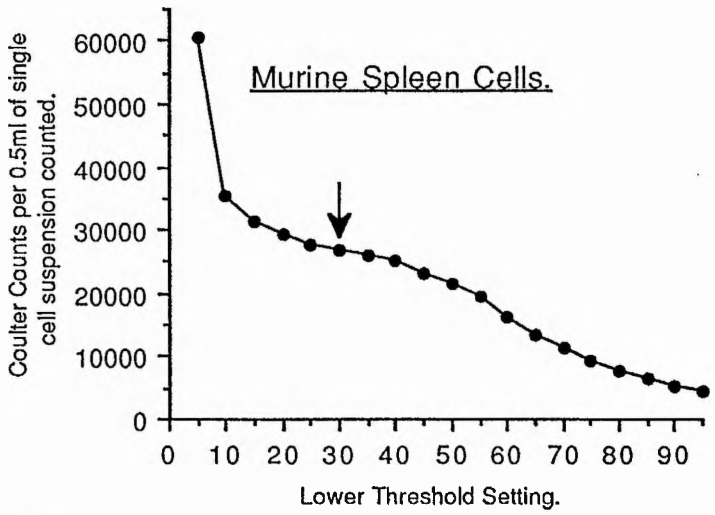
2.3 Determination of the Cellularity of Single Cell Suspensions.

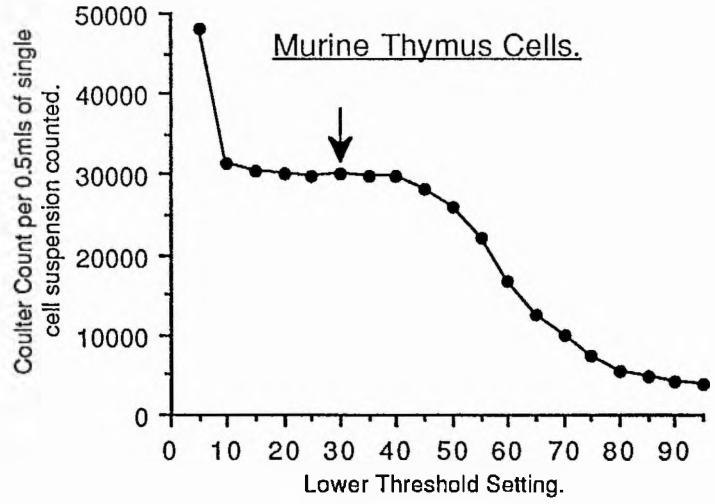
The cellularity of single cell suspensions was determined using an electronic particle counter (Coulter Counter Model ZM, Coulter Electronics). Cells are suspended in an isotonic, electrically conductive medium (Isoton II, Coulter Electronics). The medium passes through a small aperture (100μM diameter) and an electrical current is recorded. Cells in the medium produce a temporary impedance to the electrical current proportional to the cellular volume. Electronic circuitry reads the impedance as a 'pulse' and as a given volume of cell suspension (0.5ml) is drawn through the orifice, the number of pulses generated corresponds to the number of cells in that volume and the size distribution of the cells corresponds to the size distribution of the electrical pulses. With high counts a correction for 'coincidence counting', where more than one cell passes through the orifice at any one time, is made. Cell 'clumping' is a major source of potential error and as cells tend to settle with time, gentle inversion of the counting vial ensures a homogeneity of cell suspension.

Coulter Counter settings were optimized by prior calibration with the cell type of interest. Settings were adjusted until criteria for an accurate cell count were met. A calibration curve was produced [*Figure 17*] and settings chosen on the 'plateau region' to exclude

Figure 17: Coulter Counter Titration Curves For:







cell debris and platelets while giving an accurate cell count, which can be verified by use of a hemocytometer.

The following settings proved appropriate for the accurate determination of the cellularities of single cell suspensions of bone marrow and spleen and thymus tissue :

Current,I = 130 (1.3mA), Full Scale,FS = 10mA, Lower Threshold,T_L = 30, Attenuation,A = 1 and Preset Gain,PG = 2.

Routinely six counts were made and the average of three similar values taken. A background count, in the absence of cells, was taken and subtracted from this average count. From dilutions made, the cellularity of the original suspension was determined.

40µl, or 100µl of single cell suspension were added to 20mls of Isoton II (Coulter Electronics). Prior to counting, 3 drops of a red blood cell-lysing solution (Zaponin, Coulter Electronics)(25ml acetic acid/l) was added to allow a nucleated cell count and the Coulter vial gently inverted. 0.5ml, Zaponized, single cell suspension was Coulter counted and with the use of a x1000, or x400 dilution factor respectively, the cellularity of the source single cell suspension can be determined.

2.4 Cellular Staining and Cytology.

Cytology was performed on cytocentrifuged (Cytospin 2, Shandon) preparations. Single cell suspensions of tissue were produced, cellularity determined and adjusted to 2-5x10^5cell/ml. 0.5ml of single cell suspension was added to a Cytospin funnel and spun (x800 rpm, 10 mins.). Cells deposited on the slide were air dried and Jenner-Giemsa stained :-

Fix in methanol (15mins.)

Jenners stain (BDH) (15mins.)

6.8pH buffer rinse

Giemsa stain (BDH) (10mins.)

6.8pH buffer rinse

Slides were allowed to air dry and preparations can be mounted using a mounting medium (DePeX, BDH) and coverslip.

Alternatively, more rapid staining was achieved using the Diff-Quik staining system (Merz + Dade AG, Switzerland). Slides were prepared and air dried. Staining was performed by dipping slides repeatedly for 35 seconds in each of a fixative solution (Fast green in methanol), stain solution I (Eosin G) and stain solution II (Thiazine dye). Continuous immersion of slides gives unsatisfactory results. Excess solution was allowed to drain after each dip and between each solution. Slides were rinsed in pH 6.8 buffer and allowed to air dry. Preparations can be mounted using a mounting medium (DePeX, BDH) and coverslip.

2.5 Production of Medium Conditioned By WEHI 3B Myelomonocytic Leukaemic Cell Line and L929 Fibroblast Cell Line.

WEHI 3B myelomoncytic leukaemic cell line is a non-adherent cell line, growing as a single cell suspension. WEHI 3B cell line conditioned medium is a crude source of interleukin 3 (IL3)/multi-CSF [Warner *et al*,1969; Metcalf *et al*,1969; Ihle *et al*,1982; Lee *et al*,1982; Bazill *et al*,1983; Clark-Lewis *et al*,1985; McNiece *et al*,1985]. L929 fibroblast cell line is an adherent cell line growing as a monolayer. L929 conditioned medium is a crude source of macrophage colony-stimulating factor (M-CSF/CSF-1) [Stanley & Heard,1977; Burgess *et al*,1977; Waheed & Shadduck,1982; Burgess *et al*,1985]. Both WEHI 3B and L929 cell lines were maintained in culture at 37°C in

Dulbeccos medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate, 2 mM L-glutamine and 10% (v/v) foetal calf serum.

Medium was considered 'conditioned' after a 3 day incubation with either WEHI single cell suspension, or confluent L929 monolayer. Half the incubation medium was removed and replaced with fresh medium and incubation continued for three days before a second harvest. A maximum of 3 harvests of conditioned medium from one L929 monolayer were taken before passaging and restablishment of a fresh monolayer. In effect each harvest of WEHI conditioned medium acts as a passage.

Conditioned medium removed was centrifuged (x1000 rpm, 15 mins.) to remove cells and initially bulk frozen (-20°C). Once a sufficient volume of conditioned medium was produced, the whole was thawed, MilliPore filtered (0.22 µM), aliquoted and frozen (-20°C). The 'potency' of each batch of conditioned medium was assessed by performing a titration of the colony-stimulating activity in both the *in vitro* GM-CFC (2.9) and HPP-CFC (2.10) assays. Individual aliquots were not refrozen once thawed.

Cell lines were stored cryopreserved in liquid nitrogen (-196°C) until required. 1ml aliquots of 5-10x10^6 cell/ml in supplemented Dulbeccos medium, 10-20% (v/v) foetal calf serum and 5-10% (v/v) DMSO (Dimethyl Sulphoxide, AnalaR, BDH), were placed in cryotubes (Cel-Cult, Sterilin), chilled on ice then transfered to the gas-phase compartment of an N_2 freezer. After 12 hours cryotubes can be transfered to the liquid-phase compartment of the N_2 freezer.

When required cells were rapidly thawed from the N_2 freezer at 37°C, washed and suspended in supplemented Dulbeccos medium with 10% (v/v) foetal calf serum in 25 cm^2 tissue culture flask (Cel-

Cult, Sterilin) and incubated at 37°C to restablish the cell line before passaging.

Passaging of the non-adherent WEHI cell line was continuous as conditioned medium was harvested. L929 cell line grows as a monolayer and requires trypsin digestion to produce a single cell suspension prior to passaging. Medium was completely removed and the monolayer washed with 0.05% trypsin (Sigma) in phoshate buffered saline and 0.5M EDTA (Ethylenediamine tetraacetic Acid, Sigma). A wet film of trypsin was left over the monolayer and incubated at 37°C for 5 minutes. Addition of medium was sufficient to disrupt the monolayer and produce a single cell suspension.

Cellularities prior to passage were determined using a Coulter Counter Model ZM at settings:

WEHI 3B: Current,I = 700 (0.7mA), Full Scale, FS = 1mA, Lower Threshold, T_L = 30, Attenuation = 16, Preset Gain = 2.

L929: Current,I = 700 (0.7mA), Full Scale, FS = 1mA, Lower Threshold, T_L = 20, Attenuation = 32, Preset Gain = 2.

Medium conditioned by AF1-19T cell line was also produced. AF1-19T normal rat kidney cell line, transformed with the malignant histiocytosis sarcoma virus (MHSV) is a crude source of GM-CSF [Pragnell *et al*,1988; Kriegler *et al*,1990]. AF1-19T is an adherent cell line growing in culture as a monolayer. It was maintained in culture and passaged similarly to L929 cell line. AF1-19T cell line conditioned medium was prepared similarly to the L929 cell line conditioned medium, aliquoted and stored at -20°C.

2.6 Recombinant haematopoietic colony-stimulating factors.

In specific experiments more rigorously defined conditions were applied through the use of recombinantly derived haematopoietic colony-stimulating factors:

Murine interleukin 3 (IL3)/multi-CSF was kindly supplied by *Genetics Institute, Massachusetts, U.S.A.*, as a crude conditioned medium from recombinantly engineered CHO cells.

Human macrophage colony-stimulating factor (M-CSF/CSF-1) was kindly supplied by *Genetics Institute*, as a crude conditioned medium from recombinantly engineered CHO cells.

Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was kindly supplied by both *Genetics Institute*, as a crude conditioned medium from recombinantly engineered COS cells and *Immunex, Washington, U.S.A.*, as a lyophilized protein sample.

Human interleukin 1 (IL1) was kindly supplied by both *Genetics Institute*, as a crude conditioned medium from recombinantly engineered COS cells (IL1 alpha) and *Immunex*, as lyophilized protein samples (IL1 alpha and beta).

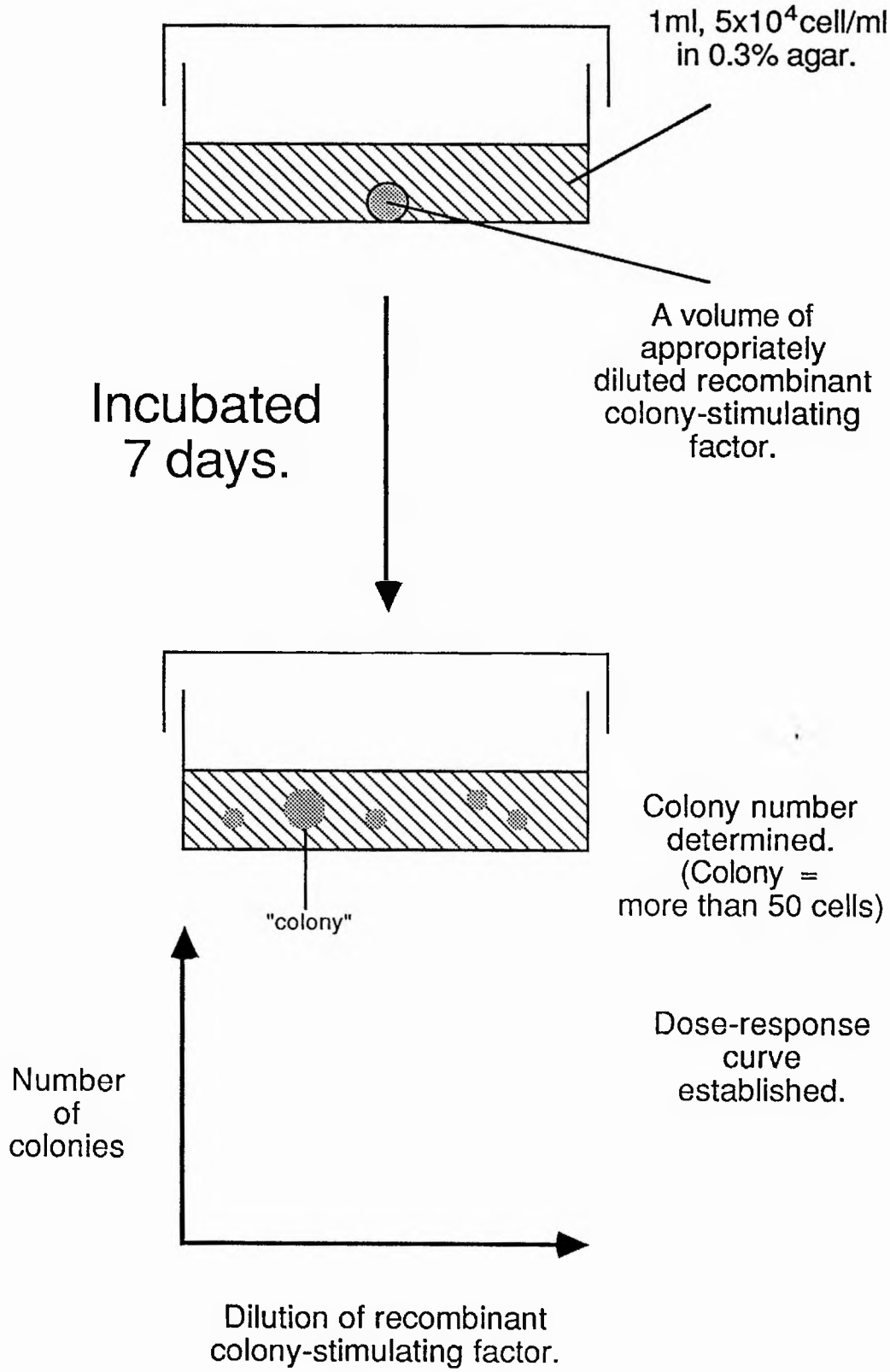
2.7 Determination of the activity of recombinant haematopoietic colony-stimulating factors.

The relative activities of recombinant murine interleukin 3 (IL3)/multi-CSF, human M-CSF/CSF-1 and murine GM-CSF were determined using two distinct assay systems:

1) The *in vitro* granulocyte-macrophage colony-forming cell assay [*Figure 18*] (2.9) and,

Figure 18: GM-CFC titration of recombinant colony-stimulating factors.

(Refer to 2.9 for GM-CFC technique)



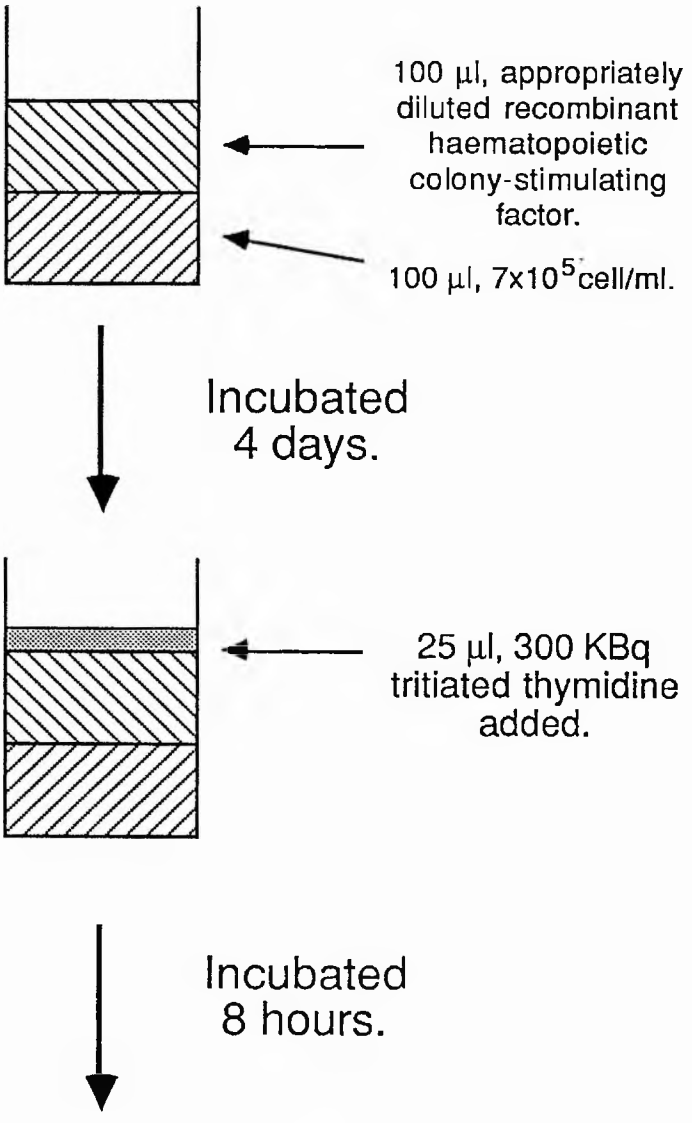
2) The *in vitro* tritiated thymidine (^{3}H-TdR) uptake assay [*Figure 19*]: The *in vitro* tritiated thymidine (^{3}H-TdR) uptake assay was used to measure cellular proliferation of murine femoral bone marrow in the presence of a range of dilutions of haematopoietic colony-stimulating factors. ^{3}H-TdR competes with native thymidine for incorporation into newly synthesizing DNA at S-phase and proliferating cells are thus radioactively labelled. (The specific activity of the ^{3}H-TdR used (185 GBq/mmol) is not sufficient for its incorporation to result in S-phase death of the cell.)

A single cell suspension of normal, murine, femoral marrow was produced in RPMI medium (Gibco) supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate, 2 mM L-glutamine and 10% (v/v) foetal calf serum (Gibco), cellularity determined and adjusted to $7x10^5$ cells/ml. 100 µl, $7x10^5$ cells/ml was aliquoted into an appropriate number of flat-bottomed wells of a 96 well microtitre plate (Nunc).

A range of haematopoietic colony-stimulating factor dilutions were prepared and 100 µl added to appropriate cell-containing wells. The final dilution of colony-stimulating factor in the 200 µl total volume was determined and cells incubated for 4 days in a 37°C, 5% CO_2 in air, fully humidified atmosphere. At t = 4 days, 25 µl of 300 KBq/ml ^{3}H-TdR (Amersham) was added to each well and incubation continued for 8 hours.

At t = 8 hours, cells were harvested using a Titertek cell harvester (Skatron, Norway) onto glass-fibre filter paper sheets (Titertek). After thorough oven-drying, individual filter discs were placed in scintillation vials and 2mls of scintillant (Optiphase 'Safe', FSA Laboratories, Loughborough) added. ^{3}H-TdR uptake by proliferating cells was determined using a liquid scintillation counter (LKB 1214 Rackbeta). Each vial was counted for a five minute period and a

Figure 19: Tritiated thymidine uptake assay as a measure of cellular proliferation.



Tritiated thymidine uptake α Cellular proliferation

'counts per minute' (CPM) value determined. The CPM figure reflects ^{3}H-TdR incorporation which inturn reflects cellular proliferation. Cellular proliferation was subsequently related to the dilution of colony-stimulating factor and a dose-response curve established.

2.8 Murine thymocyte proliferation assay of interleukin 1 activity.

[*Figure 20*] Thymic tissue was removed from 2-3 week old CBA/H mice. A single cell suspension of thymocytes was produced in RPMI medium (Gibco) supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate, 2 mM L-glutamine and 10% (v/v) foetal calf serum (Gibco), cellularity determined and adjusted to 10^{7} cell/ml.

a) Titration of concanavalin A.

Concanavalin A (Con A) will itself stimulate thymocyte proliferation. A suboptimal concentration of Con A was required to allow subsequent interleukin 1 titration.

100µl, 10^{7} thymocytes/ml were added to an appropriate number of flat-bottomed wells of a 96 well microtitre plate (Nunc) and 50 µl of variously diluted Con A added. The final concentration of Con A in the 150 µl total volume was determined and cells incubated for 3 days in a 37°C, 5%CO_2 in air, fully humidified atmosphere. At t=3 days, 25 µl of 300 KBq/ml ^{3}H-TdR (Amersham) was added to each well and incubation continued for 16 hours. At t=16 hours, cells were harvested and ^{3}H-TdR incorporation determined by scintillation counting. ^{3}H-TdR incorporation, related to cellular proliferation was subsequently related to Con A concentration and a dose-response curve established [*Figure 21*].

Figure 20: The murine thymocyte assay of interleukin 1.

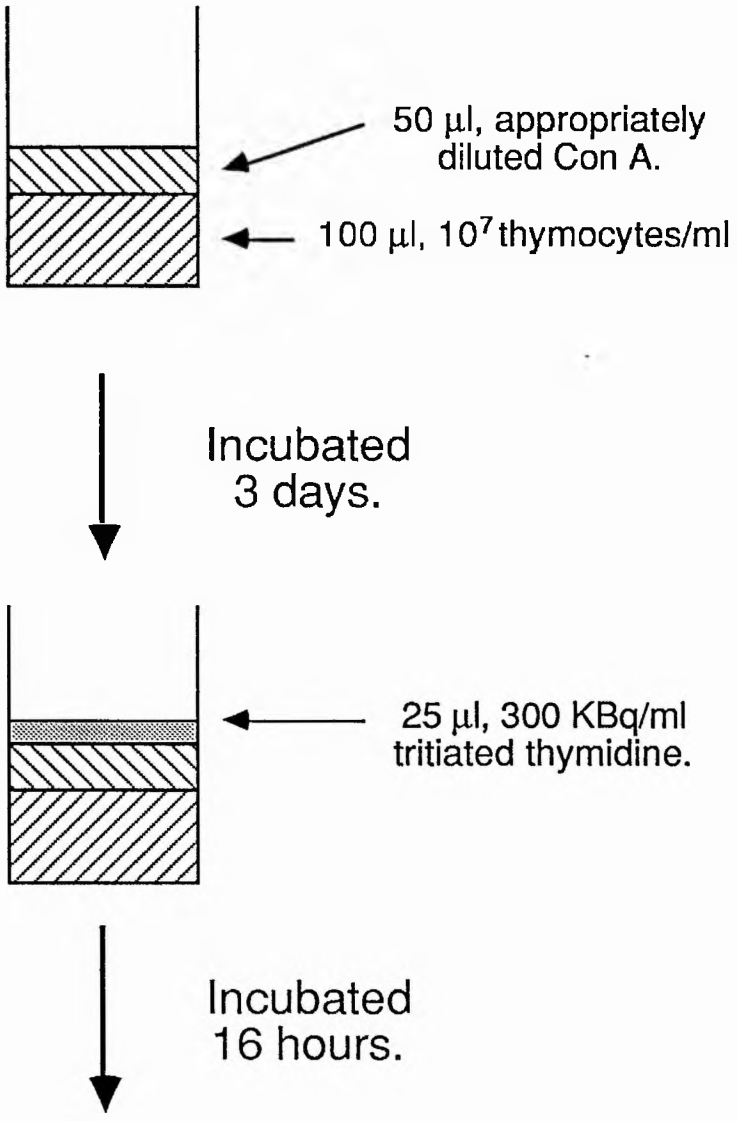
1) A subpopulation of thymocytes (T-lymphocytes), exposed to interleukin 1 produce interleukin 2 receptors.

2) A subpopulation of thymocytes interact with the Concanavalin A (Con A) antigen and release interleukin 2.

3) Interleukin 2 stimulates interleukin 2 receptor-bearing cells to undergo proliferation.

4) Thymocyte proliferation, detected by tritiated thymidine incorporation, is related to interleukin 1 levels.

Figure 21: Concanavalin A titration.



Cells harvested and tritiated thymidine uptake determined by liquid scintillation.

Tritated thymidine uptake α Thymocyte proliferation

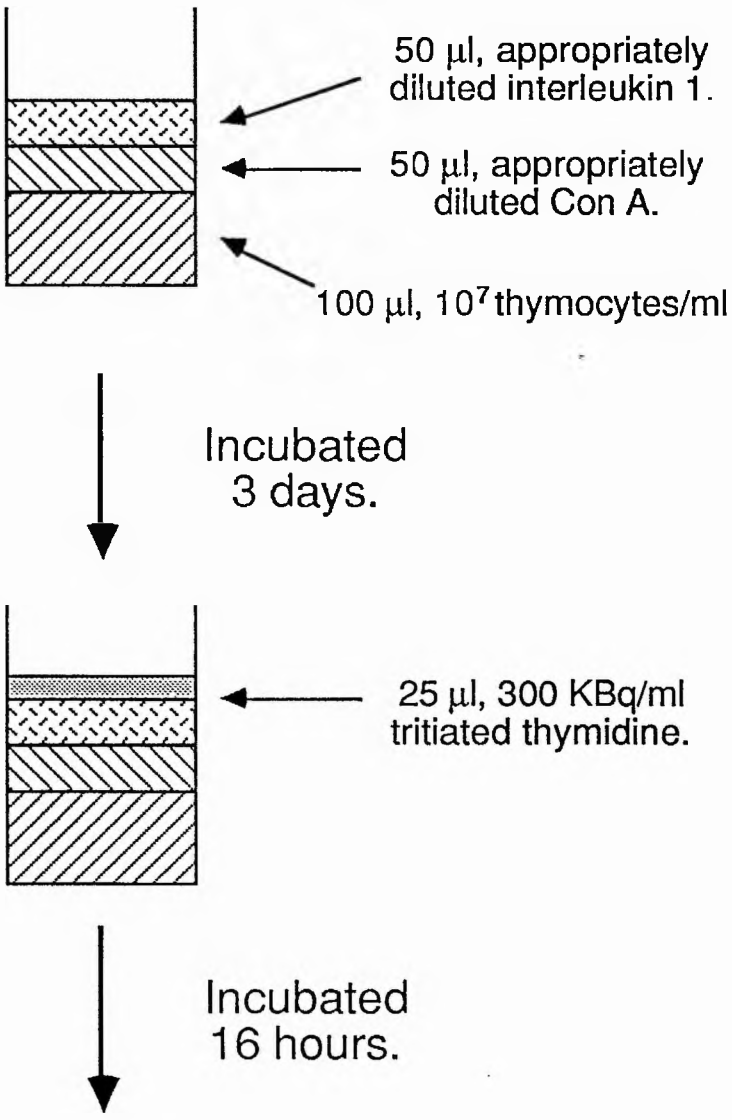
b) Titration of interleukin 1.

100µl, 10^7 thymocytes/ml were added to an appropriate number of flat-bottomed wells of a 96 well microtitre plate (Nunc) and 50 µl of Con A sufficient to produce a final concentration of 1.5 µg/ml in a 200 µl total volume was added. 50 µl of variously diluted interleukin 1 (alpha or beta) was added to appropriate wells and the final dilution of interleukin 1 in the 200 µl total volume determined. Cells were incubated for 3 days in a 37°C, 5%CO_2 in air, fully humidified atmosphere. At t=3 days, 25 µl of 300 KBq/ml ^{3}H-TdR (Amersham) was added to each well and incubation continued for 16 hours. At t=16 hours cells were harvested and ^{3}H-TdR incorporation determined by scintillation counting. ^{3}H-TdR incorporation, related to cellular proliferation was subsequently related to the dilution of interleukin 1 and a dose-response curve established [*Figure 22*].

2.9 *In Vitro* Granulocyte-Macrophage Colony-Forming Cell (GM-CFC)Assay.

A single cell suspension of haematopoietic tissue was produced in Dulbeccos medium (Gibco) supplemented with 50 I.U./ml benzyl penicillin (Glaxo), 50 µg/ml steptomycin sulphate (Evans), 2 mM L-glutamine (Flow Laboratories) and 20% horse serum (Gibco). Cellularity was determined and adjusted to $5x10^5$ cell/ml. 1ml, $5x10^5$ cell/ml and 1ml, melted 3% (w/v) agar (Difco BactoAgar) were added to 8mls of warmed (37°C), supplemented Dulbeccos medium. 1ml aliquots of the 10mls, $5x10^4$ cell/ml in 0.3% agar produced, were plated in 30mm plastic, non-tissue culture grade, triple vent, petridishes (Sterilin), over a volume of haematopoietic colony-stimulating activity. Routinely medium conditioned by WEHI 3B myelomonocytic leukaemic cell line was used (a crude source of

Figure 22: Interleukin 1 titration.



Cells harvested and tritiated thymidine uptake determined by liquid scintillation.

Tritated thymidine uptake α Thymocyte proliferation

interleukin 3 (IL3)/multi-CSF). After titration, 100µl WEHI 3B conditioned medium/1ml single cell suspension in 0.3% agar plated, was found to have an optimal colony stimulating activity (10% v/v final concentration).

A titration of medium conditioned by L929 fibroblast cell line, a crude source of macrophage colony-stimulating factor (M-CSF/CSF-1), medium conditioned by AF1-19T cell line, a crude source of GM-CSF and of recombinant colony-stimulating factors was performed using this technique.

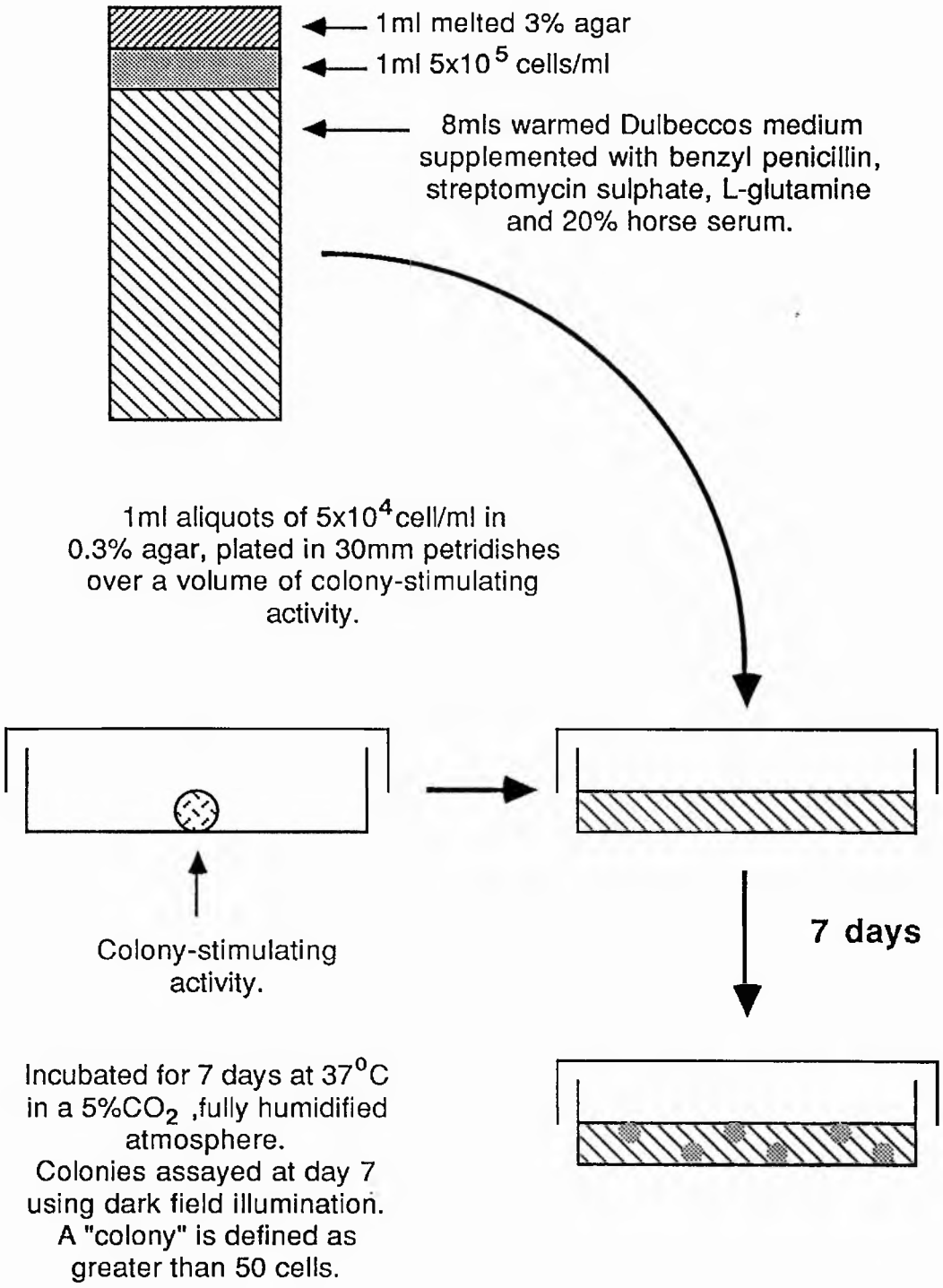
The agar was allowed to set and cultures were incubated in a 37°C, 5%CO_2 in air, fully humidified atmosphere for 7 days. Colonies were assayed on day 7 using a binocular dissecting microscope, set up to give dark field illumination and colonies in excess of 50 cells counted. Collections of less than 50 cells were considered 'clusters' and were not counted [*Figure 23*].

2.10 *In vitro* High Proliferative Potential Colony-Forming Cell (HPP-CFC) Assay.

This assay utilizes a combination of haematopoietic colony-stimulating factors and employs a bilayer agar culture technique. A 'feeder' layer containing the colony-stimulating factors underlies a cellular layer. Colony-stimulating factors diffuse from the underlying 'feeder' layer to influence the proliferation and differentiation of cells within the cellular layer.

Underlayer : A combination of haematopoietic colony-stimulating factors was added to a volume of 37°C Dulbeccos medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml steptomycin sulphate, 2 mM L-glutamine and 20% horse serum, sufficient to produce a total of 9mls and 1ml, 5% (w/v) melted agar (Difco

Figure 23: The granulocyte-macrophage colony-forming cell (GM-CFC) assay.



BactoAgar) was added. 1ml/2ml aliquots of the 10ml medium in 0.5% agar containing the combination of colony-stimulating factors were plated in 30mm/50mm plastic, non-tissue culture grade, triple vent, petridishes. The agar was allowed to set and these 'feeder' layers were stored in a 37°C, 5%CO_2 in air, fully humidified atmosphere until required.

Routinely medium conditioned by WEHI 3B myelomonocytic leukaemic cell line (a crude source of interleukin 3 (IL3)/multi-CSF) and medium conditioned by L929 fibroblast cell line (a crude source of M-CSF/CSF-1) were used in combination. Titration of conditioned media both singly and in combination demonstrated 10% (v/v) of each in combination produced optimal HPP-CFC proliferation. Thus, 1ml each of WEHI 3B and L929 conditioned media were added to 7mls of supplemented Dulbeccos medium and 1ml, 5% (w/v) agar added. In specific instances different combinations of conditioned media were used and in other cases, recombinant colony-stimulating factors were investigated.

Cellular layer : A single cell suspension of haematopoietic tissue was produced in Dulbeccos medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml steptomycin sulphate, 2 mM L-glutamine and 20% horse serum. Experimental evaluation demonstrates that the cellularity of tissue plated to produce sufficient HPP-CFC-derived colonies at assay, varies according to the source of the tissue. Normal haematopoietic tissue was diluted to 2.0-2.5x10^5 cell/ml, while haematopoietic tissue from mice subjected to whole body X-irradiation (2.13) was diluted to 5.0x10^5 cell/ml.

1ml, 2.0-2.5x10^5 normal bone marrow cells/ml, or 5.0x10^5 whole body X-irradiated bone marrow cells/ml and 1ml, 3% (w/v) melted agar were added to 8mls, 37°C, supplemented Dulbeccos medium. 1ml/2ml aliquots of the 10ml, 2.0-2.5x10^4 normal bone marrow

cells/ml, or $5.0x10^4$ whole body X-irradiated bone marrow cells/ml in 0.3% agar, were plated in 30mm/50mm petridishes over the previously prepared 0.5% agar 'feeder' layers containing the colony-stimulating factors. The agar was allowed to set and cultures incubated in a 37°C, 5% CO_2 in air, fully humidified atmosphere for 14 days, a period of incubation which, during evaluation, proved optimal for HPP-CFC proliferation.

Colonies were assayed unstained using a binocular dissecting microscope set up to give dark field illumination. Alternatively, 12-24 hours prior to assay, 0.5ml/1.0ml of an autoclaved solution of 1mg INT/ml 0.9% (w/v) NaCl was added to each 30mm/50mm culture dish and incubation continued. (INT = 2(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, BDH/Sigma) [Bol *et al*,1977]. As viable cells proliferate *in vitro*, in response to colony-stimulating factors, they convert the colourless tetrazolium salt to a water insoluble red formazan which precipitates inside cells. The contrast of colonies was increased sufficiently to allow macroscopic assay.

Colonies in excess of 2 mm diameter were established during evaluation as the population of colony-forming cells of interest a criterion applied by Pragnell *et al* [1988], Eckmann *et al* [1988] and Falk and Vogel [1988]. Colony size was determined using a 1mm grid on a clear acetate sheet underlay [*Figure 24*].

2.11 Fixation and Staining of GM-CFC and HPP-CFC-Derived Colonies.

Colonies can be fixed after assay using an overlay of a 10% (v/v) formalin solution and can be stored at +4°C, in a humidified atmosphere. Alternatively, the colony-containing agar disc can be removed from the petridish and mounted on a glass slide:

Figure 24: The high proliferative potential colony-forming cell (HPP-CFC) assay.

Underlayer.

1ml melted 5% agar

1ml WEHI conditioned medium

1ml L929 conditioned medium

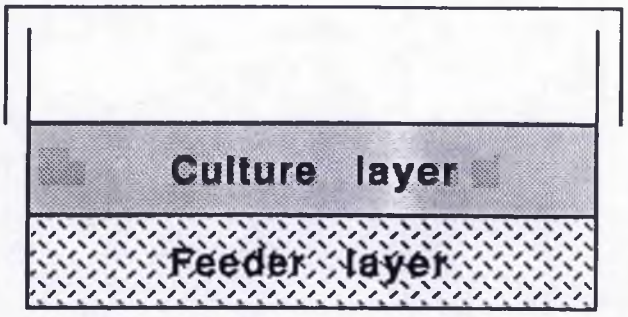
7mls warmed Dulbeccos medium supplemented with benzyl penicillin, streptomycin sulphate, L-glutamine and 20% horse serum.

Cellular layer.

1ml melted 3% agar.

1ml $2x10^5$ normal / $5x10^5$ X-irradiated cells/ml

8mls warmed Dulbeccos medium supplemented with benzyl penicillin, streptomycin sulphate, L-glutamine and 20% horse serum.



1ml/2ml, $2x10^4$ normal / $5x10^4$ X-irradiated cells/ml in 0.3% agar are plated in 30mm/ 50mm plastic petridishes over the 0.5% agar feeder layer containing 10% (v/v) each of WEHI and L929 conditioned media.

Assayed after 14 days incubation.

HPP-CFC-derived colony defined as a colony in excess of 2mm diameter.

Cultures were cooled (+4°C) to solidify the 0.3% colony-containing agar. Petridishes were then carefully submerged in a +4°C, isotonic solution (Isoton II, Coulter Electronics). If already fixed with 10% formalin, +4°C water may be used. A 25G needle was run around the edge of the agar disc and the 0.3% agar culture layer floated free from the petridish. In the case of the HPP-CFC assay, the 0.3% agar culture disc can also be floated free from the 0.5% agar 'feeder' layer.

The free-floating 0.3% agar disc was captured on a methanol-cleaned 2"x3" glass slide and carefully removed from the solution. An filterpaper disc (Whatman 541, 5.5cm) was placed over the agar disc to remove excess fluid and the whole allowed to air dry. Once dry the filter paper disc was easily removed.

If already stained with INT, HPP-CFC-derived colonies were apparent in the otherwise unstained agar disc. Unstained GM-CFC and HPP-CFC-derived agar discs can be Jenner-Giemsa stained (2.4). After staining, colonies macroscopically stain dark blue in a blue-staining agar disc. Resolution for microscopic investigation proved relatively poor due to the residual thickness and background staining of the agar. Slides can be mounted using DePeX (BDH) mounting medium and a glass coverslip.

2.12 Use of Methylcellulose as a Semi-Solid Support Medium for GM-CFC and HPP-CFC Culture.

In specific cases where colony replating experiments, cytological investigation, or cellularity determination of colonies was of interest, the 0.3% agar support medium was replaced with 0.9% methylcellulose (Methocel MC4000, Fluka). Methylcellulose allows colonies of interest to be 'plucked' from the culture environment

using a 1ml syringe and 23G needle. In replating experiments, sterile conditions were maintained. Single cell suspensions were produced from single colonies in appropriate medium and replated for subsequent GM-CFC or HPP-CFC assay. For cytological investigation cytocentrifuge preparations were produced (2.4) and colony cellularities were determined using a Coulter Counter.

Aliquots of 2% (w/v) methylcellulose in Dulbeccos medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate and 2 mM L-glutamine were stored at -20°C. For use, aliquots were thawed at room temperature.

5mls, 2% methylcellulose in supplemented Dulbeccos medium was added to 5mls, $10.0x10^4$ sublethally X-irradiated, regenerating bone marrow (RBM) cell/ml, or $5.0x10^4$ normal bone marrow (NBM) cell/ml, single cell suspension in supplemented Dulbeccos medium with 40% (v/v) horse serum. 1ml aliquots of the 10mls, $5.0x10^4$ RBM cell/ml, or $2.5x10^4$ NBM cell/ml, in supplemented Dulbeccos medium with 20% (v/v) horse serum produced, were plated in 30mm non-tissue culture grade, triple vent, plastic petridishes, over an optimal volume of colony-stimulating activity for subsequent GM-CFC assay, or over a 0.5% agar 'feeder' layer containing a combination of colony-stimulating factors, for subsequent HPP-CFC assay. Colonies were assayed after 7/14 days incubation in a 37°C, 5%CO_2 in air, fully humidified atmosphere and visualised using a binocular dissecting microscope set up to give dark field illumination.

2.13 Determination of the Proportion of Colony-Forming Cells in S-phase.

The degree of cellular proliferation was determined through the use of an S-phase "suicide" assay. Cells were incubated with a

nucleoside analogue which competes with the native nucleoside for incorporation into newly synthesizing DNA at S-phase. When the complex DNA-synthesizing enzyme system incorporates the nucleoside analogue into the elongating strand of DNA, the molecule acts as a "chain-terminator" preventing the subsequent polymerization of further nucleosides probably through stereochemical hinderance, so preventing further DNA synthesis. A failure of DNA synthesis at S-phase is a lethal event and the cell will die. If the cell is a colony-forming cell, during subsequent colony-forming cell assays, the colony-forming potential is not realised. By a comparison of the number of colonies formed from cells treated with, or without, the S-phase specific cytotoxic agent, a measure of the proportion of colony-forming cells in S-phase was obtained. The S-phase specific cytotoxic agent used was Cytosine ß-D-Arabinofuranoside (Cytosine Arabinoside, ARA-C)(Sigma), a deoxycytidine analogue [Cork *et al*,1981; Thomas *et al*,1981; Riches *et al*,1981; Wright *et al*,1982; 1985; Cork *et al*,1986].

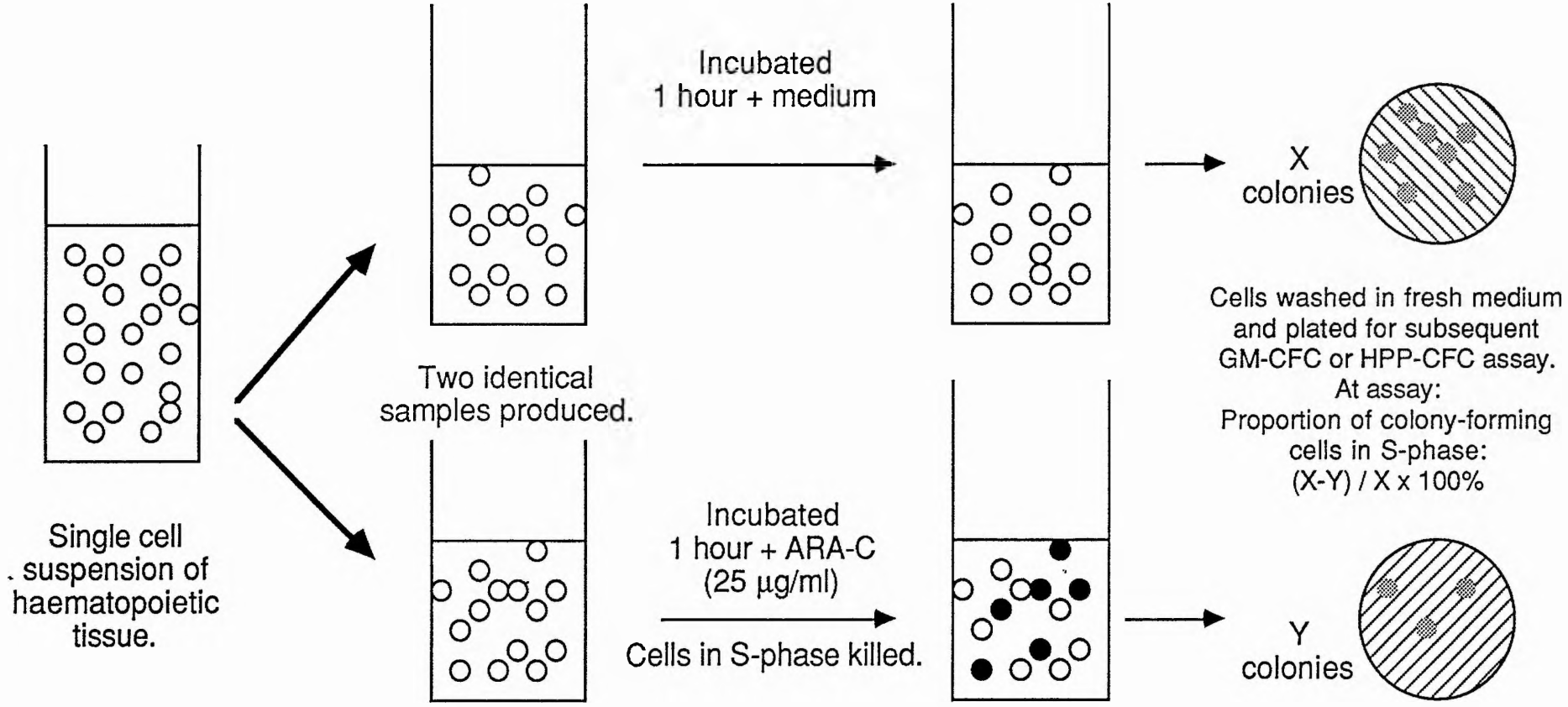
A single cell suspension was produced in appropriate medium, cellularity determined and adjusted to $5x10^6$ cell/ml. Two 1ml samples of $5x10^6$ cell/ml were produced in plastic, conical, centrifuge tubes. ARA-C, stored lyophilized at +4°C was solubilized in appropriate medium immediately prior to use as 250 µg/ml. To one 1ml sample of $5x10^6$ cell/ml, 100µl of the 250 µg ARA-C/ml was added, producing a final concentration of 25 µg ARA-C/ml (10^{-4}M). (100µl, 250 µg ARA-C/ml was added per 1ml of cell suspension) To the other 1ml sample of $5x10^6$ cell/ml, 100µl of medium alone was added as a control. Samples were incubated for 1 hour at 37°C with frequent vortex mixing. After 1 hour, cells were washed: 7mls fresh medium was added to each sample and the cell suspension centrifuged (x1000 rpm, 15 mins.). Supernatant was removed to waste, the cell pellet

resuspended in 8mls of fresh medium and recentrifuged (x1000 rpm, 15 mins.). Supernatant was removed to waste and the cell pellet resuspended in 1ml of fresh medium as a single cell suspension. Washing confines the action of the S-phase specific cytotoxic agent to the 1 hour incubation and not subsequent culture. Cellularity was determined using a Coulter Counter and adjusted as appropriate for subsequent GM-CFC (2.9) or HPP-CFC (2.10) assay [*Figure 25*].

2.14 The Production of Medium Conditioned By Normal Bone Marrow.

Normal bone marrow conditioned medium was used as a crude source of stem cell proliferation *inhibitor*. Normal bone marrow was obtained as femoral marrow from CBA/H mice and suspended as a single cell suspension in Dulbeccos medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate, 2 mM L-glutamine and 20% (v/v) horse serum. In specific cases, the 20% horse serum was ommitted to allow the production of a serum-free conditioned medium. Cellularity was determined using a Coulter Counter and adjusted as required, usually within a 15-20x10^6 cell/ml range. The single cell suspension was incubated at 37°C, with frequent vortex mixing for a 4-6 hour period [Lord *et al*,1976; 1977; 1979; Wright & Lord,1979; Wright *et al*,1980a; 1980b; Lord & Wright,1982;1984; Tejero *et al*,1984; Simmons & Lord,1985; Lord *et al*,1987; Wright & Lorimore,1987; Pojda *et al*,1988]. After incubation, the single cell suspension was centrifuged (x1000 rpm, 15 mins.). The supernatant was retained, twice recentrifuged (x2000 rpm, 15 mins. and x3000 rpm, 15 mins.), aliquoted and frozen (-20°C). Individual aliquots were not refrozen once thawed and prior to use were

Figure 25: Determination of the proportion of colony-forming cells in S-phase.



centrifuged (x3000 rpm, 15 mins.), MilliPore filtered (0.22 μM) and diluted as required [*Figure 26*].

2.15 The Production of Medium Conditioned By Regenerating/Cycling bone marrow.

Regenerating/cycling bone marrow conditioned medium was used as a crude source of stem cell proliferation *stimulator*. In mice, 8.2 Gray (Gy) whole body X-irradiation is sufficient to lethally damage the haematopoietic system and death occurs after 14 days due to haematopoietic failure. Use of 4.5 Gy whole body X-irradiation, sublethally damages the haematopoietic system and haematopoietic regeneration occurs. Regenerating femoral bone marrow was harvested from mice 7 days after 4.5 Gy whole body X-irradiation, as a source of cycling bone marrow and medium conditioned by the regenerating marrow as a crude source of the stem cell proliferation *stimulator* [Wright & Lord,1977; Lord *et al*,1977; Tejero *et al*,1984; Wright *et al*,1985].

Mice were irradiated in a perspex cassette placed a fixed distance from an X-ray source. X-rays were produced from a Siemens Stabilipan at 250KVp and 14mA and filtered through a 0.5mm copper (Cu) filter. A dose rate was established using a Farmer Dosimeter (Nuclear Enterprises, Reading) with an ionization chamber placed inside a wax mouse 'ghost' and irradiated identically to the mice. A dose rate of 0.81 Gy/min was established and a given dose obtained by varying the length of exposure.

Regenerating/cycling femoral bone marrow was obtained and suspended in Dulbeccos medium supplemented with 50 I.U./ml benzyl penicillin, 50 μg/ml streptomycin sulphate, 2 mM L-glutamine and 20% (v/v) horse serum. In specific cases the 20% horse serum

was ommitted to allow the production of a serum-free conditioned medium. Cellularity was determined using a Coulter Counter and adjusted as required usually within a 15-20x10^6 cell/ml range. The single cell suspension was incubated at 37°C with frequent vortex mixing for a 1-3 hour period. After incubation, the single cell suspension was centrifuged (x1000 rpm, 15 mins.). The supernatant was retained, twice recentrifuged (x2000 rpm, 15 mins. and x3000 rpm, 15 mins.), aliquoted and frozen (-20°C). Individual aliquots were not refrozen once thawed and prior to use were centrifuged (x3000 rpm, 15 mins.), MilliPore filtered (0.22μM) and diluted as required [*Figure 27*].

2.16 Assay of Medium Conditioned by Normal Bone Marrow.

Normal bone marrow conditioned medium is a crude source of stem cell proliferation *inhibitor*. The activity was demonstrated against haematopoietic tissue in which the proportion of stem cells in S-phase was increased. CBA/H mice were subjected to a 2Gy, sublethal whole body X-irradiation dose (X-ray unit: Siemens Stabilipan at 250KVp, 14mA and 0.5mm Cu filter. Dose rate = 0.81Gy/min.). Regenerating femoral marrow was harvested 3 days after irradiation [Lord *et al*,1977; Wright *et al*,1985].

A single cell suspension of regenerating/cycling femoral marrow was produced in Dulbeccos medium supplemented with 50 I.U./ml benzyl penicillin, 50 μg/ml steptomycin sulphate, 2 mM L-glutamine and 20% (v/v) horse serum. In specific cases the 20% horse serum was ommitted for incubation under serum-free conditions. Cells were washed once in appropriate medium to remove any endogenous regulatory activities, resuspended as a single cell suspension and

Figure 26: Production of medium conditioned by normal bone marrow.

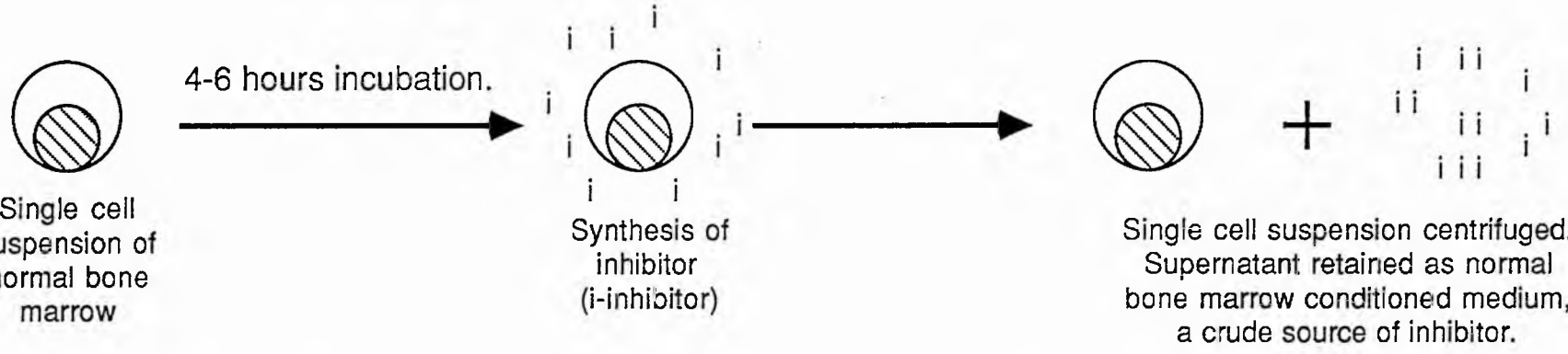
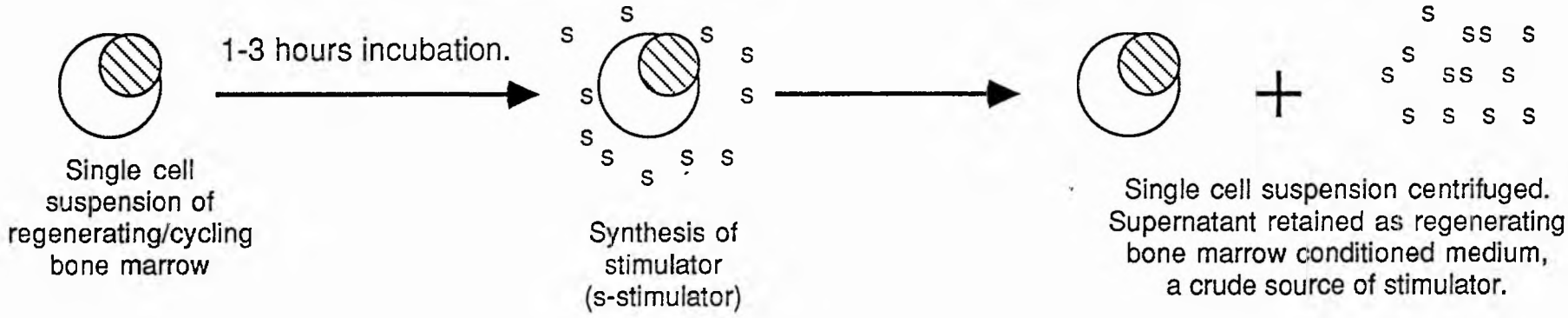


Figure 27: Production of medium conditioned by regenerating/cycling bone marrow.



cellularity determined. Cellularity was adjusted to $5x10^6$ cycling/regenerating marrow cells/ml.

Aliquots of normal bone marrow conditioned medium (2.14), stored at -20°C, were thawed, centrifuged (x3000 rpm, 15 mins.) and MilliPore filtered (0.22μM). The concentration of the proliferation *inhibitor* is related to the number of cells from which it is derived and dilution in appropriate medium produces a conditioned medium of the required 'potency'.

1ml, $5x10^6$ cycling/regenerating marrow cells/ml was incubated with 1ml of appropriately diluted normal bone marrow conditioned medium, or medium as a control, in paired, plastic, conical centrifuge tubes, for 4 hours at 37°C with frequent vortex mixing.

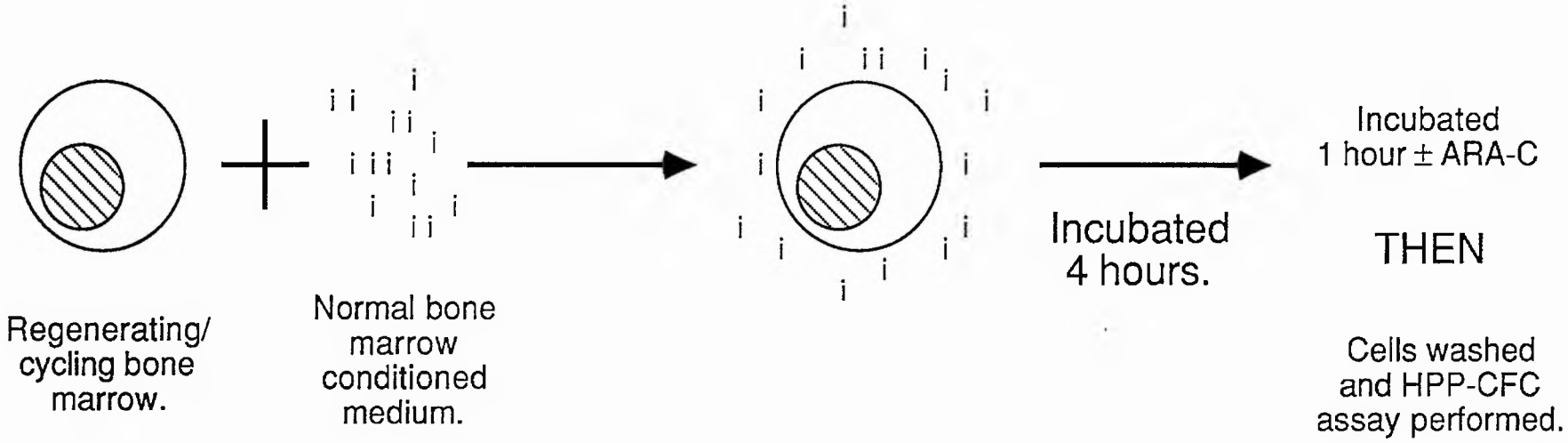
The proportion of cells in S-phase was investigated by a further 1 hour incubation in the absence or presence of ARA-C (2.13), and cells were washed, plated and subsequently cultured in the *in vitro* HPP-CFC assay (2.10) [*Figure 28*].

2.17 Assay of medium conditioned by regenerating/cycling bone marrow.

Regenerating/cycling bone marrow conditioned medium is a crude source of stem cell proliferation *stimulator*. The activity was demonstrated against haematopoietic tissue in which the proportion of stem cells in S-phase was reduced. Such haematopoietic tissue was obtained as normal femoral bone marrow [Wright *et al*,1977; Wright & Lord,1979; 1982; Wright *et al*,1982; Lord & Wright,1984; Tejero *et al*,1984; Wright *et al*,1985; Wright & Lorimore,1987].

A single cell suspension of normal CBA/H femoral marrow was produced in Dulbeccos medium supplemented with 50 I.U./ml benzyl penicillin, 50 μg/ml steptomycin sulphate, 2 mM L-glutamine and 20%

Figure 28: Assay of medium conditioned by normal bone marrow.



(v/v) horse serum. In specific cases the 20% horse serum was ommitted for incubation under serum-free conditions. Cells were washed once in appropriate medium to remove any endogenous regulatory activities, resuspended as a single cell suspension and cellularity determined. Cellularity was adjusted to $5x10^6$ normal marrow cells/ml.

Aliquots of regenerating/cycling bone marrow conditioned medium (2.15), stored at -20°C, were thawed, centrifuged (x3000 rpm, 15 mins.) and MilliPore filtered (0.22 μM). The concentration of the proliferation *stimulator* was related to the number of cells from which it was derived and dilution in appropriate medium produces a conditioned medium of the required 'potency'.

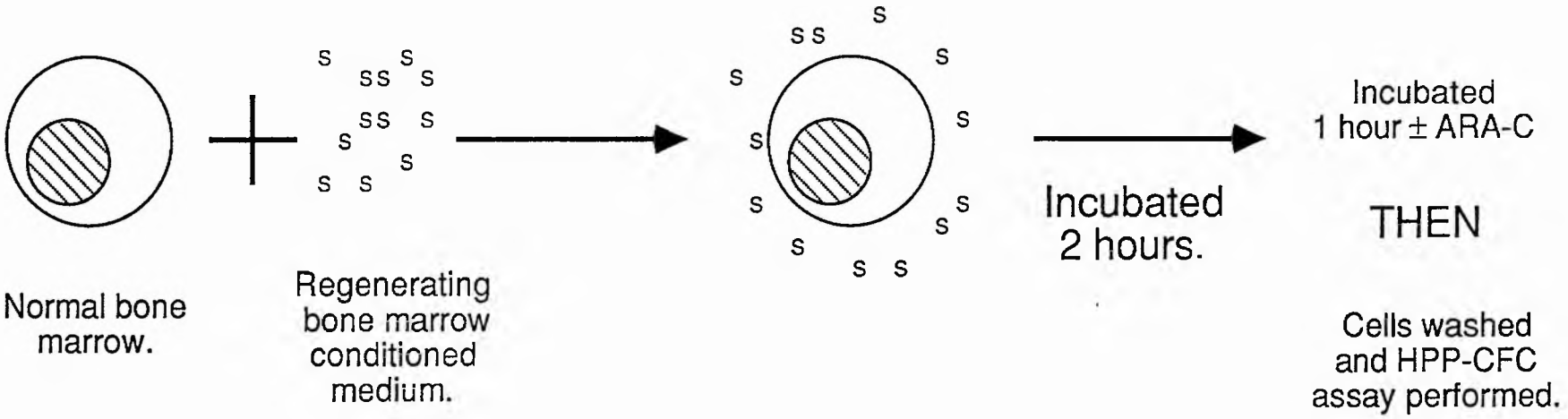
1ml, $5x10^6$ normal marrow cells/ml was incubated with 1ml of appropriately diluted regenerating/cycling bone marrow conditioned medium, or medium as a control, in paired, plastic, conical centrifuge tubes, for 2 hours at 37°C with frequent vortex mixing.

The proportion of cells in S-phase was investigated by a further 1 hour incubation in the absence or presence of ARA-C (2.13), and cells were washed, plated and subsequently cultured in the *in vitro* HPP-CFC assay (2.10) [*Figure 29*].

2.18 Derivation of primary, murine, myeloid leukaemias.

Primary myeloid leukaemias were induced in CBA/H, male mice subjected to 3Gy whole body X-irradiation (X-ray unit: Siemens Stabilipan at 250KVp 14mA and 0.5mm Cu filter. Dose rate = 0.81Gy/min.) No spontaneous leukaemias were observed in unirradiated controls [Meldrum & Mole,1982; Mole *et al*,1983; Hepburn *et al*,1987]. When mice showed ruffled fur, weight loss, stiffness of movement, lethargy and pale feet, they were killed by

Figure 29: Assay of medium conditioned by regenerating bone marrow.



ether inhalation overdose. At autopsy, leukaemias were characterized by histological examination of blood, femoral bone marrow, spleen, sternum and liver. Spleen and femoral marrow cells were injected intraperitoneally, or intravenously, into syngeneic mice to establish serial *in vivo* passage of the leukaemia [Hepburn *et al*,1987]. Two distinct morphologies subsequently developed depending on the number of leukaemic cells injected at passage. Leukaemias passaged at a low cell dose, maintain a near-primary morphology and character [Hepburn *et al*,1987]. Leukaemias passaged at a high cell dose, show a marked increase in aggressiveness, probably due to selection pressure on more rapidly growing leukaemic cells within the leukaemic population as a whole [Hepburn *et al*,1987].

Spleen and marrow cells from primary myeloid leukaemias and subsequent passages can be cryopreserved in liquid nitrogen (in supplemented medium, 10-20% (v/v) foetal calf serum and 5-10% (v/v) DMSO) and rederived as required by rapid thawing, washing and either intravenous, or intraperitoneal injection into syngeneic recipients [Hepburn *et al*,1987].

2.19 *In vivo* passage of murine, myeloid leukaemias.

Syngeneic mice injected intraperitoneally, or intravenously, with either a high, or low, cell dose of leukaemic cells, were checked daily. When mice show ruffled fur, weight loss, stiffness of movement, lethargy and pale feet, they were killed by ether inhalation overdose. At autopsy a large pale spleen was evident and was taken as indicative of leukaemic progression. Conformation of the disease was established by the production of a spleen 'touch-preparation'. The spleen was cut in two and the cut surface gently touched onto a glass slide. Cells deposited were air dried and either fixed and

Jenner-Giemsa, or Diff-Quik stained (2.4). Microscopic analysis showed the splenomegally was the result of marked leukaemic infiltration.

A crude leukaemic spleen cell suspension was produced in Fischers medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate and 2 mM L-glutamine using a 1ml syringe with no needle attatched. Allowed to stand for a short time, large pieces of debris and extracellular matrix settle out of the suspension. A single cell suspension of leukaemic spleen was produced through subsequent removal of cellular supernatant from the crude cell suspension and use of 23G and 25G needles. Cellularity was determined using a Coulter Counter, adjusted as required and injected either intraperitoneally, or intravenously, at either a high, or low, cell dose. Cytocentrifuge preparations (2.4) of the leukaemic spleen cell suspension confirm the nature of the cells injected.

2.20 Production of medium conditioned by leukaemic bone marrow.

Syngeneic mice injected intraperitoneally, or intravenously, with either a high, or low, cell dose of leukaemic cells, were checked daily. When mice show ruffled fur, weight loss, stiffness of movement, lethargy and pale feet, they were killed by ether inhalation overdose. At autopsy a large pale spleen was evident and was taken as indicative of leukaemic progression. Leukaemic bone marrow was obtained as femoral marrow and suspended as a single cell suspension in Dulbeccos medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate, 2 mM L-glutamine and 20% (v/v) horse serum. In specific cases the 20% horse serum

was ommitted to allow production of a serum-free conditioned medium. Cellularity was determined using a Coulter Counter and adjusted as required to within a 15-20x10^6 cell/ml range. The single cell suspension was incubated at 37°C with frequent vortex mixing for 1-3 hours. After incubation the single cell suspension was centrifuged (x1000 rpm, 15 mins.). The supernatant was retained and recentrifuged (x2000 rpm, 15 mins. and x3000 rpm, 15 mins.), aliquoted and frozen (-20°C). Individual aliquots were not refrozen once thawed and prior to use were centrifuged (x3000 rpm, 15 mins.), MilliPore filtered (0.22 μM) and diluted as required. Cytocentrifuge preparations of the leukaemic bone marrow confirms the nature of the cells incubated [*Figure 30*].

2.21 Assay of medium conditioned by leukaemic bone marrow.

The nature of haematopoietic stem cell proliferation regulation in myeloid leukaemic bone marrow was unknown. Medium conditioned by leukaemic bone marrow was assayed against both regenerating/cycling bone marrow (2.16) to investigate the presence/absence of proliferation *inhibitor*, and normal bone marrow (2.17), to investigate the presence/absence of proliferation *stimulator* [*Figure 31*].

2.22 Investigating the effects of leukaemic bone marrow conditioned medium on *inhibitor* and/or *stimulator* action.

Normal bone marrow (NBM) conditioned medium, a crude source of *inhibitor* (2.14) and medium conditioned by regenerating marrow (RBM) from mice 7 days after a 4.5 Gy whole body X-irradiation dose,

Figure 30: Production of medium conditioned by leukaemic bone marrow.

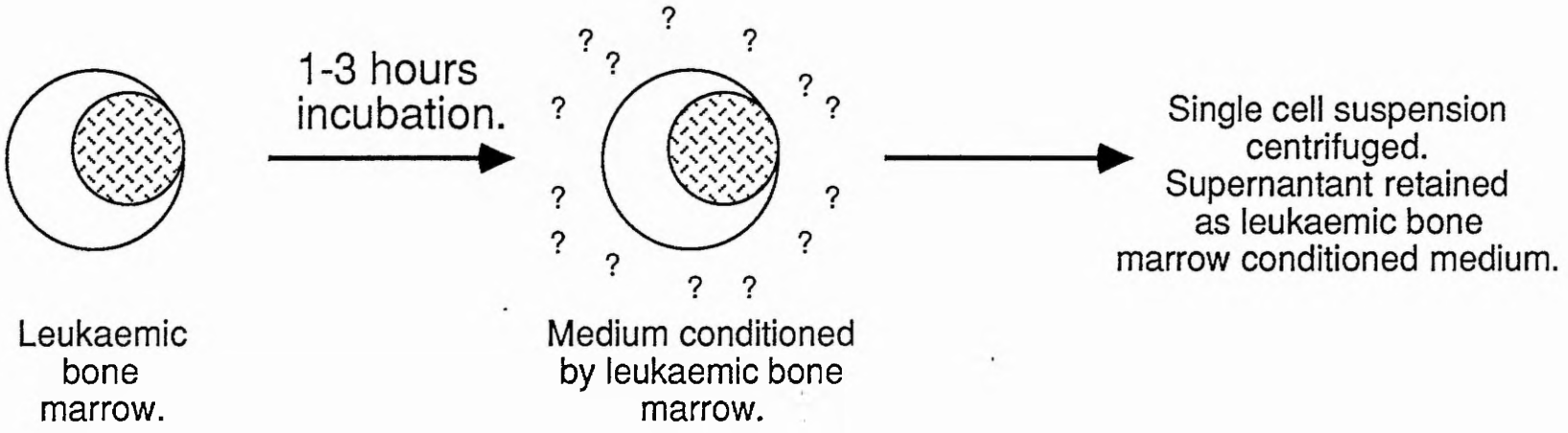
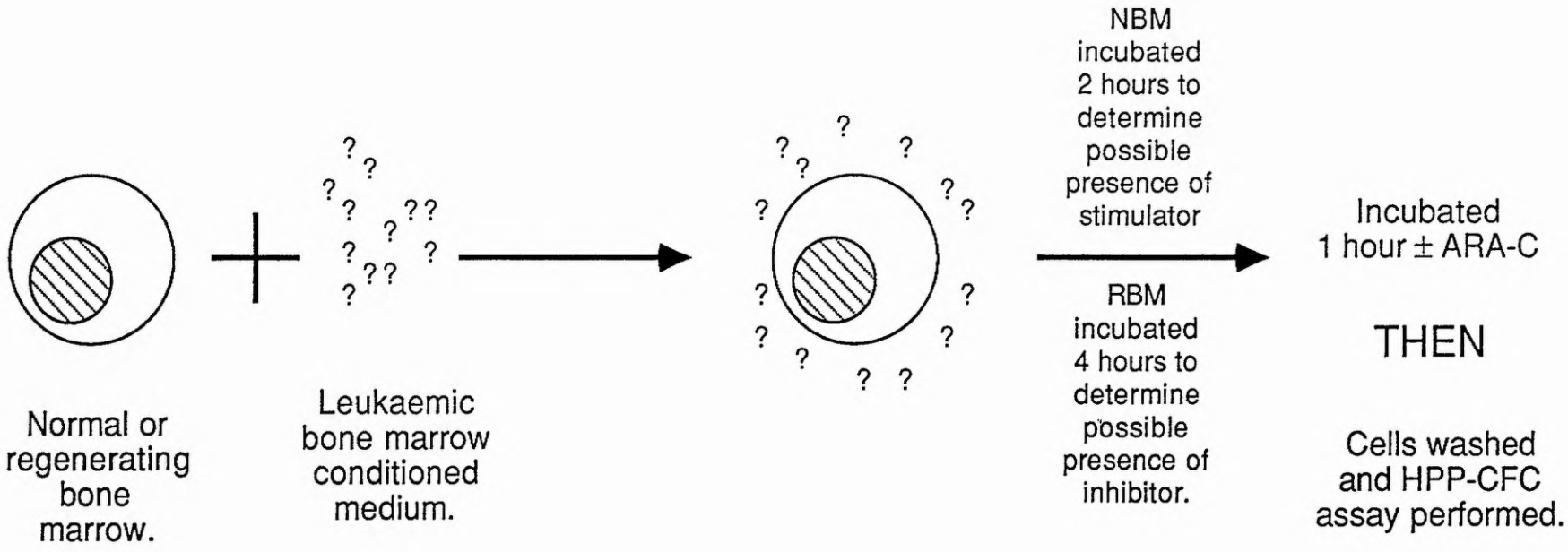


Figure 31: Assay of medium conditioned by leukaemic bone marrow.



as a crude source of *stimulator* (2.15), were produced. Conditioned medium was thawed, centrifuged (x3000 rpm, 15 mins.), MilliPore filtered (0.22 μM) and diluted so as to be derived from $15x10^6$ NBM/RBM cells/ml.

Appropriate leukaemic marrow conditioned medium (2.20) was thawed, centrifuged (x3000 rpm, 15 mins.), MilliPore filtered (0.22 μM) and diluted so as to be derived from $15x10^6$ cells/ml.

Inhibitor was assayed against regenerating bone marrow (RBM) from mice 3 days after a 2 Gy whole body X-irradiation dose (2.16). RBM cells were washed once in supplemented Dulbecco's medium and resuspended as $5x10^6$ RBM cells/ml in either leukaemic conditioned medium or normal medium [*Figure 33*].

Stimulator was assayed against normal bone marrow (NBM) (2.17). NBM cells were washed once in supplemented Dulbecco's medium and resuspended as $5x10^6$ NBM cells/ml in either leukaemic conditioned medium or normal medium [*Figure 32*].

1 ml, $5x10^6$ NBM/RBM cells/ml in either leukaemic conditioned medium or normal medium, was aliquoted into each of two paired centrifuge tubes and assay of *inhibitor* and *stimulator* performed by the addition of 1 ml of the appropriate normal, or regenerating, bone marrow conditioned medium (2.16 & 2.17). The proportion of cells in S-phase was determined during the last hour of incubation in the absence or presence of ARA-C (2.13). Cells were washed and cultured in the HPP-CFC assay (2.10).

Figure 32: Investigating the effects of leukaemic bone marrow conditioned medium on the action of STIMULATOR.

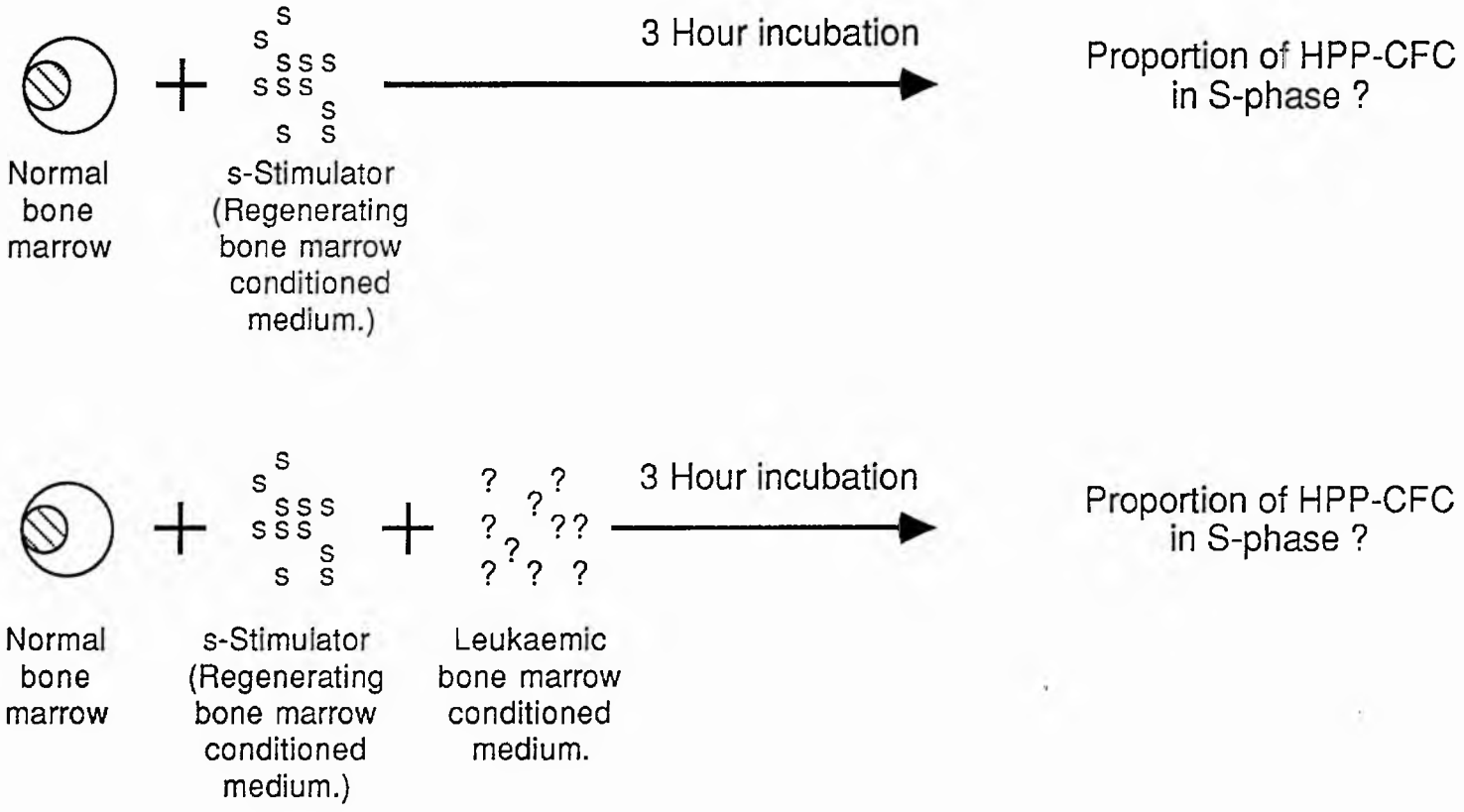
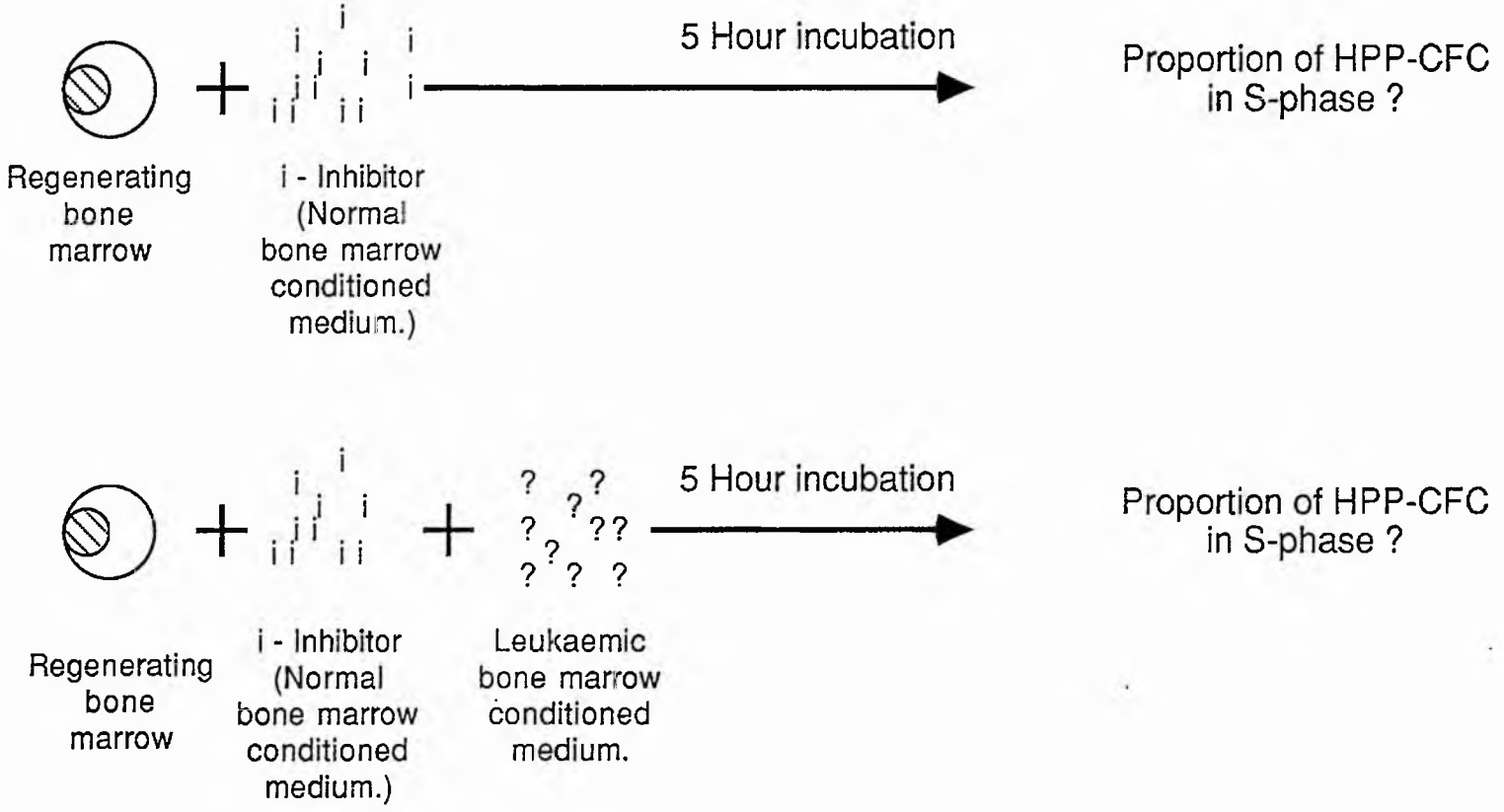


Figure 33: Investigating the effects of leukaemic bone marrow conditioned medium on the action of INHIBITOR.



2.23 Investigation of the effects of leukaemic bone marrow conditioned medium on *inhibitor* and/or *stimulator* production.

Normal bone marrow (NBM) was obtained from CBA/H mice and regenerating bone marrow (RBM) from mice 7 days after a 4.5 Gy whole body X-irradiation dose. A single cell suspension of NBM/RBM was produced in Dulbecco's medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate, 2 mM L-glutamine and 20% (v/v) horse serum.

Appropriate leukaemic bone marrow conditioned medium (2.20), stored at -20°C, was thawed, cenrifuged (x3000 rpm, 15 mins.), MilliPore filtered (0.22 µM) and appropriately diluted in supplemented Dulbecco's medium so as to be derived from $15x10^6$ cell/ml.

NBM/RBM cells were washed once in supplemented Dulbecco's medium and resuspended as a single cell suspension in either leukaemic conditioned medium, or medium. Cellularity was determined and adjusted to within $15\text{-}20x10^6$ NBM/RBM cell/ml and NBM and RBM cells were subsequently incubated for 7 and 5 hours respectively, to allow *inhibitor* and *stimulator* resynthesis [*Figures 34 & 35*].

At t=7, or 5 hours, the single cell suspension was centrifuged (x1000 rpm, 15 mins.), supernatant retained and repeatedly centrifuged (x2000 rpm, 15 mins. and 3000 rpm, 15 mins.). Medium conditioned by NBM/RBM cells in the absence and presence of leukaemic conditioned medium was aliquoted and frozen (-20°C).

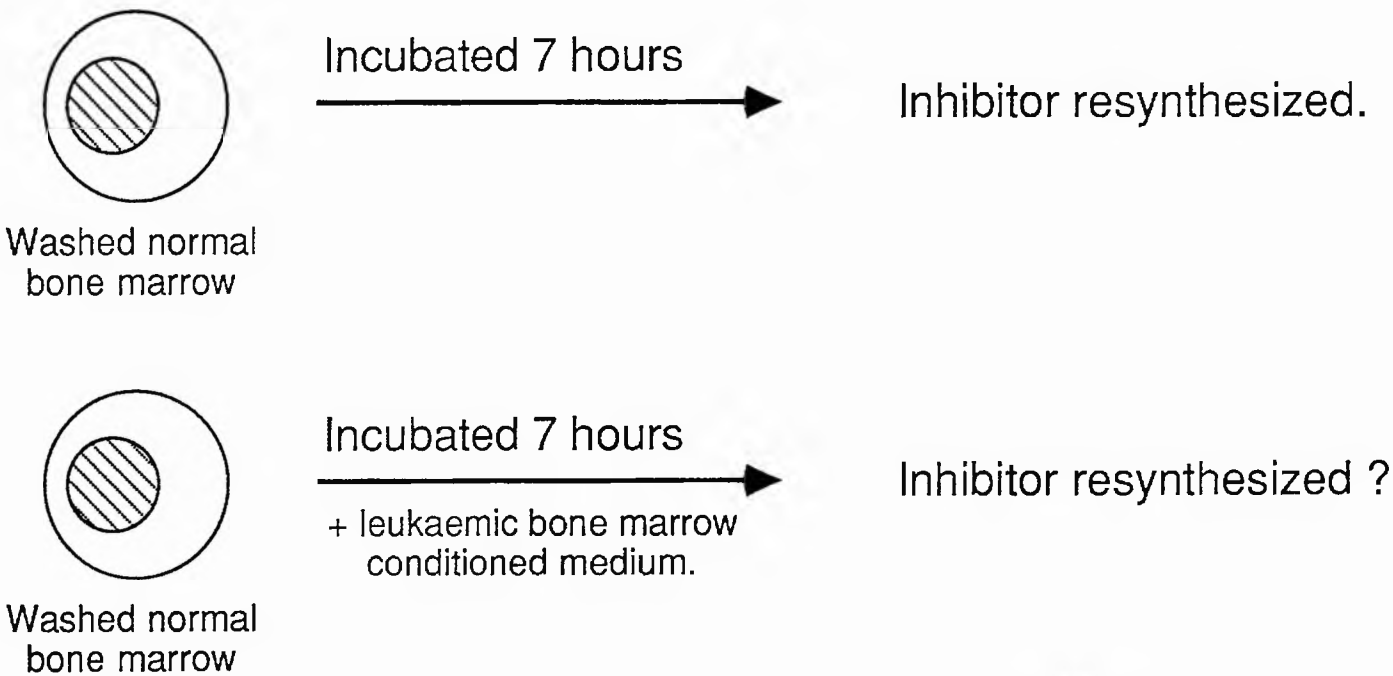


Figure 34: Investigating the effects of leukaemic bone marrow conditioned medium on inhibitor production.

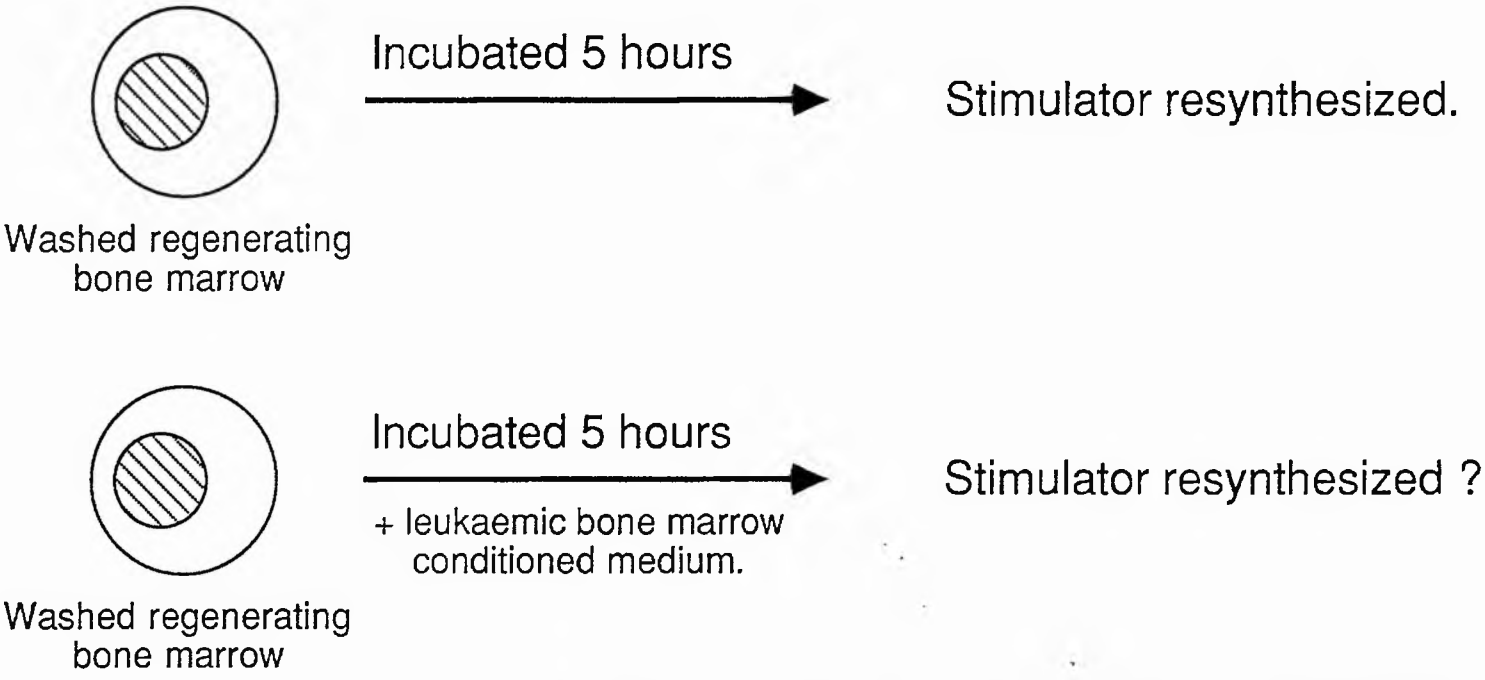


Figure 35: Investigating the effects of leukaemic bone marrow conditioned medium on stimulator production.

2.24 The low molecular weight, haemoregulatory tetrapeptide AcSDKP and tripeptide analogues.

Tetrapeptide AcSDKP and a number of tripeptide analogues were synthesized [Thierry *et al*,1990] and kindly supplied by Dr.Maryse Lenfant and Dr.Josaine Thierry (Institut de Chimie des Substances Naturelles, CNRS, Gif-sur-Yvette, France). Peptides were supplied as lyophilized HPLC fractions and stored at -20°C.

Samples were dissolved in a volume of *serum-free Dulbecco's medium sufficient to produce 100 ng peptide/ml (Approximately 10^{-7} Molar, M), aliquoted and frozen (-20°C). (*Serum is reported to enzymatically degrade the peptides (AcSDKP is reported to have a half-life of approximately 20 hours in 10% (v/v) heat inactivated foetal calf serum) and serum albumin is reported to adversely bind the peptides [M.Lenfant, personal communication]). Dissolving the peptides in the absence of serum protein, peptide manipulation was kept to a minimum and performed in plastic containers. Dissolved peptides were stored frozen (-20°C) in polypropylene cryotubes (Nunc.). Individual 100 ng/ml peptide aliquots were not refrozen once thawed and were stored at +4°C until required.

2.25 Investigation of the direct effects of the low molecular weight peptides on GM-CFC and HPP-CFC proliferation.

Direct stimulatory and/or inhibitory action of the low molecular weight peptides on both GM-CFC and HPP-CFC was investigated. Normal bone marrow (NBM) was obtained from the femora of 8-12 week old CBA/H mice and regenerating bone marrow (RBM) from mice 3 days after a 2 Gy whole body X-irradiation dose. A single cell

suspension of NBM/RBM was produced in Dulbecco's medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate and 2 mM L-glutamine, cells washed once, to remove endogenous regulatory activities, and resuspended in serum-free, supplemented Dulbecco's medium. Cellularity was determined and adjusted to $5x10^6$ NBM/RBM cells/ml.

100 ng/ml aliquots of appropriate peptide, stored at -20°C, were thawed and stored at +4°C. 1 ml, $5x10^6$ NBM/RBM cells/ml was added to each of two paired centrifuge tubes and incubated for 3 hours at 37°C with the addition of sufficient peptide to produce a final concentration of 1 ng/ml (approx. 10^{-9}M), or medium as control, at t=0, 1 and 2 hours.

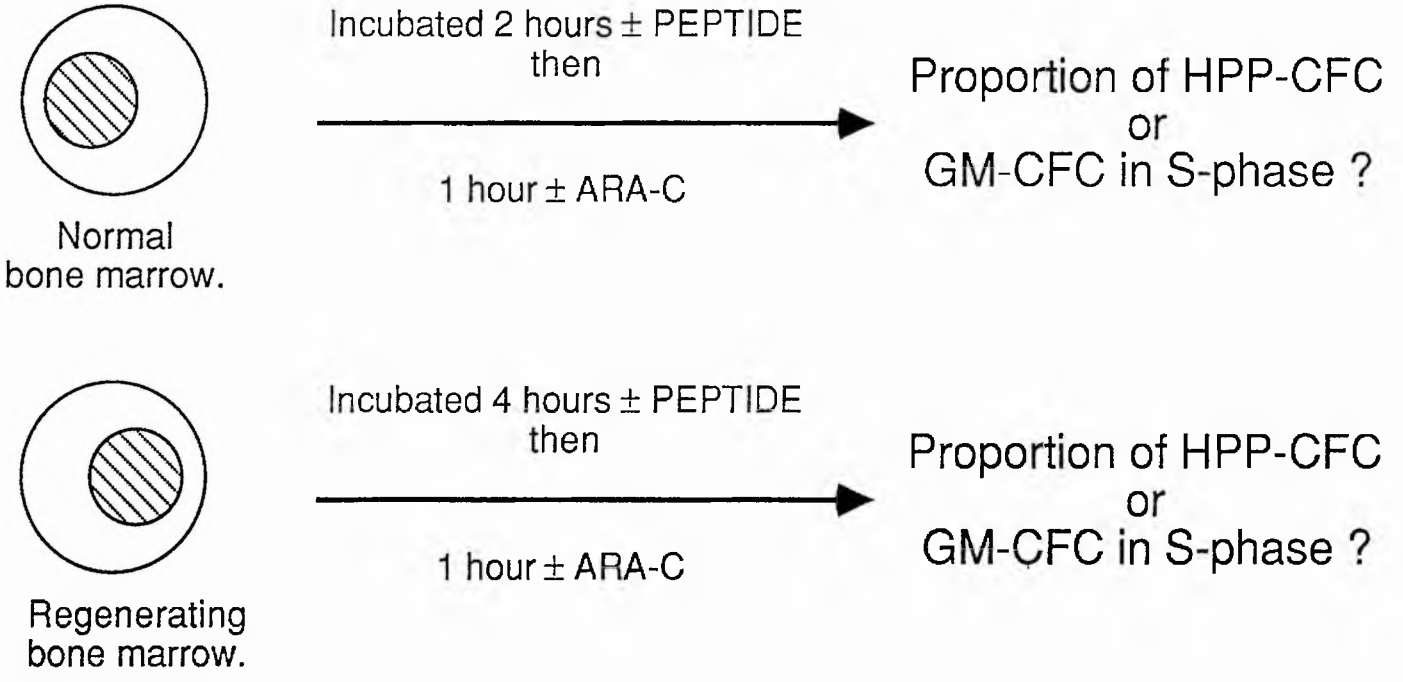
The proportion of cells in S-phase was determined by the addition of ARA-C, or medium, during the final hour of incubation (2.13). Cells were subsequently washed and cultured in the GM-CFC (2.9) and HPP-CFC (2.10) assays [*Figure 36*].

2.26 Investigation of the effects of low molecular weight peptides on *inhibitor* and/or *stimulator* action.

Normal bone marrow conditioned medium, a crude source of *inhibitor* (2.14) and medium conditioned by marrow from mice 7 days after a 4.5 Gy whole body X-irradiation dose, a crude source of *stimulator* (2.15), were produced under serum-free conditions in Dulbecco's medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate and 2 mM L-glutamine.

Inhibitor and *stimulator* were assayed as detailed (2.16 & 2.17) with the addition of a duplicate pair of centrifuge tubes to which appropriate peptide, to a final concentration of 1 ng/ml (approx. 10^{-9}M) was added at t=0 and at each subsequent hour of incubation.

Figure 36: Investigating the direct effects of haemoregulatory peptides on HPP-CFC and GM-CFC proliferation.



The final hour of incubation was performed in the absence or presence of ARA-C to determine the proportion of cells in S-phase (2.13). Cells were washed and subsequently cultured in the HPP-CFC assay (2.10) [*Figure 37*].

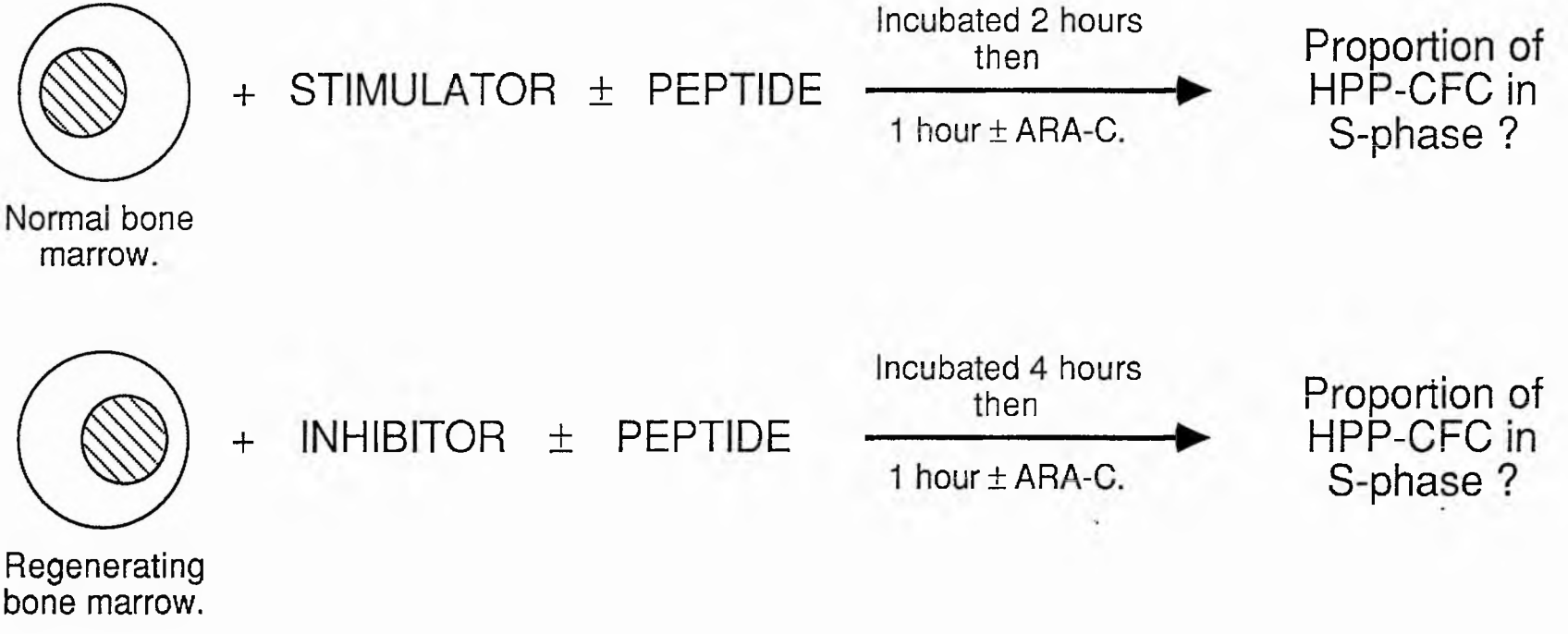
2.27 Investigation of the indirect effects of the low molecular weight peptides on *inhibitor* and/or *stimulator* production.

Normal bone marrow (NBM) was obtained from 8-12 week old CBA/H mice and regenerating bone marrow (RBM) from mice 7 days after a 4.5 Gy whole body X-irradiation dose. A single cell suspension of NBM/RBM was produced in serum-free Dulbecco's medium supplemented with 50 I.U./ml benzyl penicillin, 50 µg/ml streptomycin sulphate and 2 mM L-glutamine. Cells were washed and resuspended, cellularity determined and adjusted to 15-20x10^6 NBM/RBM cells/ml.

100 ng/ml aliquots of appropriate peptide, stored at -20°C, were thawed and stored at +4°C. NBM/RBM cells were incubated for 7/5 hours at 37°C, with either the addition of sufficient peptide to produce a final concentration of 1 ng/ml (approx. 10^{-9}M), or an equivalent volume of medium, at t=0 and at every subsequent hour of incubation.

After incubation, the NBM/RBM single cell suspension was centrifuged (x1000 rpm, 15 mins.), supernatant retained, repeatedly centrifuged (x2000 rpm, 15 mins. and x3000 rpm, 15 mins.) and frozen (-20°C). Medium conditioned by the NBM/RBM suspension in the absence and presence of peptide was subseqently assayed for *stimulator* and *inhibitor* (2.16 & 2.17) [*Figures 38 & 39*].

Figure 37: Investigation of the effects of the haemoregulatory peptides on stimulator and/or inhibitor action.



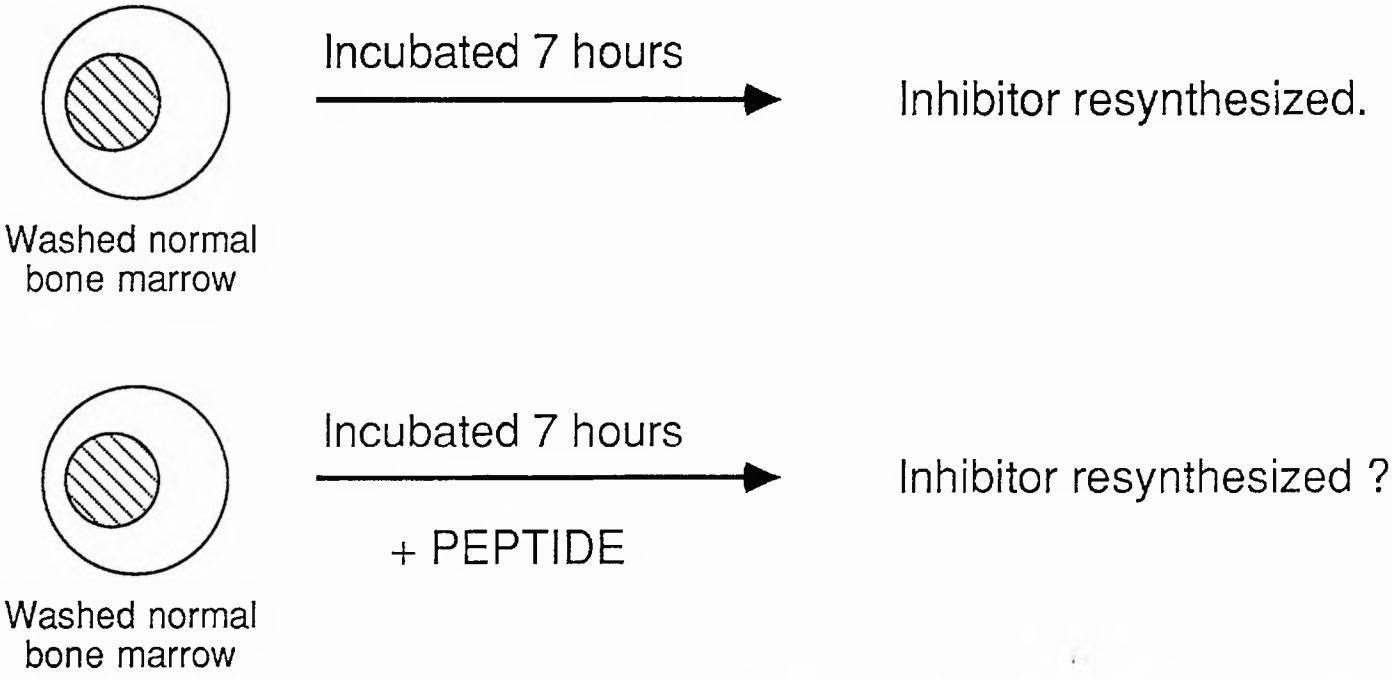


Figure 38: Investigating the effects of the haemoregulatory peptides on inhibitor production.

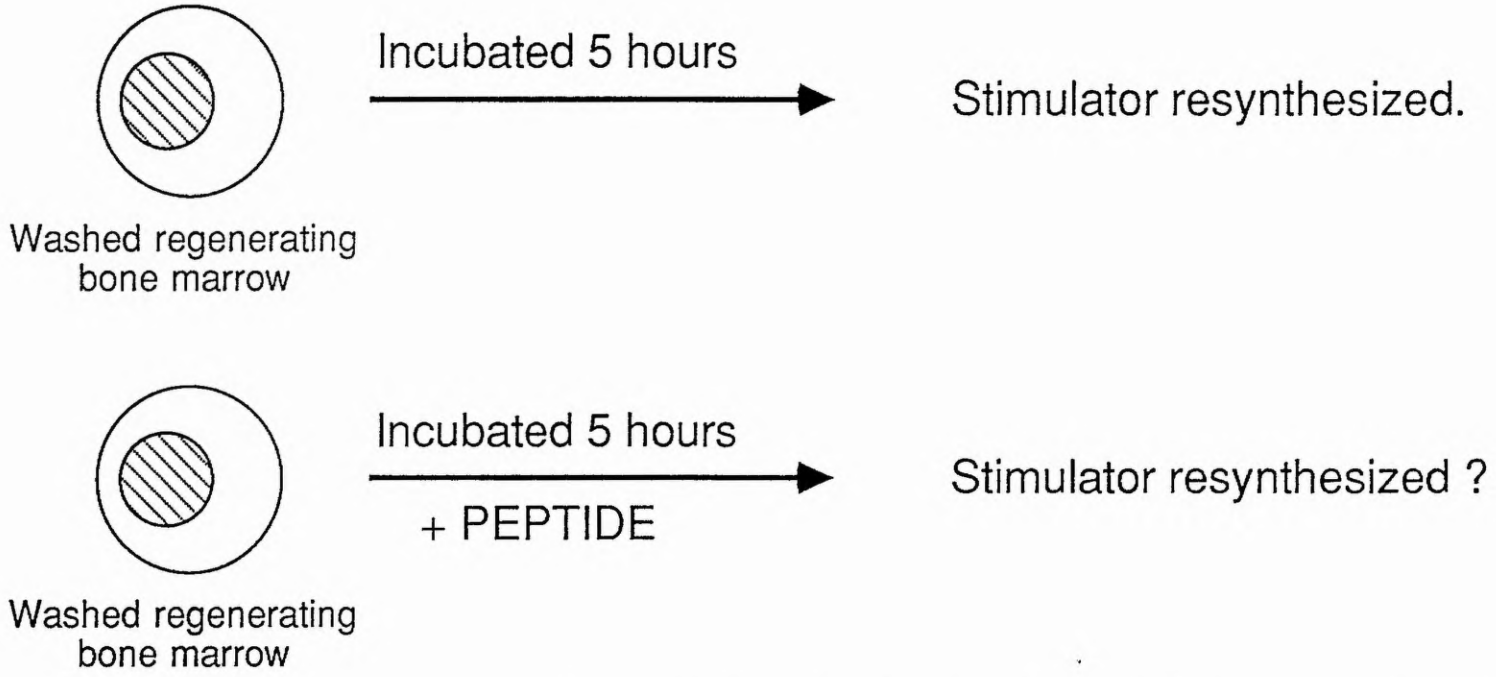


Figure 39: Investigating the effects of the haemoregulatory peptides on stimulator production.

Chapter 3:

Results.

Section 1

The characterization of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ assay.

3.1 Characterization of the potency of medium conditioned by cell lines and recombinantly-derived haematopoietic colony-stimulating factors.

3.1.1 Characterization of the colony-stimulating activity of medium conditioned by WEHI 3B, L929 and AF1-19T cell lines.

Procedure:

The colony-stimulating potential of medium conditioned by WEHI 3B myelomonocytic leukaemic cell line, L929 fibroblast cell line and AF1-19T malignant histiocytosis sarcoma virus-transformed rat kidney cell line was assessed by titration using the *in vitro* granulocyte-macrophage colony-forming cell (GM-CFC) assay (2.9).

0, 50, 100, 150 and 200 µl volumes of each conditioned medium were assayed with 1 ml, $5x10^4$ normal bone marrow cells/ml in 0.3% agar, producing a final conditioned medium concentration of 0, 5, 10, 15 and 20% respectively. At assay, "colonies" were defined as a collection of in excess of 50 cells. Collections of less than 50 cells were defined as "clusters" and were not counted. The number of colonies stimulated was related to the concentration of conditioned medium and dose-response curves established.

Results:

In the absence of conditioned medium, no cellular proliferation was observed. Increasing the concentration of the conditioned medium, initially produced a proportional increase in colony numbers, reaching a maximum, plateau value beyond which no significant increase in colony numbers was observed.

The optimal volume/concentration of conditioned medium was determined as the minimum which proved capable of stimulating near maximal colony numbers. A 100 µl/10% (v/v)

volume/concentration of conditioned medium appeared to fulfill this criterion [*Figures 40a & 40b*].

3.1.1.1 WEHI 3B melomonocytic leukaemic cell line conditioned medium.

Medium conditioned by WEHI 3B myelomonocytic leukaemic cell line is a crude source of interleukin 3 (IL3)/multi-CSF [Ihle *et al*,1982](1.21) and stimulates the proliferation of colony-forming cells of a variety of haematopoietic lineages in the GM-CFC assay. At assay this was observed in the variety of both colony sizes and colony morphologies. Colony sizes varied considerably between 'colonies' clearly in excess of 50 cells, to 'clusters' of 10-15 cells, while colony morphologies varied between compact 'tight' colonies, less compact 'loose' colonies and colonies of both a 'tight' and 'loose' morphology ('mixed' colonies). At assay, no distinction was made as to colony morphology, colony size (in excess of 50 cells) was the only criterion applied [*Plate 1, Slide C*].

3 batches of WEHI 3B cell line conditioned medium were used. Each batch was similarly titrated and no significant variation in colony-stimulating potential between individual batches was observed [*Figure 40*].

3.1.1.2 L929 fibroblast cell line conditioned medium.

Medium conditioned by the L929 fibroblast cell line is a crude source of macrophage colony-stimulating factor (M-CSF/CSF-1) [Burgess *et al*,1977](1.21). Primarily it stimulates the proliferation of lineage-committed macrophage progenitors within murine haematopoietic tissue. At assay colonies varied in cellularity between

those clearly in excess of 50 cells to 'clusters' of 10-15 cells, while colony morphology showed little variation. L929 cell line conditioned medium-stimulated colonies were characterized by a relatively compact nature [*Plate 1, Slide B & Plate 2, Slide A*].

2 batches of L929 cell line conditioned medium were used. Both batches were similarly titrated and no significant variation in colony-stimulating potential was observed [*Figure 40b*].

3.1.1.3 AF1-19T cell line conditioned medium.

Medium conditioned by AF1-19T malignant histiocytosis sarcoma virus-transformed rat kidney cell line, is a crude source of granulocyte-macrophage colony-stimulating factor (GM-CSF) [Kriegler *et al*,1990]. Primarily it stimulates the proliferation of lineage-committed granulocyte and macrophage progenitors and also bipotential granulocyte-macrophage progenitors within the murine haematopoietic tissue. This is consistent with the variety of colony morphologies observed at assay. The 'potency' of AF1-19T cell line conditioned medium proved consistently low, this may be a direct consequence of the species-specificity of GM-CSF. AF1-19T is a rat-derived cell line, while the haematopoietic tissue under assay is murine. A 100 µl/10% (v/v) volume/concentration of AF1-19T cell line conditioned medium was used. 1 batch of AF1-19T conditioned medium was used during the project [*Figure 40c*].

Figure 40a: GM-CFC titration of
WEHI 3B cell line conditioned medium.

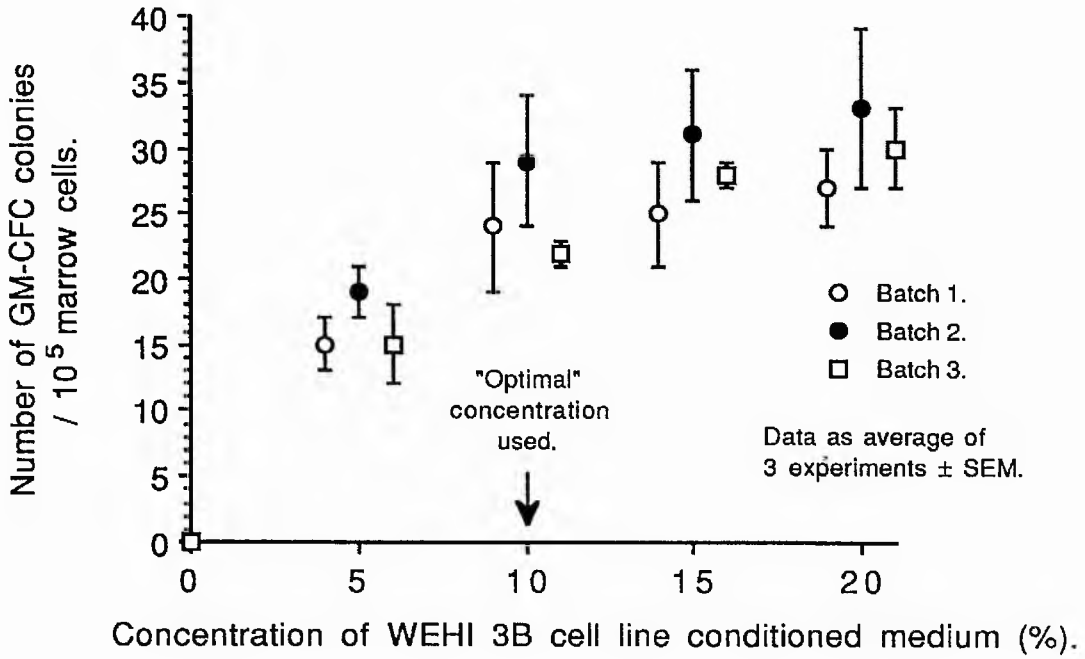


Figure 40b: GM-CFC titration of
L929 cell line conditioned medium.

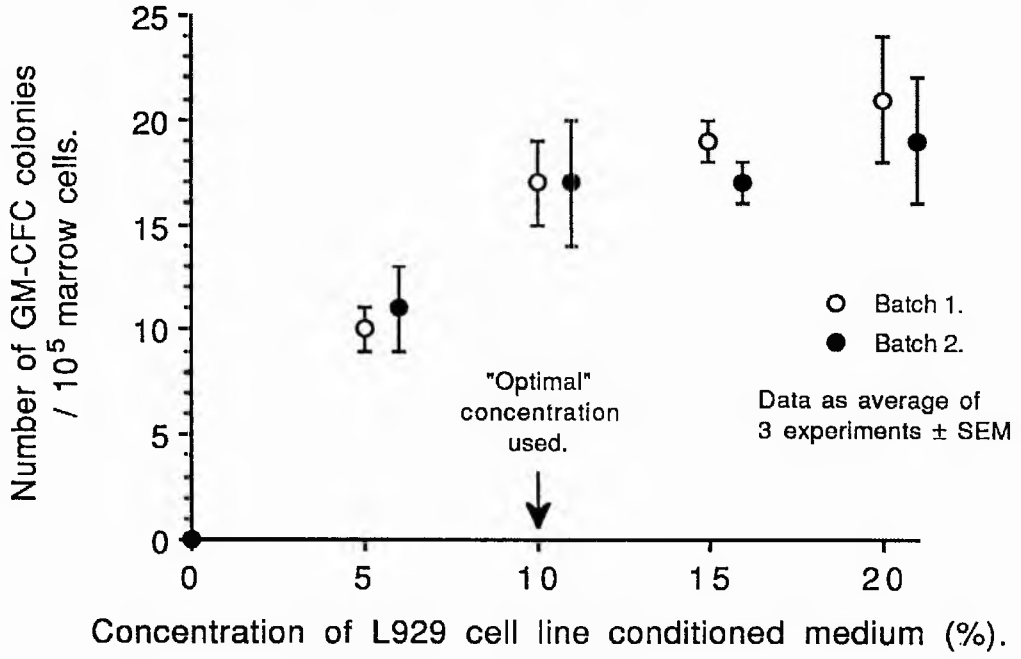
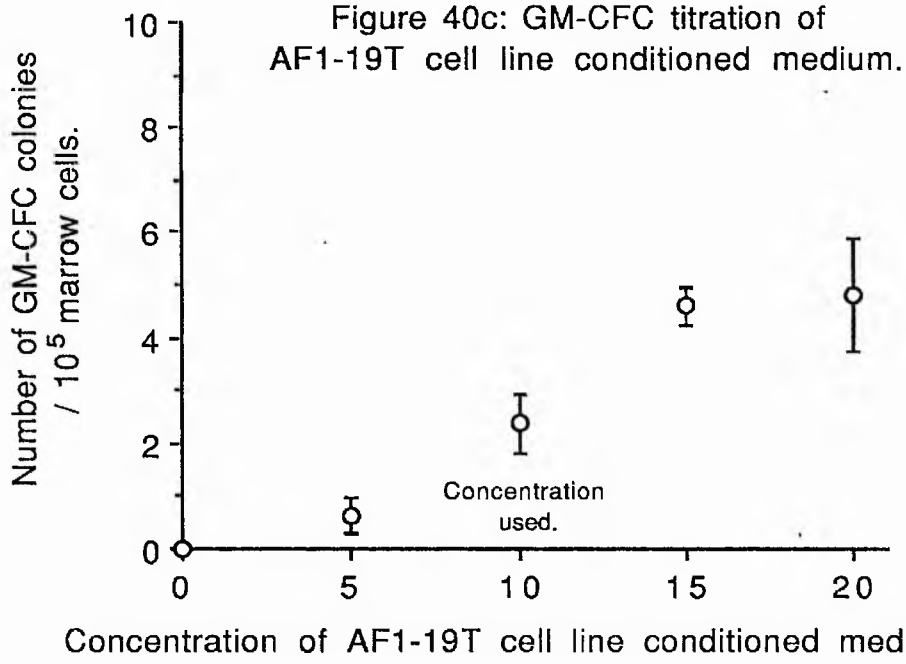


Figure 40c: GM-CFC titration of AF1-19T cell line conditioned medium.



3.1.2 Characterization of the potency of recombinantly-derived haematopoietic colony-stimulating factors.

Procedure:

The haematopoietic activity of the recombinantly-derived colony-stimulating factors was unknown and was investigated by use of both the *in vitro* GM-CFC assay (2.9) and the tritiated thymidine uptake assay (2.7).

Recombinant murine interleukin 3 (rmuIL3)/multi-CSF and recombinant human macrophage colony-stimulating factor (rhuM-CSF/CSF-1) were kindly supplied by Genetics Institute as medium conditioned by engineered CHO cells. Recombinant murine granulocyte-macrophage colony-stimulating factor (rmuGM-CSF) was kindly supplied by both Genetics Institute, as medium conditioned by engineered COS cells and Immunex, as a lyophilized protein sample.

The material was serially diluted prior to *in vitro* GM-CFC and tritiated thymidine uptake assay. For comparison, a 10% dilution of WEHI or L929 cell line conditioned medium was used as a crude source of interleukin 3 (IL3)/multi-CSF or M-CSF/CSF-1 respectively.

In the GM-CFC assay 100 µl volumes of appropriately diluted factor were incubated with 1 ml, $5x10^4$ normal bone marrow cells/ml in 0.3% agar. The final factor concentration was thus x0.1 that added. 5 dishes were produced *per* factor dilution. In the tritiated thymidine uptake assay, 100 µl volumes of appropriately diluted factor were incubated with 100 µl, $7x10^5$ normal bone marrow cells/ml. The final factor concentration was thus x0.5 that added. 4-12 microtitre wells were produced *per* factor dilution.

For each recombinant factor, GM-CFC and tritiated thymidine uptake data were related to factor dilution, and dose-response curves

established. The optimal volume/concentration of recombinantly-derived factor was determined as that which stimulated near maximal colony numbers/cellular proliferation.

Results:

3.1.2.1 Recombinantly-derived murine interleukin 3 (rmuIL3)/multi-CSF.

With reference to both GM-CFC and tritiated thymidine uptake titration data of rmuIL3 (Genetics Institute), an optimal dilution of 1:500 (of Genetics Institute rmuIL3 sample) was determined [*Figure 41a*].

3.1.2.2 Recombinantly-derived human macrophage colony-stimulating factor (rhuM-CSF)/CSF-1.

With reference to both GM-CFC and tritiatied thymidine uptake titration data of rhuM-CSF (Genetics Institute), an optimal concentration of 30 units*/ml was determined [*Figure 41b*].

3.1.2.3 Recombinantly-derived murine granulocyte-macrophage colony-stimulating factor (rmuGM-CSF).

Extensive *in vitro* GM-CFC titration of rmuGM-CSF (Immunex) had been previously performed in this laboratory and an optimal concentration of 40 units*/ml determined.

(*- rhuM-CSF (Genetics Instutute) and rmuGM-CSF (Immunex) were supplied as samples of specified units of activity. The definition of 'units' in each case was supplied, although subsequent titration of

Figure 41a: Tritiated thymidine uptake titration of rmulL3.

Data as average of 12 microtitre wells/rmulL3 diln.(±SEM).

Figure 41a: GM-CFC titration of rmulL3.

Data as average of 5 dishes /rmulL3 diln.(±SEM).

Figure 41b: Tritiated thymidine uptake titration of rhuM-CSF.

Data as average of 4 microtitre wells/rhuM-CSF diln.(±SEM).

Figure 41b: GM-CFC titration of rhuM-CSF.

Data as average of 5 dishes / rhuM-CSF concentration (±SEM).

the factors allowed the 'potency' of the factors to be determined. 'Units' were retained as reference values to assist in factor dilutions.)

3.1.3 Characterization of the activity of recombinantly-derived human interleukin 1 (rhuIL1).

IL1 activity is determined by measuring the tritiated thymidine incorporation by thymocytes cultured for 3 days in the presence of an appropriate concentration of concanavalin A and serially diluted IL1. IL1 activity is defined in units with 1 unit/ml concentration being that which stimulates 50% maximal thymocyte proliferation.

3.1.3.1 Titration of concanavalin A (Con A)(2.8a).

Procedure:

A 100 ul volume of Con A at a concentration sufficient to produce a final concentration of 0-15 µg/ml, was added to 100 µl, 10^7 thymocytes/ml, to determine the concentration of Con A which would not itself stimulate thymocyte proliferation, but which would best potentiate the interleukin 1-associated thymocyte proliferation.

Result:

A 1.5 µg/ml concentration of Con A was determined as a concentration which would best potentiate the IL1-associated thymocyte proliferation [*Figure 41c*].

3.1.3.2 Titration of recombinantly-derived human interleukin 1 (rhuIL1)(2.8b).

Procedure:

A 50 µl volume of appropriately diluted interleukin 1 (rhuIL1-alpha (Genetics Institute and Immunex) and IL1-beta (Immunex)), sufficient to produce a final concentration of 0-100 units/ml was added to 100 µl, 10^7 thymocytes/ml and 50 µl Con A at a concentration sufficient to produce a final concentration of 1.5 µg/ml, and the whole incubated for 3 days.

Results:

An optimal interleukin 1 (rhuIL1-alpha (Genetics Institute and Immunex) and IL1-beta (Immunex)) concentration of 5 units/ml was determined as active in the thymocyte proliferation assay [*Figure 41d*].

3.1.3.3 GM-CFC activity of recombinantly-derived human interleukin 1 (rhuIL1).

Procedure:

100 µl volumes of appropriately diluted rhuIL1-alpha (Genetics Institute and Immunex) and rhuIL1-beta (Immunex), sufficient to produce a final concentration of 0, 5, 10 and 20 units/ml were incubated with 1 ml, $5x10^4$ normal bone marrow cells/ml in 0.3% agar, and incubated for 7 days (2.9).

Result:

Interleukin 1, as either rhuIL1-alpha (Genetics Institute or Immunex) or rhuIL1-beta (Immunex), failed to demonstrate colony-stimulating activity over the 0-20 unit/ml concentration range assayed.

Figure 41c: Tritiated thymidine uptake titration of concanavalin A (Con A).

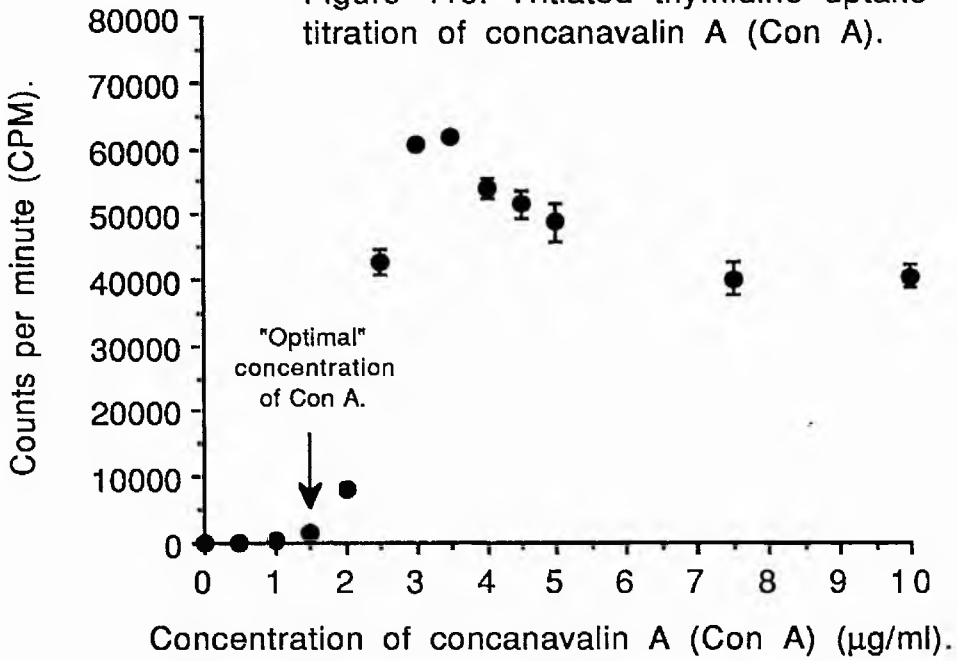
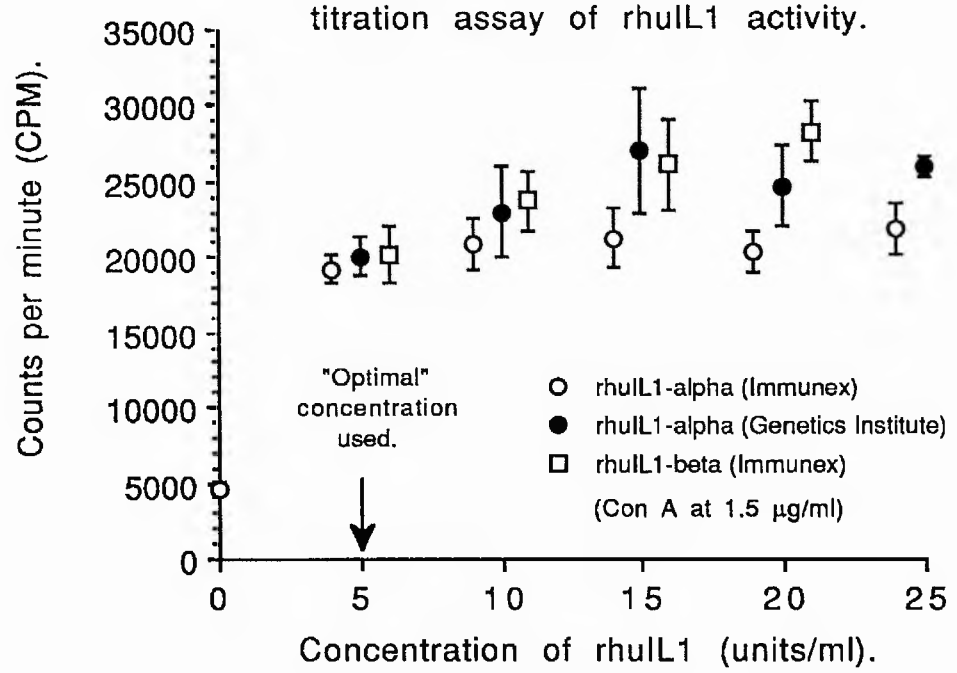


Figure 41d: Thymocyte proliferation titration assay of rhuIL1 activity.



3.1.4 Evidence of interleukin 1 activity in the WEHI 3B, L929 or AF1-19T cell line conditioned media.

3.1.4.1 Investigation of the capacity of WEHI 3B, L929 and AF1-19T cell line conditioned media to stimulate thymocyte proliferation.

Procedure:

100 ul of appropriately diluted WEHI 3B, L929 or AF1-19T cell line conditioned medium, sufficient to produce a final concentration of 0, 5, 10, 15 and 20% (v/v) was incubated with 100 µl, 10^7 thymocytes/ml, to determine the capacity of each conditioned medium to stimulate thymocyte proliferation.

Results:

WEHI 3B, L929 and AF1-19T cell line conditioned media, do not stimulate thymocyte proliferation over the 0-20% (v/v) concentation range assayed [*Figure 41e*].

3.1.4.2 Investigation of the interleukin 1 activity of WEHI 3B, L929 and AF1-19T cell line conditioned media.

Procedure:

50 ul appropriately diluted WEHI 3B, L929 or AF1-19T cell line conditioned media, sufficient to produce a final concentration of 0, 5, 10, 15 and 20% (v/v) was incubated with 100 µl, 10^7 thymocytes/ml and 50 µl Con A, at a sufficient concentration to produce a final concentration of 1.5 µg/ml. Con A-potentiated, thymocyte proliferation would be indicative of interleukin 1 activity in the conditioned media.

Results:

No evidence of Con A-potentiated thymocyte proliferation was observed implying that over a 0-20% (v/v) concentration, the conditioned media demonstrate no interleukin 1 activity [*Figure 41f*].

3.2 Characterization of haematopoietic colony-stimulating factor synergism.

Specific colony-stimulating factors are able to interact when combined, to stimulate the proliferation of a colony-forming cell population characterized by a high proliferative potential. This high proliferative potential colony-forming cell (HPP-CFC) population is only observed in the presence of combinations of colony-stimulating factors and is distinct from the colony-forming cell population stimulated in the presence of a single colony-stimulating factor. This ability of specific colony-stimulating factors to "synergize" was investigated using both crude conditioned medium and recombinantly-derived factors.

Procedure:

'Optimal' dilutions of crude and recombinant colony-stimulating factors (3.1), were combined in the *in vitro* HPP-CFC assay (2.10). Any synergism between colony-stimulating factors was investigated. In the case of colony-stimulating factor synergism, large, macroscopic colonies were evident at assay.

The ill-defined nature of medium conditioned by WEHI 3B, L929 and AF1-19T cell lines is such that, while they may prove to be crude sources of interleukin 3 (IL3)/multi-CSF, M-CSF/CSF-1 and GM-CSF respectively, other factors may be present, and responsible for establishing synergistic interactions. Use of the rigorously defined, recombinantly-derived factors was hoped to demonstrate the factors

Figure 41e: Stimulation of thymocyte proliferation by WEHI 3B, L929 or AF1-19T conditioned media.

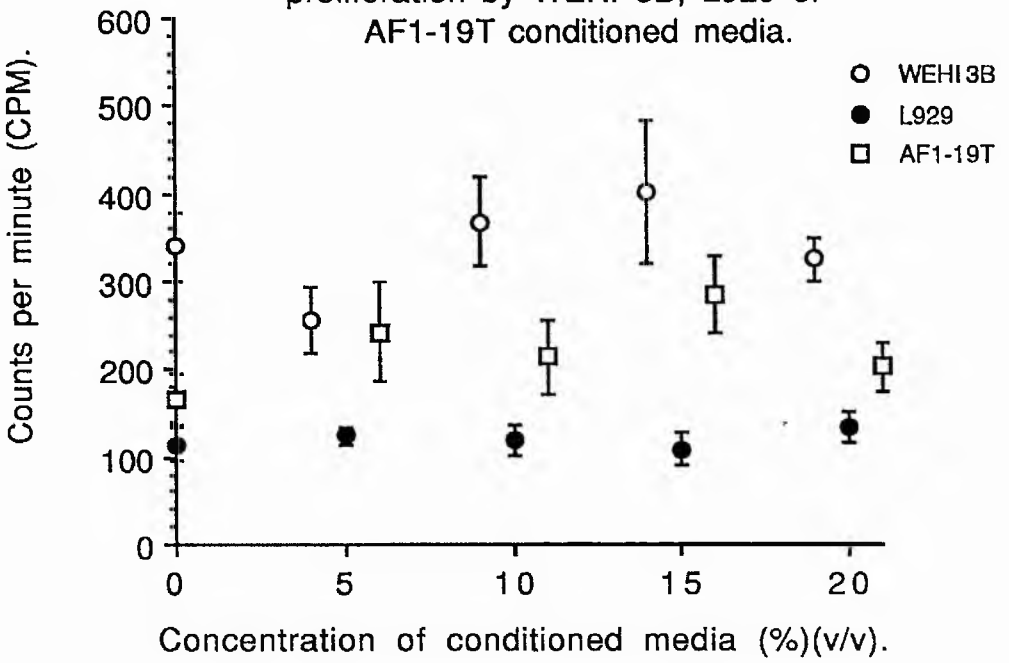
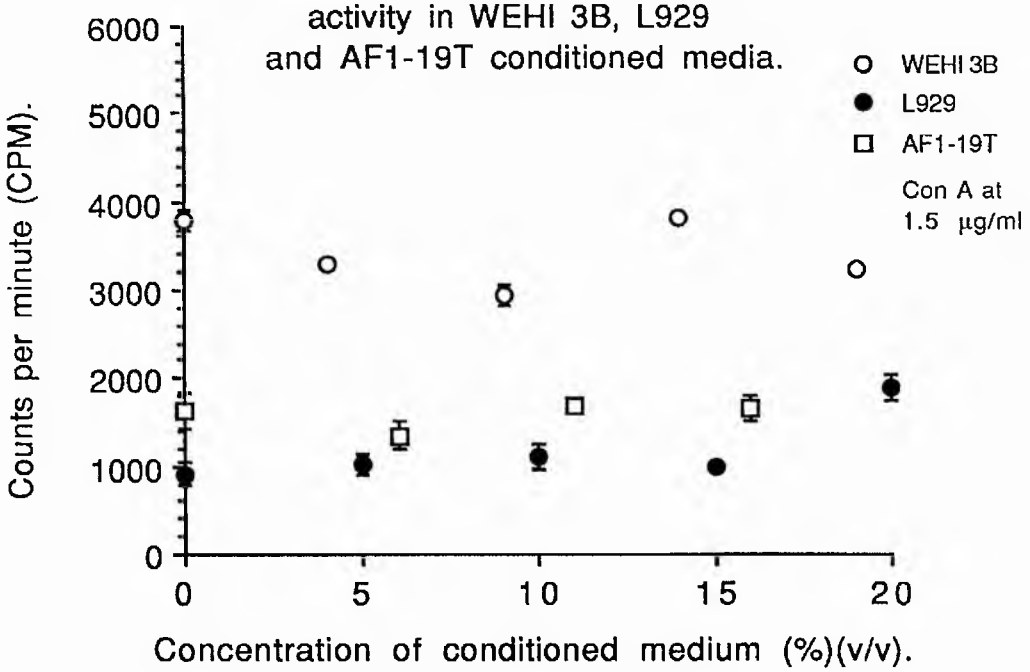


Figure 41f: Assay of interleukin 1 activity in WEHI 3B, L929 and AF1-19T conditioned media.



responsible for the stimulation of the HPP-CFC population were indeed interleukin 3 (IL3)/multi-CSF, M-CSF/CSF-1 and GM-CSF.

Results:

3.2.1 Synergism between interleukin 3 (IL3)/multi-CSF and macrophage colony-stimulating factor (M-CSF/CSF-1).

Interleukin 3 (IL3)/multi-CSF, as both medium conditioned by the WEHI 3B cell line and rmuIL3, and M-CSF/CSF-1, as both medium conditioned by the L929 cell line and rhuM-CSF were observed to synergize. Medium conditioned by the WEHI 3B cell line and rhuM-CSF, and rmuIL3 and medium conditioned by the L929 cell line, were also observed to synergize [*Plate 1, Slide D & Plate 2, Slide D*].

3.2.2 Synergism between granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF/CSF-1).

GM-CSF as both medium conditioned by the AF1-19T cell line and rmuGM-CSF, and M-CSF/CSF-1 as both medium conditioned by the L929 cell line and rhuM-CSF were observed to synergize. Significantly, despite the relatively low colony-stimulating activity of the medium conditioned by the AF1-19T cell line (3.1.1), it proved sufficient to synergize with both crude and recombinantly-derived sources of M-CSF/CSF-1. rmuGM-CSF and medium conditioned by the L929 cell line were also observed to synergize [*Plate 2, Slides B & C*].

3.2.3 Synergism between interleukin 3 (IL3)/multi-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Interleukin 3 (IL3)/multi-CSF as both medium conditioned by the WEHI 3B cell line and as rmuIL3, and GM-CSF as both medium conditioned by the AF1-19T cell line and rmuGM-CSF were observed to synergize. Both medium conditioned by the WEHI 3B cell line and rmuGM-CSF, and rmuIL3 and medium conditioned by the AF1-19T cell line were also observed to synergize.

3.2.4 Colony-stimulating factor synergism with interleukin 1 (IL1).

Interleukin 1 as either Genetics Institute rhuIL1-alpha, or Immunex rhuIL1-alpha or -beta, did *not* demonstrate a synergistic interaction with interleukin 3 (IL3)/multi-CSF, as either WEHI 3B cell line conditioned medium or rmuIL3, M-CSF/CSF-1, as either L929 cell line conditioned medium or rhuM-CSF, or GM-CSF, as either AF1-19T cell line conditioned medium or rmuGM-CSF. The pattern of colony growth observed in the presence of interleukin 3 (IL3)/multi-CSF, M-CSF/CSF-1 or GM-CSF (as either crude conditioned media or recombinantly-derived factors) was unchanged in the presence of interleukin 1.

3.3 Characterization of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ assay.

Before setting the strict criteria by which the HPP-CFC$_{IL3+CSF-1}$-derived colonies were assayed, it proved necessary to optimize the *in vitro* culture conditions. Many aspects of the HPP-CFC$_{IL3+CSF-1}$ assay

were adaptations of the GM-CFC assay and thus may not have been optimal in the HPP-$CFC_{IL3+CSF-1}$ assay.

3.3.1 The optimal cell dose for HPP-$CFC_{IL3+CSF-1}$ assay.

Procedure:

The number of normal murine bone marrow cells plated in the 0.3% agar culture layer of the bilayer HPP-$CFC_{IL3+CSF-1}$ assay, was titrated over a range $5x10^3$-$1x10^5$ normal bone marrow cells/ml.

Result:

Prior to the specific definition of HPP-$CFC_{IL3+CSF-1}$-derived colonies, a cellularity of 2.0-$2.5x10^4$ normal bone marrow cells/ml was found to give 'countable' numbers of HPP-CFC colonies. A lower cellularity gave proportionately lower HPP-$CFC_{IL3+CSF-1}$ numbers, while higher cellularities lead to colony 'overcrowding' and made subsequent colony-counting difficult. HPP-$CFC_{IL3+CSF-1}$ were also demonstrable in normal, non-haematopoietic, murine spleen, although to achieve colony numbers comparable to those of 2.0-$2.5x10^4$ normal bone marrow cells (approximately 25 HPP-CFC colonies), 10^6 spleen cells were required. HPP-$CFC_{IL3+CSF-1}$ were also demonstrable in haematopoietic, murine foetal liver, with 5.0-$10.0x10^4$ foetal liver cells producing colony numbers comparable to 2.0-$2.5x10^4$ normal bone marrow cells.

3.3.2 The concentration of colony-stimulating factors to induce optimal HPP-CFC$_{IL3+CSF-1}$ proliferation.

Procedure:

The concentration of colony-stimulating factors inducing optimal HPP-CFC$_{IL3+CSF-1}$ proliferation was investigated. As previously demonstrated, the characteristic HPP-CFC populations are only observed in the presence of combinations of colony-stimulating factors.

Results:

Titration of medium conditioned by the WEHI 3B cell line, as a crude source of interleukin 3 (IL3)/multi-CSF, against a fixed concentration of medium conditioned by the L929 cell line (10% v/v), as a crude source of M-CSF/CSF-1, demonstrated that a GM-CFC-derived 'optimal' concentration of colony-stimulating factor, was not a prerequisite for synergism. HPP-CFC$_{IL3+CSF-1}$ were demonstrable at markedly sub-optimal WEHI 3B cell line conditioned medium concentrations (as determined by the GM-CFC assay). Increasing the concentration of the WEHI 3B cell line conditioned medium, did not significantly increase the absolute number of HPP-CFC$_{IL3+CSF-1}$, although the degree of cellular proliferation *per* HPP-CFC$_{IL3+CSF-1}$-derived colony was increased, producing a stronger INT-staining colony at assay. This finding correlates with the observation that medium conditioned by the AF1-19T cell line demonstrated little colony-stimulating activity in the GM-CFC assay, although proving capable of synergism to stimulate the proliferation of the HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A" population.

A concentration of 10% (v/v) each of WEHI 3B cell line conditioned medium and L929 cell line conditioned medium (as crude sources of interleukin 3 (IL3)/multi-CSF and M-CSF/CSF-1

respectively) was determined as that which would promote HPP-$CFC_{IL3+CSF-1}$ proliferation.

3.3.3 The optimal time of assay of HPP-$CFC_{IL3+CSF-1}$.

Procedure:

The time of assay of the HPP-$CFC_{IL3+CSF-1}$ population was determined by performing a time study. Cultures of $2.5x10^4$ normal bone marrow cells/ml in 0.3% agar were incubated over a 'feeder' layer containing 10% (v/v) of WEHI 3B cell line (as a crude source of interleukin 3 (IL3)/multi-CSF), or L929 cell line (as a crude source of M-CSF/CSF-1), or a 10% (v/v) combination of each conditioned medium. Cultures were assayed after 8, 10, 12 and 14 days.

Results:

While a different pattern of colony growth was determined at as early as day 8 when comparing WEHI 3B, L929 and combined conditioned media stimuli, day 14 was chosen as a time which was considered to allow the full proliferative potential of the HPP-$CFC_{IL3+CSF-1}$ to develop and so increase the contrast between the HPP-$CFC_{IL3+CSF-1}$ and non-HPP-$CFC_{IL3+CSF-1}$-derived colonies [*Plate 3*].

3.3.4 The development of a criterion to define the HPP-$CFC_{IL3+CSF-1}$ population.

Procedure:

A criterion was required by which colonies derived from HPP-$CFC_{IL3+CSF-1}$ could be defined. The unique aspect of the HPP-CFC-derived colonies is their relatively large size in comparison to non-HPP-CFC-derived colonies. Non-HPP-CFC-derived colonies, as

stimulated by the presence of single colony-stimulating factors in the HPP-CFC assay system, were rarely observed to exceed a 2mm diameter, while HPP-CFC-derived colonies, as stimulated by the presence of a synergistic combination of colony-stimulating factors, were found to exceed a 2mm diameter.

Result:

A size criterion: "*colonies in excess of a 2mm diameter*" was thus adopted as the definition of an HPP-$CFC_{IL3+CSF-1}$-derived colony [*Plate 4 & Plates 5A & 5B*].

3.3.5 The cellular composition of the HPP-$CFC_{IL3+CSF-1}$-derived colonies.

Procedure:

In specific experiments the semi-solid 0.3% agar in the bilayer HPP-CFC assay was replaced by 0.9% methylcellulose (2.12). The use of methylcellulose allowed an investigation of the cellular composition of the HPP-$CFC_{IL3+CSF-1}$-derived colonies to be made. Individual colonies were 'plucked' from the methylcellulose after 14 days incubation in the presence of 10% (v/v) each of WEHI 3B and L929 cell line conditioned media (as crude sources of interleukin 3 (IL3)/multi-CSF and M-CSF/CSF-1 respectively) and cytospin preparations produced.

Results:

Jenner-Giemsa staining of cells derived from individual HPP-$CFC_{IL3+CSF-1}$-derived colonies revealed the majority of colonies were composed largely of macrophages. This would be consistent with the presence of the M-CSF/CSF-1 (as medium conditioned by the L929 cell line). The macrophages observed were characterized by a heavily vacuolated cytoplasm [*Plate 6A*]. In some cases, in

association with the mature macrophages, populations of immature cells were observed [*Plate 6B*]. In other cases, a morphologically distinct population of cells, characterized by a less vacuolated cytoplasm containing granules and with pale staining nuclei, were observed [*Plate 6C*].

3.3.6 The cellularity of 14 day HPP-CFC$_{IL3+CSF-1}$-derived colonies.

Procedure:

The HPP-CFC$_{IL3+CSF-1}$-derived colonies ranged in size from approximately 2-5mm in diameter. The use of 0.9% methylcellulose allowed the cellularity of individual colonies to be determined. Individual HPP-CFC$_{IL3+CSF-1}$-derived colonies were 'plucked' fom HPP-CFC assay at day 14 and cell counts performed of single cell suspensions of each colony using a Coulter Counter.

Result:

An average cellularity of $6.0(\pm 0.4)\times10^4$ cells/14 day HPP-CFC$_{IL3+CSF-1}$-derived colony was determined ($n=6$).

For comparison, a number of non-HPP-CFC-derived colonies, stimulated by either WEHI 3B (as a crude source of interleukin 3 (IL3)/multi-CSF) or L929 (as a crude source of M-CSF/CSF-1) cell line conditioned media, were investigated. Individal colonies were 'plucked' from HPP-CFC assay at day 14, pooled and a cell counts made of the resultant single cell suspension using a Coulter Counter. An average cellularity of 1.4×10^4 cells/14 day WEHI 3B cell line conditioned medium-stimulated colony and 0.8×10^4 cells/14 day L929 cell line conditioned medium-stimulated colony, was determined.

3.4 Femur cellularities and HPP-CFC$_{IL3+CSF-1}$ frequency in normal and sublethally X-irradiated CBA/H mice.

3.4.1 Femur cellularities in normal and sublethally X-irradiated CBA/H mice.

Procedure:

Femoral bone marrow was obtained from CBA/H mice as detailed (2.2). The majority of femoral marrow was expelled from the femoral cavity as a 'plug' by the initial flush of medium, however repeated flushing of the femoral cavity with medium ensured maximal marrow recovery. Average femur cellularities were determined by suspending the marrow of a known number of femora in a known volume of medium. A Coulter Count was performed on the resultant appropriately diluted, single cell suspension (2.3).

Result:

An average femoral cellularity of $14(\pm 1)x10^6$ cells ($\pm$SEM)($n=54$) was determined for normal, 9-12 week old, male CBA/H mice.

In specific experiments, CBA/H mice were subjected to sublethal, whole body X-irradiation, which induced haematopoietic damage and produced a 'regenerating' bone marrow. In CBA/H mice 3 and 7 days after sublethal whole body X-irradiation doses of 2 and 4.5 Gy respectively (2.15 & 2.16), a 57% reduction in femoral marrow cellularity was determined. In both cases, an average femur cellularity of $6(\pm 1)x10^6$ cells was observed ($\pm$SEM)($n=30$ and $n=7$ respectively).

3.4.2 HPP-CFC$_{IL3+CSF-1}$ frequency in normal and sublethally, whole body X-irradiated CBA/H femoral marrow.

3.4.2.1 HPP-CFC$_{IL3+CSF-1}$ frequency in normal CBA/H femoral marrow.

HPP-CFC$_{IL3+CSF-1}$ assay of normal CBA/H femoral marrow (assayed at 2.0-2.5x10^4 cells/ml) gave an average HPP-CFC$_{IL3+CSF-1}$ frequency of 100(±1)/10^5 marrow cells (±SEM)(n=340). In an average normal CBA/H femur of approximately 14x10^6 marrow cells, an HPP-CFC$_{IL3+CSF-1}$ frequency of 100/10^5 marrow cells, gives a total approximate femoral marrow complement of 14x10^3 HPP-CFC$_{IL3+CSF-1}$. Approximately 1 in every 1000 marrow cells will thus be a potential HPP-CFC$_{IL3+CSF-1}$ [*Figure 42*].

3.4.2.2 HPP-CFC$_{IL3+CSF-1}$ frequency in regenerating femoral marrow.

HPP-CFC$_{IL3+CSF-1}$ assay of femoral marrow from mice 3 days after a sublethal, whole body X-irradiation dose of 2 Gy (assayed at 5x10^4 regenerating bone marrow cells/ml) gave an average HPP-CFC$_{IL3+CSF-1}$ frequency of 29(±1)/10^5 regenerating marrow cells (±SEM)(n=210). In an average femur from a mouse 3 days after a 2 Gy whole body X-irradiation dose, of approximately 6x10^6 marrow cells, an HPP-CFC$_{IL3+CSF-1}$ of 29/10^5 marrow cells gives an approximate 2 Gy X-irradiated femoral marrow compliment of 2x10^3 HPP-CFC$_{IL3+CSF-1}$. Approximately 1 in every 3000 regenerating marrow cells will be a potential HPP-CFC$_{IL3+CSF-1}$.

Assayed 3 days after a whole body X-irradiation dose of 2 Gy, the total number of HPP-CFC$_{IL3+CSF-1}$ /femur is reduced by 85% in comparison to a normal non-irradiated femur. Hendry and Lajtha

[1977] reported an 80% reduction in "CFU-S" numbers in murine femora after a 1.5 Gy X-irradiation dose [*Figure 42*].

Figure 42: Summary of femur cellularities and HPP-$CFC_{IL3+CSF-1}$ frequencies in normal and X-irradiated CBA/H mice.

	Normal	*X-irradiated
Average femur cellularity ($x10^6$) (±SEM)	14(±1) (n=54)	6(±1) (n=30)
Average HPP-$CFC_{IL3+CSF-1}$ frequency (±SEM)	100(±1)/10^5 cells (n=340)	29(±1)/10^5 cells (n=210)
Approximate number of HPP-$CFC_{IL3+CSF-1}$/femur	$14x10^3$	$2x10^3$

*Marrow from mice 3 days after a whole body X-irradiation dose of 2 Gy.

Plates

Plate 1: This photograph illustrates the pattern of colony growth obtained from murine bone marrow after 14 days in semi-solid agar under different colony-stimulating factor regimes. Colonies observed are derived from $2.5x10^4$ normal bone marrow cells *per* 30mm diameter, 0.3% agar disc and have been stained with INT. Each agar disc is approximately 30mm in diameter.

Slide A: In the absence of haematopoietic colony-stimulating activity. No cellular proliferation is observed.

Slide B: In the presence of 10% (v/v) medium conditioned by the L929 fibroblast cell line, a crude source of macrophage colony-stimulating factor (M-CSF/CSF-1).

Slide C: In the presence of 10% (v/v) medium conditioned by the WEHI 3B myelomonocytic leukaemic cell line, a crude source of interleukin 3 (IL3)/multi-CSF.

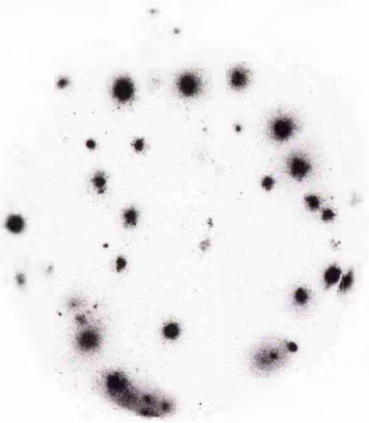
Slide D: In the presence of a combination of media conditioned by the L929 fibroblast cell line and the WEHI 3B myelomonocytic leukaemic cell line. The combination of colony-stimulating factors "synergize" to stimulate the proliferation of a colony-forming cell population characterized by a high proliferative potential. This distinct high proliferative potential colony-forming cell (HPP-CFC) population is not observed when each factor is used singly.

Slide A

Slide B

10mm

Slide C



Slide D

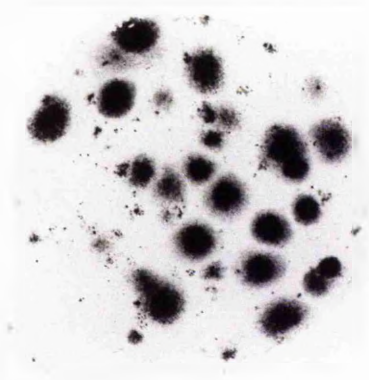


Plate 2: This photograph illustrates the pattern of colony growth from $2.5x10^4$ murine bone marrow cells after 14 days, in the presence of medium conditioned by the L929 fibroblast cell line, as a crude source of M-CSF/CSF-1 [Slide A] (colonies are stained with INT and each agar disc is approximately 30mm in diameter) and in the presence of a combination of L929 fibroblast cell line conditioned medium (M-CSF/CSF-1) and:

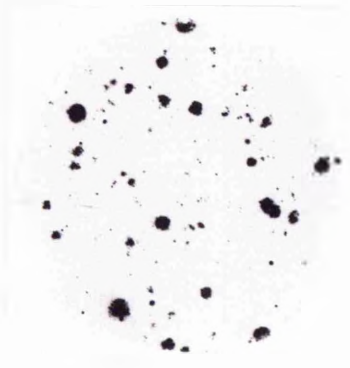
(1) AF1-19T malignant histiocytosis sarcoma virus-transformed cell line conditioned medium, a crude source of granulocyte-macrophage colony-stimulating factor (GM-CSF) [Slide B].

(2) Recombinantly-derived murine granulocyte-macrophage colony-stimulating factor (rmuGM-CSF) [Slide C].

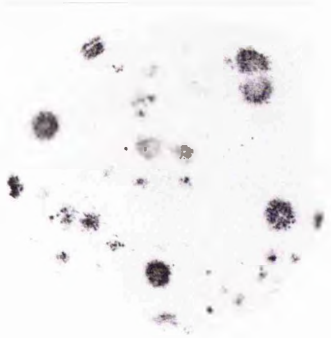
(3) WEHI 3B myelomonocytic leukaemic cell line conditioned medium, a crude source of interleukin 3 (IL3)/multi-CSF [Slide D].

GM-CSF, as either medium conditioned by the AF1-19T cell line or as rmuGM-CSF, and interleukin 3 (IL3)/multi-CSF, as medium conditioned by the WEHI 3B cell line, are demonstrated to synergise with M-CSF/CSF-1 to stimulate the proliferation of a distinct colony-forming cell population with high proliferative potential. These high proliferative potential colony-forming cell (HPP-$CFC_{GM\text{-}CSF+CSF\text{-}1}$ and HPP-$CFC_{IL3+CSF\text{-}1}$) - derived colonies, appear to differ morphologically and numerically, although the exact relationship between HPP-CFC subpopulations is, as yet, unclear.

Slide A



Slide B



10mm

Slide C

Slide D

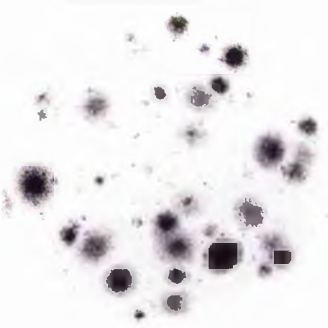


Plate 3: This photograph illustrates the pattern of colony growth by $5x10^4$ normal bone marrow cells, over a period of 14 days in the presence of medium conditioned by the L929 cell line (as a crude source of M-CSF/CSF-1), the WEHI 3B cell line (as a crude source of interleukin 3 (IL3)/multi-CSF) or a combination of the two conditioned media.

(Colonies were assayed on days 8, 10, 12 and 14 and stained with INT. Each agar disc is approximately 50mm in diameter.)

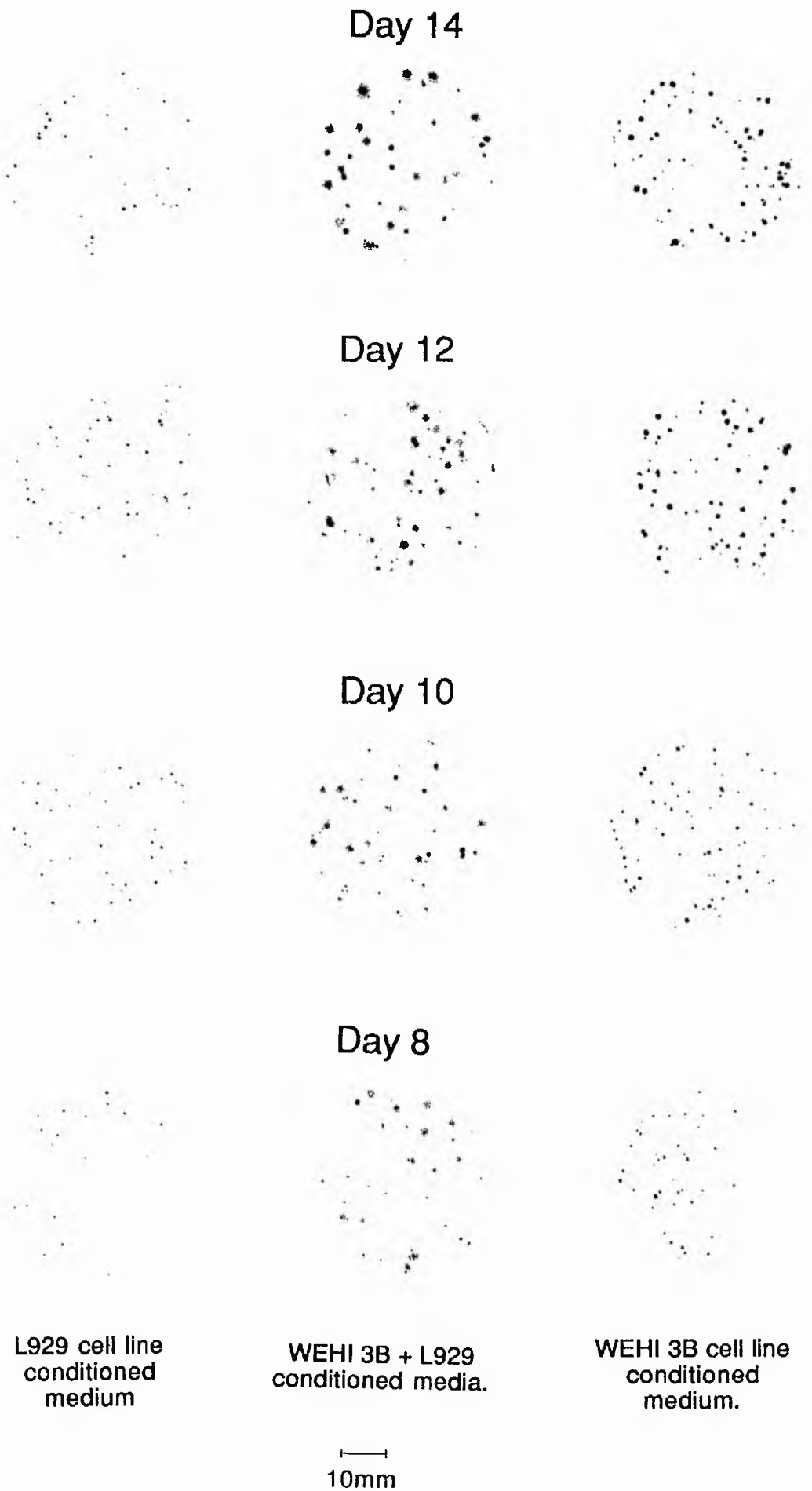
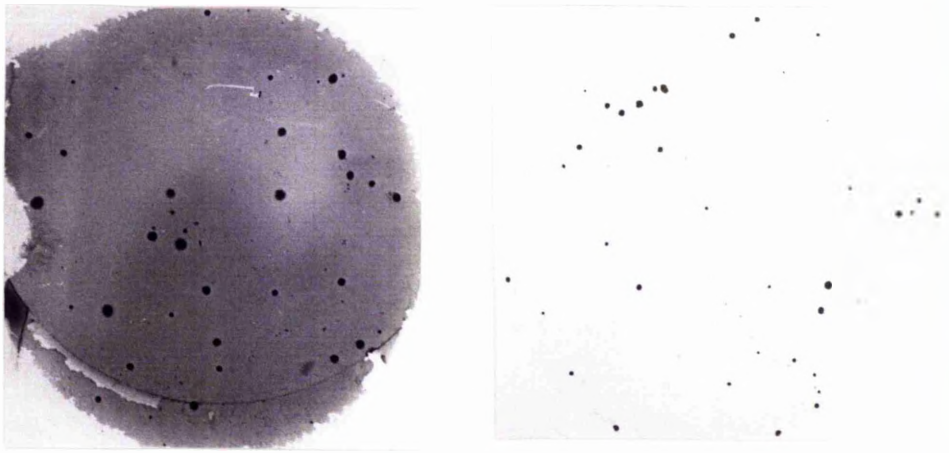

Day 14
Day 12
Day 10
Day 8
L929 cell line conditioned medium
WEHI 3B + L929 conditioned media.
WEHI 3B cell line conditioned medium.
10mm

Plate 4: This photograph illustrates the pattern of colony growth observed at day 14 after the culture of $5x10^4$ normal bone marrow cells with medium conditioned by the L929 fibroblast cell line (as a crude source of M-CSF/CSF-1), the WEHI 3B cell line (as a crude source of interleukin 3 (IL3)/ multi-CSF) or a combination of the two conditioned media.

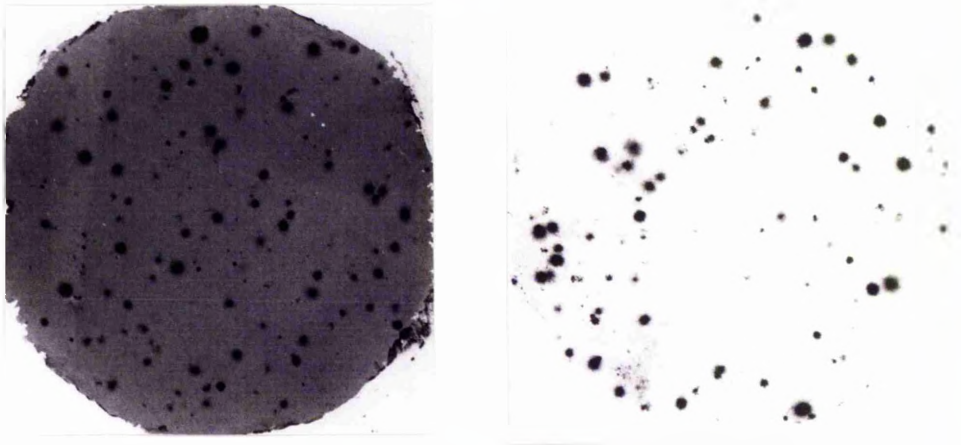
The results of both INT and Jenner-Giemsa staining techniques are illustrated (each agar disc is approximately 50mm in diameter).

Jenner-Giemsa Stained

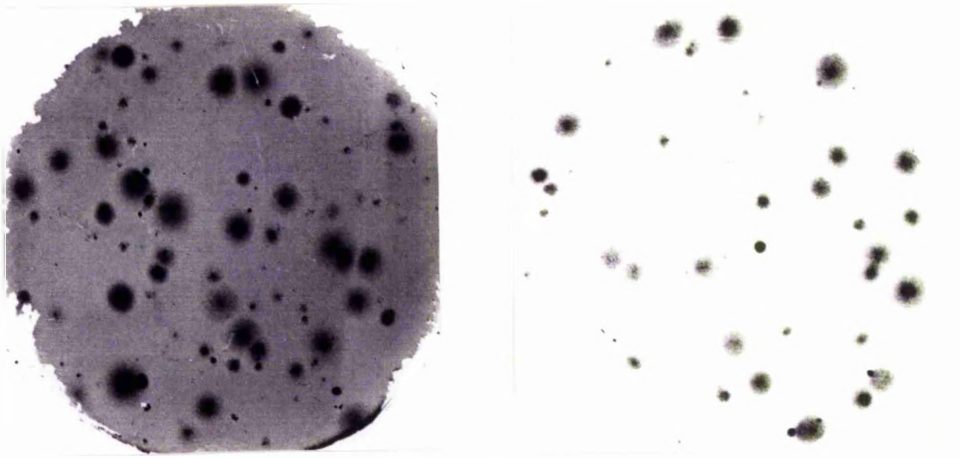
INT Stained



L929 cell line conditioned medium



WEHI 3B cell line conditioned medium.

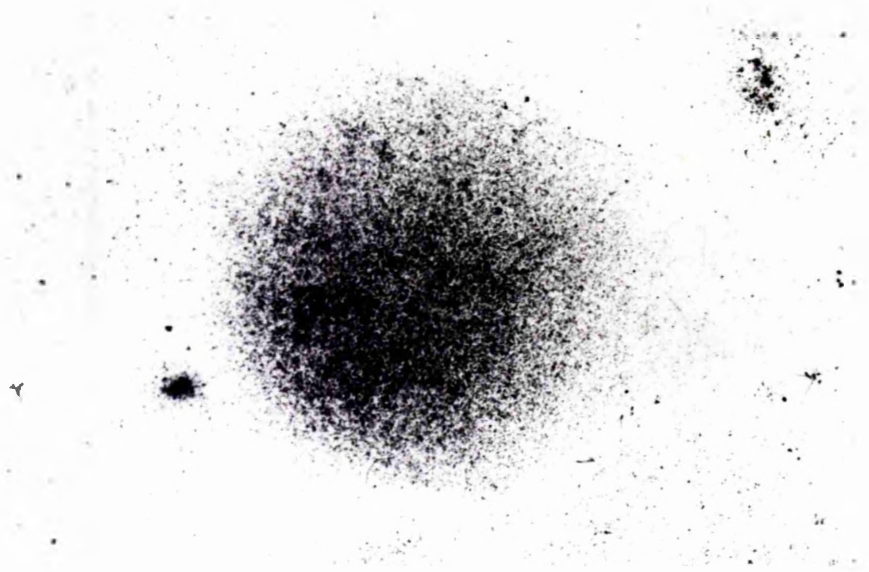


WEHI 3B + L929 conditioned media.

10mm

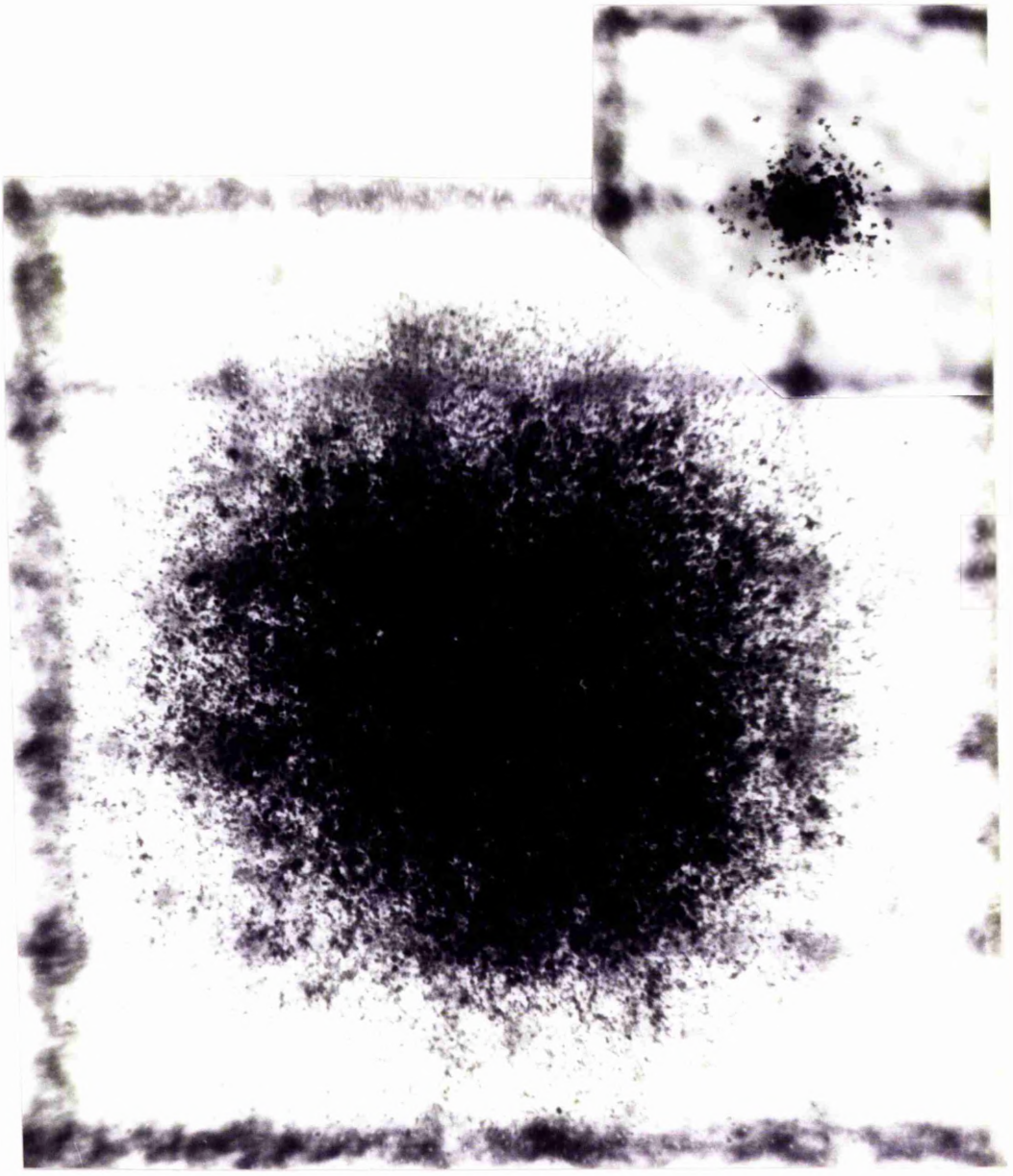
Plates 5A and 5B: These photographs illustrate, at a higher magnification, the contrast in the diameters of an HPP-CFC - derived colony, stimulated in the presence of a combination of L929 and WEHI 3B conditioned media ($HPP\text{-}CFC_{IL3+CSF\text{-}1}$), which fulfills the 2mm diameter criterion (Actual diameters of approximately: Plate 5A - 5mm, Plate 5B - 4mm) and a non-HPP-CFC - derived colony (Actual diameters of approximately: Plate 5A - 0.5mm, Plate 5B - 0.8mm). (Plate 5B has a 1mm grid background against which reference can be made. Colonies were stained with INT.)

Plate 5A



1mm

Plate 5B



1mm

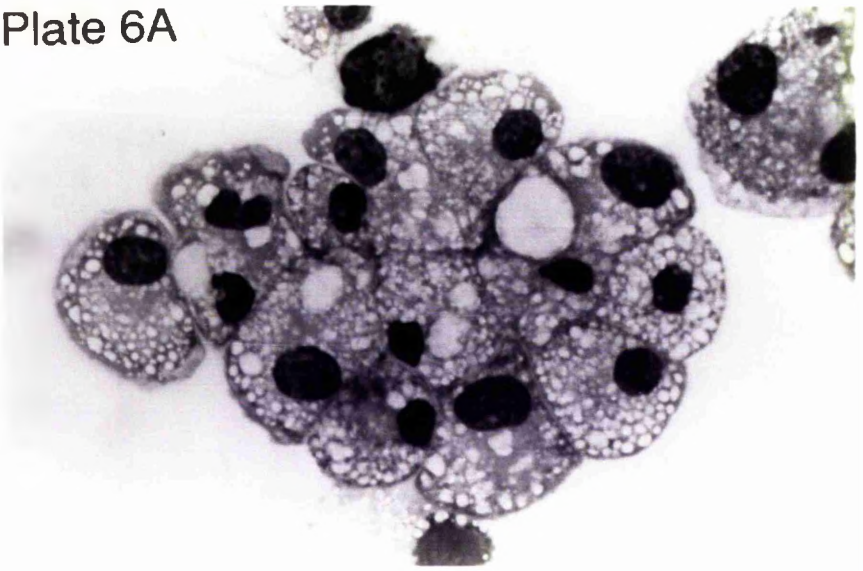
Plates 6A, 6B and 6C: This series of photomicrographs illustrate the variety of cell types observed when the cellular composition of individual HPP-$CFC_{IL3+CSF-1}$ - derived colonies was investigated. The photomicrographs are of cytocentrifudge preparations of individual 14 day HPP-$CFC_{IL3+CSF-1}$ - derived colonies cultured in 0.9% methylcellulose and cells are Jenner-Giemsa stained.

Plate 6A: This photomicrograph illustrates the heavily vacuolated nature of the macrophage population which was found to be the most abundant cell type within individual HPP-$CFC_{IL3+CSF-1}$ - derived colonies. This is consistent with the presence of M-CSF/CSF-1, as medium conditioned by the L929 fibroblast cell line.

Plate 6B: This photomicrograph illustrates the occasional finding of a relatively immature myeloid cell population, showing some degree of differentiation, together with the mature macrophages within individual HPP-$CFC_{IL3+CSF-1}$ - derived colonies.

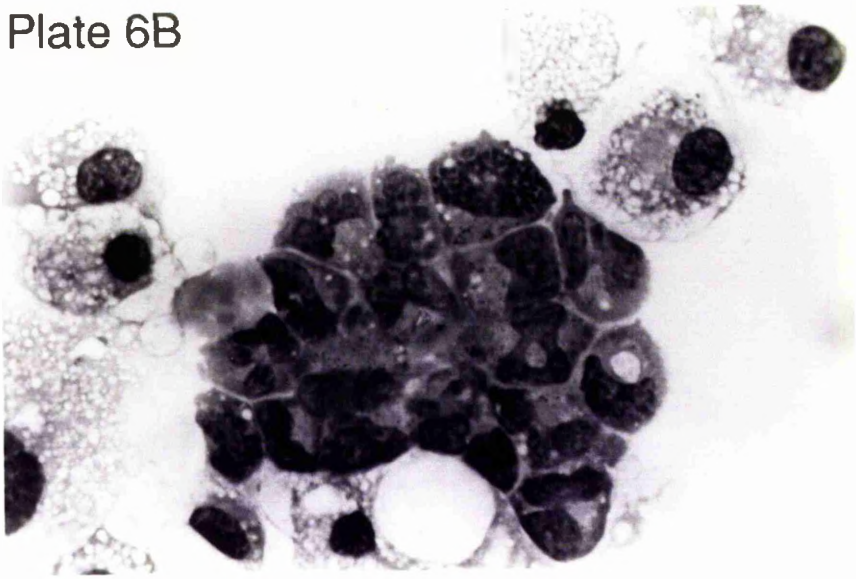
Plate 6C: This photomicrograph illustrates the occasional finding of a discrete, morphologically distinct cell population, characterized by a pale-staining nucleus and abundant cytoplasmic granules, together with the mature macrophages within individual HPP-$CFC_{IL3+CSF-1}$ - derived colonies.

Plate 6A



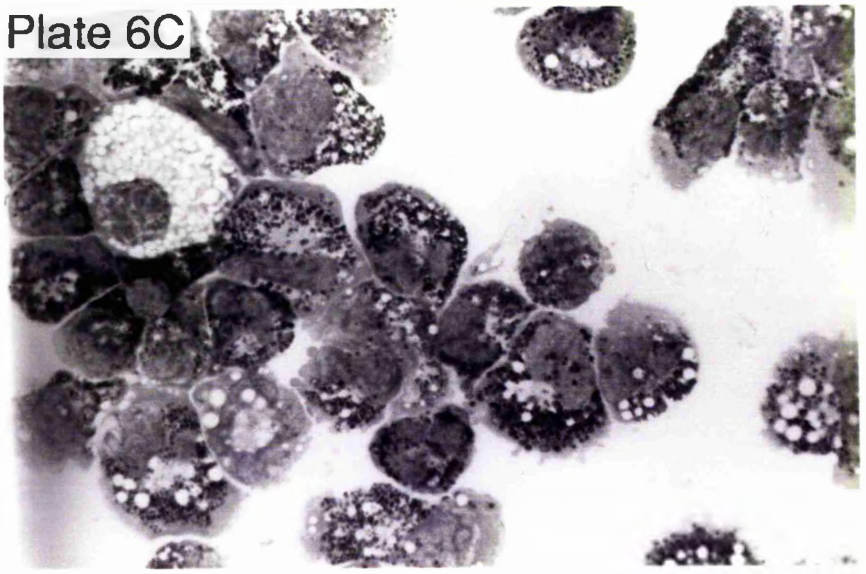
10μm

Plate 6B



10μm

Plate 6C



10μm

Section 2

An investigation of the proliferation and proliferation regulation of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population.

3.5 Determination of the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in normal and sublethally X-irradiated, regenerating, CBA/H femoral marrow.

Procedure:

The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was determined by use of the S-phase 'suicide' technique. Cells in S-phase, if incubated with the S-phase-specific cytotoxic agent *cytosine arabinoside* (ARA-C)(2.13) are killed. Thus, HPP-CFC$_{IL3+CSF-1}$ in S-phase during ARA-C incubation are killed and their colony-forming potential is not realised in the subsequent HPP-CFC$_{IL3+CSF-1}$ assay. The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase is determined by the difference in the number of HPP-CFC$_{IL3+CSF-1}$-derived colonies obtained in the absence of ARA-C and the number obtained in the presence of ARA-C.

Results:

3.5.1 Determination of the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in normal femoral marrow.

The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in normal femoral marrow was determined as 9(±1)% (±SEM)(n=255) [*Figure 43*].

In vitro GM-CFC assay of normal bone marrow demonstrates that the proportion of the more mature haematopoietic progenitor cells in S-phase is 31(±1)% (±SEM)(n=35) [*Figure 43*].

3.5.2 Determination of the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in regenerating femoral marrow.

The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in regenerating femoral marrow from mice 3 days after a whole body X-irradiation

dose of 2 Gy was determined as 40(±1)% (±SEM)(n=177) [*Figure 43*].

3.6 HPP-CFC$_{IL3+CSF-1}$ sensitivity to the stem cell-specific proliferation *inhibitor* and *stimulator*.

Procedure:

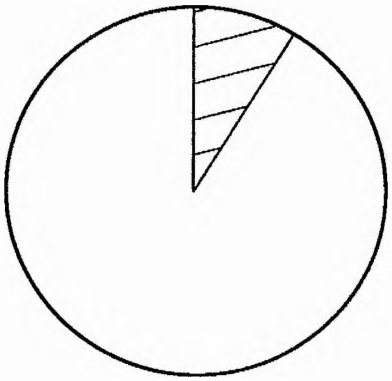
Haematopoietic stem cell-specific proliferation regulators have been identified (1.10). A stem cell proliferation *inhibitor* is present in normal bone marrow and maintains the reduced proportion of stem cells in S-phase. A stem cell proliferation *stimulator* is present in sublethally X-irradiated, regenerating bone marrow and maintains the increased proportion of stem cells in S-phase. The stem cell proliferation *inhibitor* has the capacity to reduce the proportion of stem cells in S-phase in regenerating bone marrow and similarly, the stem cell proliferation *stimulator* has the capacity to increase the proportion of stem cells in S-phase in normal bone marrow. The sensitivity of the HPP-CFC$_{IL3+CSF-1}$ population to these stem cell-specific proliferation regulators was investigated.

Results:

3.6.1 HPP-CFC$_{IL3+CSF-1}$ sensitivity to the stem cell-specific proliferation *inhibitor*.

In regenerating marrow from mice 3 days after a whole body X-irradiation dose of 2 Gy, the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was determined as 40(±1)% (3.5). If incubated with medium conditioned by normal bone marrow (2.14 & 2.16), in which the proportion of stem cells in S-phase is low and which is a crude source of stem cell proliferation *inhibitor*, the proportion of HPP-

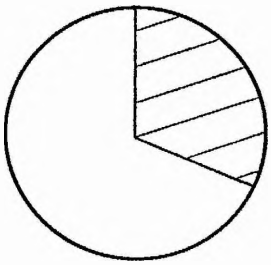
Figure 43: The proportion of HPP-CFC in S-phase in normal and X-irradiated, regenerating bone marrow.



The proportion of HPP-CFC in S-phase in normal bone marrow:

9(±1)%

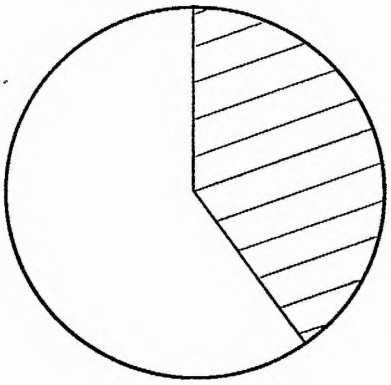
(n=255)(±SEM)



The proportion of GM-CFC in S-phase in normal bone marrow:

31(±1)%

(n=35)(±SEM)



The proportion of HPP-CFC in S-phase in X-irradiated, regenerating bone marrow:

40(±1)%

(n=177)(±SEM)

$CFC_{IL3+CSF-1}$ in S-phase was reduced from 40(±1)% (3.5) to 25(±1)% (±SEM)(n=127) [*Figure 44*]. (Medium was conditioned by normal bone marrow for 4 hours at 37°C (2.14) and prior to use was diluted so as to be derived from $15x10^6$ normal bone marrow cells/ml).

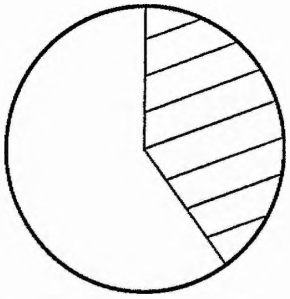
3.6.2 HPP-$CFC_{IL3+CSF-1}$ sensitivity to the stem cell-specific proliferation *stimulator*.

In normal bone marrow the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was determined as 9(±1)% (3.5). If incubated with medium conditioned by regenerating bone marrow from mice 7 days after a sublethal whole body X-irradiation dose of 4.5 Gy (2.15 & 2.17), in which the proportion of stem cells in S-phase is increased and which is a crude source of *stimulator*, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was increased from 9(±1)% (3.5) to 24(±1)% (±SEM)(n=107) [*Figure 45*]. (Medium was conditioned by regenerating bone marrow for 1 hour at 37°C (2.15) and prior to use was diluted so as to be derived from $15x10^6$ regenerating marrow cells/ml.)

No evidence of normal bone marrow conditioned medium-derived *inhibitor*, or regenerating bone marrow-derived *stimulator* toxicity was detected in the *in vitro* HPP-$CFC_{IL3+CSF-1}$ assay.

3.7 A determination of the sensitivity of the HPP-$CFC_{IL3+CSF-1}$ population to the stem cell proliferation *inhibitor* and *stimulator*.

Tejero *et al* [1984] demonstrated an 'age' related differential sensitivity to the haematopoietic stem cell proliferation regulators. More primitive components of the haematopoietic stem cell



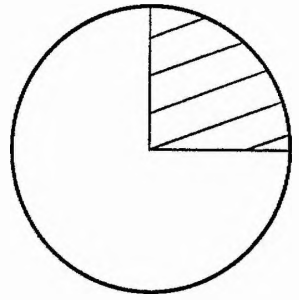
Regenerating bone marrow from mice 3 days after a whole body X-irradiation dose of 2 Gy.

40(±1)%

HPP-CFC in S-phase (n=177)(±SEM).

Medium conditioned by normal bone marrow. A crude source of INHIBITOR.

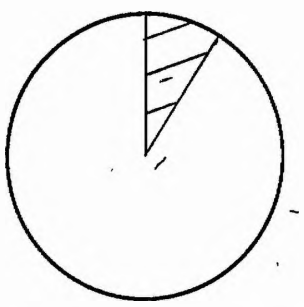
Incubated 5 hrs.



25(±1)%

HPP-CFC in S-phase (n=127)(±SEM).

Figure 44: HPP-CFC sensitivity to the stem cell proliferation inhibitor.



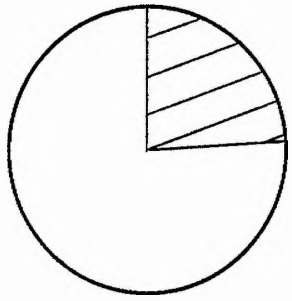
Normal bone marrow

9(±1)%

HPP-CFC in S-phase
(n=255)(±SEM).

Medium conditioned
by regenerating bone
marrow from mice
7 days after a whole
body X-irradiation dose
of 4.5 Gy.
A crude source
of STIMULATOR.

Incubated 3 hrs.



24(±1)%

HPP-CFC in S-phase
(n=107)(±SEM).

Figure 45: HPP-CFC sensitivity to the stem cell proliferation stimulator.

compartment (day 10-12 CFU-S) proved significantly more sensitive to the stem cell proliferation *inhibitor* than *stimulator*, while more mature components of the haematopoietic stem cell compartment (day 7-8 CFU-S and CFC-mix) proved significantly more sensitive to the stem cell proliferation *stimulator* than *inhibitor* (1.17) [*Figure 13*].

The sensitivity of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population to the stem cell proliferation regulators was thus investigated to allow a determination of the position of the population within the haematopoietic stem cell compartment.

3.7.1 HPP-$CFC_{IL3+CSF-1}$ sensitivity to the stem cell proliferation *inhibitor*.

Procedure:

Samples of medium conditioned for 4 hours at 37°C by 15-20x10^6 normal bone marrow cells/ml were prepared (2.14). Prior to use, samples were thawed, centrifuged (x3000 rpm, 15 mins.), MilliPore filtered (0.22 μM) and diluted with medium so as to be derived from 1, 2, 4, 6, 8 or 10x10^6 cells/ml.

Regenerating femoral marrow from mice 3 days after a sublethal whole body X-irradiation dose of 2 Gy, in which the proportion of stem cells in S-phase is increased, was washed and incubated with either medium alone, or medium conditioned by 1, 2, 4, 6, 8 or 10x10^6 normal bone marrow cells/ml (2.16).

Results:

In the presence of medium alone, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was determined as 51(±3)% (±SEM) (n=6). In the presence of an increasing concentration of normal bone marrow conditioned medium, a progressive reduction in the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was observed to a

minimum, plateau value of approximately 24% [*Figure 46*]. (Previous data (3.6.1) were obtained by incubating regenerating bone marrow with medium conditioned by $15x10^6$ normal bone marrow cells/ml and is included here: 25(±1)% (±SEM) (n=127) HPP-$CFC_{IL3+CSF-1}$ in S-phase.)

3.7.2 HPP-$CFC_{IL3+CSF-1}$ sensitivity to the stem cell proliferation *stimulator*.

Procedure:

Samples of medium conditioned for 1 hour at 37°C by 15-$20x10^6$ regenerating marrow cells/ml, from mice 7 days after a 4.5 Gy whole body X-irradiation dose, were prepared (2.15). Prior to use, samples were thawed, centrifuged (x3000 rpm, 15 mins.), MilliPore filtered (0.22 μM) and diluted with medium so as to be diluted from 2, 4, 6, 8 and $10x10^6$ cells/ml.

Normal bone marrow, in which the proportion of stem cells in S-phase is relatively low, was washed and incubated with either medium alone, or medium conditioned by 2, 4, 6, 8 and $10x10^6$ regenerating marrow cells/ml (2.17).

Results:

In the presence of medium alone, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was determined as 9(±2)% (±SEM) (n=4). In the presence of an increasing concentration of regenerating bone marrow conditioned medium, a progressive increase in the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was observed, to a maximum, plateau value of approximately 28% [*Figure 47*]. (Previous data (3.6.2) were obtained by incubating normal bone marrow with medium conditioned by $15x10^6$ regenerating bone marrow cells/ml

Figure 46: A determination of the sensitivity of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population to stem cell proliferation *inhibitor*.

Concentration of normal bone marrow conditioned medium. ($x10^6$ cells/ml)	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%).[1] (±SEM) (n=6)	
---	51(±3)	
1	49(±8)	NS (P>0.1)
2	39(±3)	S (P<0.05)
4	37(±3)	S (P<0.025)
6	30(±4)	S (P<0.005)
8	26(±5)	S (P<0.01)
10	24(±6)	S (P<0.025)
15	25(±1) (n=127)	

[1] HPP-$CFC_{IL3+CSF-1}$ derived from regenerating bone marrow.
(NS-Not significant. S-Significant)

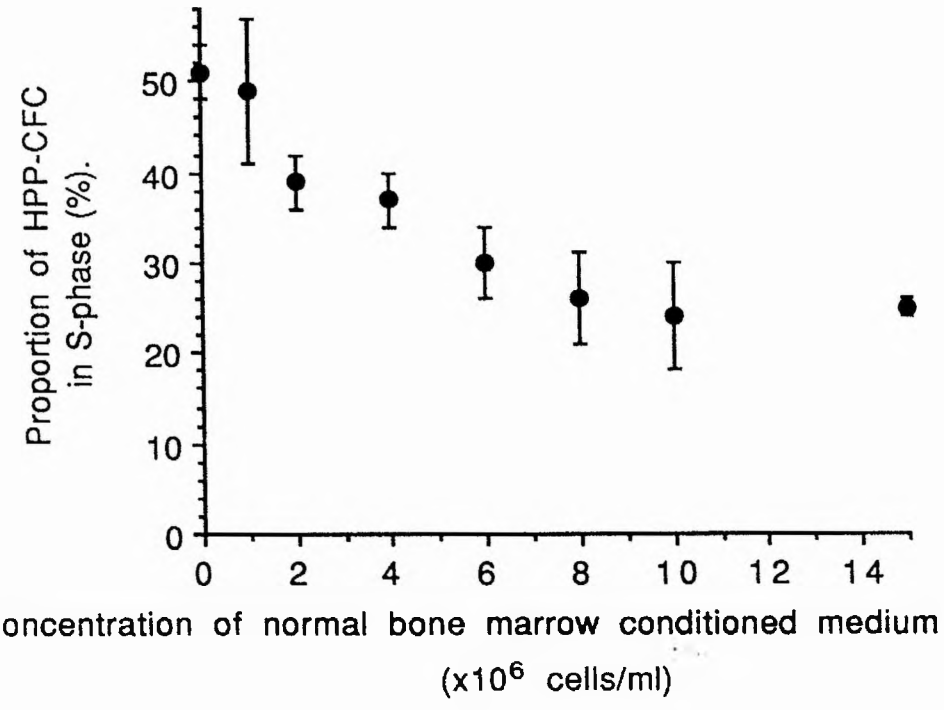
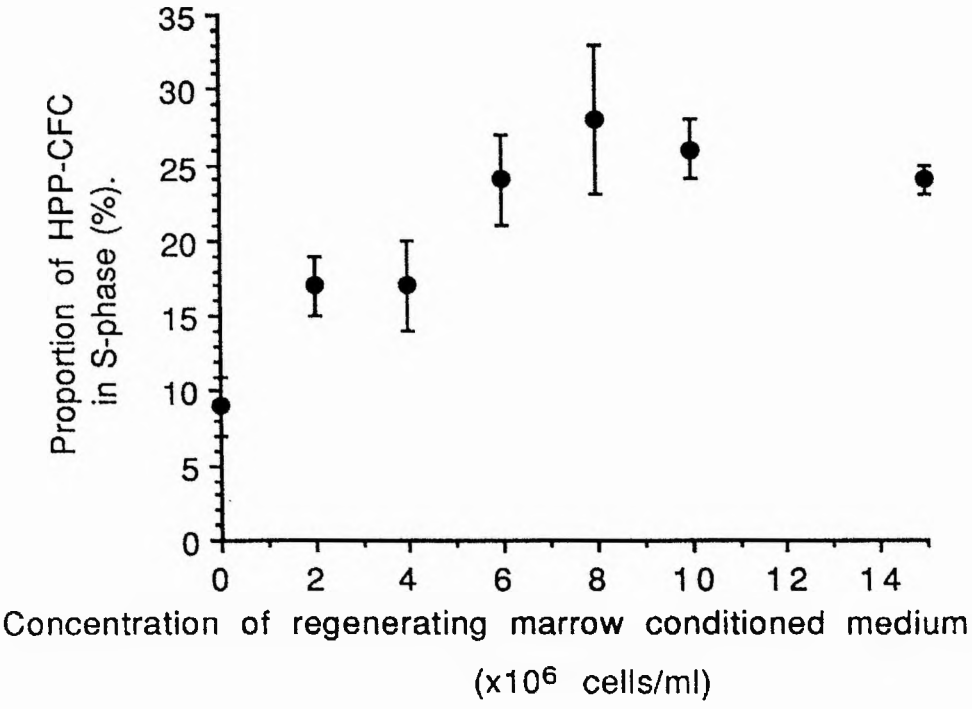


Figure 47: A determination of the sensitivity of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population to stem cell proliferation *stimulator*.

Concentration of regenerating bone marrow conditioned medium. ($x10^6$ cells/ml)	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%).[1] (±SEM) (n=4)	
---	9(±2)	
2	17(±2)	S (P<0.005)
4	17(±3)	S (P<0.005)
6	24(±3)	S (P<0.0005)
8	28(±5)	S (P<0.005)
10	26(±2)	S (P<0.005)
15	24(±1) (n=107)	

[1] HPP-$CFC_{IL3+CSF-1}$ derived from normal bone marrow. (S-Significant)



and is included here: 24(±1)% (±SEM) (n=107) HPP-CFC$_{IL3+CSF-1}$ in S-phase.)

Section 3

An investigation of proliferation *stimulator* and *inhibitor* in myeloid leukaemia.

3.8 HPP-CFC$_{IL3+CSF\text{-}1}$ investigation of the *inhibitor/stimulator* status of medium conditioned by murine, X-irradiation-induced, serially passaged, myeloid leukaemic marrow.

A number of X-irradiation-induced myeloid leukaemias have been produced in CBA/H male mice in this laboratory [Hepburn *et al*,1987] (2.18). Primary leukaemias were characterized and subsequently passaged through syngeneic mice. Passage of the primary leukaemias was at both a high and a low cell dose and two distinct leukaemic morphologies subsequently developed. Primary leukaemic cells, passaged at a relatively high cell dose (HD), produced a leukaemia characterized by a more aggressive nature than the primary leukaemia itself, while primary leukaemic cells passaged at a relatively low cell dose (LD), produced a leukaemia characterized by a near-primary morpholgy [Hepburn *et al*,1987].

Three serially passaged, X-irradiation induced, murine, myeloid leukaemias were investigated - classified as SA2, SA7 and SA8 (SA-St.Andrews). Specifically, the high cell dose-passaged SA7 myeloid leukaemia (SA7HD), the high and low cell dose-passaged SA8 myeloid leukaemia (SA8HD and SA8LD) and low and high passage numbers of the SA2 myeloid leukaemia (SA2LP and SA2HP) were investigated.

Femoral marrow was taken from overtly leukaemic mice and leukaemic marrow conditioned medium produced (2.20). Medium conditioned by the leukaemic marrows was subsequently investigated using the HPP-CFC$_{IL3+CSF\text{-}1}$ assay to establish the levels of the stem cell proliferation regulators in such aberrant haematopoiesis. Evidence for a direct-acting leukaemia-associated haematopoietic proliferation inhibitor was also sought (2.16 & 2.17).

3.8.1 HPP-CFC$_{IL3+CSF-1}$ investigation of the *inhibitor/stimulator* status of medium conditioned by high and low passage numbers of the SA2 myeloid leukaemic marrow.

Procedure:

4 batches each of medium conditioned by low and high passage numbers of the SA2 myeloid leukaemic marrow were produced (SA2LP & SA2HP)(2.20) and assayed against normal and regenerating bone marrow (2.16 & 2.17).

Results:

3.8.1.1 The *stimulator* status of medium conditioned by SA2LP and SA2HP leukaemic marrow.

The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in washed normal bone marrow was determined as 14(±1)% (±SEM) (n=8). In washed normal bone marrow, incubated with medium conditioned by low passage number SA2 myeloid leukaemic marrow (SA2LP), the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was not significantly changed (P>0.4) at 15(±1)% (n=8). In washed normal bone marrow, incubated with medium conditioned by high passage number SA2 myeloid leukaemic marrow (SA2HP), the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was not significantly changed (P>0.4) at 15(±1)% (n=8) [*Figure 48*].

No evidence for the presence of stem cell proliferation *stimulator* was observed at the concentration of conditioned medium assayed, in either the SA2LP or SA2HP leukaemic marrow conditioned media [*Figure 48*].

3.8.1.2 The *inhibitor* status of medium conditioned by SA2LP and SA2HP leukaemic marrow.

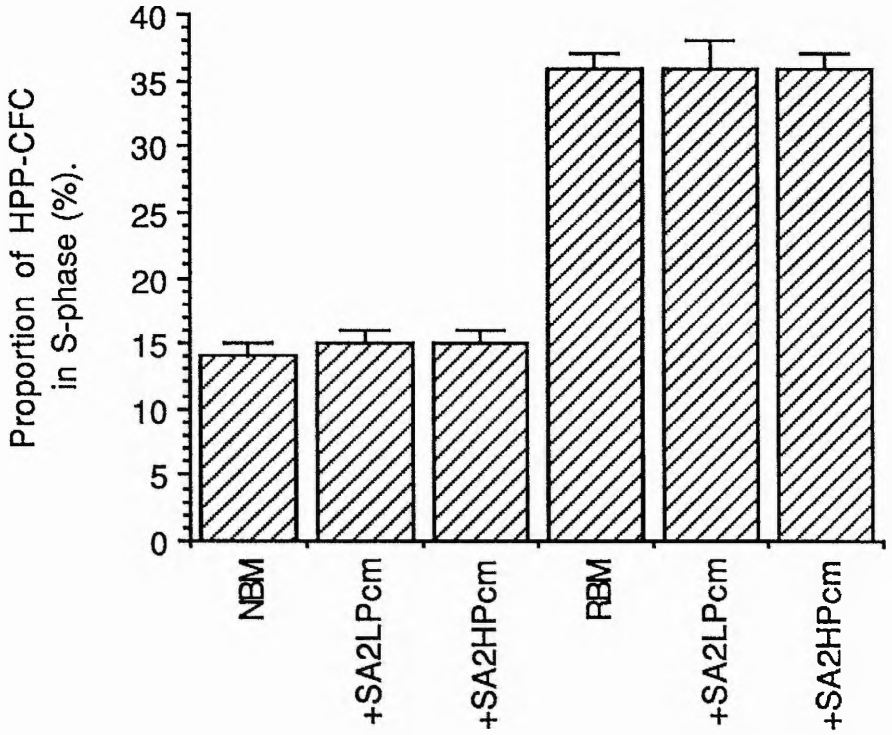
The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in washed regenerating bone marrow from mice 3 days after a 2 Gy whole body X-irradiation dose was determined as 36(±1)% (±SEM) (n=8). In washed regenerating bone marrow incubated with medium conditioned by low passage number SA2 (SA2LP), the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was not significantly changed (P>0.4) at 36(±5)% (n=8). In washed regenerating bone marrow incubated with medium conditioned by high passage number SA2 (SA2HP), the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was not significantly changed (P>0.4) at 36(±1)% (n=8) [*Figure 48*].

No evidence for the presence of stem cell proliferation *inhibitor* was observed at the concentration of conditioned medium assayed, in either the SA2LP or SA2HP leukaemic marrow conditioned media. These results also indicate that, at the concentrations of conditioned medium assayed, there was no evidence of a direct-acting leukaemia-associated haematopoietic proliferation inhibitor. Rapidly cycling HPP-CFC$_{IL3+CSF-1}$, remain rapidly cycling when incubated with the leukaemic marrow conditioned medium. No evidence of SA2LP or SA2HP leukaemic marrow conditioned media toxicity was observed.

Figure 48: HPP-$CFC_{IL3+CSF-1}$ investigation of the *inhibitor/stimulator* content of medium conditioned by high and low passage number SA2 myeloid leukaemic marrow.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%) (±SEM) (n=8)	
	Normal bone marrow (NBM	Regenerating bone marrow[1] (RBM)
Control	14(±1)	36(±1)
+SA2LP conditioned medium.(SA2LPcm)	15(±1)[2]	36(±2)[3]
+SA2HP conditioned medium.(SA2HPcm)	15(±1)[2]	36(±1)[3]

[1] Regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.
[2] No significant increase (P>0.4).
[3] No significant reduction (P>0.4).



3.8.2 HPP-CFC$_{IL3+CSF\text{-}1}$ investigation of the *inhibitor/stimulator* status of medium conditioned by high cell dose-passaged SA7 myeloid leukaemic marrow.

Procedure:

5 batches of medium conditioned by high cell dose-passaged SA7 leukaemic marrow (SA7HD) were produced (2.20) and assayed against normal and regenerating bone marrow (2.16 & 2.17).

Results:

3.8.2.1 The *stimulator* status of medium conditioned by SA7HD leukaemic marrow.

In washed normal bone marrow, the proportion of HPP-CFC$_{IL3+CSF\text{-}1}$ in S-phase was determined as 12(±1)% (±SEM) (n=11). In washed normal bone marrow, incubated with medium conditioned by SA7HD myeloid leukaemic marrow, the proportion of HPP-CFC$_{IL3+CSF\text{-}1}$ in S-phase was not significantly changed (P>0.4) at 13(±1)% (n=11) [*Figure 49*].

No evidence for the presence of stem cell proliferation *stimulator* was observed at the concentration of conditioned medium assayed.

3.8.2.2 The *inhibitor* status of medium conditioned by SA7HD leukaemic marrow.

The proportion of HPP-CFC$_{IL3+CSF\text{-}1}$ in S-phase in washed regenerating bone marrow from mice 3 days after a 2 Gy whole body X-irradiation dose, was determined as 37(±2)% (±SEM) (n=11). In washed, regenerating bone marrow, incubated with SA7HD leukaemic marrow conditioned medium, the proportion of HPP-

$CFC_{IL3+CSF-1}$ in S-phase was not significantly changed ($P > 0.1$) at 35(±2)% (n=11) [*Figure 49*].

No evidence for the presence of stem cell proliferation *inhibitor* was observed at the concentration of SA7HD leukaemic marrow conditioned medium assayed. These results also indicate that, at the concentrations of conditioned medium assayed, there was no evidence of a direct-acting leukaemia-associated proliferation inhibitor. Rapidly cycling HPP-$CFC_{IL3+CSF-1}$, remain rapidly cycling in the presence of SA7HD leukaemic marrow conditioned medium. No evidence of SA7HD leukaemic marrow conditioned medium toxicity was observed.

3.8.3 HPP-$CFC_{IL3+CSF-1}$ investigation of the *inhibitor/stimulator* status of medium conditioned by high cell dose-passaged SA8 myeloid leukaemic marrow.

Procedure:

4 batches of medium conditioned by high cell dose-passaged SA8 (SA8HD) myeloid leukaemic marrow were produced (2.20) and assayed against both normal and regenerating bone marrow (2.16 & 2.17).

Results:

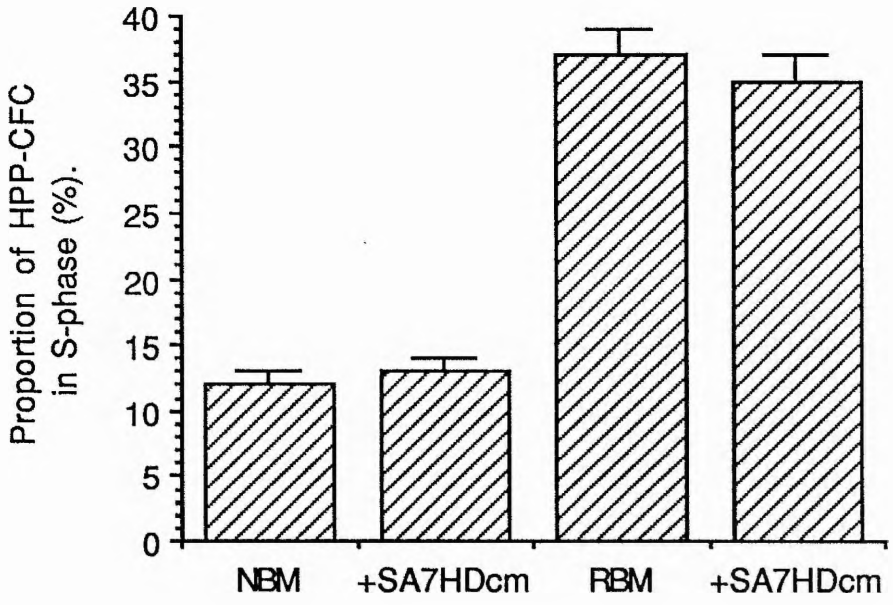
3.8.3.1 The *stimulator* status of medium conditioned by SA8HD leukaemic marrow.

The proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase in washed normal bone marrow was determined as 13(±1)% (±SEM) (n=12). In washed normal bone marrow incubated with medium conditioned by SA8HD myeloid leukaemic marrow, the proportion of HPP-

Figure 49: HPP-$CFC_{IL3+CSF-1}$ investigation of the *inhibitor/stimulator* content of medium conditioned by high cell dose-passaged SA7 myeloid leukaemic marrow.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%) (±SEM) (n=11)	
	Normal bone marrow (NBM)	Regenerating bone marrow[1] (RBM)
Control	12(±1)	37(±2)
+SA7HD conditioned medium.(SA7HDcm)	13(±1)[2]	35(±2)[3]

[1] Regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.
[2] No significant increase (P>0.4).
[3] No significant reduction (P>0.1).



$CFC_{IL3+CSF-1}$ in S-phase was not significantly changed ($P > 0.1$) at 14(±1)% (n=12) [*Figure 50*].

No evidence for the presence of stem cell proliferation *stimulator* was observed at the concentration of SA8HD leukaemic marrow conditioned medium assayed.

3.8.3.2 The *inhibitor* status of medium conditioned by SA8HD leukaemic marrow.

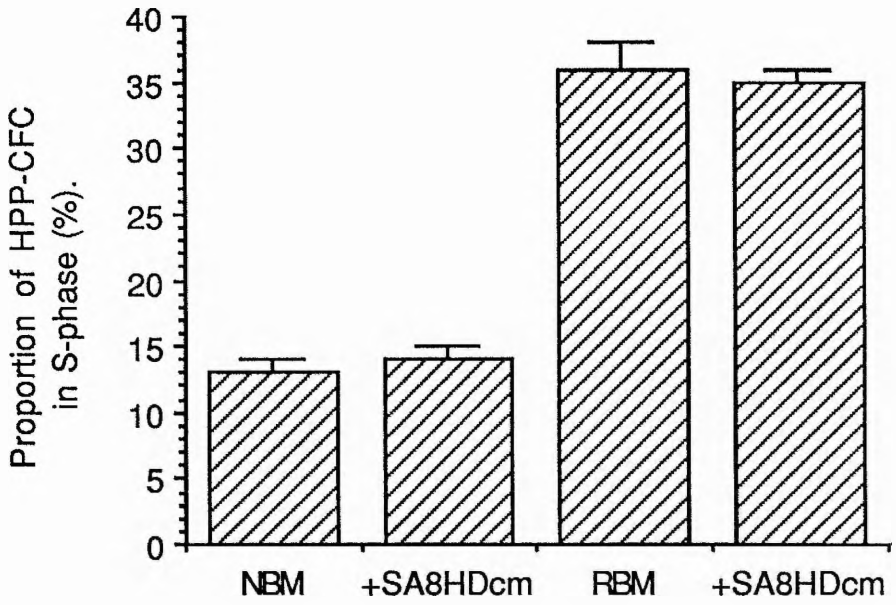
The proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase in washed regenerating bone marrow from mice 3 days after a 2 Gy whole body X-irradiation dose, was determined as 36(±2)% (±SEM) (n=12). In washed regenerating bone marrow incubated with medium conditioned by SA8HD leukaemic marrow, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was not significantly changed ($P > 0.1$) at 35(±1)% (n=12) [*Figure 50*].

No evidence for the presence of stem cell proliferation *inhibitor* was observed at the concentration of SA8HD leukaemic marrow conditioned medium assayed, nor was there any evidence for a direct-acting leukaemia-associated proliferation inhibitor. Rapidly cycling HPP-$CFC_{IL3+CSF-1}$, remained rapidly cycling in the presence of SA8HD leukaemic marrow conditioned medium. No evidence of SA8HD leukaemic marrow conditioned medium toxicity was observed.

Figure 50: HPP-$CFC_{IL3+CSF-1}$ investigation of the *inhibitor/stimulator* content of medium conditioned by high cell dose-passaged SA8 myeloid leukaemic marrow.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%) (±SEM) (n=12)	
	Normal bone marrow (NBM)	Regenerating bone marrow[1] (RBM)
Control	13(±1)	36(±2)
+SA8HD conditioned medium.(SA8HDcm)	14(±1)[2]	35(±1)[3]

[1] Regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.
[2] No significant increase (P>0.1).
[3] No significant reduction (P>0.1).



3.8.4 HPP-CFC$_{IL3+CSF-1}$ investigation of the *inhibitor/stimulator* status of medium conditioned by low cell dose passaged SA8 myeloid leukaemic marrow.

Procedure:

8 batches of medium conditioned by low cell dose-passaged SA8 (SA8LD) myeloid leukaemic marrow were produced (2.20) and assayed against both normal and regenerating bone marrow (2.16 & 2.17).

Results:

3.8.4.1 The *stimulator* status of medium conditioned by SA8LD leukaemic marrow.

The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in washed normal bone marrow was determined as 13(±1)% (±SEM) (n=29). In washed normal bone marrow incubated with medium conditioned by SA8LD myeloid leukaemic marrow, the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was not significantly changed ($P > 0.1$) at 12(±1)% (n=29) [*Figure 51*].

No evidence for the presence of stem cell proliferation *stimulator* was observed at the concentration of SA8LD leukaemic marrow conditioned medium assayed.

3.8.4.2 The *inhibitor* status of medium conditioned by SA8LD leukaemic marrow.

The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in regenerating bone marrow from mice 3 days after a 2 Gy whole body X-irradiation dose, was determined as 35(±1)% (±SEM) (n=29). In washed

regenerating bone marrow incubated with medium conditioned by SA8LD leukaemic marrow, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was not significantly changed ($P > 0.1$) at 34($\pm$1)% ($n = 29$) [*Figure 51*].

No evidence for the presence of stem cell proliferation *inhibitor* was observed at the concentration of SA8LD leukaemic marrow conditioned medium assayed, nor was there any evidence for a direct-acting leukaemia-associated proliferation inhibitor. Rapidly cycling HPP-$CFC_{IL3+CSF-1}$ remained rapidly cycling when incubated with SA8LD leukaemic marrow conditioned medium. No evidence of SA8LD leukaemic marrow conditioned medium toxicity was observed.

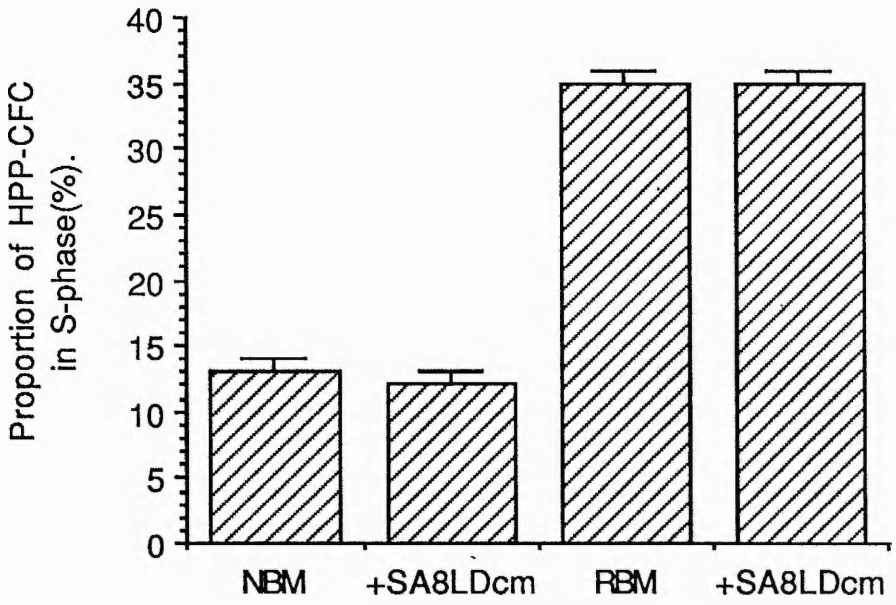
3.8.5 An investigation of the 'concentration' and duration of conditioning of leukaemic marrow conditioned medium.

The absence of evidence for stem cell proliferation *stimulator* or *inhibitor*, or of a direct-acting leukaemia-associated proliferation inhibitor in the SA2LP, SA2HP, SA7HD, SA8HD and SA8LD leukaemic marrow conditioned media, was further investigated by increasing the concentration of leukaemic marrow conditioned medium assayed and by increasing the period of time over which the medium was conditioned by the leukaemic marrow.

Figure 51: HPP-$CFC_{IL3+CSF-1}$ investigation of the *inhibitor/stimulator* content of medium conditioned by low cell dose-passaged SA8 myeloid leukaemic marrow.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%) (±SEM) (n=29)	
	Normal bone marrow (NBM)	Regenerating bone marrow[1] (RBM)
Control	13(±1)	35(±1)
+SA8LD conditioned medium.(SA8LDcm)	12(±1)[2]	35(±1)[3]

[1] Regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.
[2] No significant increase (P>0.1).
[3] No significant reduction (P>0.1).



3.8.5.1 An investigation of the 'concentration' of leukaemic marrow conditioned medium.

Procedure:

Experiments were performed in which medium conditioned by $25x10^6$ SA8HD leukaemic marrow cells/ml was assayed against both normal and regenerating bone marrow (2.16 & 2.17).

Result:

No evidence of stem cell proliferation *stimulator* or *inhibitor*, or of a direct-acting leukaemia-associated proliferation inhibitor was observed [*Data not shown*].

3.8.5.2 An investigation of the duration of conditioning of leukaemic marrow conditioned medium.

Procedure:

Experiments were also performed in which medium conditioned for 5 hours by SA8HD leukaemic marrow was assayed against both normal and regenerating bone marrow (2.16 & 2.17).

Result:

No evidence of stem cell proliferation *stimulator* or *inhibitor*, or of a direct-acting leukaemia-associated proliferation inhibitor was observed [*Data not shown*].

3.9 Time study of the progression of the SA8LD myeloid leukaemia.

The apparent absence of stem cell proliferation *inhibitor* and *stimulator* in medium conditioned by overtly leukaemic marrow from SA2LP, SA2HP, SA7HD, SA8HD and SA8LD myeloid leukaemias was further investigated by specific reference to the SA8 low cell dose-passaged (SA8LD) myeloid leukaemia.

Over a reproducible period of approximately 21 days, 9-10 week old, male, CBA/H mice injected intravenously with 10^5 SA8LD myeloid leukaemic cells, developed myeloid leukaemia (2.19). To determine whether the marked haematopoietic dysfunction associated with the development of SA8LD myeloid leukaemia was a direct consequence of abnormal stem cell proliferation regulation during disease progression, a time study of the SA8LD myeloid leukaemic progression was performed. Variations in the levels of haematopoietic stem cell proliferation *inhibitor* and *stimulator* with disease progression were investigated and evidence sought for a direct-acting leukaemia-associated proliferation inhibitor.

Procedure:

4 groups of 6-7, 9-10 week old, male, CBA/H mice *per* group were injected intravenously with 10^5 SA8LD myeloid leukaemic cells. Individual groups of mice were assayed at day 0 (day of injection), day 15, day 18 and day 21 (by which time mice were overtly leukaemic). Assay involved a determination of the total body weight of each mouse, a determination of the weight of the spleen of each mouse and a determination of the femoral marrow cellularity of each mouse. Medium was subsequently conditioned by the combined femoral bone marrow of each group (2.20) and *inhibitor* and *stimulator* assay performed on the conditioned medium (2.16 & 2.17).

Results:

3.9.1 The variation in total body weight, femoral cellularity and spleen weight with SA8LD myeloid leukaemic progression.

3.9.1.1 The variation of total body weight with SA8LD leukaemic progression.

The total body weight of 9-10 week old male CBA/H mice on the day of injection (day 0) was determined as 28(±2)g (±SD) (n=6). By day 15, the total body weight was determined as 30(±2)g (n=6) and by day 18, the total body weight was determined as 30(±2)g (n=7). By day 21 and overt leukaemia, total body weight was determined as 32(±2)g (n=7).

No significant variation in total body weight was observed with SA8LD leukaemic progression [*Figures 52 & 52a*].

3.9.1.2 The variation in femoral cellularity with SA8LD leukaemic progression.

Femoral cellularity of 9-10 week old CBA/H mice on the day of injection (day 0) was determined as 14(±2)x10^6 cells (±SD) (n=6). By day 15 femoral cellularity was determined as 15(±1)x10^6 cells (n=6) and by day 18, femoral cellularity was 13(±1)x10^6 cells (n=7). By day 21 and overt leukaemia, femoral cellularity was determined as 15(±1)x10^6 cells (n=7) [*Figures 52 & 52b*].

No significant variation in femoral cellularity was observed with SA8LD myeloid leukaemic progression, although the cellular composition of the femoral marrow was significantly changed. In normal (day 0) femoral marrow, a predominance of relatively mature

haematopoietic progenitors was observed (metamyelocytes, erythroblasts, lymphocytes and band cells [Hepburn *et al*,1987]). In overtly leukaemic SA8LD leukaemic femoral marrow (day 21), there was a predominance of immature blast cells (increased numbers of myelocytes, promyelocytes and generalized blast cells [Hepburn *et al*,1987]).

3.9.1.3 The variation in spleen weight with SA8LD leukaemic progression.

Spleen weight of 9-10 week old, male, CBA/H mice on the day of injection (day 0) was determined as 67(±7)mg (±SD) (n=6). By day 15 spleen weight was 67(±7)mg (n=6) and by day 18 spleen weight was 76(±8)mg (n=7). By day 21 and overt leukaemia, spleen weight was 138(±55)mg (n=7) [*Figures 52 & 52c*].

A significant increase in spleen weight was observed with disease progression. The increase in spleen weight was associated with a marked splenomegally, which is the result of a marked infiltration by leukaemic cells. The spleen, originally red-brown, becomes pale with infiltrated leukaemic cells with disease progression. In normal spleen, the lymphocyte is the predominant cell type. In overtly leukaemic mice the spleen becomes heavily infiltrated with non-lymphocytic, leukaemic blast cells (myelocytes, promyelocytes and generalized blast cells [Hepburn *et al*,1987]).

Figure 52: A time study of total body weight, femoral cellularity and spleen weight with SA8 low cell dose-passaged, myeloid leukaemic progression.

Progession of SA8LD myeloid leukaemia.	Total body weight (g) (±SD)	Femur cellularity (x10^6 cells) (±SD)	Spleen weight (mg) (±SD)
Control, Day 0 (n=6)	28(±2)	14(±2)	67(±7)
Day 15 (n=6)	30(±2)	15(±1)	67(±7)
Day 18 (n=7)	30(±2)	13(±1)	76(±8)
Day 21, (n=7) (overt leukaemia)	32(±2)	15(±1)	138(±55)

Figure 52a: Variation in body weight with SA8LD leukaemic progression.

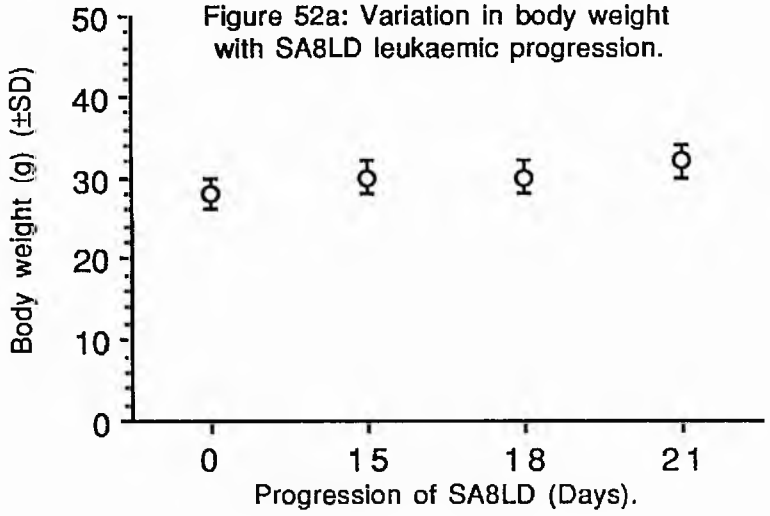


Figure 52b: Variation in femoral cellularity with SA8LD leukaemic progression.

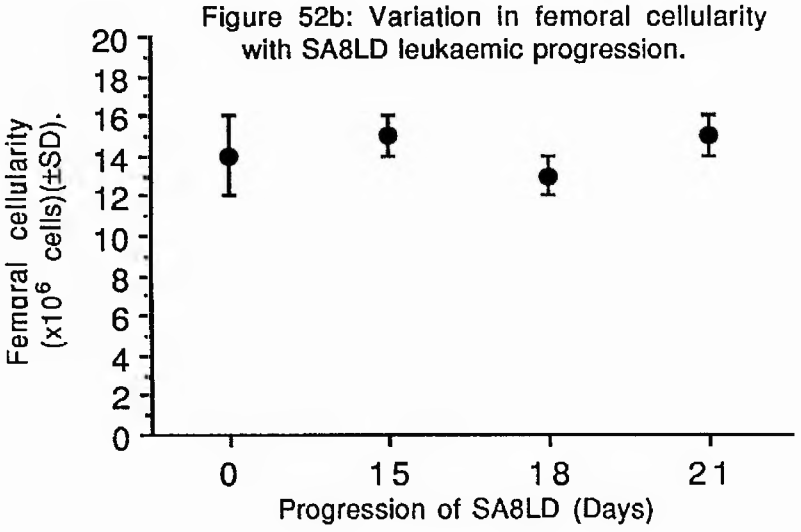
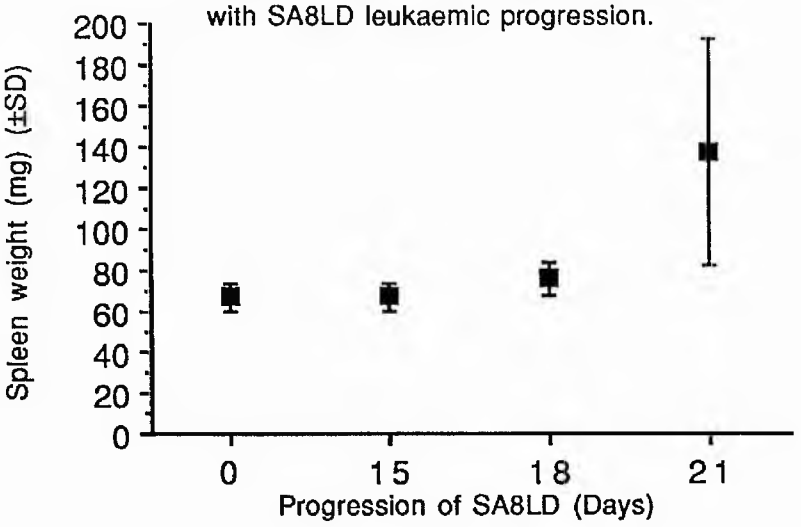


Figure 52c: Variation in spleen weight with SA8LD leukaemic progression.



3.9.2 Assay of the stem cell proliferation *inhibitor* and *stimulator* content of medium conditioned by femoral marrow after 0, 15, 18 and 21 days progression of SA8LD myeloid leukaemia.

3.9.2.1 Assay of the stem cell proliferation *stimulator* content of medium conditioned by femoral marrow after 0, 15, 18 and 21 days progression of SA8LD myeloid leukaemia.

The proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase in washed normal bone marrow was determined as 11(±1)% (±SEM) (n=8). In washed normal bone marrow incubated with medium conditioned by 'day 0' SA8LD femoral marrow the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was not significantly changed ($P>0.1$) at 12(±1)% (n=2). In washed normal bone marrow incubated with medium conditioned by 'day 15' SA8LD femoral marrow the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was not significantly changed ($P>0.1$) at 12(±1)% (n=2). In washed normal bone marrow incubated with medium conditioned by 'day 18' SA8LD femoral marrow the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was not significantly changed ($P>0.1$) at 11(±2)% (n=2). In washed normal bone marrow incubated with medium conditioned by 'day 21', overtly leukaemic SA8LD femoral marrow, the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was not significantly changed ($P>0.1$) at 11(±1)% (n=2) [*Figures 53 & 53a*].

No evidence for the presence of stem cell proliferation *stimulator* was observed during the progression of the SA8LD myeloid leukaemia.

3.9.2.2 Assay of the stem cell proliferation *inhibitor* content of medium conditioned by femoral marrow after 0, 15, 18 and 21 days progression of SA8LD myeloid leukaemia.

The proportion of HPP-CFC$_{IL3+CSF\text{-}1}$ in S-phase in washed regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose was determined as 35(±1)% (±SEM) (n=8). In washed regenerating bone marrow incubated with medium conditioned by 'day 0' SA8LD femoral marrow the proportion of HPP-CFC$_{IL3+CSF\text{-}1}$ in S-phase was significantly reduced ($P<0.025$) at 18(±3)% (n=2). In washed regenerating bone marrow incubated with medium conditioned by 'day 15' SA8LD femoral marrow the proportion of HPP-CFC$_{IL3+CSF\text{-}1}$ in S-phase was significantly reduced ($P<0.05$) at 24(±4)% (n=2). In washed regenerating bone marrow incubated with medium conditioned by 'day 18' SA8LD femoral marrow the proportion of HPP-CFC$_{IL3+CSF\text{-}1}$ in S-phase was significantly reduced ($P<0.05$) at 25(±4)% (n=2). In washed regenerating bone marrow incubated with medium conditioned by 'day 21' SA8LD femoral marrow the proportion of HPP-CFC$_{IL3+CSF\text{-}1}$ in S-phase was not significantly changed ($P>0.1$) at 34(±2)% (n=2) [*Figures 53 & 53b*].

Evidence was observed for the presence of the stem cell proliferation *inhibitor* in 'day 0' SA8LD marrow conditioned medium. This was not an unexpected finding since 'day 0' SA8LD femoral marrow was essentially 'normal bone marrow' and 'normal bone marrow conditioned medium' has been demonstrated to be a crude source of the stem cell proliferation *inhibitor* (3.6). With SA8LD myeloid leukaemic progression, the levels of proliferation *inhibitor* were observed to reduce. By 'day 21', overtly leukaemic SA8LD

Figure 53: A time study of the variation of *inhibitor* and *stimulator* in medium conditioned by marrow 0, 15, 18 and 21 days after injection of SA8LD myeloid leukaemic cells.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%)(±SEM)(n=2)	
	Normal bone marrow (NBM)	Regenerating bone marrow[1] (RBM)
Control (n=8)	11(±1)	35(±1)
+'Day 0' SA8LDcm	12(±1)[2]	18(±2)[3]
+'Day 15' SA8LDcm	12(±1)[2]	24(±3)[4]
+'Day 18' SA8LDcm	11(±2)[2]	25(±3)[4]
+'Day 21' SA8LDcm	11(±1)[2]	34(±1)[5]

[1] Regenerating bone marrow from mice3 days after a 2 Gy whole body X-irradiation dose.
[2] No significant increase ($P>0.1$).
[3] Significant reduction ($P<0.025$).
[4] Significant reduction ($P<0.05$).
[5] No significant reduction ($P>0.1$).

Figure 53a: The variation in stimulator with SA8LD leukaemic progression.

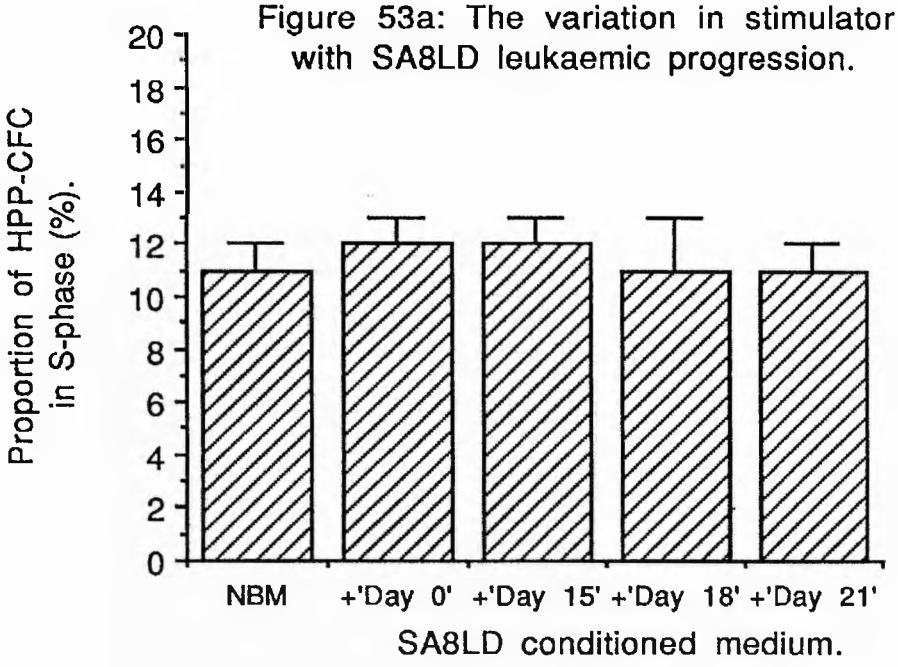
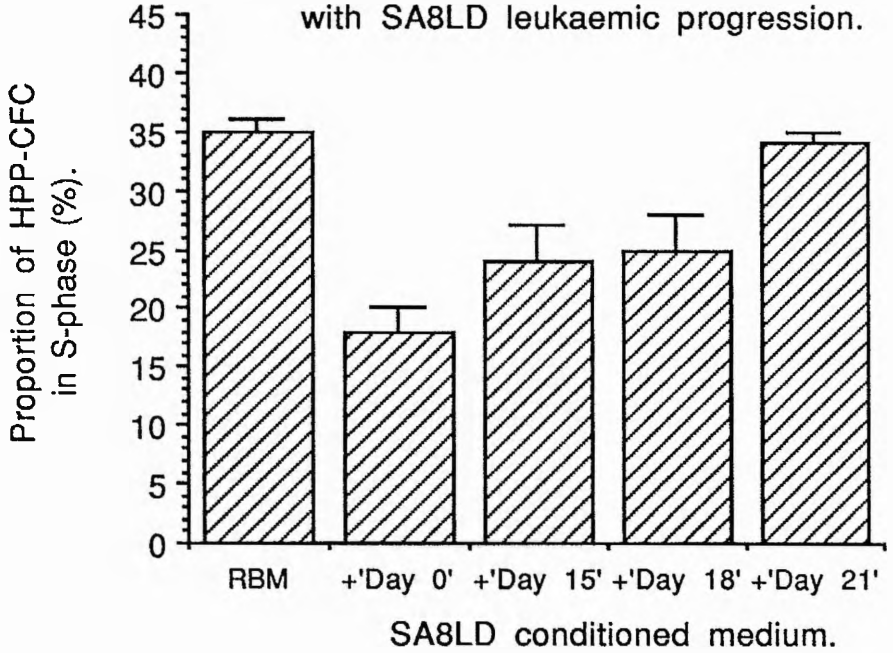


Figure 53b: The variation in inhibitor with SA8LD leukaemic progression.



femoral marrow conditioned medium demonstrated no stem cell proliferation *inhibitor*.

A progressive reduction in the detectable levels of *inhibitor* with disease progression, may explain the absence of *inhibitor* in the overtly leukaemic SA2LP, SA2HP, SA7HD, SA8HD and SA8LD marrow conditioned medium (3.8). No evidence of a direct-acting leukaemia-associated proliferation inhibitor was observed during SA8LD myeloid leukaemic progression and no evidence of SA8LD marrow conditioned medium toxicity was observed.

3.10 An investigation of the effects of medium conditioned by SA8HD leukaemic marrow on *stimulator* and *inhibitor* action.

The absence of demonstrable stem cell proliferation regulators in medium conditioned by overtly leukaemic SA2LP, SA2HP, SA7HD, SA8HD and SA8LD, may be due to an inactivation of the stem cell proliferation *stimulator* and *inhibitor*. The stem cell proliferation regulators may be produced, but their action may be blocked by the developing leukaemia. The action of medium conditioned by SA8HD leukaemic marrow, at the level of both *stimulator* and *inhibitor* action was investigated.

Procedure:

Medium conditioned by regenerating bone marrow from mice 7 days after a sublethal whole body X-irradiation dose of 4.5 Gy, as a crude source of *stimulator* (2.15) and medium conditioned by normal bone marrow, a crude source of *inhibitor* (2.14), were assayed for *stimulator* and *inhibitor* activity (2.16 & 2.17) in the absence and

presence of medium conditioned for 3 hours by SA8HD leukaemic marrow and diluted so as to be derived from $15x10^6$ cells/ml (2.22).

Results:

3.10.1 The effects of medium conditioned by SA8HD leukaemic marrow on *stimulator* action.

The proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase in washed normal marrow was determined as 10(±1)% (±SEM) (n=6). In washed normal bone marrow incubated with medium conditioned by regenerating bone marrow from mice 7 days after a 4.5 Gy whole body X-irradiation dose, as a crude source of *stimulator* (2.15), the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was significantly increased ($P < 0.0005$) at 25(±2)% (n=6). In washed normal bone marrow incubated with medium conditioned by regenerating bone marrow in the presence of medium conditioned by SA8HD leukaemic marrow cells, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was significantly increased ($P < 0.0005$) at 23(±3)% (n=4) [*Figure 54 & 54a*].

The presence of medium conditioned by SA8HD leukaemic marrow conditioned medium did not appear to impair the ability of *stimulator* to increase the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase.

3.10.2 The effects of medium conditioned by SA8HD leukaemic marrow on *inhibitor* action.

The proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase in washed regenerating bone marrow from mice 3 days after a 2 Gy whole body X-irradiation dose, was determined as 31(±1)% (±SEM) (n=2). In washed regenerating marrow, incubated with medium conditioned by normal bone marrow, as a crude source of *inhibitor* (2.14), the

proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was significantly reduced ($P < 0.05$) at 24(±3)% (n=2). In washed regenerating bone marrow incubated with medium conditioned by normal bone marrow in the presence of SA8HD leukaemic marrow, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was significantly reduced ($P < 0.05$) at 26(±1)% (n=2) [*Figure 54 & 54b*].

The presence of the SA8HD leukaemic marrow conditioned medium, did not appear to impair the ability of the *inhibitor* to reduce the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase.

3.11 An investigation of the effects of medium conditioned by SA8HD leukaemic marrow on *stimulator* and *inhibitor* production.

The absence of detectable stem cell proliferation *inhibitor* and *stimulator* in medium conditioned by SA2LP, SA2HP, SA7HD, SA8HD and SA8LD leukaemic marrow, was further investigated to determine whether the leukaemic marrow conditioned medium was capable of inhibiting the production of the stem cell proliferation regulators. A mechanism similar to that previously discussed (1.14), where *inhibitor* is capable of preventing *stimulator* production and similarly *stimulator* is capable of preventing *inhibitor* production, may exist.

3.11.1 The effects of medium conditioned by SA8HD leukaemic marrow on *stimulator* production by regenerating bone marrow.

Procedure:

Regenerating femoral marrow was produced from mice 7 days after a 4.5 Gy whole body X-irradiation dose. Cells were washed once and resuspended in either fresh medium or medium conditioned for 3

Figure 54: An investigation of the effects of medium conditioned by SA8HD leukaemic marrow on *stimulator* and *inhibitor* action.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%) (±SEM)	
	Normal bone marrow (NBM)(n=6)	Regenerating bone marrow[1] (RBM)(n=2)
Control	10(±1)	31(±1)
+RBMcm[2] (*Stimulator*)	25(±2)[5]	---
+RBMcm+SA8HDcm[3]	23(±1)[5]	---
+NBMcm[4] (*Inhibitor*)	---	24(±2)[6]
+NBMcm+SA8HDcm	---	26(±1)[6]

[1] Regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.

[2] Regenerating bone marrow conditioned medium from mice 7 days after a 4.5 Gy whole body X-irradiation dose.

[3] Medium conditioned by SA8HD leukaemic marrow.

[4] Medium conditioned by normal bone marrow.

[5] Significant increase ($P<0.0005$).

[6] Significant reduction ($P<0.05$).

Figure 54a: The effect of SA8HD leukaemic marrow conditioned medium on stimulator action.

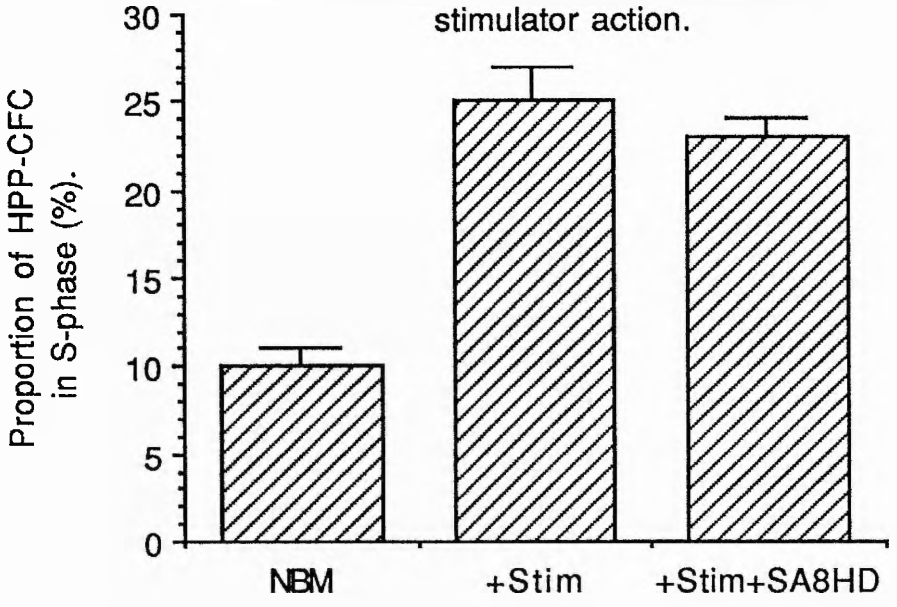
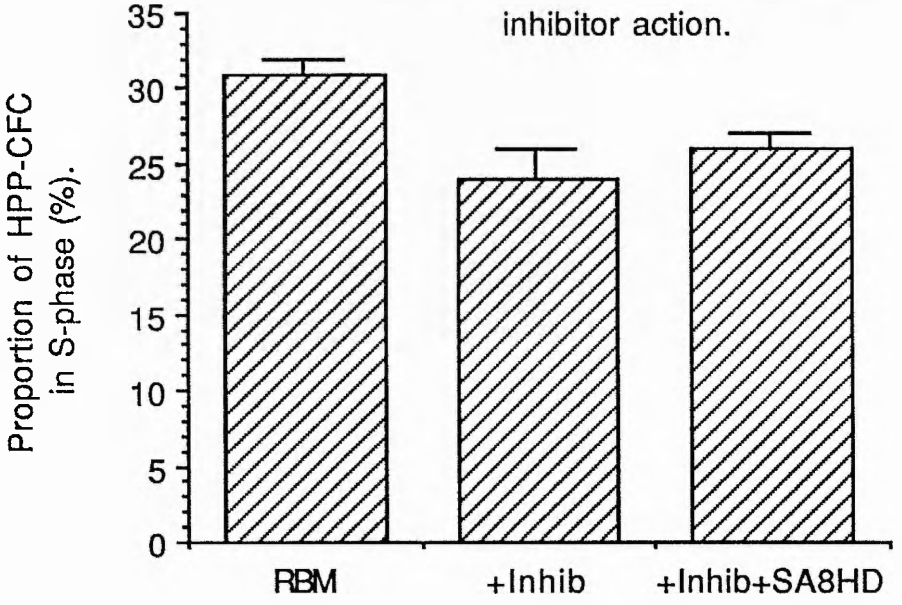


Figure 54b: The effect of SA8HD leukaemic marrow conditioned medium on inhibitor action.



hours by $15x10^6$ SA8HD leukaemic marrow cells/ml. The regenerating marrow was subsequently incubated for 4 hours in the presence of fresh or SA8HD leukaemic marrow conditioned medium, to allow *stimulator* synthesis. 3 batches of regenerating bone marrow conditioned medium and regenerating bone marrow conditioned medium conditioned in the presence of SA8HD leukaemic marrow conditioned medium, were produced and assayed for *stimulator* content (2.17).

Results:

In washed normal bone marrow the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was determined as 15(±1)% (±SEM) (n=11). In washed normal bone marrow, incubated in the presence of regenerating bone marrow conditioned medium, the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was significantly increased ($P < 0.0005$) at 24(±1)% (n=11). In washed normal bone marrow incubated with regenerating bone marrow conditioned medium, conditioned in the presence of SA8HD leukaemic marrow conditioned medium, the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was not significantly changed ($P > 0.1$) at 14(±2)% (n=11) [*Figure 55 & 55a*].

The presence of SA8HD leukaemic marrow conditioned medium appears to impair the production of the stem cell proliferation *stimulator* by regenerating bone marrow.

3.11.2 The effects of medium conditioned by SA8HD leukaemic marrow on the production of *inhibitor* by normal bone marrow.

Procedure:

Normal bone marrow cells were washed and resuspended in either fresh medium or in medium conditioned for 3 hours by $15x10^6$ SA8HD leukaemic marrow cells/ml. The normal bone marrow was

subsequently incubated for 6 hours in the presence of fresh medium, or medium conditioned by SA8HD leukaemic marrow, to allow *inhibitor* synthesis. 3 batches of normal bone marrow conditioned medium and normal bone marrow conditioned medium conditioned in the presence of SA8HD leukaemic marrow conditioned medium, were produced and assayed for *inhibitor* (2.16).

Results:

In washed regenerating bone marrow, from mice 3 days after a 2 Gy whole body X-irradiation dose, the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was determined as 37(±1)% (±SEM) (n=12). In washed regenerating bone marrow incubated in the presence of normal bone marrow conditioned medium, the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was significantly reduced ($P<0.0005$) at 26(±1)% (n=12). In washed regenerating bone marrow incubated with normal bone marrow conditioned medium, conditioned in the presence of SA8HD leukaemic marrow conditioned medium, the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was not significantly changed ($P>0.1$) at 38(±1)% (n=12) [*Figure 55 & 55b*].

The presence of SA8HD leukaemic marrow conditioned medium appears to impair the production of the stem cell proliferation *inhibitor* by normal bone marrow.

This finding may explain the absence of detectable levels of stem cell proliferation regulators in the SA2LP, SA2HP, SA7HD, SA8HD and SA8LD leukaemic marrow conditioned medium. An inhibition of stem cell proliferation regulator production may explain the apparent inability of the normal haematopoietic tissue to respond to the deterioration in the quality of the peripheral blood which is associated with leukaemic progression, and may also explain the leukamia-associated suppression of normal haematopoiesis, allowing the leukaemic cells to develop a proliferative advantage.

Figure 55: An investigation of the effects of medium conditioned by SA8HD leukaemic marrow on *stimulator* and *inhibitor* production.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%) (±SEM)	
	Normal bone marrow (NBM)(n=11)	Regenerating bone marrow[1] (RBM)(n=12)
Control	15(±1)	37(±1)
+RBMcm[2] (*Stimulator*)	24(±1)[7]	---
+[RBMcm+SA8HDcm[3]][4]	14(±1)[8]	---
+NBMcm[5] (*Inhibitor*)	---	26(±1)[9]
+[NBMcm+SA8HDcm][6]	---	38(±1)[10]

[1] Regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.

[2] Regenerating bone marrow conditioned medium from mice 7 days after a 4.5 Gy whole body X-irradiation dose.

[3] Medium conditioned by SA8HD leukaemic marrow.

[4] Regenerating bone marrow conditioned medium, conditioned in the presence of SA8HD leukaemic marrow conditioned medium

[5] Medium conditioned by normal bone marrow.

[6] Normal bone marrow conditioned medium conditioned in the presence of SA8HD leukaemic marrow conditioned medium.

[7] Significant increase ($P<0.0005$).

[8] No significant increase ($P>0.1$).

[9] Significant reduction ($P<0.0005$).

[10] No significant reduction ($P>0.1$).

Figure 55a: The effect of SA8HD leukaemic marrow conditioned medium on stimulator production.

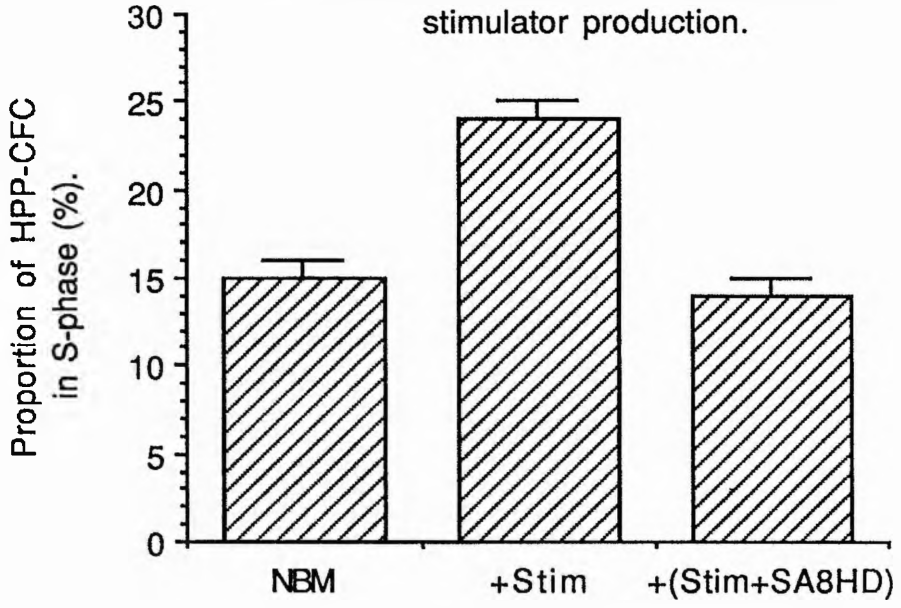
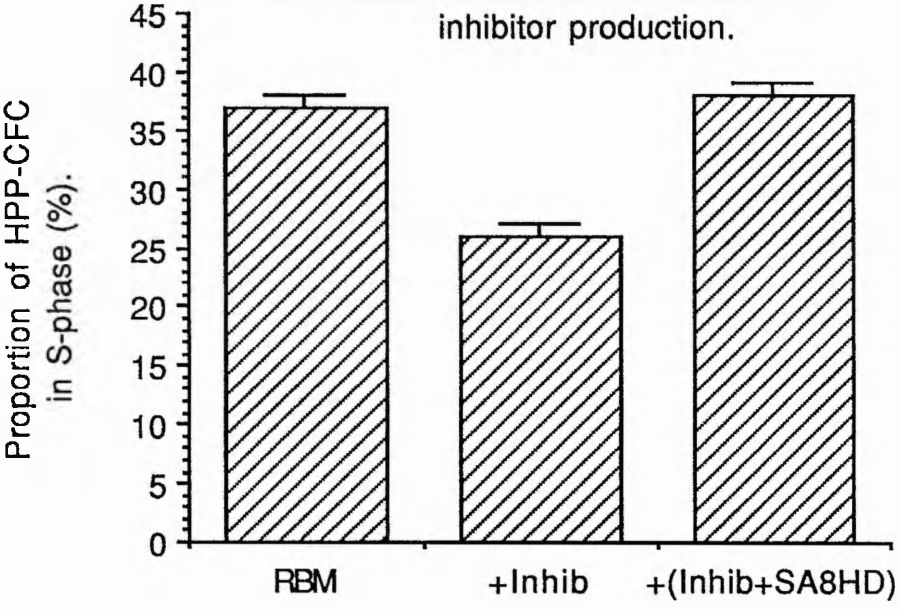


Figure 55b: The effect of SA8HD leukaemic marrow conditioned medium on inhibitor production.



Section 4

An investigation of the possible mechanism of action of the low molecular weight, haemoregulatory peptide Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP).

3.12 An investigation of the possible mechanism of action of the low molecular weight, haemoregulatory peptide Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP) using the minimal active amino acid sequence Ser-Asp-Lys (SDK).

The low molecular weight haemoregulatory tetrapeptide Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP, M_r=487amu)(1.20) is reported to reduce the haemotoxicity of S-phase-specific cytotoxic agents by preventing the 'recruitment' of haematopoietic stem cells into S-phase. The mechanism of action of the tetrapeptide AcSDKP was investigated using the *in vitro* HPP-$CFC_{IL3+CSF\text{-}1}$ assay and the minimal active amino acid sequence Ser-Asp-Lys (SDK, M_r=348amu). (AcSDKP was not initially available to this laboratory).

Procedure:

Preliminary experiments were performed to determine the inherent inhibitory, or stimulatory characteristics of the haemoregulatory peptide.

3.12.1 An investigation of the direct effects of tripeptide SDK against the *in vitro* GM-CFC population.

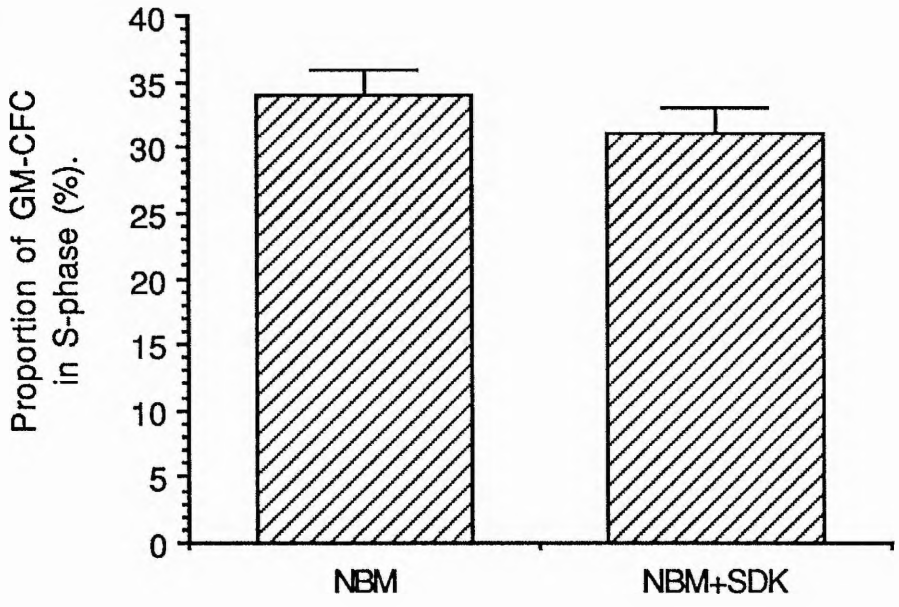
The proportion of GM-CFC in S-phase in normal washed bone marrow was determined as 34(±2)% (±SEM) (n=16) (2.25). In normal washed bone marrow incubated for 3 hours in the presence of SDK, the proportion of GM-CFC in S-phase was not significantly changed ($P>0.1$) at 31(±8)% (n=16). (SDK was added at t=0, 1 and 2 hours to a final concentration of 1 ng/ml, 2.87×10^{-9}M) [*Figure 56*].

At 1 ng/ml, 2.87×10^{-9}M, the tripeptide SDK does not demonstrate inherent inhibitory or stimulatory activity against the haematopoietic

Figure 56: An investigation of the direct inhibitory or stimulatory effects of tripeptide Ser-Asp-Lys (SDK) on the proportion of GM-CFC in S-phase.

	Proportion of GM-CFC in S-phase.(%)(±SEM)(n=16)
Normal bone marrow (NBM)	34(±2)
NBM + SDK (1 ng/ml, 2.87×10^{-9}M)	31(±2)[1]

[1] No significant change (P>0.1).



progenitor GM-CFC population. No evidence of SDK toxicity was observed.

3.12.2 An investigation of the direct effects of tripeptide SDK against the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population.

3.12.2.1 An investigation of the direct stimulatory effects of tripeptide SDK against the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population.

The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in normal bone marrow was determined as 12(±1)% (±SEM) (n=19). In normal bone marrow incubated for 3 hours in the presence of SDK (2.25), the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was not significantly changed (P>0.1) at 12(±1)% (n=19). (SDK was added at t=0, 1 and 2 hours to a final concentration of 1 ng/ml, 2.87x10^{-9}M) [*Figure 57*].

At 1 ng/ml, 2.87x10^{-9}M, the tripeptide SDK does not demonstrate inherent stimulatory activity against the HPP-CFC$_{IL3+CSF-1}$ population. No evidence of SDK toxicity was observed.

3.12.2.2 An investigation of the direct inhibitory effects of tripeptide SDK against the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population.

The proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase in washed regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose, was determined as 33(±1)% (±SEM) (n=6). In washed regenerating marrow incubated for 3 hours in the presence of SDK, the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was not significantly changed (P>0.1) at 32(±2)% (n=6). (SDK was added at

t=0, 1 and 2 hours to a final concentration of 1 ng/ml, $2.87x10^{-9}$M) [*Figure 57*].

At 1 ng/ml, $2.87x10^{-9}$M, the tripeptide SDK does not demonstrate inherent inhibitory activity for the haematopoietic precursor HPP-$CFC_{IL3+CSF-1}$ population. No evidence of SDK toxicity was observed.

3.13 An investigation of the effects of tripeptide SDK on stem cell proliferation *inhibitor* and *stimulator* action.

3.13.1 Investigating the effects of SDK on *stimulator* action.

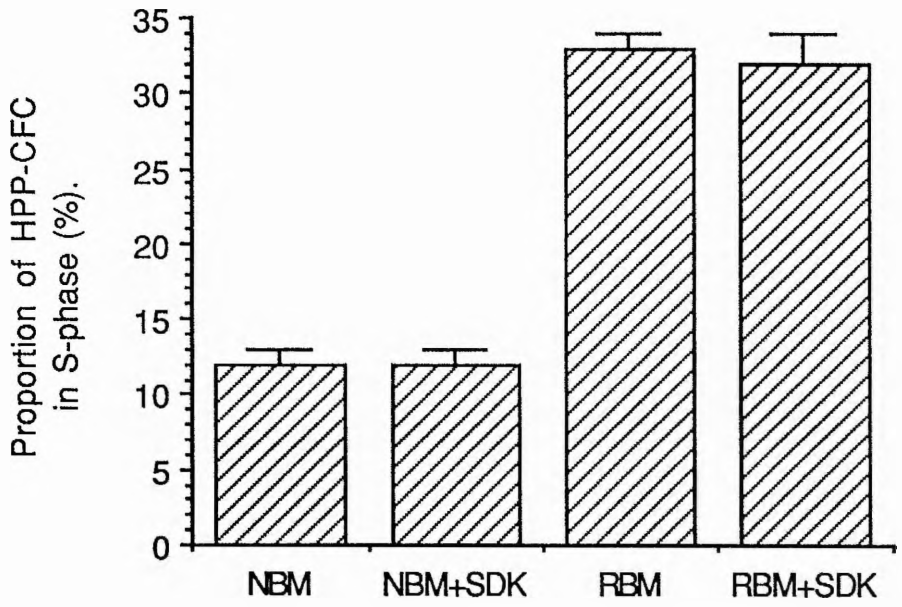
The proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase in washed normal bone marrow was determined as 14(±1)% (±SEM) (n=11). In washed normal bone marrow incubated with medium conditioned by regenerating bone marrow from mice 7 days after a 4.5 Gy whole body X-irradiation dose, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was significantly increased ($P<0.0005$) at 28(±1)% (n=11). In washed normal bone marrow, incubated with medium conditioned by regenerating bone marrow and in the presence of SDK (2.26), the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was not significantly changed ($P>0.1$) at 13(±1)% (n=11). (SDK was added at t=0 and at each subsequent hour of incubation, to a final concentration of 1 ng/ml, $2.87x10^{-9}$M) [*Figure 58 & 58a*]

The presence of SDK at 1 ng/ml, $2.87x10^{-9}$M, appears to impair the capacity of *stimulator*, derived from regenerating bone marrow conditioned medium, to increase the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase. No evidence of SDK toxicity was observed.

Figure 57: An investigation of the direct inhibitory or stimulatory effects of tripeptide Ser-Asp-Lys (SDK) on the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%)(±SEM)	
	Normal bone marrow (NBM) (n=19)	Regenerating bone marrow[1] (RBM) (n=6)
Control	12(±1)	33(±1)
+ SDK (1 ng/ml, 2.87×10^{-9}M)	12(±1)[2]	32(±2)[2]

[1] Regenerating bone marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.
[2] No significant change (P>0.1).



3.13.2 Investigating the effects of SDK on *inhibitor* action.

The proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase in washed regenerating bone marrow from mice 3 days after a 2 Gy whole body X-irradiation dose was determined as 37(±3)% (±SEM) (n=7). In washed regenerating marrow incubated with medium conditioned by normal bone marrow, as a crude source of *inhibitor*, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was significantly reduced ($P<0.005$) at 23(±2)% (n=7). In washed regenerating bone marrow incubated with medium conditioned by normal bone marrow and in the presence of SDK (2.26), the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was not significantly changed ($P>0.1$) at 36(±3)% (n=7). (SDK was added at t=0 and at each subsequent hour of incubation, to a final concentration of 1 ng/ml, $2.87x10^{-9}$M) [*Figure 58 & 58b*]

The presence of SDK at 1 ng/ml, $2.87x10^{-9}$M, appears to impair the capacity of *inhibitor*, derived from normal bone marrow conditioned medium, to reduce the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase. No evidence of SDK toxicity was observed.

3.14 An investigation of the effect of SDK on stem cell proliferation *inhibitor* and *stimulator* production.

3.14.1 The effect of SDK on *stimulator* production by regenerating bone marrow.

Procedure:

Regenerating femoral marrow was produced from mice 7 days after a whole body X-irradiation dose of 4.5 Gy. Washed regenerating marrow was resuspended in serum-free medium and split into 2 samples. To one a volume of SDK was added sufficient to produce a

Figure 58: An investigation of the effects of tripeptide Ser-Asp-Lys (SDK)on *stimulator* and *inhibitor* action.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%) (±SEM)	
	Normal bone marrow (NBM) (n=11)	Regenerating bone marrow[1] (RBM) (n=7)
Control	14(±1)	37(±3)
+RBMcm[2] (*Stimulator*)	28(±1)[5]	---
+RBMcm+SDK[3]	13(±1)[6]	---
+NBMcm[4] (*Inhibitor*)	---	23(±1)[7]
+NBMcm+SDK[3]	---	36(±1)[8]

[1] Regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.

[2] Regenerating bone marrow conditioned medium from mice 7 days after a 4.5 Gy whole body X-irradiation dose.

[3] SDK present at 1 ng/ml, 2.87×10^{-9}M

[4] Medium conditioned by normal bone marrow.

[5] Significant increase ($P<0.0005$).

[6] No significant increase ($P>0.1$).

[7] Significant reduction ($P<0.005$).

[8] No significant reduction ($P>0.1$).

Figure 58a: The effects of tripeptide SDK on stimulator action.

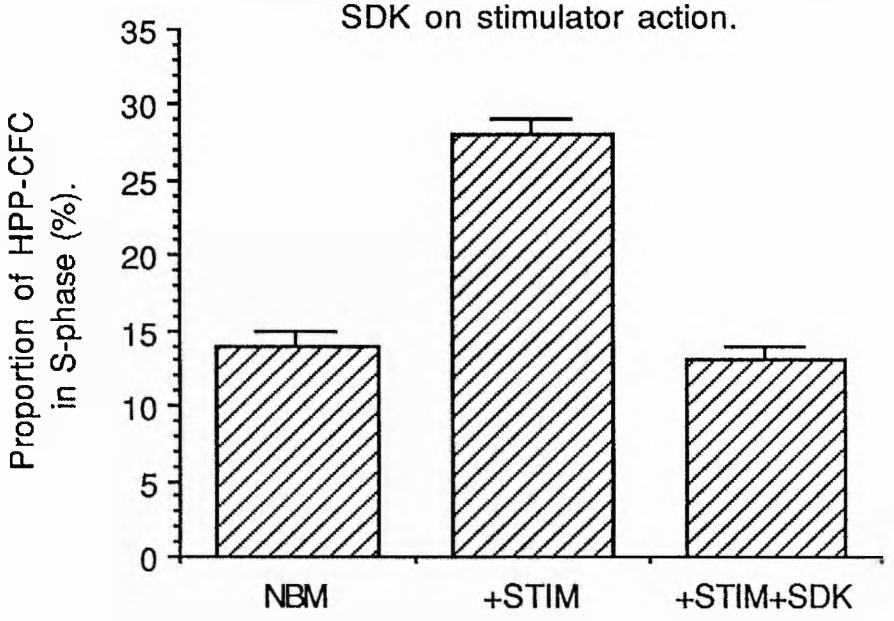
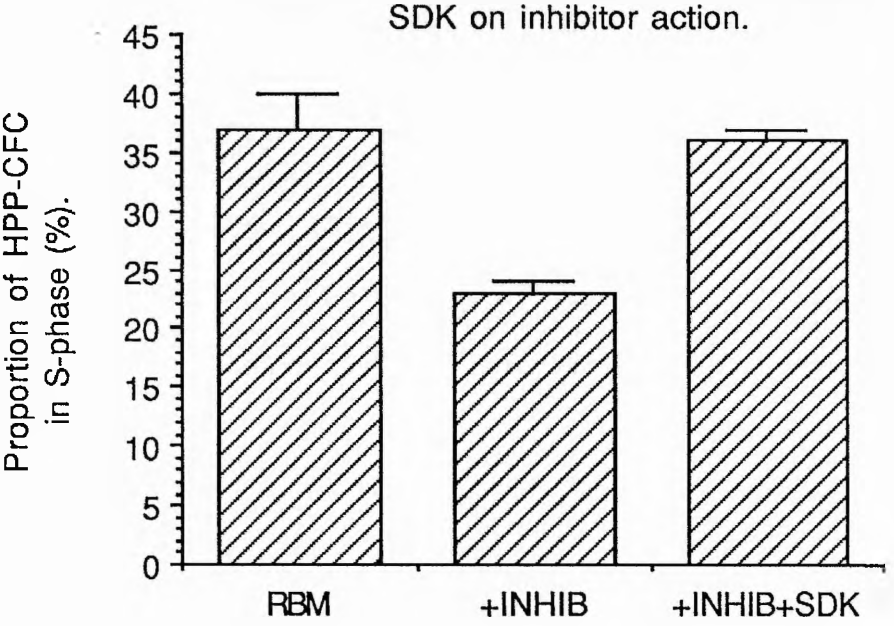


Figure 58b: The effects of tripeptide SDK on inhibitor action.



final concentration of 1 ng/ml, 2.87×10^{-9}M, to the other an equivalent volume of serum-free medium was added. The regenerating marrow was subsequently incubated for 5 hours to allow *stimulator* synthesis (2.27). (SDK was added at t=0 and at each hour of subsequent incubation to a final concentration of 1 ng/ml, 2.87×10^{-9}M). 2 batches of regenerating bone marrow conditioned medium and regenerating bone marrow conditioned medium conditioned in the presence of SDK, were produced and assayed to determine their *stimulator* content (2.17).

Results:

In washed normal bone marrow the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was determined as 16(±1)% (±SEM) (n=5). In washed normal bone marrow incubated with medium conditioned by regenerating bone marrow, a crude source of *stimulator*, the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was significantly increased ($P < 0.005$) at 28(±2)% (n=5). In washed normal bone marrow, incubated with medium conditioned by regenerating bone marrow conditioned in the presence of SDK, the proportion of HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase was significantly increased ($P < 0.05$) at 22(±4)% (n=5) [*Figure 59 & 59a*].

While the presence of SDK at 1ng/ml, 2.87×10^{-9}M, does appear to impair the capacity of regenerating bone marrow to synthesize *stimulator*, these results should be considered in view of the findings of (3.13.1) where SDK was reported to impair the action of *stimulator*. The apparent impairment of *stimulator* synthesis by SDK may actually be due to the presence of residual SDK in the regenerating bone marrow conditioned medium during the *stimulator* assay. If *stimulator* synthesis was unaffected by the presence of SDK, during subsequent *stimulator* assay any residual SDK may act to impair *stimulator* action, giving the impression of an absence of *stimulator*. Before any

conclusion as to the effects of SDK on *stimulator* production by regenerating bone marrow can be drawn, removal of residual SDK from the regenerating bone marrow conditioned medium prior to *stimulator* assay would be necessary. The low molecular weight of SDK should enable it to be readily removed from the regenerating bone marrow conditioned medium by dialysis or Amicon ultrafiltration.

3.14.2 The effect of SDK on *inhibitor* production by normal bone marrow.

Procedure:

Normal bone marrow was washed, resuspended in serum-free medium and split into 2 samples. To one, a volume of SDK sufficient to produce a final concentration of 1 ng/ml, 2.87×10^{-9}M was added, to the other, an equal volume of serum-free medium was added. The normal bone marrow was subsequently incubated for 7 hours to allow *inhibitor* synthesis, with the addition of SDK at each hour of incubation, to a final concentration of 1 ng/ml, 2.87×10^{-9}M, or an equivalent volume of serum free medium (2.27). 1 batch of normal bone marrow conditioned medium and normal bone marrow conditioned medium conditioned in the presence of SDK, was produced.

Results:

In normal regenerating bone marrow, from mice 3 days after a 2 Gy whole body X-irradiation dose, the proportion of $HPP\text{-}CFC_{IL3+CSF\text{-}1}$ in S-phase was determined as 40($\pm$1) ($\pm$SEM) ($n=2$). In washed regenerating bone marrow incubated with normal bone marrow conditioned medium, as a crude source of *inhibitor*, the proportion of $HPP\text{-}CFC_{IL3+CSF\text{-}1}$ in S-phase was significantly reduced ($P < 0.05$) at

26(±6)% (n=2). In washed regenerating bone marrow incubated with medium conditioned by normal bone marrow in the presence of SDK, the proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase was significantly reduced ($P<0.05$) at 26(±4)% (n=2) [*Figure 59 & 59b*].

The presence of SDK at 1 ng/ml, $2.87x10^{-9}$M, does not appear to impair the synthesis of *inhibitor* by normal bone marrow. Concern over the presence of residual SDK in the normal bone marrow conditioned medium and SDK effects in the subsequent *inhibitor* assay, could be answered by dialysis or Amicon ultrafiltration of the normal bone marrow conditioned medium to remove the low molecular weight SDK prior to *inhibitor* assay (3.13.2).

3.15 A comparative analysis of the tripeptide Ser-Asp-Lys (SDK) with tetrapeptide Acetyl-Ser-Asp-Lys-Pro (AcSDKP), and tripeptides Asp-Lys-Pro (DKP) and Ala-Asp-Lys (ADK).

In the first instance our laboratory was only given access to the minimal active amino acid sequence of the AcSDKP moiety, the tripeptide Ser-Asp-Lys (SDK). Latterly, access was given to the tetrapeptide AcSDKP and a number of tripeptide analogues (ADK and DKP). A limited number of experiments were performed to compare the relative activities of SDK and AcSDKP, ADK and DKP.

3.15.1 Investigation of the direct effects of tetrapeptide AcSDKP against the haematopoietic progenitor *in vitro* GM-CFC population.

The proportion of GM-CFC in S-phase in normal washed bone marrow was determined as 28(±1)% (±SEM) (n=2). In normal bone marrow incubated for 3 hours in the presence of AcSDKP, the

Figure 59: An investigation of the effects of tripeptide Ser-Asp-Lys (SDK) on *stimulator* and *inhibitor* production.

	Proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase (%) (±SEM)	
	Normal bone marrow (NBM) (N=5)	Regenerating bone marrow[1] (RBM) (n=2)
Control	16(±1)	40(±1)
+RBMcm[2] (*Stimulator*)	28(±1)[6]	---
+[RBMcm+SDK][3]	22(±4)[7]	---
+NBMcm[4] (*Inhibitor*)	---	26(±1)[8]
+[NBMcm+SDK][5]	---	26(±4)[8]

[1] Regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.

[2] Regenerating bone marrow conditioned medium from mice 7 days after a 4.5 Gy whole body X-irradiation dose.

[3] Regenerating bone marrow conditioned medium, conditioned in the presence of SDK at 1 ng/ml, 2.87×10^{-9}M

[4] Medium conditioned by normal bone marrow.

[5] Normal bone marrow conditioned medium conditioned in the presence of SDK at 1 ng/ml, 2.87×10^{-9}M.

[6] Significant increase ($P<0.005$).

[7] Significant increase ($P<0.05$).

[8] Significant reduction ($P<0.05$).

Figure 59a: The effects of tripeptide SDK on stimulator production.

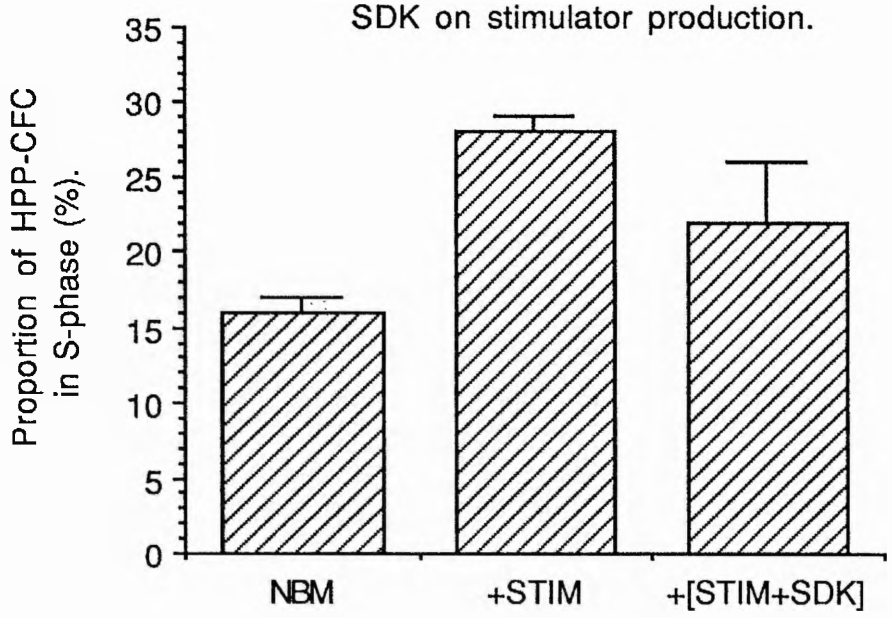
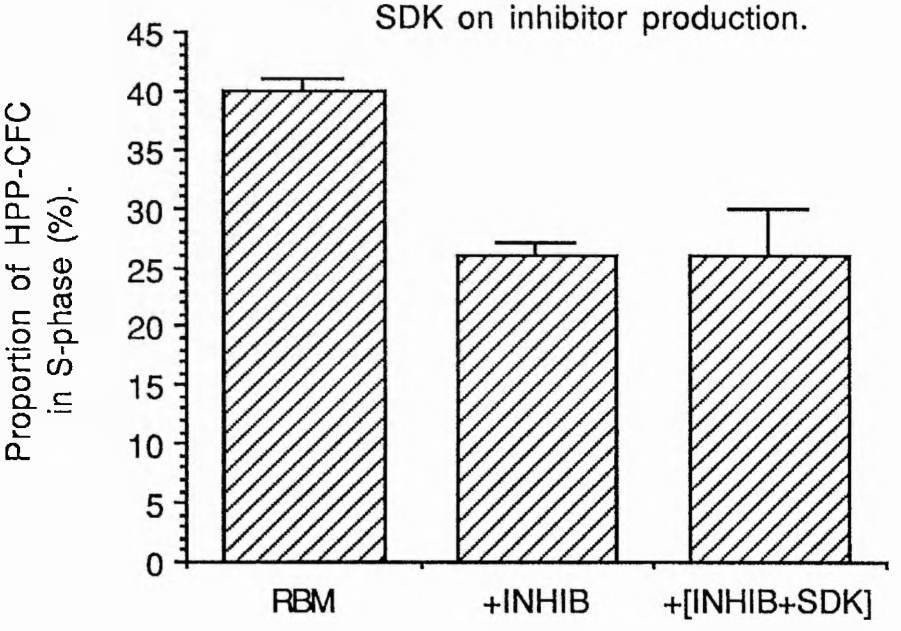


Figure 59b: The effects of tripeptide SDK on inhibitor production.



proportion of GM-CFC in S-phase was not significantly changed ($P > 0.4$) at 28(±4)% (n=2). (AcSDKP was added at t=0, 1 and 2 hours to a final concentration of 1 ng/ml, 2.05×10^{-9}M) [*Figure 60*].

At 1 ng/ml, 2.05×10^{-9}M, AcSDKP does not demonstrate either inherent inhibitory or stimulatory activity against the haematopoietic progenitor GM-CFC population. No evidence of AcSDKP toxicity was observed.

3.15.2 Investigation of the direct stimulatory effects of tetrapeptide AcSDKP and tripeptide ADK against the haematopoietic precursor $HPP\text{-}CFC_{IL3+CSF\text{-}1}$ population.

The proportion of $HPP\text{-}CFC_{IL3+CSF\text{-}1}$ in S-phase in washed normal bone marrow was determined as 12(±1)% (±SEM) (n=15). In normal bone marrow incubated for 3 hours in the presence of AcSDKP, or ADK, the proportion of $HPP\text{-}CFC_{IL3+CSF\text{-}1}$ in S-phase was not significantly changed at 13(±1)% ($P > 0.1$) (n=15) and 11(±1)% ($P > 0.1$) (n=13), repectively. (AcSDKP and ADK were added at t=0, 1 and 2 hours to a final concentration of 1 ng/ml or 2.05×10^{-9}M and 3.01×10^{-9}M respectively.) [*Figure 61*].

At 1 ng/ml neither the tetrapeptide AcSDKP, or the tripeptide ADK demonstrated inherent stimulatory activity for the haematopoietic precursor $HPP\text{-}CFC_{IL3+CSF\text{-}1}$ population. No evidence of AcSDKP or ADK toxicity was observed.

3.16 Investigation of the effects of AcSDKP, ADK and DKP on *stimulator* action.

The proportion of $HPP\text{-}CFC_{IL3+CSF\text{-}1}$ in S-phase in washed normal bone marrow was determined as 8(±1)% (±SEM) (n=7). In washed

Figure 60: An investigation of the direct inhibitory or stimulatory effects of tetrapeptide Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP) on the proportion of GM-CFC in S-phase.

	Proportion of GM-CFC in S-phase (%)(±SEM)(n=2)
Normal bone marrow (NBM)	28(±1)
NBM + AcSDKP (1 ng/ml, 2.05×10^{-9}M)	28(±4)[1]

[1] No significant change (P>0.4)

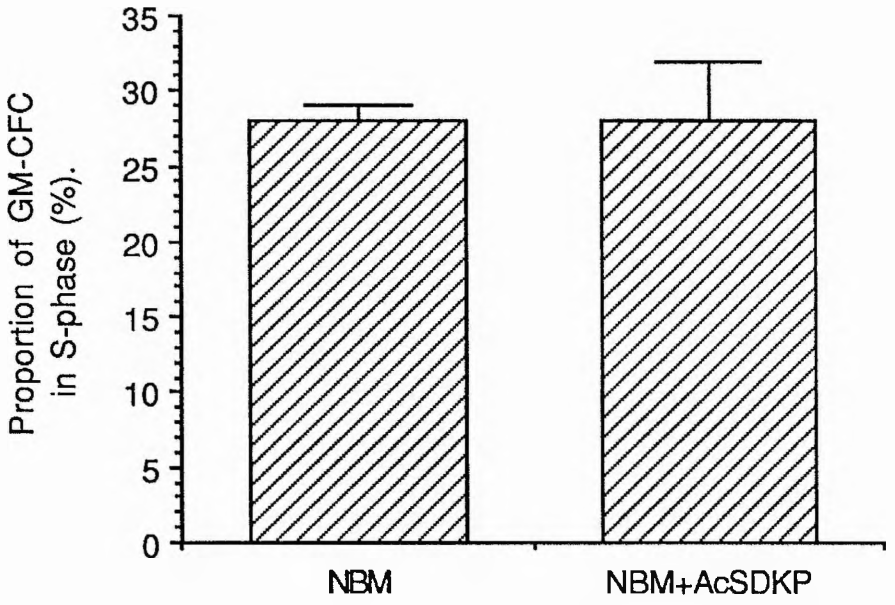
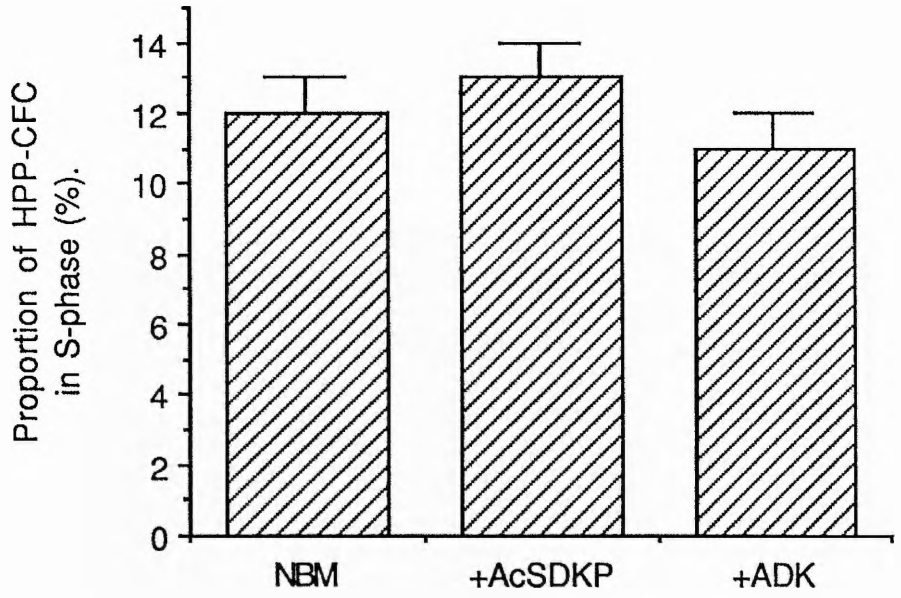


Figure 61: An investigation of the direct stimulatory effects of tetrapeptide Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP) and tripeptide Ala-Asp-Lys (ADK) on the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase.

	Proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase (%)(±SEM)
Normal bone marrow (NBM) (n=15)	12(±1)
NBM + AcSDKP (1 ng/ml, 2.05×10^{-9}M) (n=15)	13(±1)[1]
NBM + ADK (1 ng/ml, 3.01×10^{-9}M) (n=13)	11(±1)[1]

[1] No significant change (P>0.1).



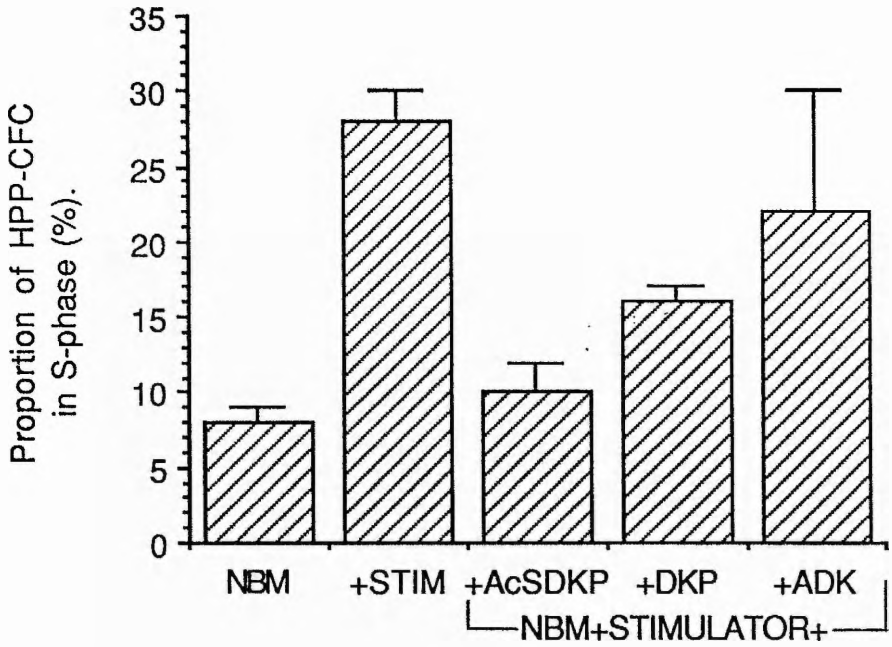
normal bone marrow incubated with medium conditioned by regenerating bone marrow from mice 7 days after a 4.5 Gy whole body X-irradiation dose, as a crude source of *stimulator*, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was significantly increased ($P < 0.0005$) at 28(±2)% (n=7). In washed normal bone marrow incubated with medium conditioned by regenerating bone marrow and in the presence of AcSDKP, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was not significanty changed ($P > 0.1$) at 10(±2)% (n=7). In washed normal bone marrow incubated with medium conditioned by regenerating bone marrow and in the presence of DKP, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was not significantly changed ($P > 0.05$) at 16(±1)% (n=3).In washed normal bone marrow incubated with medium conditioned by regenerating bone marrow and in the presence of ADK, the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase was significantly increased ($P < 0.025$) at 22(±8)% (n=4). (AcSDKP,DKP and ADK were added at t=0 and at each hour of subsequent assay, to a final concentration of 1 ng/ml, 2.05×10^{-9}M, 2.79×10^{-9}M and 3.01×10^{-9}M respectively.) [*Figure 62*].

The presence of both AcSDKP and DKP at 1 ng/ml appeared to impair the capacity of *stimulator*, derived from regenerating bone marrow conditioned medium, to increase the proportion of HPP-$CFC_{IL3+CSF-1}$ in S-phase. The presence of ADK at 1 ng/ml, however, gives a less clear result and may imply that the tripeptide ADK is a less efficient *stimulator*-blocking moiety than SDK, AcSDKP or DKP. No evidence of AcSDKP, DKP or ADK toxicity was observed.

Figure 62: An investigation of the effects of tetrapeptide AcSDKP and tripeptides DKP and ADK on *stimulator* action.

	Proportion of HPP-CFC$_{IL3+CSF-1}$ in S-phase (%)(±SEM) Normal bone marrow (NBM).
Control (n=7)	8(±1)
+ RBMcm [1](*Stimulator*) (n=7)	28(±2)[2]
Stimulator + AcSDKP (n=7) (1 ng/ml, 2.05x10^{-9}M)	10(±2)[3]
Stimulator + DKP (n=3) (1 ng/ml, 2.79x10^{-9}M)	16(±1)[4]
Stimulator + ADK (n=4) (1 ng/ml, 3.01x10^{-9}M)	22(±8)[5]

[1] Medium conditioned by marrow 7 days after a 4.5 Gy whole body X-irradiation dose.
[2] Significant increase (P<0.0005).
[3] No significant change (P>0.1).
[4] No significant change (P>0.05).
[5] Significant increase (P<0.025)



Chapter 4:

Discussion.

Section 1

The characterization of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ assay.

4.1.1 Characterization of haematopoietic colony-stimulating factor synergism.

In the presence of interleukin 3 (IL3)/multi-CSF, macrophage colony-stimulating factor (M-CSF/CSF-1), or granulocyte-macrophage colony-stimulating factor (GM-CSF), colony-forming cells of limited proliferative potential were stimulated. In the presence of combined haematopoietic colony-stimulating factors, the proliferation of a morphologically distinct colony-forming cell population, characterized by a high proliferative potential, was stimulated. The stimulation of the high proliferative potential colony-forming cell (HPP-CFC) population, is a demonstration of haematopoietic colony-stimulating factor *synergism.* A *synergistic interaction* may be broadly described as one in which the end product of combining two (or more) components, proves significantly different to the sum of each of the component parts acting singly. Combinations of interleukin 3 (IL3)/multi-CSF and M-CSF/CSF-1, GM-CSF and M-CSF/CSF-1, and interleukin 3 (IL3)/multi-CSF were demonstrated to synergize to stimulate the proliferation of distinct HPP-CFC populations. The relationship between these distinct HPP-CFC subpopulations is, as yet, unclear. The capacity of well defined, recombinantly-derived haematopoietic colony-stimulating factors to synergistically interact with each other and to replace the synergistic activities in the appropriate cell line conditioned media, is strong evidence to suggest that the interleukin 3 (IL3)/multi-CSF, M-CSF/CSF-1 and GM-CSF are the synergizing activities in the WEHI 3B, L929 and AF1-19T cell line conditioned media, although these results do not exclude the possible contribution of factors contained in the ill-defined horse serum (20% v/v) also present during *in vitro* HPP-CFC assay. In the absence of horse serum no colony formation was

observed, even in the presence of colony-stimulating factors; while horse serum alone failed to demonstrate colony-stimulating activity. Putative factors present in the horse serum may not be colony-stimulating activities, rather they may be colony-potentiating/ initiating factors, enhancing colony-stimulating factor synergism by a possible stimulation of colony-stimulating factor receptor expression by factor-sensitive colony-forming cells.

4.1.2 Possible mechanism of synergism between haematopoietic colony-stimulating factors.

The capacity of 'sub-optimal' concentrations of haematopoietic colony-stimulating factors to synergize and stimulate the proliferation of high proliferative potential colony-forming cell populations, may suggest a possible mechanism for the synergistic interaction. The *in vitro* GM-CFC assay, is effectively a measure of the colony-forming cell proliferation induced by a colony-stimulating activity. The finding that 'sub-optimal' concentrations of haematopoietic colony-stimulating factors, as determined by the GM-CFC assay, were effective in synergistic interactions, implies that the factors may be acting not as proliferation '*stimuli*', rather, they may be acting as proliferation '*initiators*'. A model can be proposed in which M-CSF/CSF-1 effectively acts to promote the proliferation of interleukin 3 (IL3)/multi-CSF- , or GM-CSF- 'initiated' colony-forming cells, which are subsequently capable of the high proliferative potential. The putative colony-forming cell 'initiation' by interleukin 3 (IL3)/multi-CSF, or GM-CSF may involve the stimulation of the expression of cell surface receptors for the more lineage-specific haematopoietic colony-stimulating factors, for example M-CSF/CSF-1. HPP-CFC populations sensitive to interleukin 3 (IL3)/multi-CSF, or GM-CSF

initiation, may subsequently develop increased M-CSF/CSF-1 receptors and, in the presence of M-CSF/CSF-1 undergo the marked proliferation characteristic of the HPP-CFC population.

During the assay of murine marrow in the presence of interleukin 3 (IL3)/multi-CSF alone, colonies in excess of 1mm, but less than 2mm, were occasionally observed after 14 days of culture. These relatively high proliferative potential colony-forming cells may be interleukin 3 (IL3)/multi-CSF initiated colony-forming cells which, in the absence of M-CSF/CSF-1, do not subsequently receive as strong a proliferation stimulus from the interleukin 3 (IL3)/multi-CSF and do not realise their full proliferative potential. The presence of the interleukin 3 (IL3)/multi-CSF may induce increased M-CSF/CSF-1 receptor expression, however, in the absence of M-CSF/CSF-1, subsequent proliferation is limited to that stimulated by the interleukin 3 (IL3)/multi-CSF alone.

4.1.3 The absence of 'small colonies' in the *in vitro* HPP-CFC assay.

When appropriate haematopoietic colony-stimulating factors were combined *in vitro*, in addition to stimulating the proliferation of the high proliferative potential colony-forming cell population, a consistent finding was the absence of a population of relatively small colonies, which were evident in the presence of M-CSF/CSF-1 alone. McNiece *et al* [1988b] suggest that the presence of the second synergizing factor as either interleukin 3 (IL3)/multi-CSF, or GM-CSF, may act on the small colony-forming cell population to stimulate granulocytic rather than macrophagic differentiation. Granulocytic colonies are less 'stable' than macrophagic colonies in 14 day semi-solid agar culture and degenerate to be absent at subsequent assay. In the presence of M-CSF/CSF-1 alone, 'stable' macrophagic colonies

would develop to be observed as the small colony population at assay. Alternatively, the developing HPP-CFC population may elaborate factors which act to suppress the development of the small colony background.

4.1.4 The optimal time of assay of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population.

The optimal time of assay of the HPP-CFC$_{IL3+CSF-1}$ population was investigated. At assay, sufficient cellular proliferation was required to allow a distinction to be made between the HPP-CFC$_{IL3+CSF-1}$-derived colonies and the non-HPP-CFC$_{IL3+CSF-1}$-derived colonies. While differences in colony morphologies were evident at as early as day 7-8 of culture when comparing WEHI 3B, L929 and combined cell line conditioned media as stimuli, a 12-15 day assay period was considered to best emphasize the HPP-CFC$_{IL3+CSF-1}$/non-HPP-CFC$_{IL3+CSF-1}$-derived colony distinction. Assay at day 14 of culture was determined as the standard time of HPP-CFC$_{IL3+CSF-1}$ assay.

A number of other groups report a similar 12-15 day assay period when investigating a number of high proliferative potential colony-forming cell populations stimulated by a variety of combinations of haematopoietic colony-stimulating factors [Bradley & Hodgson,1979; Kriegler *et al*,1982; 1990; Bradley *et al*,1985; McNiece *et al*,1986; 1987a; 1987b; 1988a; 1988b; 1989a; 1989b; Quesenberry *et al*,1987; Williams *et al*,1987; Falk & Vogel,1988; Bartelmez *et al*,1989; Niskanen *et al*,1990; Bot *et al*,1990]. Pragnell *et al* [1988], Eckmann *et al* [1988] and Lorimore *et al* [1990], report the assay of the HPP-CFC population stimulated by the combination of GM-CSF and M-CSF/CSF-1 (HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A") on day 11 of culture.

4.1.5 A criterion by which to define the HPP-CFC$_{IL3+CSF-1}$ population.

The development of a criterion by which to define the HPP-CFC$_{IL3+CSF-1}$-derived colonies at assay, was of considerable importance. The unique aspect of the HPP-CFC-derived colonies is their relatively large size in comparison to non-HPP-CFC-derived colonies in *in vitro* semi-solid agar culture. A size criterion was thus used to distinguish between HPP-CFC and non-HPP-CFC-derived colonies. HPP-CFC$_{IL3+CSF-1}$-derived colony diameter was effected by the number of marrow cells plated. During the titration of the cell dose required to achieve 'optimal' numbers of HPP-CFC$_{IL3+CSF-1}$, it was observed that while low cell numbers gave few HPP-CFC$_{IL3+CSF-1}$-derived colonies, those which did form were occasionally observed to exceed 5mm in diameter. With increasing cell numbers, the diameters of the HPP-CFC$_{IL3+CSF-1}$-derived colonies was observed to reduce. This observation may be a consequence of limiting *in vitro* culture conditions, alternatively the HPP-CFC population may elaborate factors which, in the absence of physical overlapping of colonies, serve to restrict cellular proliferation, in a similar manner to that suggested for the inhibition of small colony formation.

A cell dose of $2.5x10^4/5.0x10^4$ normal marrow cells *per* 30mm/50mm petri dish was found to give 'countable' HPP -CFC$_{IL3+CSF-1}$ numbers (10-15/20-30). At this cell dose a direct comparison was made between the sizes of the colonies observed after 14 days in the presence of WEHI 3B, L929 or a combination of the cell line conditioned media. In the presence of the factors acting singly, colonies rarely exceeded a 1.0-1.5mm diameter, and never exceeded a 2.0mm diameter. A 2mm diameter criterion was thus set to preferentially select the HPP-CFC$_{IL3+CSF-1}$-derived colonies, which generally exceeded a 2.0-3.0mm diameter. Pragnell *et al* [1988],

Eckmann *et al* [1988], Falk and Vogel [1988] and Lorimore *et al* [1990] apply a 2mm diameter criterion in the characterization of the HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A"-derived colonies.

In contrast to the 2mm diameter criterion, set as the definition of an HPP-$CFC_{IL3+CSF-1}$ - and HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" - derived colony [Pragnell *et al*,1988; Eckmann *et al*,1988; Falk & Vogel,1988; Lorimore *et al*,1990], a number of laboratories apply a 0.5mm diameter as the criterion for the definition of an HPP-CFC derived colony. Colonies of less than 0.5mm diameter are considered derived from colony-forming cells of a low proliferative potential (LPP-CFC) [Bradley & Hodgson,1979; Kriegler *et al*,1982; Bradley *et al*,1985, McNiece *et al*,1986; 1987; 1988a; 1988b; 1989; Williams *et al*,1987; Bertoncello *et al*,1989; Bartelmez *et al*,1989; Niskanen *et al*, 1990; Morris *et al*,1990]. However, the application of a 0.5mm diameter criterion as a definition of an HPP-CFC-derived colony would not have been appropriate in this instance. Such a criterion would not have been effective in distinguishing between HPP-$CFC_{IL3+CSF-1/GM-CSF+CSF-1}$ and non-HPP-$CFC_{IL3+CSF-1/GM-CSF+CSF-1}$ derived colonies. Non HPP-$CFC_{IL3+CSF-1/GM-CSF+CSF-1}$ derived colonies, stimulated in the presence of interleukin 3 (IL3)/multi-CSF, M-CSF/CSF-1 or GM-CSF alone, were often observed to exceed a 0.5mm diameter and occasionally reached a 1.0-1.5mm diameter.

Significantly, confidence in the 2mm diameter criterion in distinguishing between HPP-$CFC_{IL3+CSF-1}$ and non-HPP-$CFC_{IL3+CSF-1}$-derived colonies was justified when the effects of incubating normal murine femoral marrow with the S-phase-specific cytotoxic agent *cytosine arabinoside* (ARA-C) were investigated. Colony-forming cells in S-phase during incubation with ARA-C are killed and their colony-forming potential is not realised in subsequent colony-forming cell assays. The proportion of colony-forming cells in S-

phase is determined by the difference in the colony numbers obtained in the absence and presence of ARA-C treatment. The number of colonies in excess of 2mm diameter in normal murine marrow was only slightly reduced in the presence of ARA-C, implying that the proportion of the colony-forming cells giving rise to these 2mm diameter colonies in S-phase is relatively low. The number of 'sub-2mm' diameter colonies was markedly reduced by the presence of ARA-C, implying the proportion of colony-forming cells giving rise to these 'sub-2mm' diameter colonies in S-phase is relatively high. The difference in the proliferative potential and proportion of colony-forming cells in S-phase implies that the 'sub-2mm' and 'in excess of 2mm' diameter colonies are derived from distinct populations of colony-forming cells, and that the 2mm criterion is able to distinguish between HPP-CFC and non-HPP-CFC-derived colonies.

4.1.6 The cellular composition of the HPP-CFC$_{IL3+CSF-1}$-derived colonies.

The use of 0.9% methylcellulose as a semi-solid support medium, allowed the cellular composition and cellularity of individual HPP-CFC$_{IL3+CSF-1}$-derived colonies to be investigated. The majority of HPP-CFC$_{IL3+CSF-1}$-derived colonies were composed of large, mature macrophages, characterized by a heavily vacuolated cytoplasm. This would be consistent with the presence of M-CSF/CSF-1. Occasionally, in association with the large mature macrophages, a population of relatively immature, haematopoietic progenitors and a population of mature cells characterized by a pale-staining nucleus and a cytoplasm containing abundant, uniform granules, were observed. In some cases, all three cell types were observed within an individual HPP-CFC$_{IL3+CSF-1}$-derived colony. Individual HPP-

$CFC_{IL3+CSF-1}$-derived colonies are the product of the proliferation of an individual HPP-$CFC_{IL3+CSF-1}$ and the presence of at least two morphologically distinct mature cell populations and a relatively immature cell population, implies that the HPP-$CFC_{IL3+CSF-1}$ population is at least bipotential.

Pragnell *et al* [1988], Eckmann *et al* [1988] and Lorimore *et al* [1990], report the finding of at least four morphologically distinct blood cell lineages in day 11 HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A"-derived colonies. Macrophages, granulocytes, early erythroid cells, megakarocytes and cells with a 'blast-like morphology' are reported. These 'mixed' colonies, which demonstrate the multipotency of the HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" population, were only demonstrable under defined culture conditions: a 37°C, fully humidified, 10% CO_2, 5% O_2, 85% N_2 atmosphere. Pragnell *et al* [1988], Eckmann *et al* [1988] and Lorimore *et al* [1990], report that the use of a 37°C, fully humidified, 5% CO_2 in air atmosphere proved less effective at demonstrating the formation of 'mixed' colonies. A 37°C, fully humidified, 5% CO_2 in air atmosphere was used throughout this project in the culture of 14 day HPP-$CFC_{IL3+CSF-1}$ and if these culture conditions are limiting, this does not rule out the possible multipotent nature of the HPP-$CFC_{IL3+CSF-1}$ population.

An average 14 day HPP-$CFC_{IL3+CSF-1}$-derived colony, in excess of 2mm in diameter, was determined to have a cellularity of $6.0(\pm 0.4)x10^4$ cells. By comparison, colonies obtained after 14 days culture in the presence of either L929 cell line or WEHI 3B cell line conditioned medium, were composed of an average of $0.8x10^4$ and $1.4x10^4$ cells respectively.

Pragnell *et al* [1988], Eckmann *et al* [1988], Falk and Vogel [1988] and McNiece *et al* [1988b], report similar cellularities for individual HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A"-derived colonies, while Bradley and

Hodgson [1979], Kriegler *et al* [1982] and McNiece *et al* [1988b] report similar cellularities for individual M-CSF/CSF-1-derived colonies.

4.1.7 The frequency of the HPP-CFC$_{IL3+CSF-1}$ population.

At assay the average HPP-CFC$_{IL3+CSF-1}$ frequency in normal 9-12 week old CBA/H mice was determined as 100(±1)/10^5 normal marrow cells. In a normal CBA/H femur of 14(±1)x10^6 marrow cells, a total femoral compliment of 14x10^3 HPP-CFC$_{IL3+CSF-1}$ would be predicted. Approximately 1 in every 1000 femoral marrow cells will be a potential HPP-CFC$_{IL3+CSF-1}$.

In comparison, Pragnell *et al* [1988] report the average HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A" frequency in NIHOla, BD26F1 and BALB/c mice to be 197(±22), 145(±16) and 180(±14) *per* 10^5 normal marrow cells respectively. By combining a range of typical values quoted by a number of laboratories for a wide range of mouse strains [Boggs *et al*,1982], an average *in vivo* CFU-S$_{day\ 8\text{-}10}$ frequency of approximately 10-40 *per* 10^5 marrow cells is reported. A seeding factor of approximately 8% [Playfair & Cole,1965] gives the absolute number of CFC-S *per* 10^5 normal marrow cells as 125-500 and a total femoral compliment of approximately 10^4 CFC-S. The frequency of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population in femoral marrow compares favourably with the frequency of the *in vitro* HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A" and *in vivo* CFU-S$_{day\ 8\text{-}10}$ populations.

In femoral marrow from 9-12 week old CBA/H mice, 3 days after a sublethal whole body X-irradiation dose of 2 Gy, the average HPP-CFC$_{IL3+CSF-1}$ frequency was determined as 29(±1)/10^5 regenerating marrow cells and femoral cellularity was significantly reduced to 6(±1)x10^6 cells, giving a total femoral HPP-CFC$_{IL3+CSF-1}$ compliment

of approximately $2x10^3$. Approximately 1 in every 3000 regenerating marrow cells will be a potential HPP-$CFC_{IL3+CSF-1}$.

An 85% reduction in the femoral complement of HPP-$CFC_{IL3+CSF-1}$ was observed 3 days after a whole body X-irradiation dose of 2Gy. Interestingly, Hendry and Lajtha [1977] report an 80% reduction in the femoral marrow complement of CFU-S after a 1.5 Gy whole body X-irradiation dose.

4.1.8 *In vitro* HPP-CFC self-renewal and relationship with the *in vivo* CFU-S population.

While providing a continuous and varying cellular output to meet the haematological requirements of the body, haematopoietic stem cells must, of necessity, be capable of maintaining their own numbers. This implies the existance of a stem cell self-renewal capacity. Siminovitch *et al* [1963] demonstrated that individual CFU-S-derived colonies contained a number of CFU-S giving evidence of CFU-S self-renewal. By replating individual HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A"-derived colonies, Pragnell *et al* [1988] and Eckmann *et al* [1988], report evidence of HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" self-renewal. However, attempts to demonstrate a self-renewal capacity for the HPP-$CFC_{IL3+CSF-1}$ population in this laboratory proved inconclusive. Replating individual day 7 HPP-$CFC_{IL3+CSF-1}$-derived colonies, cultured in 0.9% methylcellulose, gave rise to only sub-2mm, non-HPP-$CFC_{IL3+CSF-1}$-derived colonies. While this does not apparently represent evidence of HPP-$CFC_{IL3+CSF-1}$ self-renewal, it does not rule out the possibility that the HPP-$CFC_{IL3+CSF-1}$ population is giving rise to other HPP-CFC subpopulations for which the combination of interleukin 3 (IL3)/multi-CSF and M-CSF/CSF-1 is not an optimal stimulus. This would correlate with the findings of McNiece *et al*

[1987b], who report the replating of individual HPP-CFC$_{SF-1+CSF-1}$-derived colonies ("SF-1" - 'Synergistic Factor-1') gives rise to a more mature HPP-CFC subpopulation (HPP-CFC-2), which is optimally stimulated by the presence of interleukin 3 (IL3)/multi-CSF and M-CSF/CSF-1 (HPP-CFC$_{IL3+CSF-1}$). It may be that the sub-2mm colonies observed on the replating of individual HPP-CFC$_{IL3+CSF-1}$ may be a more mature HPP-CFC subpopulation (HPP-CFC-3), for which the combination of other haematopoietic colony-stimulating factors is required to allow optimal proliferation. Alternatively, in day 7 HPP-CFC$_{IL3+CSF-1}$-derived colonies, any *in vitro* HPP-CFC$_{IL3+CSF-1}$, generated by the self-renewal mechanism, may have undergone maturation and differentiation with a resultant reduction in the high proliferative potential of the colony-forming cell population, producing colonies of less than 2mm in diameter. Further experiments would be required to determine the optimal time at which to best illustrate HPP-CFC$_{IL3+CSF-1}$ self-renewal within individual HPP-CFC$_{IL3+CSF-1}$-derived colonies.

In conclusion, the *in vivo* CFU-S and *in vitro* HPP-CFC populations demonstrate a high proliferative potential and multipotential character. In this respect they fulfill a number of the criteria expected of a component of the heterogenous haematopoietic stem cell compartment. McNiece *et al* [1987b] and Pragnell *et al* [1988], further demonstrated the relationship between the *in vivo* CFU-S and the *in vitro* HPP-CFC-1 (HPP-CFC$_{SF1+CSF-1}$) and HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A" populations, respectively. The injection of a suspension of pooled cells of day 4 HPP-CFC-1-derived colonies into lethally X-irradiated recipient mice (approximately 200 cells/mouse), lead to the development of day 13 CFU-S (0-6 colonies/spleen), while the injection of a suspension of day 5 HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A"-derived colonies into lethally X-irradiated mice, lead to the development of day 12 CFU-S (approximately 6, day

12 CFU-S/ day 5 HPP-CFC$_{GM\text{-}CSF+CSF\text{-}1}$ "CFU-A"-derived colony). Further evidence of the close correlation between the *in vivo* CFU-S and *in vitro* HPP-CFC$_{IL3+CSF\text{-}1}$ and HPP-CFC$_{GM\text{-}CSF+CSF\text{-}1}$ "CFU-A" populations is observed when the kinetics of the *in vivo* and *in vitro* populations are compared.

Section 2

An investigation of the proliferation and proliferation regulation of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population.

4.2.1 The proportion of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population in S-phase.

The proportion of the HPP-CFC$_{IL3+CSF-1}$ population in S-phase in normal and sublethally X-irradiated femoral marrow was determined using the S-phase specific cytotoxic agent *cytosine arabinoside* (ARA-C) (1.17). The proportion of the HPP-CFC$_{IL3+CSF-1}$ population in S-phase in normal femoral marrow was determined as 9(±1)%, while the proportion of the GM-CFC population in S-phase was determined as 31(±1)%.

Pragnell *et al* [1988], Eckmann *et al* [1988] and Lorimore *et al* [1990], report the proportion of the HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A" population in S-phase as approximately 8% and the proportion of the GM-CFC population in S-phase as approximately 30% in normal femoral marrow.

The proportion of the HPP-CFC$_{IL3+CSF-1}$ population in S-phase in S-phase in regenerating marrow from mice 3 days after a 2 Gy whole body X-irradiation dose, was determined as 40(±1)%. The proportion of the GM-CFC population in S-phase in the regenerating marrow was not significantly changed at approximately 30%.

Pragnell *et al* [1988], Eckmann *et al* [1988] and Lorimore *et al* [1990], used a regimen of subcutaneously-injected phenylhydrazine hydrochloride to induce haemolytic anaemia and produce a haematopoietically-stressed marrow. The proportion of the HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A" population in S-phase in the haematopoietically-stressed marrow was determined as approximately 28%.

It is significant that the *in vitro* HPP-CFC$_{IL3+CSF-1}$ and *in vitro* HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A" populations show similar kinetic changes in normal and haematopoietically-stressed marrow, changes not

observed with the *in vitro* GM-CFC population. Of greater significance is the fact that the kinetic changes observed for the *in vitro* HPP-$CFC_{IL3+CSF-1}$ and HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" populations mirror those observed for the *in vivo* CFU-S population. The proportion of the *in vivo* CFU-S population in S-phase in normal femoral marrow is reported as approximately 10% [Becker *et al*,1965], increasing to 40-50% in S-phase in regenerating marrow from mice subjected to sublethal whole body X-irradiation [Guzman & Lajtha,1970] and 30-50% in S-phase in marrow from mice subjected to phenylhydrazine hydrochloride treatment [Rencricca *et al*,1970] [*Figure 63*].

The favourable comparison of the kinetic changes observed in the normal and haematopoietically-stressed marrow, for both the *in vitro* HPP-CFC and *in vivo* CFU-S populations, together with evidence of a similar high proliferative potential and multipotency, is further evidence of the relatively primitive nature of the *in vitro* HPP-CFC subpopulations within the haematopoietic system.

4.2.2 HPP-$CFC_{IL3+CSF-1}$-sensitivity to the stem cell proliferation regulators.

The proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase in regenerating bone marrow from mice 3 days after a 2 Gy whole body X-irradiation dose was determined as 40(±1)%. When incubated with medium conditioned by normal bone marrow, in which the proportion of stem cells in S-phase is relatively low, the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase was significantly reduced from 40(±1)% to 25(±1)% [*Figure 64*].

Similarly, the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase in normal marrow was determined as 9(±1)%. When incubated with medium conditioned by marrow from mice 7 days after a 4.5 Gy

Figure 63: A comparison of the proportion of the *in vitro* HPP-$CFC_{IL3+CSF-1}$, *in vitro* HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" and *in vivo* CFU-S populations in S-phase in normal and haematopoietically-stressed marrow.

Colony-forming cell population.	Proportion of colony-forming cells in S-phase (±SEM)	
	Normal Bone Marrow	Regenerating Bone Marrow
In vitro HPP-$CFC_{IL3+CSF-1}$	9(±1)% (n=255)	40(±1)% (n=177) [1]
In vitro HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" [2]	~8%	~28% [3]
In vivo CFU-S	~10% [4]	30-50% [1 & 3,5]

~ Approximately.
[1] Marrow from mice subjected to sublethal whole body X-irradiation.
[2] Pragnell *et al* [1988] and Eckmann *et al* [1988].
[3] Marrow from mice pretreated with phenylhydrazine hydrochloride.
[4] Becker *et al* [1965].
[5] Guzman and Lajtha [1970] and Rencricca *et al* [1970].

whole body X-irradiation dose, in which the proportion of the haematopoietic stem cell population in S-phase is relatively high, the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase was significantly increased from 9(±1)% to 24(±1)% [*Figure 64*].

The *in vitro* HPP-$CFC_{IL3+CSF-1}$ population demonstrates a sensitivity to both the normal bone marrow-derived stem cell proliferation *inhibitor* and the regenerating bone marrow-derived proliferation *stimulator*. Significantly, the changes in the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase under the influence of appropriate stimuli, mirror those observed for the *in vivo* CFU-S population, and in common with the *in vivo* CFU-S population, no evidence of either stem cell proliferation *inhibitor* or *stimulator* toxicity was observed against the HPP-$CFC_{IL3+CSF-1}$ population.

Pragnell *et al* [1988], Eckmann *et al* [1988] and Lorimore *et al* [1990], report a similar HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A"-sensitivity to the stem cell proliferation regulators. In haematopoietically-stressed marrow from mice subjected to phenylhydrazine hydrochloride-treatment, the proportion of the HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" population in S-phase was determined as approximately 28%. When incubated with a source of the stem cell proliferation *inhibitor*, the proportion of the HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" population in S-phase was reduced from approximately 28% to approximately 11%. Similarly, the proportion of the HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" population in S-phase in normal bone marrow was determined as approximately 8%. When incubated with a source of stem cell proliferation *stimulator*, the proportion of the HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" population in S-phase was increased from approximately 8% to approximately 49% [*Figure 64*].

The demonstration of both *in vitro* HPP-$CFC_{IL3+CSF-1}$ and *in vitro* HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A"-sensitivity to the *in vivo* CFU-S-

Figure 64: A comparison of the proportion of the *in vitro* HPP-$CFC_{IL3+CSF-1}$, *in vitro* HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" and *in vivo* CFU-S populations in S-phase in the absence and presence of the stem cell proliferation regulators..

Colony-forming cell population.	Proportion of colony-forming cells in S-phase (±SEM)			
	Regenerating Bone Marrow [1]	RBM + *inhibitor* [2]	Normal Bone Marrow	NBM + *Stimulator* [3]
In vitro HPP-$CFC_{IL3+CSF-1}$	40(±1)% (n=177)	25(±1)% (n=127)	9(±1)% (n=255)	24(±1)% (n=107)
In vitro HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" [4]	~28%	~11%	~8%	~49%
In vivo CFU-S [5]	30-50%	~10%	~10%	30-40%

~ Approximately.
[1] Haematopoietically-stressed marrow.
[2] Medium conditioned by normal bone marrow.
[3] Medium conditioned by haematopoietically-stressed marrow.
[4] Pragnell *et al* [1988] and Eckmann *et al* [1988] and Lorimore *et al* [1990].
[5] Becker *et al* [1965], Guzman and Lajtha [1970] and Rencricca *et al* [1970].

characterized proliferation regulators, in addition to the evidence of a high proliferative potential, multipotency and kinetic changes similar to those observed for the *in vivo* CFU-S population, is further evidence for the *in vitro* HPP-CFC$_{IL3+CSF-1}$ and HPP-CFC$_{GM-CSF+CSF-1}$ "CFU-A" populations being relatively primitive haematopoietic precursors, possibly components of the stem cell compartment within the haematopoietic system.

4.2.3 The relative sensitivity of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population to the stem cell proliferation regulators.

Tejero *et al* [1984], Wright *et al* [1985] and Lorimore *et al* [1990], demonstrate an 'age-related' variation in the sensitivity of the various components of the haematopoietic stem cell population to the stem cell proliferation *inhibitor* and *stimulator* (1.17). Day 10-12 CFU-S, believed to be a more primitive component of the haematopoietic stem cell compartment, demonstrate a significantly increased sensitivity to the stem cell proliferation *inhibitor*. In comparison, day 7-8 CFU-S, believed to be a more mature component of the haematopoietic stem cell compartment, demonstrate a significantly increased sensitivity to the stem cell proliferation *stimulator* [*Figure 13*].

The sensitivity of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population to the stem cell proliferation regulators was investigated in an attempt to determine the relative position of the population within the stem cell compartment. Assay of the stem cell proliferation regulators against the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population, demonstrated that both normal and regenerating bone marrow-derived *inhibitor* and *stimulator*, are titratable activities. Increasing the 'concentration' of the medium conditioned by the normal, or regenerating marrow,

resulted in an appropriate reduction, or increase, to a minimum/maximum plateau value, of the proportion of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population in S-phase. Similar observations are reported by Wright *et al* [1982] and Lord and Wright [1982], when assaying similarly-derived proliferation *inhibitor* and *stimulator* against the *in vivo* CFU-S population.

A number of groups report the use of lyophilized Amicon ultrafiltered fractions of normal and regenerating marrow extracts, as sources of ***NBME-IV*** (stem cell proliferation *inhibitor*) and ***RBME-III*** (stem cell proliferation *stimulator*), and the use of increasing weights of lyophilized material similarly demonstrates that both factors are titratable activities against the *in vivo* CFU-S population [Lord *et al*,1981; Wright & Lord,1979; Wright *et al*,1980a; Cork *et al*,1981; Tejero *et al*,1984; Wright *et al*,1985; Wright and Lorimore,1987; Lorimore *et al*,1990].

In addition to the demonstration that both *inhibitor* and *stimulator* are titratable activities, it is significant that the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population demonstrates an approximately equal sensitivity to the stem cell proliferation regulators. In comparison to the day 10-12 CFU-S population, which proves more sensitive to the stem cell proliferation *inhibitor*, and the day 7-8 CFU-S population, which proves more sensitive to *stimulator*, a cell population which demonstrates an equal sensitivity to each factor should theoretically prove to be more mature than the day 10-12 CFU-S population, and more primitive than the day 7-8 CFU-S population. In this manner, an approximate position for the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population within the already established *in vivo* CFU-S hierarchy within the haematopoietic stem cell population, can be proposed [*Figure 65*].

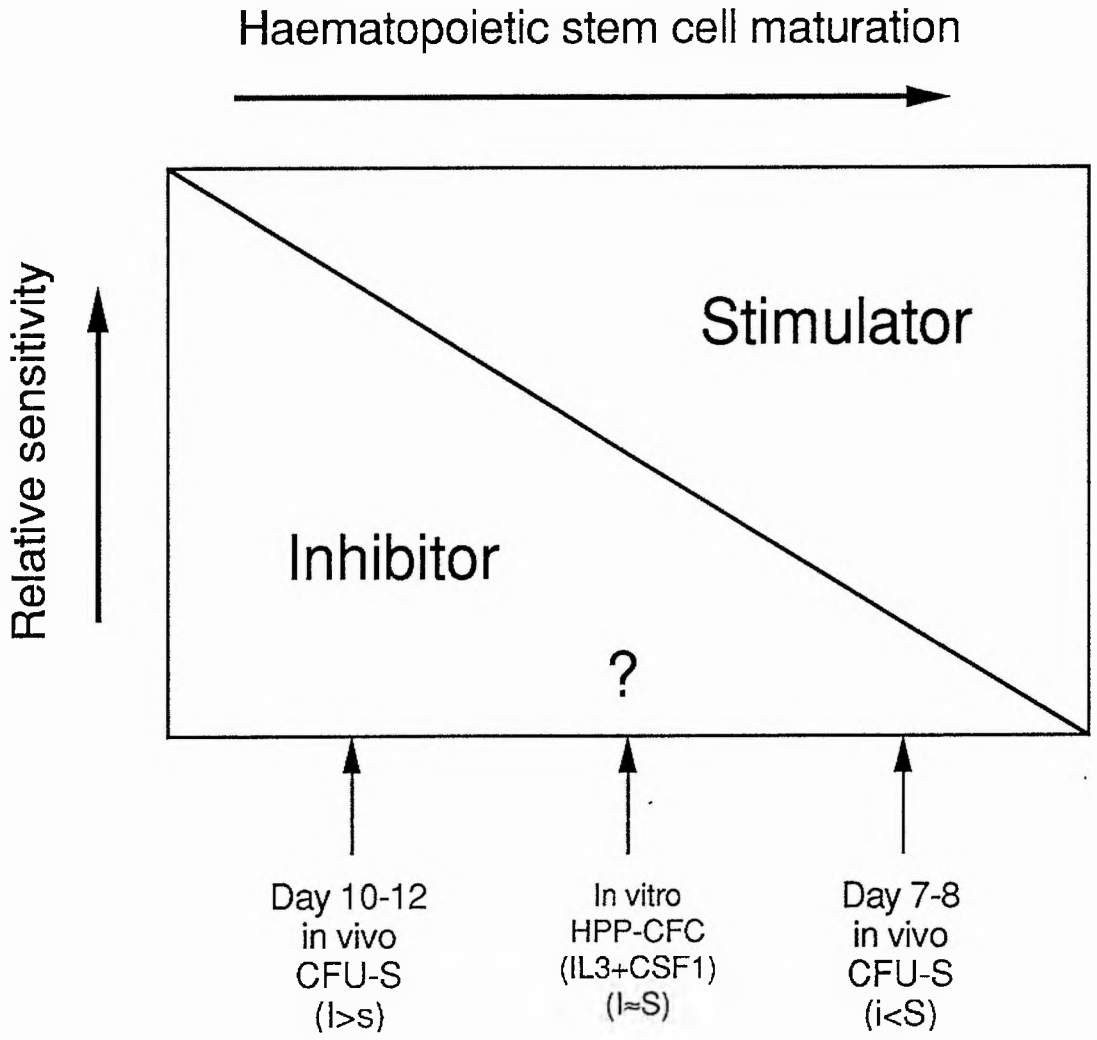


Figure 65: A diagramatic representation of the variation in inhibitor and stimulator sensitivity with haematopoietic stem maturation.

Lorimore *et al* [1990], report a direct comparison between *inhibitor* and *stimulator* sensitivities of day 12 CFU-S, day 7 CFU-S and *in vitro* HPP-CFC$_{GM\text{-}CFC+CSF\text{-}1}$ "CFU-A" population. Using lyophilized Amicon ultrafiltered *NBME-IV* and *RBME-III*, as sources of stem cell proliferation *inhibitor* and *stimulator*, a significant difference in the HPP-CFC$_{GM\text{-}CSF+CSF\text{-}1}$ "CFU-A" sensitivity to the stem cell proliferation regulators was reported. The HPP-CFC$_{GM\text{-}CSF+CSF\text{-}1}$ "CFU-A" population demonstrated a markedly increased sensitivity to *inhibitor* and a reduced sensitivity to the stem cell proliferation *stimulator*. This pattern of stem cell proliferation regulator sensitivity proved similar to that of the day 12 CFU-S population, believed to be a more primitive component of the haematopoietic stem cell compartment, rather than the day 7 CFU-S population and implies that the *in vitro* HPP-CFC$_{GM\text{-}CSF+CSF\text{-}1}$ "CFU-A" population is a relatively primitive haematopoietic precursor within the haematopoietic stem cell hierarchy.

The age-related variation in the relative sensitivity of the haematopoietic stem cell population to *inhibitor* and *stimulator*, may subsequently allow the positions of the various *in vitro* HPP-CFC populations within the haematopoietic stem cell hierarchy, to be established. Since the HPP-CFC-2 (HPP-CFC$_{IL3+CSF\text{-}1}$) population appears to demonstrate an approximately equal sensitivity to *inhibitor* and *stimulator*, the more primitive HPP-CFC-1 (HPP-CFC$_{SF1+CSF\text{-}1}$) population might be expected to demonstrate an increased *inhibitor* sensitivity, while the more mature HPP-CFC-3 population might be expected to demonstrate an increased *stimulator* sensitivity.

Section 3

An investigation of proliferation *stimulator* and *inhibitor* in myeloid leukaemia.

4.3.1 An investigation of haematopoietic stem cell proliferation regulators in murine myeloid leukaemia.

The *in vitro* HPP-CFC$_{IL3+CSF-1}$ assay technique was subsequently used to investigate the regulation of the haematopoietic stem cell population in the haematopoietic, neoplastic proliferative disorder, *myeloid leukaemia*. '*Leukaemia*', a term applied to a group of malignant haematopoietic proliferative disorders, is characterized by an inappropriate production of white blood cells (1.28). In the case of '*myeloid leukaemia*' there is an inappropriate production of abnormal myeloid precursors. One particularly significant aspect of most leukaemias, is an apparent leukaemia-associated suppression of normal haematopoiesis. As a result, with disease progression, the 'quality' of the peripheral blood deteriorates and anaemia, haemorrhage and infection are common secondary symptoms. The apparent suppression of normal haematopoiesis by the leukaemia allows the leukaemic population to develop a proliferative advantage over the normal haematopoietic tissue. The possible mechanism(s) by which the leukaemias may suppress normal haematopoiesis were investigated using a number of X-irradiation-induced, murine myeloid leukaemias [Hepburn *et al*,1987] and the *in vitro* HPP-CFC$_{IL3+CSF-1}$ assay.

Initial investigations were performed to determine the levels of the stem cell proliferation *inhibitor* and *stimulator* in media conditioned by overtly leukaemic marrow samples (2.20). Media conditioned by overtly leukaemic marrow was assayed against normal marrow, to investigate the presence of the stem cell proliferation *stimulator*, and regenerating marrow to investigate the presence of the stem cell proliferation *inhibitor* OR the presence of a direct-acting leukaemia-associated proliferation inhibitor. No

evidence of the presence of the haematopoietic stem cell proliferation *stimulator* was detected in media conditioned by the murine myeloid leukaemias. The proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase in normal marrow was not increased by incubation with the leukaemic marrow conditioned media. Similarly, no evidence of the presence of the haematopoietic stem cell proliferation *inhibitor*, or of any direct-acting leukaemia-associated proliferation inhibitor, was detected in the media conditioned by the murine myeloid leukaemias. The proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase in regenerating marrow was not reduced by incubation with leukaemic marrow conditioned media. The absence of stem cell proliferation *stimulator* and *inhibitor*, or of any direct-acting leukaemia-associated proliferation inhibitor, was not apparently a consequence of either the concentration of the leukaemic marrow conditioned medium, or the length of time used in the conditioning of the medium.

A time study of leukaemic progression was performed to investigate the variation in the levels of the haematopoietic stem cell proliferation regulators with disease progression.

No evidence of the stem cell proliferation *stimulator* was observed during the progression of the murine myeloid leukaemia, however, significant changes in the levels of detectable stem cell proliferation *inhibitor* were observed with disease progression.

A possible explanation of such a finding may be to relate the levels of the stem cell proliferation *inhibitor* to the way in which the cellular composition of the femoral marrow changes with disease progression. In normal murine femoral marrow, there is a predominance of relatively mature haematopoietic progenitors: metamyelocytes, erythroblasts, lymphocytes and band cells [Hepburn *et al*,1987]. In overtly leukaemic SA8LD marrow, there is a

predominance of relatively immature haematopoietic progenitors: myelocytes, promyelocytes and generalized blast cells [Hepburn *et al*,1987]. The change in cellular composition of the marrow may also include the cell population responsible for the production of the stem cell proliferation *inhibitor*. The *inhibitor*-producing cell population may become 'diluted' as the marrow becomes more leukaemic, with the possible result that the observed 'concentration' of detectable *inhibitor* would reduce. However, while this dilution effect may play a role, subsequent evidence implies that the reduction in the levels of the stem cell proliferation *inhibitor*, may be a direct consequence of the leukaemic cell population.

The mechanism(s) by which the levels of detectable proliferation *inhibitor* were reduced with leukaemic progression was investigated to determine whether the reduction was inherently linked to the developing leukaemia. Was the leukaemic population reducing the levels of detectable *inhibitor* by either acting to prevent it's action, or acting to prevent it's production ? The effects of medium conditioned by overtly leukaemic marrow on the action of both the haematopoietic stem cell proliferation *inhibitor* and *stimulator* was investigated by performing assays of normal and regenerating bone marrow-derived *inhibitor* and *stimulator* in the absence or presence of medium conditioned by overtly leukaemic marrow.

The presence of the medium conditioned by the overtly leukaemic marrow, did not significantly impair the capacity of the normal bone marrow-derived *inhibitor* to reduce the proportion of the HPP-$CFC_{IL3+CSF\text{-}1}$ population in S-phase, or regenerating bone marrow-derived *stimulator* to increase the proportion of the HPP-$CFC_{IL3+CSF\text{-}1}$ population in S-phase. These results suggest that the reduction in the level of the proliferation *inhibitor* observed with disease progression is not apparently due to a leukaemia-associated

inactivation of the haematopoietic stem cell proliferation regulator. *Stimulator* action is similarly unaffected by the presence of medium conditioned by overtly leukaemic marrow.

The effects of medium conditioned by overtly leukaemic marrow on the production of the haematopoietic stem cell proliferation regulators was investigated. Normal bone marrow conditioned medium was conditioned in the absence, or presence of medium conditioned by overtly leukaemic marrow and similarly, medium was conditioned by regenerating marrow from mice 7 days after a 4.5 Gy whole body X-irradiation dose in the absence or presence of medium conditioned by overtly leukaemic marrow.

Proliferation *inhibitor*, produced from normal bone marrow reduced the proportion of the HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase. Medium conditioned by normal bone marrow in the presence of medium conditioned by overtly leukaemic marrow, did not reduce the proportion of the HPP-$CFC_{IL3+CSF\text{-}1}$ population in S-phase. Similarly, proliferation *stimulator*, produced from regenerating marrow increased the proportion of the HPP-$CFC_{IL3+CSF\text{-}1}$ population in S-phase. Medium conditioned by regenerating bone marrow in the presence of medium conditioned by overtly leukaemic marrow, did not increase the proportion of the HPP-$CFC_{IL3+CSF\text{-}1}$ population in S-phase. The presence of medium conditioned by overtly leukaemic marrow appears to significantly impair the capacity of normal bone marrow to produce the stem cell proliferation *inhibitor* and regenerating bone marrow to produce the stem cell proliferation *stimulator*.

4.3.2 Possible mechanisms of murine myeloid leukaemic progression.

These results suggest that the leukaemic population may be able to manipulate the levels of endogenous stem cell proliferation *inhibitor* and *stimulator* by apparently impairing their production. Normally, stem cell proliferation *inhibitor* and *stimulator* subtly interact to coordinate the proportion of the haematopoietic stem cell population in S-phase and so tailor haematopoietic output to the haematopoietic demands of the body. If the production of the stem cell proliferation regulators was impaired, this subtle regulatory interaction could not occur and the coordination of haematopoietic output to haematopoietic demand would fail. With the additional haematopoietic stress induced during leukaemic progression and the inappropriate production of abnormal white blood cells by a population of cells able to proliferate presumably independently of *inhibitor* and *stimulator* regulation, the 'quality' of the peripheral blood will deteriorate leading to anaemia, haemorrhagic episodes and infection due to the reduced levels of functional erythrocytes, platelets and granulocytes and macrophages.

The action of the leukaemic population in apparently blocking the production of the stem cell proliferation regulators may be a modification of an already existing mechanism. For example, distinct *inhibitor*- and *stimulator*-producing cell populations exist (1.11) and are both present regardless of the proliferative state of the haematopoietic tissue. Only one proliferation regulator is however predominant at any one time, which implies a distinct coordinating mechanism must exist. Lord and Wright [1982], demonstrated that isolated *inhibitor*-producing cells cease *inhibitor* production in the presence of exogenous *stimulator*. Similarly, Lord and Wright [1982]

and Riches and Cork [1987] demonstrated that *stimulator*-producing cells cease *stimulator* production in the presence of exogenous *inhibitor* (1.14). One could envisage a slight modification of this regulatory mechanism could be exploited to the advantage of the leukaemic population. A putative leukaemic factor could be produced which mimics the proliferation *stimulator* and *inhibitor*, but which is itself inactive. In this way both *inhibitor* and *stimulator* production would cease, to give the observed absence of regulators in overtly leukaemic marrow. With the characterization of the stem cell proliferation regulators and identification of appropriate receptors, it may prove possible to perform competitive-binding studies between appropriate factors, factor-producing cells and the putative leukaemic factor, to further investigate the possible mechanism by which this apparent, leukaemia-associated suppression of the proliferation regulators occurs.

Similarly, a modification of the 'stem cell feedback factor' (SCFF) could be developed to leukaemic advantage. SCFF is reported to be produced by the haematopoietic stem cell population and acts to maintain stem cell numbers [Lord,1986] (1.15). If stem cell numbers are 'sufficient', levels of SCFF inhibit the production of *stimulator* by *stimulator*-producing cells. If stem cell numbers are reduced, the levels of SCFF reduce and the inhibition of *stimulator* production is lifted. Increased *stimulator* production acts to increase the proportion of haematopoietic stem cells in S-phase and stem cell numbers are restored through a self-renewal mechanism. Increased stem cell numbers, increase SCFF production, which inturn increases the inhibition of *stimulator* production, enabling the subsequent production of the proliferation *inhibitor*. The production of a leukaemia-associated SCFF-mimic could thus be envisaged to modulate the production of the stem cell proliferation regulators.

While the leukaemic population may inhibit the production of the stem cell proliferation regulators, by possibly modifying and subsequently exploiting these existing regulatory mechanisms to it's advantage, this does not rule out the possibility that the leukaemic population may use an, as yet unclear, novel mechanism. Future studies might involve attempts to characterize the putative leukaemia-associated, proliferation regulator production-inhibiting factor, perhaps through Amicon ultrafiltration. If such a factor could be isolated, it may be possible to exploit it to clinical advantage. By preventing the production of the stem cell proliferation *stimulator*, and so preventing the recruitment of haematopoietic stem cells into S-phase, it may be possible to reduce certain aspects of chemotherapeutic haemotoxicity.

In conclusion, if the leukaemia-associated suppression of normal haematopoiesis and the subsequent development of a leukaemic proliferative advantage over the normal haematopoietic tissue, is in part due to a suppression of *stimulator* production, would the controlled reintroduction of *stimulator*, as an exogenous factor, assist in the management of leukaemia ? Provided the leukaemic population is not itself sensitive to the stem cell proliferation *stimulator*, a controlled reintroduction of the factor may allow sufficient 'activation' of the marrow to allow a partial restoration of the 'quality' of the peripheral blood, which would inturn, reduce the secondary, often debilitating symptoms of anaemia, haemorrhage and infection. The 'activation' of the normal, suppressed haematopoietic tissue may also act to overcome the leukaemic proliferative advantage, which may inturn act to impair the progression of the disease and give a better disease prognosis. Such disease management may lead to essentially healthier (less anaemic, less haemorrhagic and less susceptible to infection) leukaemic

patients, more able to withstand the subsequent rigors of chemo- and radiotherapeutic regimes.

Section 4

An investigation of the possible mechanism of action of the low molecular weight, haemoregulatory peptide Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP).

4.4.1 Stability and activity of AcSDKP.

Information about the stability and activity of AcSDKP was initially very limited. Caution was advised against the use of serum in medium used to dilute and incubate AcSDKP, due to the presence of degradative enzymes, and while heat inactivated serum (serum incubated at 56°C for 30 mins.) proved less degradative, AcSDKP activity was still reduced. The use of serum albumin was also precluded by adverse binding of the AcSDKP to the molecule [Lenfant, personal communication]. After consideration, dilution and subsequent incubations involving the peptides were performed under serum-free conditions. Manipulation of diluted peptides was kept to a minimum and dilutions performed in polypropylene vials (Nunclon) to reduce loss of peptides by adhesion to plastic. Stock solutions of 100 ng/ml of each peptide were produced, aliquoted and frozen (-20°C) and individual 100 ng/ml aliquots were not refrozen once thawed. The stability and activity of AcSDKP, SDK, ADK and DKP under incubation conditions and in the presence of marrow cells was uncertain, and addition of peptides at t=0 and at each subsequent hour of incubation was hoped to maintain at least a minimum concentration of peptide present should degradation occur. A standard concentration of 1 ng/ml (approximately 10^{-9}M) was adopted for each of the peptides, following reference to reports of Monpezat and Frindel [1989] and Lauret *et al* [1989]. Subsequent reports of Guigon *et al* [1990] demonstrated maximal AcSDKP activity at a 10^{-9}-10^{-10}M (approximately 1 ng/ml) concentration against a human progenitor cell population.

[Recent research performed by Lenfant *et al*,[unpublished], has characterized the enzyme present in serum which is responsible for AcSDKP degradation as "*angiotensin converting enzyme*" (ACE). ACE

has been previously characterized in the renin-angiotensin system, responsible for the regulation of blood pressure. *Angiotensinogen*, an inactive precursor found in the plasma and tissue fluid, is converted to *angiotensin I*, an inactive 10 amino acid peptide, by *renin*. Inactive *angiotensin I* is converted to an active, 8 amino acid peptide, *angiotensin II*, by the carboxydipeptidase activity of *angiotensin converting enzyme* (ACE), which is widely distributed in tissue. ACE also apparently acts to degrade the active AcSDKP peptide by enzymatic cleavage, and the behaviour and regulation of ACE in normal and aberrant haematopoiesis is currently under investigation by Lenfant *et al* in collaboration with this laboratory. The half-life of AcSDKP in serum has subsequently been reported as approximately 80 mins. After 4 hours no AcSDKP activity is detectable. In heat inactivated serum, the AcSDKP half-life is reported as approximately 20 hours [Lenfant & Wdzieczak-Bakala, personal communication].]

4.4.2 The direct and indirect haemoregulatory activity of SDK.

The direct, inherent stimulatory or inhibitory activity of SDK was investigated against both the *in vitro* HPP-$CFC_{IL3+CSF\text{-}1}$ and the *in vitro* GM-CFC populations. No evidence of inherent stimulatory or inhibitory activity against either the *in vitro* HPP-$CFC_{IL3+CSF\text{-}1}$ or *in vitro* GM-CFC populations was observed. Significantly, no evidence of SDK toxicity against either the *in vitro* HPP-$CFC_{IL3+CSF\text{-}1}$ or *in vitro* GM-CFC population was observed at 1 ng/ml, 2.87×10^{-9}M.

The activity of SDK against *stimulator* and *inhibitor* action was investigated. The proportion of the HPP-$CFC_{IL3+CSF\text{-}1}$ in S-phase in normal bone marrow was increased in the presence of medium

conditioned by regenerating marrow, as a crude source of *stimulator*. However, in the presence of *stimulator* and SDK at 1 ng/ml, $2.87x10^{-9}$M, no significant increase in the proportion of the HPP-$CFC_{IL3+CSF-1}$ in S-phase was observed. Similarly, the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase in regenerating marrow was reduced in the presence of medium conditioned by normal marrow, as a crude source of *inhibitor*. However, in the presence of *inhibitor* and SDK at 1 ng/ml, $2.87x10^{-9}$M, no reduction in the proportion of the HPP-$CFC_{IL3+CSF-1}$ was observed.

These results imply that SDK at 1 ng/ml, $2.87x10^{-9}$M is apparently effective at blocking the action of the stem cell proliferation regulators. In the presence of SDK, *stimulator* fails to increase the proportion of the HPP-$CFC_{IL3+CSF-1}$ in S-phase and *inhibitor* fails to reduce the proportion of the HPP-$CFC_{IL3+CSF-1}$ in S-phase.

The activity of SDK against *stimulator* and *inhibitor* production was investigated. The proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase was increased in the presence of medium conditioned by regenerating marrow. Incubated with medium conditioned by regenerating bone marrow in the presence of 1 ng/ml, $2.87x10^{-9}$M SDK, the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase was similarly increased. The proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase was reduced in the presence of medium conditioned by normal bone marrow. Incubated with medium conditioned by normal bone marrow in the presence of 1 ng/ml, $2.87x10^{-9}$M SDK, the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase was similarly reduced. These results imply that SDK at 1 ng/ml, $2.87x10^{-9}$M does not act to block the production of the stem cell proliferation regulators. In the presence of SDK, regenerating bone marrow continues to produce *stimulator* and normal bone marrow continues to produce *inhibitor*.

4.4.3 The direct and indirect haemoregulatory activity of AcSDKP.

A limited number of experiments were performed with the parent molecule AcSDKP. No evidence of an inherent stimulatory activity for the *in vitro* HPP-$CFC_{IL3+CSF-1}$, or of an inherent stimulatory, or inhibitory activity for the *in vitro* GM-CFC population was observed for AcSDKP at 1 ng/ml, $2.05x10^{-9}$M over a 3 hour period. In addition, no evidence of AcSDKP toxicity against either the *in vitro* HPP-$CFC_{IL3+CSF-1}$, or *in vitro* GM-CFC was observed.

The effect of AcSDKP on the action of the stem cell proliferation *stimulator* was also investigated. The proportion of the HPP-$CFC_{IL3+CSF-1}$ in S-phase was increased on incubation with medium conditioned by regenerating marrow as a crude source of *stimulator*. In the presence of *stimulator* and AcSDKP at 1 ng/ml, $2.05x10^{-9}$M, the proportion of the HPP-$CFC_{IL3+CSF-1}$ in S-phase was not increased, implying that, in common with the findings of SDK, AcSDKP appears to block the action of the stem cell proliferation *stimulator*.

4.4.4 The direct and indirect haemoregulatory activity of DKP and ADK.

A limited number of further experiments were performed using the tripeptide analogues DKP and ADK. The proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase was not increased by the presence of 1 ng/ml, $3.01x10^{-9}$M ADK for 3 hours and no evidence of ADK toxicity against the HPP-$CFC_{IL3+CSF-1}$ population at 1 ng/ml, $3.01x10^{-9}$M was observed.

The effects of both ADK and DKP on the action of the stem cell proliferation *stimulator* were investigated. The proportion of the HPP-$CFC_{IL3+CSF-1}$ in S-phase was increased in the presence of medium

conditioned by regenerating marrow as a crude source of *stimulator*. In the presence of *stimulator* and 1 ng/ml, 2.79×10^{-9}M DKP, the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase was not increased. No evidence of DKP toxicity against the HPP-$CFC_{IL3+CSF-1}$ population of 1 ng/ml, 2.79×10^{-9}M was observed. In the presence of *stimulator* and 1 ng/ml, 3.01×10^{-9}M ADK, however, the proportion of the HPP-$CFC_{IL3+CSF-1}$ population was increased. These results imply that while DKP proved effective at blocking the action of the haematopoietic stem cell proliferation *stimulator*, ADK appeared to be a less effective *stimulator*-'blocking' agent. In conclusion, these results suggest that the low molecular weight, haemoregulatory tetrapeptide AcSDKP, may act by blocking the action of the haematopoietic stem cell proliferation *stimulator*. A number of observations involving AcSDKP may now be explained in light of this suggestion.

4.4.5 Possible mechanism of action of the haemoregulatory tetrapeptide AcSDKP.

Lauret *et al* [1989b], report the production of a polyclonal antiserum against an extended AcSDKP peptide, which cross-reacts with the AcSDKP tetrapeptide. Use of this antiserum *in vivo* in mice is reported to markedly increase the proportion of the haematopoietic stem cell population in S-phase [Monpezat & Frindel,1989]. If as postulated, AcSDKP acts to block *stimulator* action, the removal of endogenous AcSDKP by the neutralizing antiserum, would remove the block to *stimulator* action, and allow a subsequent increase in the proportion of haematopoietic stem cells in S-phase, as was observed.

The exogenous administration of AcSDKP *in vivo* to mice, as crude dialysate of foetal liver or bone marrow, semi-purified factor or synthetic peptide, is reported to prevent the recruitment of haematopoietic stem cells into S-phase and so acts to protect the haematopoietic system from the potentially haemotoxic effects of the S-phase-specic cytotoxic agent *cytosine arabinoside* (ARA-C) [Guigon & Frindel,1978; Guigon *et al*,1980; 1981; 1982; 1989; Wdzieczak-Bakala *et al*,1983]. If as postulated AcSDKP acts to block the action of the haematopoietic stem cell proliferation *stimulator*, then the exogenous addition of AcSDKP will act to further increase the block in *stimulator* action. With the cellular damage induced as a result of an S-phase cytotoxic drug challenge, levels of endogenous *stimulator* will rise to increase the proportion of stem cells in S-phase and begin tissue regeneration. However, with the continued presence of the S-phase cytotoxic agent, an increase in the proportion of stem cells in S-phase would lead to severe tissue damage. The presence of increased levels of AcSDKP would be envisaged to block the action of *stimulator*, prevent G_0-G_1 transition of the haematopoietic stem cells, so 'protecting' them from the persistant effects of the S-phase-specic cytotoxic agent.

Wdzieczak-Bakala *et al* [1990], report cyclical changes in the proliferative state of the haematopoietic stem cell population in *in vitro* long-term bone marrow cultures with refeeding, and offered an explanation in terms of levels of AcSDKP in the culture medium. Removal of exhausted medium at the refeeding of a long-term bone marrow culture and its replacement with fresh medium, would reduce the endogenous levels of AcSDKP and allow an increased proportion of haematopoietic stem cells to enter S-phase. With time, levels of endogenous AcSDKP would be restored, inhibition of stem cell G_0-G_1 transition would be restablished and a reduction in the proportion of

the haematopoietic stem cell population in S-phase would occur. This can be compared with the observations of Dexter *et al* [1977], Toksoz [1980] and Cashman *et al* [1985], who also report cyclical changes in long-term bone marrow cultures at refeeding. They interpret these observations as the result of changing levels of the stem cell proliferation *inhibitor* and *stimulator.* Cellular depletion of the haematopoietic culture, leads to the production of *stimulator* and an appropriate increase in the proportion of the haematopoietic stem cell population in S-phase. An increase in the cellular output and cellular repopulation of the haematopoietic layer, subsequently leads to the production of *inhibitor* and a reduction in the proportion of the haematopoietic stem cells in S-phase. The two distinct models of proliferation regulation may be reconsiled with the finding that AcSDKP does not act directly, rather it interacts with the stem cell proliferation *stimulator.* At refeeding exhausted medium is removed and replaced with fresh medium. AcSDKP levels in the medium after refeeding are reduced. The cellular depletion of the layer leads to the production of *stimulator.* The low levels of AcSDKP allow *stimulator* produced to be active and to increase the proportion of the haematopoietic stem cell population and so increase the cellular output and repopulation of the layer. With time the levels of AcSDKP increase with an associated increase in the blocking of *stimulator* action. A block in *stimulator* action may subsequently allow *inhibitor* production with the result that the proportion of the haematopoietic stem population in S-phase is reduced.

It is significant that the use of a neutralizing polyclonal antiserum against AcSDKP and the exogenous addition of AcSDKP elicit haematopoietic responses *in vivo.* This is strong evidence for the physiological role of the tetrapeptide AcSDKP. However, the precise mechanism by which the action of the haematopoietic stem cell

proliferation *stimulator* is compromised, is at present unclear, although a number of possible mechanisms can be suggested.

Stimulator-sensitive cells, which are likely to include the haematopoietic stem cell population and possibly the *inhibitor*-producing cell population, are likely to express membrane-bound *stimulator* receptors. An oversimplified illustration of the *stimulator*/ *stimulator* receptor interaction may be that of a '*lock and key*' [*Figure 66a*]. Subtle molecular interactions will occur between the active sites of both the *stimulator* and the *stimulator* receptor and with the formation of a *stimulator*/ *stimulator*-receptor complex intracellular mechanisms are likely to elicit secondary messenger activity with subsequent signal transduction to the nucleus and the stimulation of the haematopoietic stem cell population into S-phase. One possible mechanism of AcSDKP action may be to directly interact with either the *stimulator* molecule or the receptor. In the interaction of AcSDKP and SDK in the inhibition of rosette formation between human Jurkat T-cells and sheep erythrocytes the addition of exogenous AcSDKP, or SDK, is proposed to compete with the presence of an endogenous -SDK- sequence in a region of the T-cell CD_2 receptor which subsequently determines the interaction with the sheep erythrocyte. The presence of exogenous AcSDKP, or SDK, is thought to impair rosette formation by competing with the intramolecular interaction of the putative native -SDK- sequence and disrupting the tertiary structure of the T-cell receptor moiety so that no interaction with the sheep erythrocyte can occur [Lenfant, personal communication]. A similar mechanism may exist in the *stimulator*/ *stimulator* receptor system, with native -SDKP-, or -SDK-, sequences being potentially significant in the interaction between agonist and receptor. The presence of free AcSDKP may compete with these putative native -SDKP-, or -SDK-, sequences at the level of either the *stimulator* or the

stimulator receptor, to impair the access of the one molecule to the other. Whether the AcSDKP interacts with either *stimulator* [*Figure 66b*] or the *stimulator* receptor [*Figure 66c*] is, at present unclear, although Wdzieczak-Bakala *et al* [personal communication] report the low affinity binding of radiolabelled AcSDKP to a small population of, as yet unclassified, haematopoietic precursors in murine bone marrow. This may be interpreted as an interaction of AcSDKP at the level of the membrane bound *stimulator* receptor [*Figure 66c*]. This low affinity binding of the AcSDKP to the putative *stimulator* receptor could imply that the interaction is possibly reversible. The use of unlabelled AcSDKP is reported to displace the radiolabelled AcSDKP, suggesting that the binding of the AcSDKP to certain cells is relatively specific.

To investigate evidence for an AcSDKP interaction with *stimulator* [*Figure 66b*], a purified source of *stimulator* (*RBME-III*) could be incubated with radiolabelled AcSDKP. Dialysis of the combination of the *stimulator* and AcSDKP should remove unbound AcSDKP and the binding of AcSDKP to *stimulator* could be investigated by measuring the retained radioactivity. The specificity of any interaction could be investigated by the simultaneous addition of unlabelled AcSDKP and the displacement of labelled AcSDKP observed.

Alternatively, the AcSDKP molecule may not interact within the active sites of the *stimulator* and receptor, but may bind to the appropriate molecule and indirectly alter the 3-dimensional conformation of the active site. Binding of AcSDKP may alter critical intermolecular bonds within the molecule, sufficient to disrupt the tertiary structure of the molecule and impair the interaction of agonist with receptor [*Figures 66d & 66e*].

The above hypotheses are significant since they do not require the presence of discrete AcSDKP receptors. An alternative

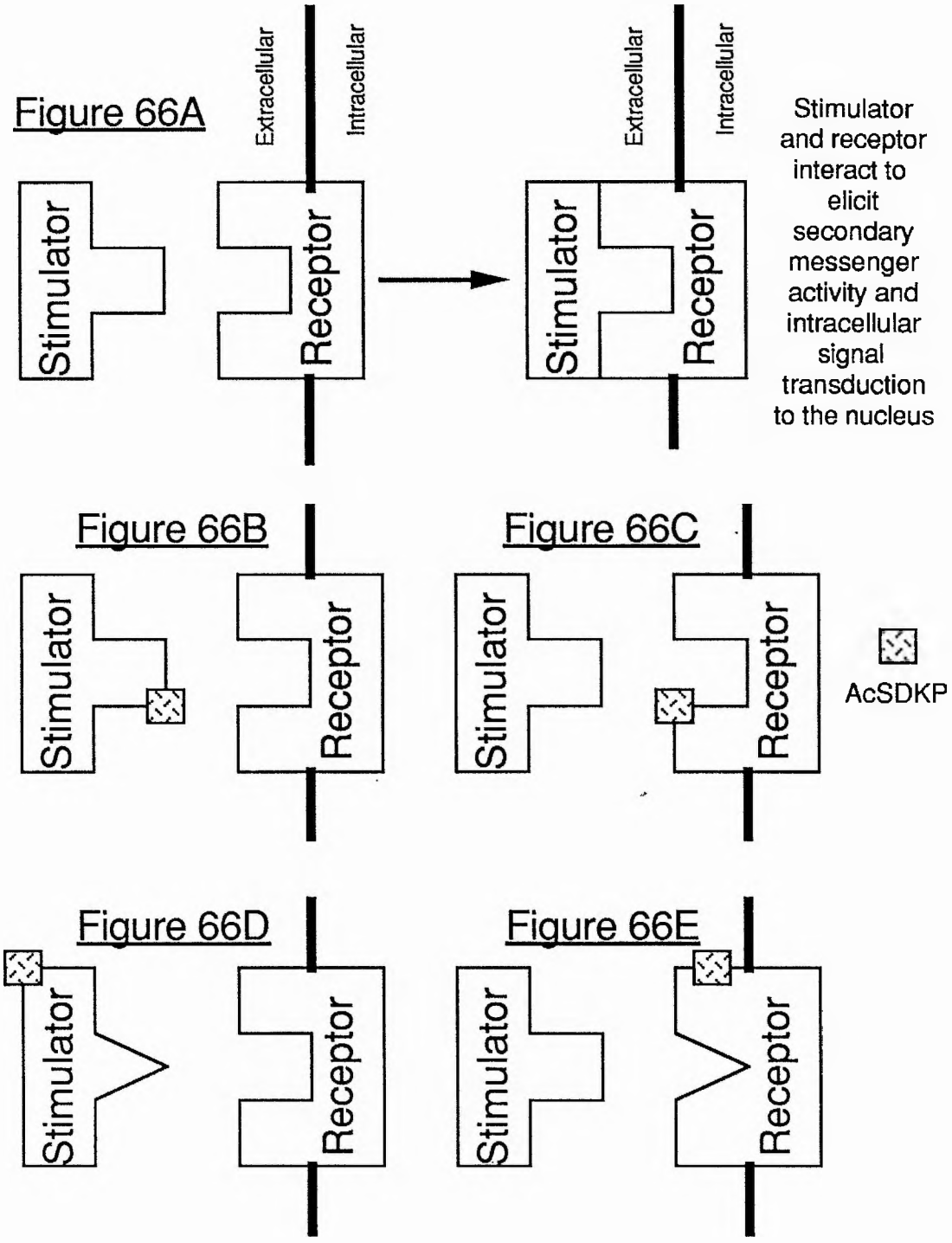


Figure 66: The possible mechanism by which the tetrapeptide AcSDKP may act to impair the action of the stem cell proliferation stimulator.

interpretation of the reports of Wdzieczak-Bakala *et al* [personal communication] of low affinity binding of radiolabelled AcSDKP to an unclassified population of haematopoietic precursors, may be evidence of AcSDKP receptors. If discrete AcSDKP receptors exist, the binding of AcSDKP to the receptor may elicit a number of intracellular responses amongst which may be the inhibition of expression of *stimulator* receptors on the extracellular surface of the cell membrane. Other possible mechanisms may be envisaged.

The attraction of a haemomodulatory role for the low molecular weight tetrapeptide AcSDKP is its great potential flexibility. An increase in the levels of endogenous AcSDKP could be produced by increasing the activity of *endoproteinase Asp-N*, and so the cleavage of AcSDKP from the putative precursor *Thymosin ß4* and/or a reduction in the AcSDKP-degrading activity of *angiotensin converting enzyme*. Similarly, a reduction in the levels of AcSDKP could be produced by reduced *endoproteinase Asp-N* activity and/or increased *angiotensin converting enzyme* activity. In this manner, a less coarse regulatory mechanism can be envisaged than were changes in the proportion of the stem cell population in S-phase solely reliant on changes in the production of *inhibitor* and *stimulator*. How the levels of AcSDKP are coordinated, possibly through the variation in the relative activities of *endoproteinase Asp-N* and *angiotensin converting enzyme*, to tailor the appropriate activity of the haematopoietic stem cell proliferation *stimulator* to the haematopoietic demands of the body is, as yet unclear.

In addition to thymosin ß4, the -SDKP- amino acid sequence is also reported in tumour necrosis factor-alpha (TNF-alpha) [Lenfant *et al*,1989a; Pradelles *et al*,1990]. TNF-alpha is reported to demonstrate haemoregulatory activity [Old,1985; Broxmeyer *et al*,1986; Munker *et al*,1987; Peetrie *et al*,1988; Slordal *et al*,1989] and in this respect the -

SDKP- sequence may be significant. Similarly, the minimal active amino acid sequence of the AcSDKP tetrapeptide, the SDK tripeptide sequence is found in murine leukaemia inhibitory factor (muLIF) [Gearing *et al*,1987]. LIF is also reported to demonstrate haemoregulatory activity [Gearing *et al*,1987; Leary *et al*,1990; Metcalf *et al*,1990; Verfaille & McGlave,1991] and in this respect, the presence of the -SDK- sequence may be significant. With the characterization of other haematopoietically active molecules, the significance of the -SDKP-,or -SDK-, amino acid sequences may become clearer.

4.4.6 Another low molecular weight haemoregulatory peptide: pEEDCK/HP5b/SP1.

In addition to AcSDKP, another low molecular weight haematopoietically active peptide is reported. A pentapeptide of amino acid sequence pyroGlu-Glu-Asp-Cys-Lys (pEEDCK) has been isolated from mature granulocytes and produced synthetically as "*haemoregulatory pentapeptide*" (HP5b), or "*synthetic pentapeptide-1*" (SP1) [Laerum *et al*,1987; Foa *et al*,1987; Lu *et al*,1989; Paukovits *et al*,1990a; Paukovits *et al*,1990b]. The pentapeptide is reported to be non-species-specific, of low toxicity and is active *in vivo* and *in vitro* against both normal and murine myeloid progenitor cells and human and murine leukaemic cell lines. The molecule is reported to inhibit colony-formation by haematopoietic colony-forming cells and to suppress "myelopoietic stem cells" in a non-toxic, reversible manner. Paukovits *et al* [1990] have suggested HP5b/SP1 may prove to be a potential myelosuppressive agent effective during S-phase-specific cytotoxic chemotherapy, keeping the haematopoietic stem cell population out of a drug sensitive S-phase. Laerum *et al* [1990] have identified the EEDCK amino acid sequence of HP5b/SP1 in a

subclass of G-protein, suggesting a possible interference by HP5b/SP1 with G-protein-mediated intracellular signal transduction. This is additional evidence that low molecular weight peptides may be able to interfere with mechanisms involved in proliferation regulation and that small, low molecular weight peptides can effectively mimic the binding sites of larger molecules. pEEDCK/HP5b/SP1 has also been demonstrated to possess a similar C-terminal chemical structure to the tripeptide Gly-Cys-Glu (GCE, "*glutathione*"), isolated from calf spleen and reported to regulate granulopoiesis [Fetsch & Maurer,1990]. Both pEEDCK and GCE possess similarly configured -COOH, -SH and -COOH and $-NH_2$ groups which may be significant in understanding their similar biological activity. Glutathione is reported to exist in a reduced, monomeric form and an oxidised, dimeric form. The former is reported to be a granulopoietic proliferation inhibitor, the latter is reported to be a granulocytic proliferation stimulator [Fetsch & Maurer,1990]. Neither pEEDCK/HP5b/SP1, or GCE/glutathione, show any structural similarity to AcSDKP.

Further evidence of the significance of low molecular weight, potent regulatory peptides in biological systems can also be drawn from studies of the nervous system [Cottrell & Bewick,1989; Price *et al*,1990]. A number of potent low molecular weight neuroactive peptides have been isolated in the invertebrate nervous system. One group of neuroactive peptides are characterized by a common terminal -Phe-Met-Arg-Phe-NH_2 (-FMRFamide, M_r approximately 600 amu) motif. The FMRFamide tetrapeptide is the minimal active amino acid sequence of the peptide family, and amino acid extensions of the tetrapeptide to a penta-, hexa-, or heptapeptide appears to play a role in modulating the activity of the FMRFamide moiety.

Section 5

Potential clinical applications.

4.5.1 An *in vitro* assay of a primitive haematopoietic precursor.

It has been demonstrated that the high proliferative potential colony-forming cell population stimulated by the synergistic interaction between interleukin 3 (IL3)/multi-CSF and macrophage colony-stimulating factor (M-CSF/CSF-1) (HPP-$CFC_{IL3+CSF-1}$) shares many behavioural and regulatory similarities with the *in vitro* HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" population and more significantly with the *in vivo* "CFU-S" population. This is considerable evidence to imply that the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population is a relatively primitive haematopoietic precursor and is possibly a component of the stem cell compartment within the haematopoietic system.

The *in vivo* CFU-S assay has allowed considerable investigation of the behaviour and regulation of the haematopoietic stem cell population, however, a major drawback of this assay is it's use of large numbers of mice. Since each mouse has only one spleen, the numbers of mice required to achieve statistically significant results can be large. The development of an *in vitro* assay of a cell population which shares many behavioural and regulatory similarities with the *in vivo* CFU-S population would be significant, and while the replacement of the *in vivo* CFU-S assay with the *in vitro* HPP-$CFC_{IL3+CSF-1}$ or HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" assay, is not being advocated, these relatively simple, reproducible *in vitro* assays of a primitive haematopoietic precursor, should provide powerful tools with which to investigate haematopoietic stem cell regulation and behaviour in normal and aberrant haematopoiesis.

The sensitivity of the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population to the haematopoietic stem cell proliferation regulators may, as previously demonstrated in the case of the X-irradiation-induced, murine,

myeloid leukaemias, allow the levels of the endogenous stem cell proliferation *stimulator* and *inhibitor* in aberrant haematopoiesis to be investigated. Of particular significance is the possible use of the murine *in vitro* HPP-$CFC_{IL3+CSF-1}$ or HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" populations to investigate the levels of the stem cell proliferation regulators in aberrant human haematopoiesis, since *inhibitor* and *stimulator* apparently demonstrate a non-species specificity (1.18). If human haematopoietic dysfunction could be related to abnormal levels of the haematopoietic stem cell proliferation regulators, it may prove possible to develop novel treatment regimes, perhaps involving the administration of exogenous *inhibitor* and *stimulator*.

At present, no assay of the human haematopoietic stem cell population exists. However, McNiece *et al* [1989b] report the demonstration of a colony-forming cell population with a high proliferative potential from human haematopoietic tissue. If, as in the murine HPP-$CFC_{IL3+CSF-1}$ and HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" assays, the human HPP-CFC population could be demonstrated to be a primitive haematopoietic precursor, this may significantly assist the direct investigation of human haematopoietic stem cell behaviour and regulation.

It is significant that the *in vitro* HPP-$CFC_{IL3+CSF-1}$ and HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" population prove sensitive to the haematopoietic stem cell proliferation *inhibitor* and *stimulator*, and the *in vitro* HPP-$CFC_{GM-CSF+CSF-1}$ "CFU-A" played a significant role in the identification and characterization of the stem cell proliferation *inhibitor* as "*macrophage inflammatory protein-1 alpha*" (MIP-1 alpha) [Graham *et al*,1990]. Similar procedures are likely to identify and characterize the haematopoietic stem cell proliferation *stimulator*.

The clinical implications of a number of positive and negative haemoregulatory activities have been reviewed [Moore,1991].

Specifically, the clinical significance of the identified and characterized haematopoietic stem cell proliferation *inhibitor* and *stimulator* will be great. The controlled administration of apparently non-toxic proliferation regulators, may allow the manipulation of the haematopoietic system to clinical advantage.

4.5.2 Potential clinical applications of the stem cell proliferation *inhibitor*.

The use of exogenous stem cell proliferation *inhibitor* may be particularly useful in reducing the proportion of the haematopoietic stem cell population in S-phase during chemo- or radiotherapeutic regimes. A reduction in the proportion of the haematopoietic stem cell population in S-phase prior to the administration of S-phase-specific chemotherapeutic agents, or radiotherapy, could markedly reduce the damage sustained by the haematopoietic system, damage which is usually a significant limiting factor of the dose and frequency of therapy. A potential reduction in the haemotoxicity of specific chemo- or radiotherapeutic regimes, may allow increased, or more frequent, and potentially more effective levels of treatment to be used. With a potentially greater tumour kill and reduced haemotoxicity, more effective chemo- and radiotherapeutic regimes may be developed.

Lord and Wright [1980], demonstrated the administration of exogenous stem cell proliferation *inhibitor*, prior to the administration of potential haemotoxic doses of tritiated thymidine, hydroxyurea or cytosine arabinoside, was able to 'protect' the murine haematopoietic system, sufficient to allow survival. Lord *et al* [1987], report similar findings in the administration of the stem cell proliferation *inhibitor* to *in vitro* long-term bone marrow cultures, prior to the addition of

cytosine arabinoside. Cultures exposed to cytosine arabinoside alone were markedly suppressed, while those subjected to cytosine arabinoside after pretreatment with the haematopoietic stem cell proliferation *inhibitor*, were observed to perform at least as well as control cultures not exposed to either *inhibitor* or cytosine arabinoside.

4.5.3 Potential clinical applications of the stem cell proliferation *stimulator*.

The clinical potential of the stem cell proliferation *stimulator* may also be significant. The only sucessful treatment of a number of haematological disorders with the failure of chemo- and radiotherapeutic regimes, is haematopoietic transplantation. Prior to haematopoietic transplantation patients are subjected to a dose of whole body irradiation and/or chemotoxic drugs sufficient to ablate their haematopoietic tissues and subsequently receive haematopoietic tissue intravenously. However, while the haematopoietic tissue becomes established and haematopoietic reconstitution occurs, the patient develops a marked pancytopenia. During this period of pancytopenia, patients are subjected to marked anaemia and haemorrhagic episodes and a particularly high vulnerability to infection. With successful transplantation, after this period of marked pancytopenia, haematopoietic regeneration will occur and restore the quality of the peripheral blood. Management of this period of pancytopenia, specifically the neutropenia, by the administration of individual haematopoietic colony-stimulating factors, has met with considerable success and the potential use of the haematopoietic stem cell proliferation *stimulator*, may allow a further reduction in the period of pancytopenia. The addition of

exogenous stem cell proliferation *stimulator* to donor tissue *in vitro*, prior to its intravenous transplantation, may serve to 'prime', or 'activate' the haematopoietic stem cell population.

The controlled administration of the haematopoietic stem cell proliferation *stimulator*, may also serve to assist haematopoietic reconstitution after sublethal haemosuppressive therapies. Chemo- and radiotherapeutic regimes, by their nature, will unavoidably damage normal as well as target tissue. The haematopoietic system is particularly sensitive to such agents and the damage to the haematopoietic system is often a significant limiting factor to the dose and frequency of therapy. Provided the tumour system is not itself sensitive to the haematopoietic stem cell proliferation *stimulator*, it's use may allow a more rapid haematopoietic recovery after and between treatments and may subsequently allow more frequent and potentially more effective levels of therapy to be employed.

The potential efficacy of the appropriate use of controlled levels of both the stem cell proliferation *inhibitor* and *stimulator* to manipulate the normal haematopoietic tissue to clinical advantage during both chemo- and radiotherapy, may be of great significance. The use of the haematopoietic stem cell proliferation *stimulator* may also be significant in assisting the management of certain diseases which present with haematological suppression. Leukaemia-associated suppression of normal haematopoiesis has been discussed, and is largely responsible for the deterioration in the 'quality' of the peripheral blood. Leukaemic patients often present with anaemia, haemorrhage and increased susceptibility to infection which can be severely debilitating. The controlled use of the haematopoietic stem cell proliferation *stimulator*, may serve to eleviate these symptoms and improve the 'quality of life' for the

patient. The use of *stimulator* would however, be precluded if the leukaemic cell population proved sensitive to it's action. In managing the disease in this manner, 'healthier' patients, more able to tolerate more intense and/or frequent treatment regimes may allow a more optimistic disease prognosis.

4.5.4 Potential clinical applications of the low molecular weight haemoregulatory tetrapeptide AcSDKP.

Many of the beneficial aspects of the use of the stem cell proliferation *inhibitor* and *stimulator* involving the manipulation of the haematopoietic system to clinical advantage, are equally applicable in discussing the clinical potential of the haemoregulatory tetrapeptide AcSDKP. AcSDKP appears to modulate haematopoietic stem cell proliferation at the level of haematopoietic stem cell proliferation *stimulator* action. The addition of exogenous AcSDKP has been demonstrated to block the recruitment of haematopoietic stem cells into S-phase *in vivo* in mice [Frindel & Guigon,1977; Guigon & Frindel,1978; Guigon *et al*,1980; 1981; 1982; 1989; Wdzieczak-Bakala *et al*,1983], while removal of endogenous AcSDKP by a polyclonal antiserum has been demonstrated to increase the proportion of haematopoietic stem cells in S-phase [Frindel & Monpezat,1989].

The use of exogenous AcSDKP to prevent the recruitment of haematopoietic stem cells into S-phase has been demonstrated to protect the murine haematopoietic system from potentially haemotoxic doses of the S-phase-specific cytosine arabinoside [Frindel & Guigon,1977; Guigon & Frindel,1978; Guigon *et al*,1980; 1981; 1982; 1989; Wdzieczak-Bakala *et al*,1983]. The manipulation of the proportion of the haematopoietic stem cell population in S-phase,

through the use of the non-species-specific, non-toxic, low molecular weight tetrapeptide AcSDKP to clinical advantage could thus be envisaged.

Increasing the concentration of AcSDKP, by the addition of exogenous factor, would increase the impairment of *stimulator* action, and prevent the recruitment of haematopoietic stem cells into S-phase and would be particularly effective in protecting the haematopoietic stem cell population from the effects of both chemotherapeutic agents and radiation.

A reduction in the levels of AcSDKP, by the administration of anti-AcSDKP polyclonal antiserum, would reduce the blocking effect on the action of *stimulator* and the proportion of haematopoietic stem cells in S-phase would increase. Such increased haematopoietic stem cell proliferation would assist in haematopoietic recovery after an episode of chemo- or radiotherapy, or else may be effective in the priming of haematopoietic tissue *in vitro* prior to transplantation, and so to reduce the period of pancytopenia. Similarly, the clinical manipulation of AcSDKP levels in diseases which present with haematological dysfunction may assist in disease management.

Liozon *et al* [1991] report evidence which may implicate a pathological role for AcSDKP in a number of human myeloproliferative disorders. Increased levels of AcSDKP are reported, which may act to suppress normal haematopoiesis to the advantage of the pressumably AcSDKP-, or *stimulator*-insensitive aberrant myeloproliferative disease. The physiopathology of AcSDKP may prove significant and the roles of the AcSDKP-liberating enzyme *endoproteinase Asp-N* and AcSDKP-degrading enzyme *angiotensin converting enzyme*, may also play an important role in certain disease mechanisms. It may prove possible to manipulate the relative activities of these two enzymes to indirectly manipulate the

endogenous levels of AcSDKP to clinical advantage, or to correct possible pathologically high or low enzyme activities.

4.5.5 In conclusion.

The manipulation of the haematopoietic stem cell population to clinical advantage would be a powerful technique. By manipulation of either the levels of the haematopoietic stem cell proliferation *stimulator* or *inhibitor*, and/or levels of the low, molecular weight, haemoregulatory tetrapetide AcSDKP, appropriate changes in the proportion of the haematopoietic stem cell population in S-phase could be induced. However, while, at present, no alternative role has been attributed to AcSDKP, the identification and characterization of the stem cell proliferation *inhibitor* as *macrophage inflammatory protein-1 alpha* (MIP-1 alpha) [Graham *et al*,1990], might suggest that the administration of exogenous *inhibitor*, with a view to reducing the proportion of the haematopoietic stem cell population in S-phase, may also elicit an inflammatory response and macrophage activation. MIP-1 alpha is also reported to be active against clonogenic epidermal cells. A monocyte/ lymphocyte-derived Langerhans cell population within the murine skin 'epidermal proliferative unit' (EPU), is reported to produce MIP-1 alpha and to inhibit EPU proliferation [Dr.M.Plumb, *Instutute for Cancer Studies, University of Leeds*, personal communication]. These alternative roles for MIP-1 alpha may somewhat restrict it's potential clinical use *in vivo*.

Evidence of MIP-1 alpha activity in a number of different tissues implies that the molecule is an important, conserved proliferation regulator, and its effects may subsequently be identified in other tissue systems. To act separately in these different tissues and elicit an appropriate proliferative response, MIP-1 alpha must be produced

and active locally. Evidence of the local haematopoietic action of MIP-1 alpha has been identified by part body X-irradiation studies [Croizat *et al*,1970; Gidali & Lajtha,1972] (1.9). The localization of MIP-1 alpha to either bone marrow, skin EPU or foci of inflammation, may be maintained by a blood-bone marrow/ EPU barrier, similar to that demonstrated for M-CSF/CSF-1 [Shadduck *et al*,1989]. However, while the presence of such a localizing barrier is precluded by evidence that the intravenous administration of the stem cell proliferation *inhibitor* to mice demonstrated a haematopoietic response [Lord & Wright,1980], such a compartmentalized system would allow levels of MIP-1 alpha to vary independently between the various MIP-1 alpha sensitive tissues. By comparison, the low molecular weight haemoregulatory tetrapeptide AcSDKP, has been demonstrated to be present in both serum and within the haematopoietic system, using an AcSDKP enzyme immunoassay technique [Pradelles *et al*,1990] and it's relatively low molecular weight would allow it to cross any putative blood-bone marrow barrier to effect a haematopoietic response. The local concentration and subseqent activity of AcSDKP could be regulated by the presence of the AcSDKP-degrading enzyme activity, ACE, within the haematopoietic microenvironment. The intravenous administration of AcSDKP to effect a haematopoietic response may thus prove less problematic than the use of the multiple activity MIP-1 alpha. Whether alternative roles for AcSDKP will subsequently be identified and whether similar multiple functions will be attributable to the haematopoietic stem cell proliferation *stimulator*, once identified and characterized, will be of considerable interest.

Section 6

Summary.

1) Characterization of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population.

a) In conclusion, the combination of interleulin 3 (IL3)/multi-CSF and macrophage colony-stimulating factor (M-CSF/CSF-1), as either media conditioned by the WEHI 3B myelomonocytic leukaemic cell line and L929 fibroblast cell line, or recombinantly-derived factors, stimulates the proliferation of a distinct colony-forming cell population from murine haematopoietic tissue *in vitro*. The colony-forming cell population, not observed when either factor is used singly, is characterized by a high proliferative potential. The high proliferative potential colony-forming cell (HPP-CFC$_{IL3+CSF-1}$) population has the capacity to produce macroscopic colonies in excess of 2mm diameter and of approximately $6x10^4$ cells after 14 days in semi-solid agar culture. The HPP-CFC$_{IL3+CSF-1}$-derived colonies are occassionally composed of cells of more than one haematopoietic lineage, which implies a degree of HPP-CFC$_{IL3+CSF-1}$ multipotency.

b) An HPP-CFC$_{IL3+CSF-1}$ frequency of approximately $100/10^5$ normal CBA/H femoral marrow cells is determined, which, in an average femoral marrow cellularity of $14x10^6$ cells, gives an approximate total CBA/H femoral marrow compliment of $14x10^3$ HPP-CFC$_{IL3+CSF-1}$. In marrow from CBA/H mice 3 days after a 2 Gy sublethal whole body X-irradiation dose an HPP-CFC$_{IL3+CSF-1}$ frequency of approximately $29/10^5$ regenerating marrow cells is determined, which, in an average femoral marrow cellularity of approximately $6x10^6$ cells, gives an approximate total femoral marrow compliment of $2x10^3$ HPP-CFC$_{IL3+CSF-1}$.

c) The proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase in normal femoral marrow was determined as approximately 9% and increases to approximately 40% in marrow from mice 3 days after a 2 Gy whole body X-irradiation dose.

d) The *in vitro* HPP-$CFC_{IL3+CSF-1}$ population proves sensitive to the previously characterized haematopoietic stem cell-specific proliferation regulators. *Stimulator*, derived from regenerating bone marrow from mice 7 days after a 4.5 Gy whole body X-irradiation dose, significantly increased the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase from approximately 9% to approximately 24%, while *inhibitor*, derived from normal bone marrow, significantly reduced the proportion of the HPP-$CFC_{IL3+CSF-1}$ population in S-phase from approximately 40% to approximately 25%. No evidence of stem cell proliferation *stimulator* or *inhibitor* toxicity against the HPP-$CFC_{IL3+CSF-1}$ population was observed.

e) Titration of the stem cell proliferation regulators demonstrated that the HPP-$CFC_{IL3+CSF-1}$ population proved equally sensitive to both *stimulator* and *inhibitor*.

f) A high proliferative potential, evidence of multipotency and a sensitivity to the stem cell proliferation regulators, is strong evidence to suggest that the *in vitro* HPP-$CFC_{IL3+CSF-1}$ population is a relatively primitive haematopoietic precursor, possibly a component of the heterogenous stem cell compartment.

2) *In vitro* HPP-CFC$_{IL3+CSF-1}$ investigation of haematopoietic stem cell regulation in murine, myeloid leukaemia.

a) The use of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ assay to investigate the levels of the haematopoietic stem cell proliferation regulators in a number of X-irradiation-induced murine myeloid leukaemias, demonstrated the absence of detectable levels of the haematopoietic stem cell proliferation *inhibitor* and *stimulator*, and no evidence of a direct-acting leukaemia-associated proliferation inhibitor was observed, in medium conditioned by overtly leukaemic marrow.

b) Medium conditioned by overtly leukaemic marrow did not impair the capacity of *stimulator*, derived from regenerating bone marrow, to increase the proportion of the HPP-CFC$_{IL3+CSF-1}$ population in S-phase or, the capacity of *inhibitor*, derived from normal bone marrow, to reduce the proportion of the HPP-CFC$_{IL3+CSF-1}$ population in S-phase.

c) Medium conditioned by overtly leukaemic marrow did appear to impair the capacity of regenerating bone marrow to produce *stimulator* or, of normal bone marrow to produce *inhibitor*.

d) The mechanism by which the leukaemic population impairs the production of the haematopoietic stem cell proliferation regulators is at present unclear. Characterization of this mechanism may allow the development of more effective disease treatment and management regimes.

3) *In vitro* HPP-CFC$_{IL3+CSF-1}$ investigation of the possible mechanism of action of the low molecular weight, haemoregulatory tetrapeptide AcSDKP.

a) Use of the *in vitro* HPP-CFC$_{IL3+CSF-1}$ population to investigate the possible mechanism(s) of action of the haemoregulatory tetrapeptide acetyl-N-Ser-Asp-Lys-Pro (AcSDKP, M_r = 487 amu) and the minimal active tripeptide sequence Ser-Asp-Lys (SDK, M_r = 348 amu), demonstrate that neither peptide is inherently a stimulatory activity. The proportion of the HPP-CFC$_{IL3+CSF-1}$ population in S-phase is not significantly increased by the presence of AcSDKP, or SDK, at 1 ng/ml (2.05×10^{-9}M and 2.87×10^{-9}M respectively) for 3 hours.

b) The presence of AcSDKP, or SDK at 1 ng/ml (2.05×10^{-9}M and 2.87×10^{-9}M respectively), did however, impair the capacity of the stem cell proliferation *stimulator* to significantly increase the proportion of the HPP-CFC$_{IL3+CSF-1}$ population in S-phase.

c) The tripeptide Asp-Lys-Pro (DKP, M_r = 358 amu, at 1 ng/ml, 2.79×10^{-9}M) proved less effective in impairing the action of the stem cell proliferation *stimulator* than either AcSDKP, or SDK while the tripeptide Ala-Asp-Lys (ADK, M_r = 332 amu, at 1 ng/ml, 3.01×10^{-9}M) did not significantly impair *stimulator* action.

d) Once the mechanism of action of AcSDKP and SDK has been characterized, a further investigation of other peptide analogues can be made in association with molecular modelling techniques, to produce potentially more potent peptides, or peptides more resistant to enzymatic degradation.

Publications,

Published Abstracts

and

Conference

Presentations

Publications

Robinson,S and Riches,A. 1991. Haematopoietic stem cell proliferation regulators investigated using an *in vitro* assay.

***Journal of Anatomy*, 174, 153.**

Robinson,S, Lenfant,M and Riches,A. [Submitted]. The mode of action of the tetrapeptide AcSDKP in regulating haematopoietic stem cell proliferation. [Submitted].

Published Abstracts

Robinson,S and Riches,A. 1990. Haematopoietic stem cell proliferation regulators investigated using an *in vitro* assay of a primitive haematopoietic precursor.

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Robinson,S and Riches,A. 1990. Kinetic properties of a primitive haematopoietic precursor following sublethal X-irradiation assayed *in vitro*.

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Robinson,S, Lenfant,M and Riches,A. [In Press]. The mode of action of the tetrapeptide AcSDKP in regulating haematopoietic stem cell proliferation.

***Journal of Anatomy*,[In Press].**

Conference Presentations

Robinson,S and Riches,A. Haematopoietic stem cell regulation investigated using an *in vitro* assay of a primitive haematopoietic precursor.

At: The Joint Spring Meeting of the British Society of Cell Biologists and British Society of Developmental Biologists, University of St.Andrews, April 1989.

Robinson,S and Riches,A. Kinetic properties of a primitive haematopoietic precursor following sublethal X-irradiation assayed *in vitro*.

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At: The Anatomical Society of Great Britain and Ireland and Nederlandse Anatomen Vereniging Joint Summer Meeting, University of Limburg, Holland, July 1990.

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