Evaluation Of In Vitro Bone Marrow Culture As A Tool For Assessing Mechanisms Of Haematotoxicity

R. Fagg

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Abstract

Dose limiting haematotoxicity has been associated with a range of therapeutic agents used for the treatment of a number of different conditions. Haematotoxicity is usually assessed as part of the preclinical safety studies in experimental animals, where changes in peripheral blood cell numbers and bone marrow cellularity are determined at the end of the study. Often no information on the mechanism of the haematotoxicity is revealed. This thesis demonstrates how *in vitro* bone marrow cultures can be utilized to assist in the assessment of haematotoxicity by two different approaches; firstly, *in vitro* bone marrow cultures can be used to assess the haematopoietic lineage specificity of vincristine sulphate, vinblastine sulphate, hydroxyurea and anagrelide hydrochloride using clonogenic cultures, enabling ranking of these compounds according to their haematotoxicity. Secondly, using *in vitro* assays only, elucidate the mechanism(s) of the megakaryocytic lineage specific inhibition of anagrelide hydrochloride. To this end both clonogenic cultures and LTBMC offer the ability to elucidate mechanisms of action on multipotent stem cells, lineage specific cells and effects on the bone marrow microenvironment following single and repeated administration. In addition, the combination of cell identification techniques flow cytometry and light microscopy was shown to provide a more detailed understanding of the different cell populations within the non-adherent cell layer.

In vivo AN reduces platelet counts only, however, the mechanism of the megakaryocyte specific toxicity by AN is not understood. In these studies, the mechanism (s) of the megakaryocytic lineage haematotoxicity of AN was examined using the established human clonogenic and LTBMC. The action of AN was shown to be focused at a late stage in megakaryocyte (Mk) colony development. Ranking the potential mechanisms of action of AN by concentration at which they were noted, the inability to organize the microtubules appears to be secondary to 1) alteration in cell cycling, 2) surface receptor expression and 3) inhibition in achieving high (greater than 8N) ploidy number. However, identification of the primary mechanism based solely on concentration seems to be very crude and most probably reflects a limitation of *in vitro* systems. The inhibition of platelet production by AN is most likely a result from a combination of mechanisms; inhibition of cell cycling, disruption in the expression of cell surface receptors, inhibition of the ability of the cells to increase ploidy number and an associated inability to organize microtubules leading to a reduction in platelet

release.

This work also demonstrated the importance of the selection of the source of bone marrow used in the cultures. The concentration at which 50 percent of Mk colony growth was inhibited (IC50) by AN for murine cells was markedly (46 fold) different (88.6μM) compared to the IC50 with human cord blood (hCB) (1.92μM). This disparity is indicative of differences in species sensitivity possibly related to AN having a greater affinity towards the human c-mpl thrombopoietin (TPO) receptor than the equivalent murine receptor as suggested by McCarty et al (2006).

This work highlights the utility of *in vitro* bone marrow cultures as a tool for investigating the lineage specific haematotoxicity by evaluating compounds used in the treatment of ET. In addition *in vitro* haematopoietic cultures can successfully be used as a tool to investigate potential mechanism(s) of haematotoxicity as demonstrated herein by providing an insight to mechanism of platelet count reduction by AN.

List of Contents

Abbreviations

Name	Abbreviation
Acidic isoferritins	AIFs
Acquired Immunodeficiency	AIDS
Alkaline phosphtase anti-Alkaline phosphotase	APAAP
Anagrelide Hydrochloride	AN
Anagrelide metabolite	AM
Azidothymidine	AZT
Bone marrow	BM
Burst Forming Units- Erythroid	BFU-E
Cluster of Differentiation	CD
Cobblestone area forming cells	CAFC
Colony Forming Unit	CFU
Colony Forming Unit- Erythroid	CFU-E
Colony Forming Unit-Megakaryocyte	CFU-Mk
Colony Forming Unit- Mix	CFU-Mix
Colony Forming Unit- Granulocytic, monocytic	CFU-GM
Colony Stimulating Factor	CSF
Colony Forming Unit- Granulocytic, erythrocytic/ monocytic	CFU-GEMM
Colony Stimulating Factor- Megakaryocyte	Meg-CSF
Colony Stimulating Factor- Granulocytic/Monocytic	GM -CSF
Colony Stimulating Factor- Monocytic	M-CSF
Dimethylsulfoxide	DMSO
Extracellular matrix Molecules	EMM
Erythropoietin	Epo
Essential Thrombocythemia	ET
Fetal bovine serum	FBS
GlaxoSmithKline	GSK
Glycoprotein	GP
High proliferative potential colony forming units	HPP-CFU
Hydroxyurea	HU
Inhibition of colony growth by 50%	IC50
Interleukin	IL
Low proliferative potential colony forming units	LPP-CFU
Long Term Bone Marrow Cultures	LTBMC
Long term culture initiating cells	LTC-IC
Name	**Abbreviation**

Megakaryocyte	Mk
Myeloproliferative disorder	MPD
Receptor tyrosine Kinases	RTK
Stem Cell Factor	SCF
Thrombopoietin	Tpo
Vinblastine	VB
Vincristine	VC

Chapter 1

1.0 Introduction

1.1 The Haematopoietic System

All blood cells are derived from a small common pool of totipotent cells, called haematopoietic stem cells. The production of all the blood cells is strictly regulated by the haematopoietic microenvironment, which includes stromal cells, extracellular matrix molecules and soluble regulatory factors.

Blood cells composing the haematopoietic system can be classified into two main classes, lymphoid (B, T and natural killer lymphocytes) and myeloid (erythrocytes, megakaryocytes, granulocytes and monocytes). The life -span of the fully differentiated mature forms of blood cells vary considerably, from several hours for some cells (granulocytes), and several weeks (erythrocytes) to several years (lymphocytic and monocytic memory cells) for others. The haematopoietic system is extraordinarily complex, since not only are huge numbers of new mature cells produced per day, but there is also a need for the maintenance of a pool of undifferentiated cells and for a rapid response to situations of acute stress (Kiel et al 2006; Ogawa et al, 1993).

During ontogeny, there are a number of haematopoietic sites. Studies in mice and birds have shown that an early intra-embryonic site of haematopoiesis is found in the para-aortic splanchnopleura (Cumano et al, 1996) and the aorta, gonads, (Medvinsky et al, 1996). It is believed that haematopoietic stem cells arise in these two structures and also in blood islands of the yolk sac, enter the embryonic circulation and colonize the newly formed liver rudiment. The fetal liver is the site of definitive haematopoiesis early during embryonic development. From birth onwards, and throughout adult life, the bone marrow with its intersinusoidal spaces is the site responsible for the generation of blood cells. The mechanisms responsible for these ontogenic shifts are not well known, and may involve changes in the developing haematopoietic cells (Houssaint and Hallet, 1988) or in the haematopoietic microenvironment (Slaper-Cortenbach et al, 1987; Friedrich et al 1996; Kiel et al, 2006). Recently, researchers have demonstrated that once in the bone marrow, the HSCs reside in vascular niches containing reticular cells that secrete CXCL12, a chemokine that promotes HSC maintenance

(Sugiyama et al 2006). The consistent presence of HSCs adjacent to CXCL12-secreting reticular cells, irrespective of whether the HSCs were in vascular or endosteal locations, provides a functional link between vascular and endosteal niches.

1.2 Differentiation in the Haematopoietic System

As mentioned above, all different types of blood cells are derived, through a series of maturational cell divisions regulated by the haematopoietic microenvironment, from a small pool of totipotent cells called haematopoietic stem cells. The stem cell concept originated from the work of Till and McCulloch in 1961 showing the formation of nodules of haematopoietic cells in the spleens of lethally irradiated mice after reconstitution with bone marrow from normal syngeneic donors (Orlic and Bodine, 1994). In spite of much effort since then, stem cells are still elusive entities. These cells are of interest not only because of their developmental capacity but also because of their potential usefulness for the treatment of hematological disorders and as vectors for gene therapy (Scott and Gordon, 1995; Lord and Dexter, 1995; Kiel et al, 2006). The most widely used criterion for their identification, that of a multipotential cell capable of self-renewal as well as of reconstituting long-term haematopoiesis after marrow ablation, presents theoretical as well as practical problems (Lansdorp, 1995). Besides these two main characteristics, other well-established features of human stem cells include (Morrison et al, 1995; Zhixing and Linheng, 2006):

a) They constitute a very small compartment, with estimates varying from less than 0.05% to up to 0.5% of cells in the bone marrow.
b) The majority of stem cells are normally quiescent, as shown by their resistance to treatment with 5-fluorouracil or 4-hydroperoxycyclophosphamide, which spare them and eliminate dividing cells without adversely affecting the long-term repopulating ability of bone marrow. Estimates of periodicity of mitosis vary widely (once a month to once in a few years), and the direct examination of the cell cycle of the long-term cells (quiescent totipotent stem cells) indicates that at any moment only 4% of them are in the $S/G_2/M$ phases.
c) The surface phenotype, which will be discussed fully later.
d) Stem cells cannot only be found in the bone marrow, but also in umbilical cord blood and in peripheral blood, particularly after "mobilization" treatments, such as Colony

Stimulating Factor- granulocytic/ monocytic.

When stem cells divide, they may return to the G_0 phase of the cell cycle generating more stem cells; alternatively, they may generate large numbers of committed progenitors with a progressively restricted differentiation potential. The mechanisms involved in the decision between self-renewal versus differentiation of the stem cell, at each division, are poorly understood. Till and colleagues proposed a model of stem cell functions in which the decision of the stem cell to self -renew and differentiate was depicted as stochastic process (Till et al, 1961). They tested the proposed stochastic model by performing a computer simulation based on the generation of random numbers and analyzing the colony forming units in the spleen. Recently, homeotic genes have been identified that appear to be of fundamental importance in these and other cellular processes (Moore et al, 2006).

Most models assume that the self-renewal potential of stem cells is finite, as evidenced by serial transfer experiments (Keller et al, 1998). Stochastic and deterministic models (Nakahata et al, 1982) attribute different weights to the roles played by intrinsic and micro-environmental factors in the decision between self-renewal and differentiation.

The transition from stem cells to mature haematopoietic cells occurs through several intermediate steps characterized by the progressive loss of the self-renewal capacity and the commitment to a specific cell lineage. The progenitor cell compartment which can be identified by the expression of surface antigens specific to each lineage has the potential to differentiate into any of the haematopoietic lineages (Kaushansky, 2006).
The developmental potential of the pluripotent progenitor cells is generally limited to only one or two of the haematopoietic lineages and these cells progressively display the antigenic, biochemical, and morphological features characteristic of the mature cells of the appropriate lineages, losing their capacity for self-renewal. Their proliferation is normally tightly controlled and coupled to their maturation, so those cells leaving the bone marrow usually possess little or no proliferative potential.

The expression of different receptors on the surface of haematopoietic progenitors permits the interaction with various regulatory elements present in their environment, which includes stromal cells, extracellular matrix molecules (EMM) and soluble regulatory factors (cytokines

and growth/differentiation factors). Although the role played by the microenvironment in the determination of the stem cell fate is still unclear, its pivotal role in the regulation of the amplification of the progenitor cell compartment is well established. Further differentiation of cells into one of several lineages critically depends on the nature of factors acting on these cells at a particular time and at a particular concentration. Figure 1 .1 shows the differential pathway of totipotent haemopoietic stem cells.

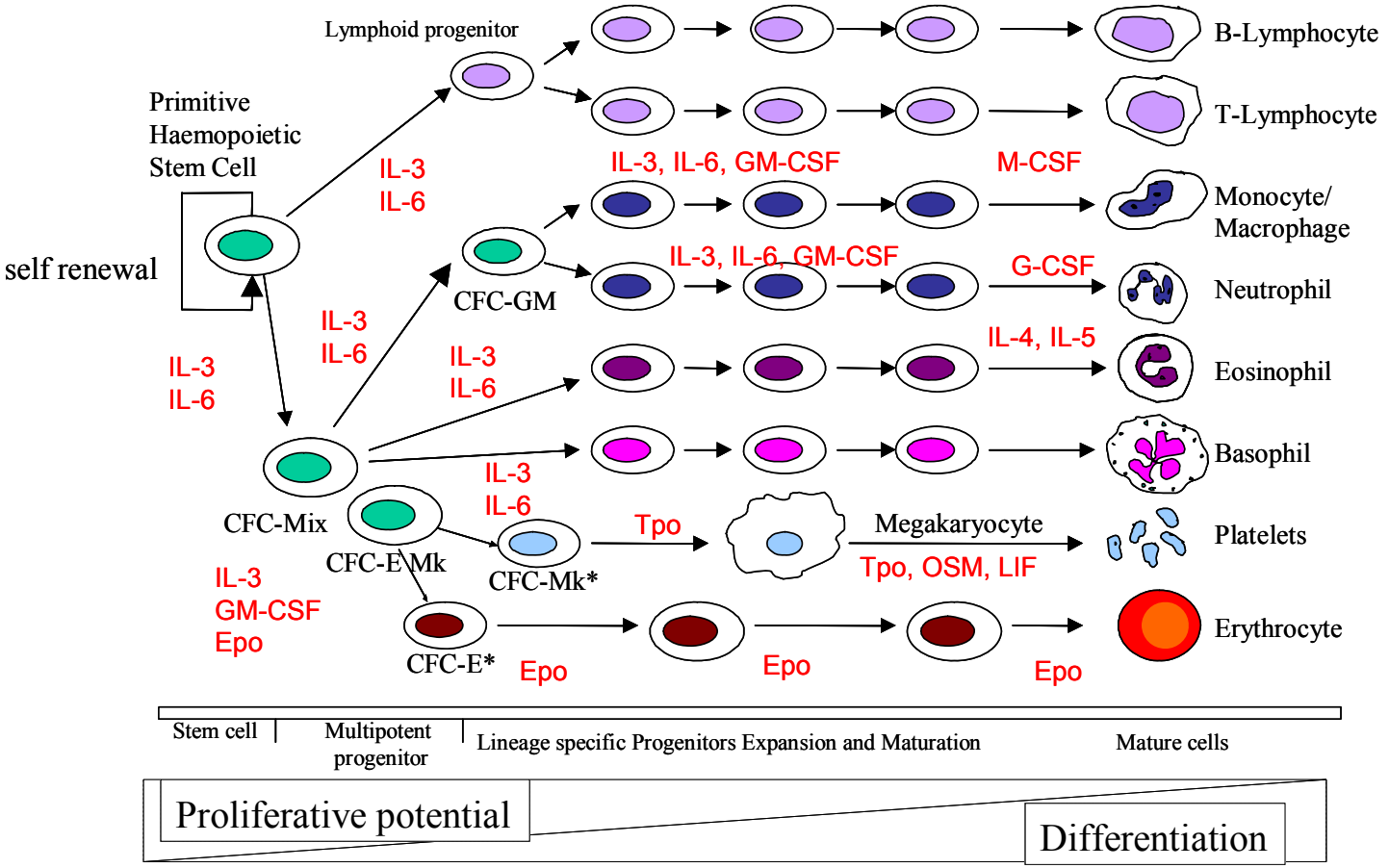


Figure 1. 1 The Differential Pathway of Totipotent Haemapoietic Stem Cells

The totipotent haemopoietic stem cells have the potential to differentiate to mature lymphocytic, erythroid, megakaryocytic, monocytic and granulocytic cell lineages. Proliferative potential reduces as the cells differentiate to give rise to the mature cell lineages. Key cytokines involved in differentiation are shown. Abbreviations: CFC-GEMM - colony forming cells-granulocytic, erythrocytic, monocytic; CFC-MIX colony forming cells-granulocytic/ monocytic, erythrocytic; CFC-E Mk – colony forming cell erythroid/ megakaryocytic; CFC-E - colony forming cell-erythrocytic; CFC-Mk - colony forming cell-megakaryocytic; IL - Interleukin; GM-CSF- Granulocytic/monocytic Colony Stimulating Factor; M-CSF - monocytic Colony Stimulating Factor; G-CSF - Granulocytic Colony Stimulating Factor; Epo- erythropoietin; Tpo - thrombopoietin.

1.3 Megakaryopoiesis

Megakaryopoiesis results in the production and release of platelets. The erythroid and megakaryocyte lineages are closely related. There is increasing evidence that the erythroid and megakaryocyte lineages are derived from a common bipotent progenitor, called the Burst forming units –erythroid / megakaryocyte forming units (BFU-E/MK) that gives rise to colonies containing a large majority of erythroblasts and a minority of MKs (Debiliet al, 1996). In the current scheme of hematopoietic development, the BFU-E/MK progenitors are considered to derive from the CFC-Mix (totipotent stem cells. However, a controversial model suggests that the BFU-E/MK can derive directly from HSC (Forsberg et al, 2006). As with the other lineages it is a multistage process of cellular differentiation and maturation regulated by a variety of cytokines (George, 2004).

Although megakaryopoiesis is normally confined to the bone marrow from the end of the third trimester of pregnancy, in all mammals, megakaryocytes (Mk) are found in large numbers in the lungs (Kickler, 2005). It has been shown that pulmonary Mk originate from the bone marrow and are carried to the lungs via the blood supply and retained there (Debili et al, 1992; Kickler, 2005). Furthermore, the contribution of the pulmonary Mk to the body platelet pool can be between 7 and 25 percent (Debili et al, 1992; Deutsch and Tomer, 2006).

The development and maturation of the committed megakaryocytic stem cells is unique in that they develop a highly polyploid DNA content which is associated with giant multi-lobed nuclei. Formation of platelets results from the projection of megakaryocytic cytoplasm through the sinusoidal endothelium (Deutsch and Tomer, 2006).

1.3.1 Megakaryopoiesis and the endomitotic process

The megakaryocytes are polyploid during normal differentiation. Polyploidy enables platelet production as the MK cytoplasm volume increases in parallel with ploidy. MK can stop DNA duplication at any stage between 2 N to 64 N, and possibly until 128 N. In humans the modal ploidy is 16 N (about 50% of the MK). During polyploidization and at the end of this process, the MK cells increase the synthesis of platelet specific proteins and of important proteins for platelet formation and function. This leads to an 81-fold increase in MK volume and,

theoretically, in the number of platelets produced. It has been suggested that only MKs with a ploidy over 4 N are capable of forming proplatelets. Endomitosis was originally given to the polyploidization mechanism because it was thought that mitosis was occurring without rupture of the nuclear envelope (Ravid et al, 2002). Endomitosis corresponds to a mitosis that skipped anaphase B and cytokinesis (Vitrat et al, 1998) An endomitosis, similarly to a mitosis, begins with the duplication of the centrosomes, the development of a mitotic spindle, the prophase with chromatin condensation, the rupture of the nuclear envelope, the alignment of the chromosomes on the equatorial plate during the metaphase and finally the separation of the sister chromatids at anaphase. However, the spindle of a polyploid MK is multipolar with the number of poles corresponding to the ploidy level. In MK polyploidy, the spindle remains short and does not elongate as in a normal mitosis. Chromatids move towards each pole and appear as a round mass circling each pole at anaphase, but each DNA mass remains tight as a consequence of the absence of spindle elongation. Nevertheless, a true midzone develops as during normal mitosis (Geddis et al, 2006). At the end of endomitosis, the MK contains a single nucleus with a single nuclear membrane. Each nuclear lobe corresponds to each pole of the multipolar spindle and their number is the direct reflection of the ploidy (Roy et al, 2001). The cell cycle during MK polyploidization is clearly composed of a succession of G1, S, G2 and M phases, but the M phase is incomplete (Ravid et al, 2002). After M phase, MKs re-enter into G1 to initiate a subsequent cell cycle in order to duplicate their DNA. None of the molecular mechanisms suggested at the origin of the endomitotic process are involved in MK polyploidization. Endomitosis appears to be related to a failure in cytokinesis and thus may be due to an abnormal depolymerization of microtubules and/or a defect in contractile forces related to the actin/myosin II complex. MK cells express very high levels of cyclin D3 and that an ectopic increase in cyclin D1 raises the MK ploidy level (Sun et al, 2001).

Microtubule reorganization is considered one of the key events in the release of platelets from Mk cells. Tubulin –α has been shown to be associated with microtubule structures in platelets and interact with tubulin -β. Tubulin-β1 has been demonstrated to be concentrated in specialized microtubule structures in mature megakaryocytes and platelets (Radley and Scurfield 1980). Tubulin-β1 KO mice have been found to produce up to 60% less platelets when compared with wild type (Levin et al 1993). An overview of megakaryocyte production of platelets is provided in Figure 1.2. Similar to the development of erythrocytes, the nucleus is extruded. This strongly supports a close etiology of the erythroid and megakaryocytic cell lineages (Tavassoli,1980).

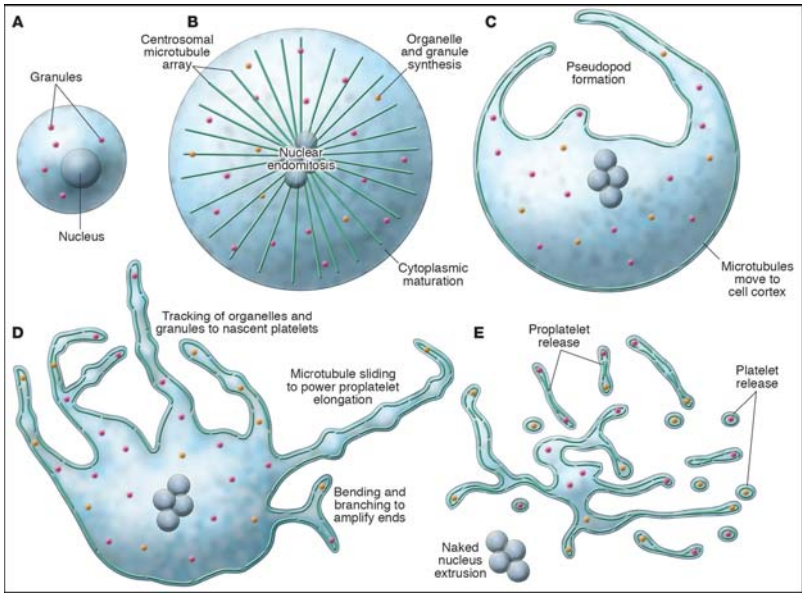


Figure 1. 2 Megakaryocyte production of platelets, (Patel et al, 2005)

Megakaryocytes transition from immature cells (A) to released platelets (E), a systematic series of events occurs. (B) The cells first undergo nuclear endomitosis, organelle synthesis, and a cytoplasmic maturation and expansion, while a microtubule array, emanating from centrosomes, is established. (C) Prior to the onset of proplatelet formation, centrosomes disassemble and microtubules translocate to the cell cortex. Proplatelet formation commences with the development of thick pseudopods. (D) Sliding of overlapping microtubules drives proplatelet elongation as organelles are tracked into proplatelet ends, where nascent platelets assemble. Proplatelet formation continues to expand throughout the cell while bending and branching amplify existing proplatelet ends. (E) The entire megakaryocyte cytoplasm is converted into a mass of proplatelets, which are released from the cell. The nucleus is eventually extruded from the mass of proplatelets, and individual platelets are released from proplatelet ends.

Mk progenitors express the CD34 antigen, but mature Mk cells do not. Additionally the precise stage along the Mk differentiation at which the CD34 is lost is not known. Purified marrow CD34+ cells give rise within 4 days in culture to rare mature Mk, suggesting that some Mk precursors bear the CD34 antigen (Chang et al, 2007). By flow cytometry, CD34+ cells bearing platelet glycoproteins (GP) could be detected, but at a low frequency (less than 2% of the marrow CD34 positive (+) cells. These cells gave rise within another 4 days in culture to numerous Mk (up to 50%), showing that these CD34+ cells were greatly enriched in Mk precursors. This was confirmed by ultrastructural studies that showed the presence of typical megakaryoblasts. By flow cytometry, three populations of small cell size can be defined: CD34+ CD61- (development stage I), CD34+ 61+ (development stage II) and CD34-

CD61+ive cells(development stage III). The two CD61+ ive populations are almost pure immature MK. Alpha-Granules, organelles containing alkaline phosphatase, are rare in the CD34+ ive CD61+ ive cells, whereas they are more developed in the CD34- ive CD61+ ive cells, which also exhibited demarcation membranes (Erickson et al, 1993; Deutsch and Tomer, 2006). Recent flow cytometric studies have demonstrated that VWF is strongly expressed by early (2 and 4 N) marrow MKs, enabling their complete resolution from the other marrow cells at a level superior to that achieved with GPIIb/IIIa as a lineage-specific marker (Tomer, 2004).

A diagrammatic representation of megakaryopoiesis is shown figure 1.3.

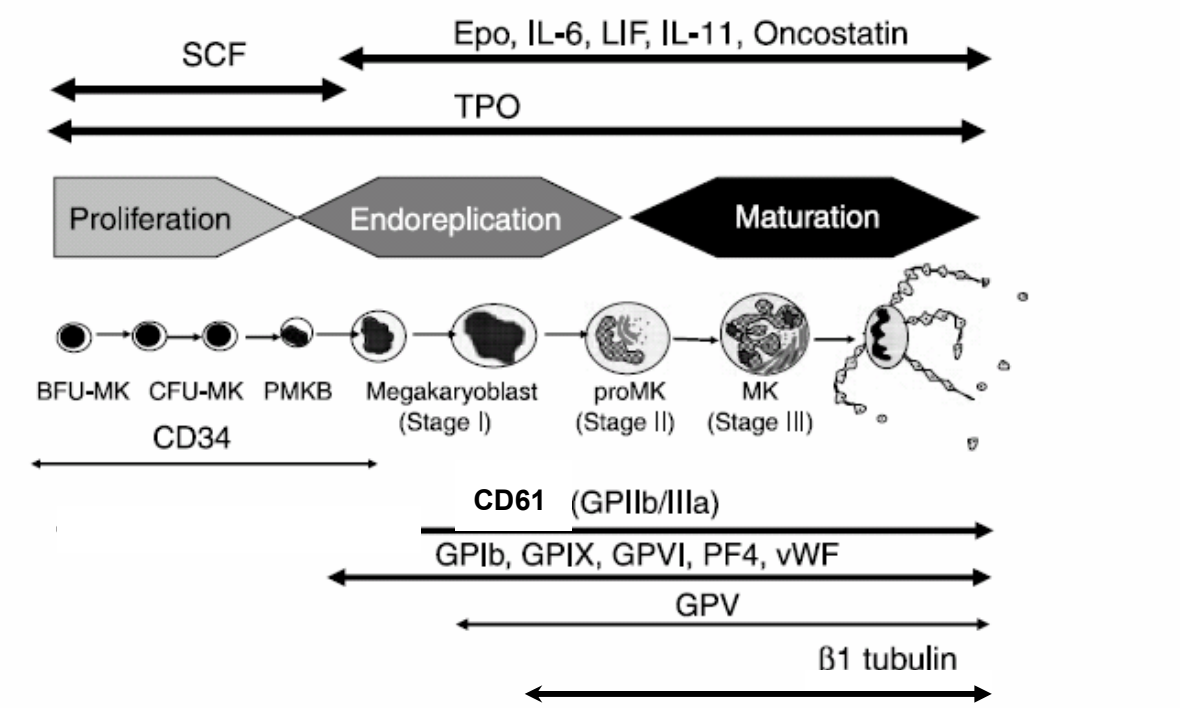


Figure 1. 3 Diagrammatic representation of Megakaryopoiesis (Chang, et al, 2007)

Mk colonies derive from a bi-potent BFU-E/Mk progenitor. Stem cell factor (SCF), interleukins 3, 6 and 11 (IL-3,IL-11, IL-6), leukocyte inhibitory factor (LIF) thrombopoietin (Tpo) and erythropoietin (Epo) drive the maturation and differentiation to give rise to mature megakaryocytes (MK stage III) cells. Also shown are the surface antigens expressed during megakaryopoiesis; CD34+ CD61- (development stage I), CD34+ 61+ (development stage II) and CD34-CD61+ive cells (development stage III). B-tubulin is detectable intracellularly in all three development stages.

1.4 Regulation of Haematopoiesis

Haematopoiesis is controlled by a number of proteins, growth factors and cytokines that define the lineage of the differentiating cells. In the last decade it has become clear that homeobox containing genes (HOX genes) not only play a significant role in regulating body formation, but in addition, they are contributing to organization and regulation of haematopoiesis. The HOX genes are organized into four genomic clusters (A, B, C and D).
Analysis of human and mouse haematopoietic cell lines revealed that some HOX genes were expressed in several haematopoietic cell lineages (HOXC8, HOXA7 and HOXB7), while others were limited to a single cell lineage; HOXA10 which is restricted to myelomonocytic cell lines. Furthermore, expression of all HOXB genes was mainly associated with cell lines of the erythroid lineage (Oostveen et al, 1999).

Over expression of HOXB4 in totipotent haemopoietic cells results in the increased self-renewal potential suggest that it involved in controlling the self-renewal versus differentiation decision processes (Abramovich et al, 2005; Moore et al, 2006).
A number of growth factors and cytokines act through binding to high affinity receptor tyrosine kinases (RTKs). Twenty different RTKs have been identified of which many are involved in the regulation of haematopoiesis. Table 1.1 shows the key RTKs involved in haematopoiesis.

Receptor Tyrosine Kinase	Role in Haematopoiesis
RTK CLASS II (Insulin Receptor Family)	Regulate the proliferation and differentiation of haematopoietic cells via specific receptors located on erythrocytes, monocytes, as well as B and T lymphocytes.
RTK CLASS III (PDGF Receptor Family) - cKit	Binding of c-KIT ligand, stem cell factor (SCF), results in c-KIT dimerization and subsequent activation of the JAK-STAT, PI-3 kinase and MAP kinase pathways that promote cell growth and differentiation
RTK CLASS III (PDGF Receptor Family)- FLT3	FLT3 (FMS-like tyrosine kinase, CD135 is expressed in multipotential haematopoietic stem cells and progenitors, suggesting a critical role in stem cell development and differentiation.
RTK CLASS III (PDGF Receptor Family)- PDGFRα and β	Expressed on myeloid lineage; over activation results in myeloproliferative disorder with eosinophilia, eosinophilic leukaemia or chronic myelomonocytic leukaemia
RTK CLASS IV (FGF Receptor Family)	Expressed on most haemopoietic cell lineages; over activation resulting in myeloproliferative disorder with eosinophilia,
RTK CLASS V (VEGF Receptor Family)	Clonogenic assays reveal that VEGF inhibits total colony formation from less mature progenitor cells and at the same time promotes the formation of myeloid, mixed and erythroid colonies from lineage-committed progenitors. VEGFR-1 has also been suggested as regulating the cell cycle and differentiation of haematopoietic stem cells and promoting haematopoietic cell mobilization.
RTK CLASS VII (TRK Receptor Family)	Regulate immature erythroblasts (TrkA), eosinophilic metamyelocytes and polymorphonuclear cells (TrkB), as well as promyelocytes, myelocytes and megakaryocytes (TrkC).

Table 1. 1 Receptor tyrosine Kinases and their regulatory role in haematopoiesis

The key receptor tyrosine kinases (RTKs) expressed on normal and malignant cell lineages and their potential role in the regulation of haematopoiesis are shown.

The generation of haematopoietic colonies is absolutely dependent on the continuous presence of so-called colony-stimulating factors (CSF), and the in vitro haematopoietic cultures were the key to the original discovery and characterization of many of the haematopoietic growth factors. Colony formation assays, therefore, allow the study of the influences of given growth factors or cytokines on the determination of the lineage along which colony-forming cells differentiate. Growth factors are denominated according to the colonies originating under their influence - M-CSF or GM-CSF, for instance, for factors inducing colonies composed of monocytes or granulocytes and monocytes, respectively. The cytokine families share some common features, for example, they are all found in the extracellular compartment, that interact with specific target cells to stimulate growth or differentiation. There are very few

responses that are mediated by just the one cytokine and many responses can be achieved by several cytokines (Nicola et al, 1994; Keil et al, 2000; Kaushansky, 2006). Many of the cytokines also overlap in the target population they control. This ensures that cell production continues if one or more pathways are inhibited. It has also been shown that the impact of combinations of cytokines can induce a greater than additive proliferative response - synergism. For example, work conducted using factor dependent cell lines have demonstrated stimulatory synergy between granulocytic/ monocytic - colony stimulating factor (GM-CSF), interleukin -3 (IL-3) and erythropoietin (Epo) (Hendrie et al, 1991; Kaushansky, 2006). A number of other synergies have been identified, including synergies between interleukin-6 (IL-6) and interleukin 11. Also synergy has been demonstrated to be important between Epo and thrombopoietin (Tpo) *in vivo* (Ogawa et al, 1993; Kaushansky, 2006). The cytokine network also has an inhibitory / negative feedback mechanism (Figure 1.4). These systems ensure that cell proliferation and cell maturation is kept under tight control. For example, changes in the oxygen potential of body organs act as a feed back loop on the production of Epo. When oxygen tension is high Epo release by the kidney is reduced. Other inhibitory mechanisms result from cell to cell interactions. For example, acidic isoferritins (AIFs), found in mature granulocytes, monocytes, lymphocytes and erythrocytes contribute to a feedback mechanism affecting colony forming units - granulocytic / monocytic (CFU-GM) and burst forming units -erythroid (BFU-E) (Jacobs et al, 1983). A similar inhibitory effect on CFU-GM and BFU-E colonies has been demonstrated by the production of increased levels of interferons (Gasner et al,1985; Kaushansky, 2006). Also lactoferrin present in neutrophils has been implicated in controlling release of the cytokine GM-CSF. A detailed list of cytokines grouped according to their known target lineages is shown in Table 1.2.

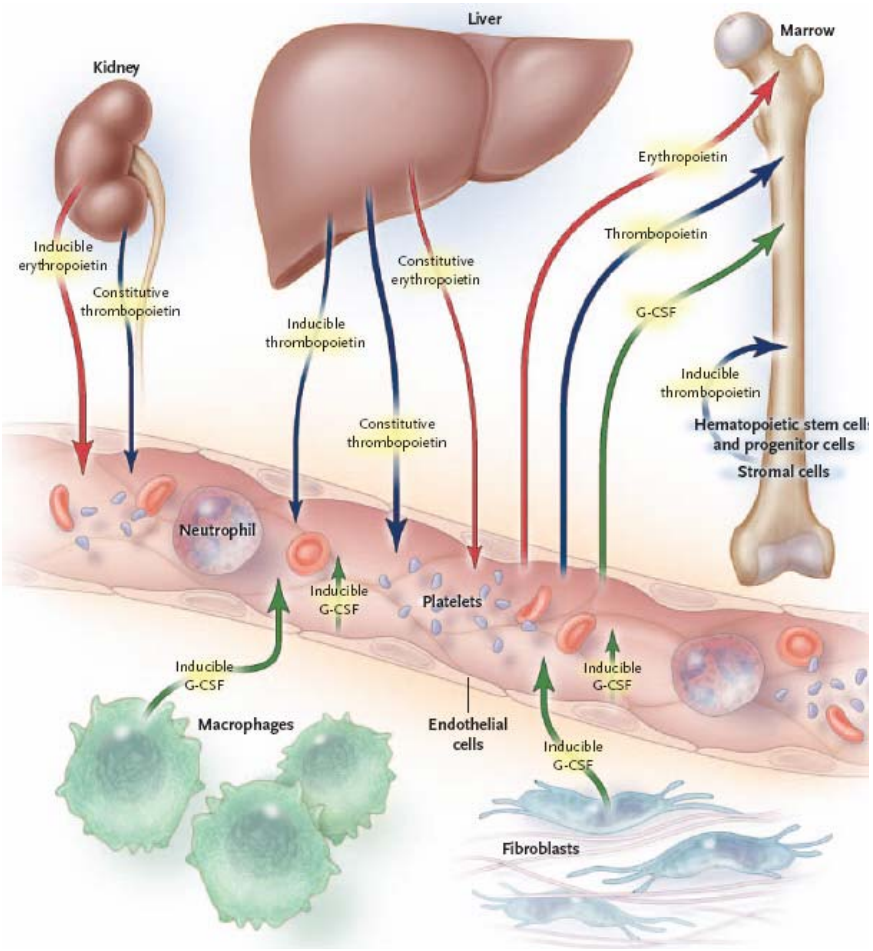


Figure 1. 4 Regulation of the Production of Haematopoietic Growth Factors (modified from Kaushansky, 2006)

The production of growth factors by various tissues and cells (arrows) is both constitutive and inducible and subject to adsorption by receptor-bearing cells. Production of erythropoietin (red arrows) is inducible by hypoxia in the kidney and constitutive in the liver (10 percent of total body erythropoietin), and it is excreted in the urine. G-CSF (green arrows) is mostly inducible in tissues from fibroblasts and macrophages and from endothelial cells by inflammatory mediators (interleukin-1, interleukin-6, and tumor necrosis factor α. Thrombopoietin (blue arrows) is produced constitutively by the liver (accounting for approximately 50 percent of baseline thrombopoietin levels) and kidney (accounting for a minority of the total amount) and is inducible in the liver by inflammatory mediators (especially interleukin-6) and from bone marrow stromal cells by thrombocytopenia. In addition, both G-CSF and thrombopoietin are removed from the circulation by receptor-mediated uptake and destruction by the mature cells that bear the corresponding receptors on neutrophils and platelets. Arrows indicate the production and secretion of haematopoietic growth factors.

Lineage	Cytokines
Stem cells	SCF, IL-1, IL-6, IL-3, GM-CSF, G-CSF, M-CSF, IL-12, IL-4
Granulocytes	IL-6, IL-3, GM-CSF, G-CSF, SCF, IL-11, IL-4
Granulocyte subpopulations:	
Neutrophils	IL-6, IL-3, GM-CSF, G-CSF, SCF, IL-11, IL-4
Eosinophils	IL-6, IL-3, GM-CSF, G-CSF, SCF, IL-11, IL-4, IL-5
Basophils	IL-6, IL-3, GM-CSF, G-CSF, SCF, IL-11, IL-4
Monocytes/ Macrophages	M-CSF, IL-3, GM-CSF, IL-4
Megakaryocytes	SCF, IL-3, GM-CSF, IL-6, Tpo, Epo, Oncostatin M, IL-6, LIF
Erythroid cells	IL-3, SCF, Epo
T -lymphocytes	IL-1, IL-2, IL-4, IL-10, IL-7
B-lymphocytes	SCF, IL-7, IL-6, IL-2
Dendritic cells	GM-CSF

Table 1. 2 Haematopoietic Growth Factors

Cytokines are grouped according to their known target lineages. Many of the cytokines also overlap in the target population they control. This ensures that cell production continues if one or more pathways are inhibited. The impact of combinations of cytokines can induce a greater than additive proliferative response – synergy Abbreviations: SCF- Stem Cell Factor, IL - Interleukin, GM-CSF- Granulocytic/monocytic Colony Stimulating Factor, M-CSF - monocytic Colony Stimulating Factor, G-CSF - Granulocytic Colony Stimulating Factor, Epo- erythropoietin, Tpo - thrombopoietin.

1.5 Regulation of Megakaryopoiesis

A number of transcription factors regulate the expression of genes specific for the platelet/MK lineages, genes involved in the polyploidization process, and genes implicated in proplatelet formation, have been identified. The GATA-1 and FOG -1 proteins have been detected in MKs (Chang et al, 2007). The directed knock-out of these proteins in the MK lineage has permitted the generation of mice presenting marked abnormalities in megakaryocytopoiesis characterized by a profound macro-thrombocytopenia with an excess of small immature MKs in the marrow and combined with an increase in cell proliferation. The bHLH transcription factor TAL-1 is expressed in MKs. Recent data indicate that TAL-1 plays a major role in platelet production during stress thrombopoiesis by regulating NF-E2 transcriptional activity. The ETS family of transcription factors includes more than 30 different members; four of them, ETS-1, PU.1/SPI-1, FLI-1 and GABPa, are expressed in the MK lineage and are able to

transactivate different promoters of MK genes in vitro. Furthermore, Fli-1 knock-out is lethal in the embryo due to a defect in vascular development and megakaryocytopoiesis, characterized by a marked thrombocytopenia with an excess of small immature MKs undergoing apoptosis (Chang et al, 2007).

c-Mpl is a member of the type I haematopoietic growth factor receptor family and is expressed by stem cells, erythroid colony forming units and megakaryocytes, their precursors, and their progeny. For the most part, c-Mpl is constitutively expressed in these cell types although receptor display is modulated by thrombopoietin binding and receptor internalization. In addition, co-expression of the signaling kinase JAK2 is vital for haematopoietic cytokine receptor expression (Saharinen, et al, 2003). Once c-Mpl is activated by thrombopoietin engagement, its multiple effects on HSCs, megakaryocytes, and platelets are mediated by a series of biochemical signaling events. Thrombopoietin activates both JAK2 and TYK2 in c-Mpl–bearing cell lines, although only JAK2 is essential for signaling and is the predominant isoform activated in primary megakaryocytes (Kaushansky, 2005c). By generating a complex composed of the phosphatase SHP2, a scaffolding Gab/IRS protein, and the p85 regulatory subunit of PI3K, thrombopoietin stimulation of megakaryocytes and their precursors activates PI3K and its immediate downstream effector Akt (PKB) (Kaushansky, 2005c). Blocking this pathway inhibits thrombopoietin-induced cell survival and proliferation (Kaushansky, 2005c). In the mature platelet, the hormone enhances α-granule secretion and aggregation induced by thrombin in a PI3K-dependent fashion.

A number of cytokines play a pivotal role in the differentiation and proliferation of platelets. Stem cell factor (SCF) has been found to synergize with interleukin-3 (IL-3), interleukin-6 (IL-6) interleukin-11 (IL-11), leukocyte inhibitory factor (LIF), erythropoietin (Epo) and oncostatin M to increase the number of megakaryocytic colonies (Adams,1997; Deutsch and Tomer 2006), *in vitro*. Thrombopoietin (Tpo) is a major hormonal regulator of megakaryocytopoiesis both *in vitro* and *in vivo*, and, thus, blood platelet production. Existing data show that the action, chemical nature, and immunologic properties of thrombopoietin from animal sources are similar, if not identical. The cloning and characterization of thrombopoietin, a ligand for the receptor encoded by the c-Mpl proto-oncogene, provides new insights into the humoral regulation of megakaryocytopoiesis and platelet production. Consistent with the proposed role as a major physiological regulator of megakaryocytopoiesis, thrombopoietin has potent effects on megakaryocytopoiesis *in vitro* and *in vivo*. Tpo initiates

its biologic effects by binding to the Mpl receptor, which is a member of the haemapoietic receptor family.

In addition to the original supposition that thrombopoietin functions as a late-acting megakaryocyte maturation factor, recombinant thrombopoietin proves also to be a potent stimulator of haematopoietic progenitor cells, inducing them to undergo proliferation and differentiation into megakaryocytic colonies (Goncalves et al, 1997; McDonald et al, 1987; Kaushansky, 2005a).

Recent studies have shown that not only does Tpo poteniate the action of megakaryocytic colony stimulating factor (meg-CSF), but it also has a direct effect on precursor cells to increase the number of megakaryocytic colonies. Other *in vitro* work showed that Tpo stimulates precursor cells directly to differentiate into megakaryocytic colonies (Ivanovic et al, 2006). *In vivo*, Tpo increases megakaryocyte size and number; it causes an elevation in the number of precursor cells in mouse marrow and increases the maturation of megakaryocytes. Moreover, Tpo increases the endomitosis of megakaryocytes in the marrow of mice, along with elevating the number of megakaryocytic colonies in spleens of lethally irradiated bone marrow reconstituted mice (Broudy et al, 1997). MK volume and ploidy attained predictable maximum values simultaneously; MK ploidy is an accurate measure of the Mpl-ligand stimulation of megakaryopoiesis (Tomer & Harker, 1996). TPO can also act in synergy with other haematopoietic cytokines and has been utilised effectively to expand human HSC and MK progenitor cells in vitro (Kaushansky, 2005b; Ivanovic et al, 2006).

Platelet production is also stimulated in mice by Tpo as evidenced by elevated isotopic incorporation into platelets; it increases platelet sizes, and when administered in high doses Tpo elevates platelet counts. Full development of colonies of megakaryocytes may depend on two growth factors. It has been hypothesized that one factor, meg-CSF, is effective in clonal expansion whereas a second factor is predominately involved in the endomitotic phase of megakaryocyte development. Multifactoral regulation has been observed for the other cell lineages (Deutsch and Tomer, 2006). The Mpl receptor is not limited to the megakaryocytic lineage, but also includes progeny of BFU-E. The binding affinity of the Mpl receptor for Tpo is high, with relatively few receptors displayed per cell. These findings suggest that Tpo, can speed red blood cell recovery after myelosuppressive therapy, in vivo, and enhance colony-

forming unit-erythroid generation, in vitro. (Goncalves et al 1997; Siticka et al , 1996; Williams et al, 1990; Yang et al, 1995 : Deutsch and Tomer, 2006).

1.6 Surface Antigen Phenotype of Haemopoietic Stem Cells

In the 1980's the identification of surface antigens - so called glycoproteins- on the surface of all haemopoietic cells greatly enhanced the understanding of haemopoietic cell maturation. Surface antigens were identified that were found exclusively on totipotent stem cells and others that were specific to mature cells of specific cell lineages. The expression of these surface antigens was found to change during the maturation events. As the totipotent haemopoietic cells matures the surface antigen specific for the totipotent potential of the cells is lost. As maturation continues and lineage specific cells emerge, lineage specific surface antigens develop. The lineage- or function-specific surface antigens are called Clusters of Differentiation (CD). The identification and isolation of lineage-specific cells has been greatly enhanced by the commercial availability of monoclonal antibodies raised against such surface antigens. Flow cytometric cell sorting and magnetic bead separation used in conjunction with the lineage-specific monoclonal antibodies has enabled pre-selection of the totipotent haemopoietic cells for addition to *in vitro* cultures. Table 1.3 summarizes the expression of haemopoietic surface markers by murine haemopoietic cells modified from Morrison et al, 1995.

Cell Surface Marker	Major lineage of expression	Multipotent progenitor
Thy-1	T cells	Low
Sca-1	T cells	High
c-kit	Pluripotent stem cells	High
Mac-1	Granulocytes and monocytes	Negative or low
CD4	T cells	Negative or low
CD3	T cells	Negative
CD5	T and B cells	Negative
CD8	T cells	Negative
B220	B cells	Negative
Gr-1	granulocytes	Negative
CD41	megakaryocytes	Negative
Ter119	Erythroid progenitor cells	Negative
CD34	Multipotent progenitor cells	High
H-2K	All haemopoietic cells	High
Ly5	All haemopoietic cells	High

Table 1. 3 The expression of haematopoietic cell surface markers by murine cells (modified from Morrison et al, 1995)

The lineage specific surface antigens expressed on pluripotent, lineage committed progenitors and mature murine haemopoietic cells used to identify these cell lineages by flow cytometry. The table highlights the surface antigens that are expressed on progenitor cells alone, such as CKit and Sca-1 and others that are expressed on all cell lineages such as H-2K and Ly5. Others shown are expressed exclusively by committed cell lineages such as CD4, CD3 and CD8 on T cells. CD- Clusters of differentiation.

1.7 The Experimental Analysis of Haematopoietic Differentiation

Although histological observations of the haematopoietic system, *in vivo*, continue to be the primary source of information on the process of differentiation (Metcalf and Moore, 1971) several *in vitro* assays have also been employed in the study of the haematopoietic system.

Till and Mc Culloch established in 1961 the first quantitative assay for cells with a radioprotective effect, cells that are not undergoing cell replication. These non -replicating cells were initially considered to be the most primitive haematopoietic cells. However, it is now been recognised that these cells were predominantly Spleen Colony-Forming Units (CFU-S) and do not represent the more primitive stem cells. *In vivo* assays in which human haematopoietic cells are engrafted in immunodeficient mice, have demonstrated the existence of human pluripotent cells either by limiting dilution analysis or by clonal integration of a

retroviral marker gene (Namikawa et al , 1990; Larochelle et al 1996; Bhatia et al 1997). The term marrow repopulating ability, derived from *in vivo* studies, refers to primitive totipotent haematopoietic stem cells with self-renewal capacity that are capable of repopulating the bone marrow of lethally irradiated mice. Two different types of cells with marrow repopulating ability have been distinguished in the mouse. Initial engraftment (short-term repopulation) is due to CFU-S. Long-term engraftment is attributed to a different cell type but is possible only if the animals also receive short-term repopulating cells (Cope, 1998).

However, it was the development of *in vitro* short- term culture systems for the study of haematopoiesis (Nishijima et al, 1997) that allowed the identification and quantification of several different types of precursor cells. The colony formation assay (Metcalf et al, 1997) allows the enumeration of early progenitors capable of forming colonies when cultured under appropriate conditions in semisolid medium. In these short- term cultures, cells are grown *in vitro* in soft agar or other highly viscous media, containing, for example, methylcellulose, plasma gel or fibrin clots. These semisolid media reduce cell movement and allow individual cells to develop into cell clones that are identified as single clusters (<50 cells) or colonies (>50 cells) of differentiated cells after a culture period of 7 to 14 days. These colonies are the progeny of single cells called Colony-Forming Units (CFU), and the composition of the colonies determines which CFU is being assessed. Thus, Colony-Forming Units granulocytes, erythrocytes, monocytes and megakaryocytes (CFU-GEMM) correspond to pluripotent progenitor cells identified by the production of multi-lineage colonies (granulocytes, erythrocytes, monocytes and megakaryocytes). This system has also enabled the identification of the high proliferative potential Colony-Forming Cells (HPP-CFC), defined by their ability to form very large colonies containing approximately 50,000 cells and including progenitor and mature haematopoietic cells of the granulocyte, macrophage, erythroid lineages (Cheng et al, 2006). Cells that give rise to colonies smaller than 1 mm, on the other hand, are usually referred to as low proliferative potential colony-forming cells (LPP-CFC). These primitive haematopoietic stem cells are considered to comprise cell types such as Burst Forming Unit-erythrocyte (BFU-E) and Colony-Forming Units granulocytes, erythrocytes, monocytes (CFU-GM). Until the availability of recombinant thrombopoietin (Tpo), the evaluation of megakaryopoiesis has not been very easy, *in vitro*. Tpo amplifies the action of colony stimulating factor-megakaryocyte, but it also has a direct effect on precursor cells to increase the number of megakaryocytic colonies. Other *in vitro* work showed that Tpo stimulates

precursor cells directly to differentiate into megakaryocytic colonies (Broudy et al, 1997; Kaushansky 2006).

1.8 Long-term Bone Marrow Cultures

As described above, differentiation into various lineages can be observed in agar-based assays. Sustained production or self-renewal of clonogenic cells, however, has not been possible with standard semisolid culture systems. The long-term bone marrow cultures (LTBMC), were originally described by Dexter and colleagues for murine cells (Dexter et al, 1974) and later adapted for human cells (Gartner and Kalpan, 1980; Coulombel et al, 1983), and make use of a rich culture medium containing high concentrations of horse serum and hydrocortisone. The rich culture conditions enable self-renewal of stem cells over a period of several months in the presence of a supportive microenvironment. The long-term culture of bone marrow cells employs primary adherent layers of stromal cells as an important source of cytokines and low molecular weight substances required for the controlled differentiation and proliferation of haematopoietic progenitor cells (Figure 1.5). Stromal cells provide a complex functional EMM allowing direct cell-to-cell contacts between different cell types. These, alone or in synergy with defined cytokines, can conserve primitive stem cells, induce early differentiation of a fraction of the primitive progenitors, and prevent their terminal differentiation. In addition to CFU-S and HPP-CFC, pluripotent stem cells and early precursors can be identified by the LTBMC as cobblestone area-forming cells (CAFC) (Weilbaecher et al, 1991).

Work has been conducted to improve the basic LTBMC protocol by ensuring good stromal layer development and increasing the longevity of the progenitor cells. The quality of the horse serum added to LTBMC has long been known to affect the quality of and time taken to establish a healthy stromal layer. The variability in the horse serum has been identified to be due to the level of interleukin-6 (IL-6) present in the particular batch of horse serum in use. Horse serum containing high levels of IL-6 supported healthy stromal cell development. This suggests that IL-6 is one of the factors that plays a role in the formation and function of haematopoietically active LTBMC (Hauser et al, 1997). Addition of IL-6 to LTBMC could reduce horse serum batch to batch variance. The addition of Stem Cell Factor (SCF) has been shown to establish haematopoietic activity more rapidly, and reduce the decline in cellularity

and progenitor cell content normally observed during the first few weeks of culturing (Dunlop et al, 1993). Exogenous addition of other growth factors, for example, interleukin-3 result in only moderate expansion of progenitor cells and increase mature cells and enable healthy stromal layer development (Carlo-Stella and Rozzoli, 1990; Yaroslavskiy et al, 1998).

1.9 The Haematopoietic Stroma

To obtain sustained haematopoiesis, primitive haematopoietic cells must interact with an adequate microenvironment, which includes, as already mentioned, stromal cells, EMM components and soluble regulatory factors (Eaves et al, 1991; Zhixing et al, 2006). The experimental analysis of haematopoiesis, *in vitro*, has provided much of the present knowledge on the role played by the stroma in the process.

The term "stromal cells" is used rather loosely and the true histogeneic origin of these mesenchyme-derived cells is still uncertain. Stromal cells, which mechanically support the differentiating haematopoietic cells, include adventitial reticular cells (adipocytes), macrophages, fibroblasts, and osteoclasts and are frequently defined as non-haematopoietic cells (Dexter et al, 1976; Derubeis and Cancedda, 2004). The following cell types are found in the stroma:

Adventitial reticular cells - These cells reside on the adluminal surface of venous endothelial cells, which branch through the medullary cavity, and appear to provide a reticular network that supports haematopoietic cells. Marrow adipocytes possess the mechanical function of controlling haematopoietic volume: impaired haematopoiesis is associated with increased accumulation of fat inclusions, whereas accelerated haematopoiesis is associated with loss of fat vacuoles and the provision of increased space for haematopoietic cells (Tavassoli et al, 1984). Adipocytes may play an additional role in blood cell production as a reservoir for lipids needed in cell metabolism during proliferation.

Macrophages, fibroblasts and osteoclasts - Macrophages, fibroblasts and osteoclasts, cells derived from haematopoietic precursor cells and osteoblasts, may also play important roles in the haematopoietic microenvironment. Macrophages are important in removing defective cells resulting from ineffective erythropoiesis and in the removal of the nuclear pole, produced during the process. Fibroblasts release cytokines, which control the proliferation and maturation of the totipotent progenitor cells (Derubeis and Cancedda, 2004).

Stromal cells represent a highly dynamic structure, which plays an active role in haematopoiesis by producing EMM components and both soluble and membrane-associated growth factors (Fernandes et al, 1996; Zhixing and Linheng, 2006). Stromal cells are rare in the marrow (approximately 0.125% of the marrow cellularity). Adherent stromal cell layers in LTBMC, established by growing bone marrow cells over a period of several weeks, are considered to mimic many characteristics of the marrow microenvironment. However, it has not yet been established conclusively that these cultures encompass all types of stromal cells identified *in vivo* and that they retain all of their functional properties *in vitro*.

Haematopoietic stromal precursors have been described, besides adult bone marrow, in fetal liver and fetal bone marrow, with reported differences in the anatomic and ultrastructural characteristics. Although the *in vivo* structures of the stroma, seen in the fetal liver and fetal bone marrow, vary from that seen in adult bone marrow, the variations do not affect their ability to support haematopoiesis (Riley et al, 1987; Van Den Heuvel et al, 1991; Zhixing and Linheng, 2006).

The ontogeny of stromal cells is currently very poorly understood. Based on the well-established generation of multiple mesenchymal cell types from bone marrow cells, the existence of stromal stem cells has been proposed (Owen et al, 1988; Prockop, 1997; Zhixing and Linheng, 2006). Besides gaps in our knowledge of the biology of these cell populations, information at the molecular level is also lacking. At least 16 *Hox* genes and 5 genes with homeobox domains, that are thought to be involved in the maintenance of the stromal layer cellular composition, have already been identified, although their temporal expression has not yet been determined (Simmons et al, 1987; Abramovich et al, 2005).

The transplantation capability of stromal cells is also a controversial subject. Some studies have indicated that the stroma of bone marrow receptors is developed from the host's precursor cells (Laver, et al, 1987; Tanaka et al, 1994). Others have shown that stromal cells in LTBMC of transplanted patients originate from the donor marrow (Keating et al, 1982). Using an *in vivo* system, it has been reported that murine bone marrow mesenchymal precursor cells, expanded in culture, were detected in the bone, cartilage and lungs of recipient mice at 1 to 5 months after transplantation. Recently, the migration of bone marrow-derived cells to areas of induced muscular degeneration, followed by differentiation along the myogenic pathway and

participation in the regeneration of damaged fibers, has been reported in mice (Ferrari et al, 1998).

The existence of stromal precursors outside the bone marrow in adults is highly controversial, in that while some studies have detected their presence in peripheral blood (Sutkowski et al, 1995; Shi et all, 1998 ; Fernandez, 1997; Abramovich et al, 2005) others have reported negative results (Ojeda-Uribe et al, 1993; Lazarus et al, 1997). The existence of stromal precursors in cord blood under normal conditions is the subject of intense discussion (Mayani et al, 1998; Ye et al, 1994; Nieda et al, 1997; Prindull et al; 1987; Hows et al, 1992). A study (Rios and Williams, 1990), reported a deficiency in the myeloid progenitor cell growth in LTBMC of umbilical cord blood, suggesting that this may be due to the impaired development of an adherent layer. Other researchers (Clausen et al, 2000) have described the establishment of an adherent layer from human umbilical cord blood capable of supporting the proliferation of haematopoietic clonogenic cells. The variable results are probably based on the varying experimental conditions employed.

The haematopoietic stroma is defined by its ability to support haematopoiesis. This functional characteristic is most probably a result of interactions between the adherent cells and haematopoietic cells. Although stromal layers can be developed by *in vitro* cultivation of different organs, only those derived from bone marrow were shown to support the proliferation of more primitive haematopoietic cells (Miller et al, 1998; Zhixing and Linheng, 2006). Although stem cells adhere to stroma developed from other organs, their haematopoietic potential is not maintained.

1.10 Haematotoxicity

Dose limiting haematotoxicity has been associated with a range of therapeutic agents. The haemapoietic cell lineage that is often affected *in vivo* is the megakaryocyte lineage. For a number of years, *in vitro* haemapoietic cultures have been available for the evaluation of the potential myelotoxicity of compounds, with the emphasis on the erythroid and myeloid lineages. Only since the commercial availability of recombinant thrombopoietin in 1994, has it been possible to study megakaryopoiesis *in vitro*.

Two mechanisms often attributed to the sensitivity of haematopoiesis to these classes of

therapeutic agents are the high mitotic rate within the bone marrow and the consequence of direct exposure via systemic administration. For example, methotrexate and docetaxel, are known to block cell cycling (Botta et al, 1999). As a consequence of the blockade of cell cycling, haematopoietic cell differentiation and maturation is disrupted, resulting in a decrease in the number of mature cells released into blood circulation. Also just as critical, self-renewal of the totipotent stem cells can be disrupted (Botta et al, 1999). Although the modes of action of different therapeutic agents maybe similar, differences in the sensitivity of each specific haematopoietic cell lineages to them, have been reported both *in vivo* and *in vitro*. For example, granulocytic/monocytic cell lineage maturation and differentiation is disrupted at lower exposure concentrations than that reported to affect the erythroid cell lineage (Williams et al, 1990).

Hence, the ability to determine the mechanism underlying the lineage specific haematotoxicity would be of great value, if haematopoietic cultures could be developed with a similar sensitivity and lineage specificity to *in vivo* human bone marrow.

An important advantage of a liquid LTBMC system over *in vitro* short-term cultures is the ability to evaluate any potential effects on the stromal layer, which is not present in the latter culture system. Additionally, stromal cells have been shown to exhibit some drug metabolizing activities. Therefore, evaluation of any potential haematotoxicity of metabolites of the parent compound is possible. Also any direct toxicity affecting the ability of the stromal layer cells to produce cytokines that direct differentiation and maturation can be evaluated. For example, it has been reported that therapeutics such as doxorubicin, accumulate in stromal cells subsequently affecting their ability in supporting haematopoiesis (Pessina et al, 1999). Other agents, such as methotrexate, have been reported, *in vitro*, to increase the proliferation of the stromal macrophage cell population (Corraza et al, 2004). The increase in macrophage cell population resulted in an increase in the production and release of colony stimulating factors. Although an increase in the colony counts of granulocytic/monocytic cell lineage would be expected, no such increase was found. Researchers found that the granulocytic/monocytic cells were unable to utilize the cytokines (Williams et al, 1998; Corraza et al, 2004). The mechanism of the cytokine blockade is unknown.

1.11 Comparison of In Vitro Haematotoxicity Using Murine, Human Adult and Human Cord Haematopoietic Cells

The integration of *in vitro* bone marrow studies into the discovery process would facilitate early evaluation for potential haematotoxicity. *In vitro* murine haematopoietic cultures have been shown to be predictive for human haematotoxicity associated with anti- cancer and anti-viral therapeutics (Scheding, 1994). Other compounds, such as lead and catechol, have been found to be more haematotoxic to human haematopoietic cells than murine haematopoietic cells (Van Den Heuvel et al, 1999). Lead was found to be 10 to 15 times more toxic to human Burst Forming Units - Erythroid (BFU-E) than murine BFU-E, based on IC50 concentrations. Human CFU-GM cell lineage was 6 times more sensitive to Catechol than murine CFU-GM cell lineage. For adryamicin, methoxy-morpholinyldoxorubicin, Flavopiridol and Taxol, no differences in the *in vitro* haematotoxicity, based on IC 50 values, have been demonstrated between data derived from human adult bone marrow and human cord blood derived cells (Corsini et al, 2000). The use of human cord blood stem cells in the *in vitro* cultures would be advantageous for predicting human haematotoxicity potential of new chemical entities and thereby circumvent the issue of differences in species sensitivity.

1.12 Role of Platelets

Platelets are produced in the bone marrow megakaryocytes stimulated by the growth factor thrombopoietin (George, 2004). Platelets are found in peripheral blood circulation at concentrations ranging from 150 to 450 x 10^9/L.

Haemostasis is the complex physiologic process that leads to the arrest of bleeding. It consists of the following components: platelets; plasma proteins, blood vessels and endothelial cells. With injury to a vessel, all the components are very quickly activated. Blood vessels constrict, platelets aggregate to form a plug, the plug is stabilized by the formation of insoluble fibrin due to the action of thrombin on soluble fibrin (Kickler, 2005). At the site of vessel damage, subendothelial structures come into contact with the blood, where collagen is the more abundant thrombogenic substance to which platelets adhere and by which they become activated. In the high shear arterial system, platelets slow down by reversibly interacting or "rolling" over collagen-bound von Willebrand Factor (VWF), mainly via the platelet

glycoprotein complex GPIb-IX-V. The platelets then directly interact with collagen via their collagen receptors, of which glycoprotein (GP)VI and integrin α2β1 are considered to be the more important ones. Platelets bind to each other. Formation of this platelet 'web' is the result of the binding of fibrinogen to glycoprotein IIb–IIIa, an integrin molecule that only binds fibrinogen after the molecule undergoes a conformational change. The activated platelet membrane permits the binding of clotting proteins Factor V and X complex, leading to greatly increased catalytic activity of these plasma factors.

1.13 Known Mechanisms of Drug Induced Platelet Count Reduction

Drug induced thrombocytopenia (reduction in peripheral blood circulating platelet cell counts with or without reductions in bone marrow megakaryocyte numbers) has been described with a wide range of compounds including anti-neoplastic agents, anti-coagulants, anti-viral agents (Gynn et al, 1972; Kickler, 2005). The known primary pharmacology of such therapies include:

1. Direct inhibition of cell cycling of all replicating cells, including haematopoietic cells. Anti retroviral therapies also specifically target cell cycling.
2. Inhibition of the formation of microtubules resulting in cell cycle arrest at metaphase has been attributed to vinca alkaloid agents such as Vinblastine and Vincristine. Interestingly, both these two agents have been used in the treatment of essential thrombocythemia (discussed in more detail in chapter 3).
3. Inhibition of dihydrofolate reductase (folate antagonist). This mode of action is noted with anti-neoplastic and rheumatoid arthritis agent methotrexate (Storb et al, 1986; Kickler, 2005).

The consequence of the pharmacology of these therapies results in the reduction of both circulating and bone marrow haematopoietic cells.

Platelet destruction may also be caused by direct toxic effect of the drug on the platelets or, more commonly, through an immunological mechanism (Van Den Bemt et al, 2004). Three mechanisms of action have been described:

1. Hapten-Dependent Antibodies: Although infrequent, penicillins and cephalosporins can cause thrombocytopenia. These betalactam antibiotics – or haptens – bind covalently to autologous proteins (in this case a cell membrane protein of the thrombocyte) to form novel antigens. Antibodies are subsequently formed, directed against this hapten-protein complex and ultimately result in cell destruction (Van den

Bemt et al, 2004).

2. Drug-Induced, Platelet-Reactive Auto-Antibodies: Platelet-specific auto-antibodies appear to be involved in the thrombocytopenia caused by gold and procainamide, and occasionally by quinine and quinidine. These auto-antibodies are induced by these drugs but bind to platelet membrane targets without a requirement for added drugs (Van Den Bemt et al, 2004).
3. Drug-Dependent Antibodies: Although this mechanism has been recognized for some time, the trigger for the induction of antibody formation is unknown. One hypothesis proposes the binding of the drug to the platelet glycogen protein complex. The resultant complex initiates an antibody response. The favored glycoproteins are the GPIb/IX/V and the GPIIB/IIa glycoprotein complexes (Van Den Bemt et al, 2004).

1.14 Essential Thrombocythemia

Essential Thrombocythemia (ET) shares with other myeloproliferative disorders (MPD) such as chronic myeloid leukemia, Polycythemia vera, myelofibrosis and myeloid metaplasia of the spleen, a molecular pathogenesis leading to overproduction of mature blood cells. ET is defined as a disease where the platelet count of an individual is elevated above 600,000/mL platelets, as a direct result of over production. Essential thrombocythemia is currently defined as a persistent thrombocythemia state that is neither reactive nor associated with an otherwise defined chronic myeloid disorder (Briere, 2007).

ET patients are at risk from spontaneous thrombotic events (blood clots) occurring in the blood circulation. The genetic causes of the most common MPD remained unknown until the identification of mutations that activate Janus kinase 2 (JAK2) signalling in most patients with myeloproliferative disorders including ET (Briere, 2007). In 2005, several independent groups identified a recurrent mutation in the JAK2 tyrosine kinase in most patients with ET. JAK2 is a member of the Janus family of cytoplasmic non-receptor tyrosine kinases, which also includes JAK1, JAK3 and TYK2 (Levine, et al, 2007). The mutation is a guanine-to-thymidine substitution, which results in a substitution of valine for phenylalanine at codon 617 of JAK2 (JAK2V617F) (Kralovics, et al,2005), gain -of -function mutation of JAK2 in myeloproliferative disorders. How the JAK2V617F mutation sustains the increased phosphorylation of JAK2 in the presence of serum, but in the absence of interleukin-3, and the

factor or factors in serum that mediate this effect remain unknown. (Kralovics et al, 2005). The mutation is present in haematopoietic cells but not germline. DNA from ET patients demonstrated that JAK2V617F is a somatic mutation that is acquired in the haematopoietic compartment. In addition, the JAK2V617F allele can occasionally be present in different haematopoietic compartments including B and T lymphoid cells. These findings suggest that the mutation might occur in the pluripotent haematopoietic stem cell (Levine, et al, 2007). However, the consequence of the presence of the presence of the JAK2V617F in the B and T cells is unknown.

Acquisition of the constitutively active Janus kinase 2 (JAK2)V617F mutation occurs in 65–97% of patients with polycythemia vera (homozygous in 25–33%), 35–57% with myelofibrosis with myeloid metaplasia (MMM) (homozygous in 9–29%), and 23–57% with ET (homozygous in 0–7%) (James et al, 2005). JAK2V617F is a constitutively active tyrosine kinase that activates signal transducer and activator of transcription (Stat), mitogen activated protein kinase (MAPK) and phosphotidylinositol 3-kinase (PI3K) signalling pathways, and transforms haematopoietic progenitors. Gain-of-function mutations in JAK2 exon 12 and in the thrombopoietin receptor are observed in some patients with JAK2V617F-negative myeloproliferative disorders, suggesting constitutive activation of JAK2 signalling is central to the pathogenesis of PV and ET (Levine, et al, 2007). Negative regulation of JAK2 signalling is normally mediated by suppressor of cytokine signalling (Socs) proteins, most notably SOCS1 and SOCS3; recent data indicate that instead of acting as a suppressor, SOCS3 enhanced the proliferation of cells expressing JAK2 V617F allele. Additionally, although SOCS1 and SOCS2 are degraded in the presence of JAK2 V617F, turnover of SOCS3 is inhibited by the JAK2 mutant kinase and this correlated with marked tyrosine phosphorylation of SOCS3 protein. These findings suggest that the JAK2 V617F has overcome normal SOCS regulation by hyperphosphorylating SOCS3, rendering it unable to inhibit the mutant kinase. Thus, JAK2 V617F may even exploit SOCS3 to potentiate its myeloproliferative capacity (Hookham, et al, 2007). The mechanism of activation of JAK2 kinase activity by mutations in the JAK2 signalling pathway is shown in figure 1.5.

Clinical phenotype

Median age at diagnosis in ET is estimated at 55 years. The female to male ratio is about 2:1. Thrombohemorrhagic complications cap the clinical course and major thrombosis, which is

mostly arterial, occurs in 11–25% of patients at diagnosis and in 11–22% during follow-up (Tefferi, 2006). In contrast, the incidence of major hemorrhage is only 2–5% at diagnosis and 1–7% during follow-up. Similarly, hemorrhage is a rare cause of death in ET whereas thrombosis might account for 13–27% of deaths. Abdominal large vessel thrombosis is particularly prevalent in ET affecting approximately 10% of ET patients. Non-life-threatening complications in ET include microvascular disturbances (headaches, acral paresthesia, erythromelalgia) and first trimester miscarriages (Tefferi, 2006). The former are relatively frequent but easily managed by low-dose aspirin therapy. The miscarriages occur in 30–40% of pregnant women with ET and do not appear to be increased by either a higher platelet count or aspirin therapy (Tefferi, 2006).

Treatment

The majority of patients with ET can have a normal life expectancy in the first 15 years of the disease. Thereafter, survival is shortened because of increased attrition from disease transformation into AML, which occurs in less than 5% and 20% of the cases in the first and second decades of the disease, respectively (Tefferi, 2006). Leukemic transformation in ET is considered a natural progression of the disease and not a result of drug therapy. Both anti-platelet (aspirin) and cytoreductive agents are used in ET to either alleviate microvascular symptoms or prevent thrombo-hemorrhagic complications, respectively (Tefferi, 2006). Hydroxyurea is the first line cytoreductive agent used; vincristine, vinblastine and anagrelide are considered second line therapies.

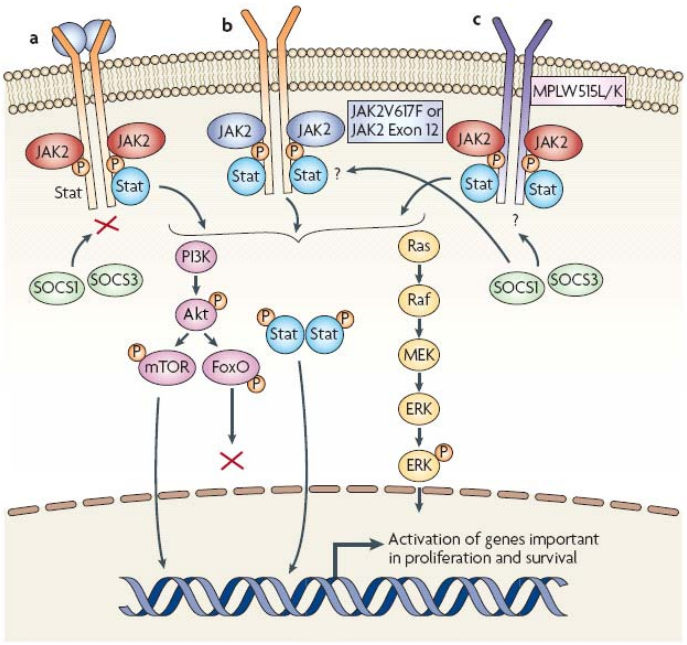


Figure 1. 5 Mechanism of activation of JAK2 kinase activity by mutations in the JAK2 signalling pathway, Levine et al, 2007. A) Cytokine ligands normally bind cytokine receptors, which results in Janus kinase 2 (JAK2) phosphorylation, recruitment of signal transducer and activator of transcription (Stat) signalling proteins and phosphorylation and activation of downstream signalling pathways including Stat transcription factors, mitogen activated protein kinase (MAPK) signalling proteins, and the phosphotidylinositol 3-kinase (PI3K)–Akt pathway. b) The JAK2V617F and JAK2 exon 12 mutant kinases bind cytokine receptors and are phosphorylated in the absence of ligand, and lead to ligand-independent activation of downstream signalling pathways. c | By contrast, MPLW515L/K mutant thrombopoietin receptors are able to phosphorylate wild-type JAK2 in the absence of thrombopoietin, and result in the activation of signalling pathways downstream of JAK2. Negative regulation of JAK2 signalling is normally mediated by suppressor of cytokine signalling (Socs) proteins, most notably SOCS1 and SOCS3; recent data indicate that the JAK2V617F allele might escape negative feedback by SOCS3.

1.15 Anagrelide Hydrochloride

Anagrelide (AN) (Figure 1.6) was initially developed in 1992, by Roberts laboratories Inc (Eatontown, USA) as a potential anti-thrombotic therapy by inhibiting platelet aggregation (Petrides, 2006). The anti-thrombotic activity of AN was assessed in various animal models including the biolaser induced rabbit thrombosis model, electrically induced dog thrombosis model and the ellagic acid-induced rat thrombosis model. In all thrombosis models evaluated AN was a potent anti-aggregation agent (SBA, 1997). Furthermore, *in vitro* human platelet aggregation studies demonstrated that AN prevented platelet aggregation by increasing the

phosphorylation of platelet proteins and it also inhibited thrombin-induced increase of intra-platelet calcium ions.

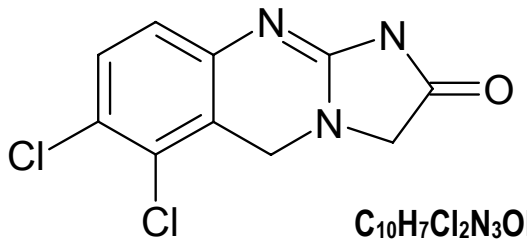


Figure 1. 6 Anagrelide Hydrochloride Structure

Platelet count reduction: Mechanism of action

After single, oral dose administration of up to and including a dose of 25mg, no reduction in peripheral blood platelet count was noted. In the initial single dose study the volunteers receiving single administrations were followed for 14 days following dosing. No reduction in platelet counts were noted following this post treatment period (SBA, 1997). However, initial repeat dose studies in healthy volunteers highlighted the ability of AN to reduce platelet counts. Doses of 1mg were noted to cause a reduction in peripheral blood platelet count (SBA, 1997).

Pre and post AN treatment bone marrow aspirates from patients with Essential Thrombocythemia (ET) demonstrated no alteration in the number of megakaryocytes (SBA, 1997). Additionally, doses of AN that reduced platelet counts in the pre-clinical toxicology studies caused no significant reduction in megakaryocytes (SBA, 1997). Therefore the reduction in circulating platelets was not a direct result of a reduction in the number of megakaryocytes within the bone marrow (SBA, 1997). The metabolites of AN were not identified in the SBA. Four metabolites have subsequently been identified but their structures and their platelet aggregation and Mk colony inhibition activities have not been reported by Roberts Pharmaceuticals. It has been demonstrated, however, that all four major metabolites are produced in the rat, dog, monkey and humans.

In vitro clonogenic cultures using human bone marrow-derived stem cells showed that concentrations between 32nM - 161μM inhibited megakaryocytic colony formation with an

IC50 concentration of 32μM (SBA, 1997). Mazur et al (1992) demonstrated that AN at concentrations of 32 μM to 161μM, resulted in an increase (65%) in the percentage of megakaryocytes in developmental stages I and II compared to concurrent controls (41.5% in developmental stages I and II). This shift in the developmental stage of cultured human megakaryocyte is a morphological change only, and in terms of megakaryocyte function is unclear.

Lane et al, (2001) hypothesized that all *in vitro* and *in vivo* studies carried out with AN's thrombocytopenic effects have been complicated, for a number of reasons. Firstly, the water-insoluble nature of AN (solubility of AN in water was 2μg/mL) has, in their opinion, hindered *in vitro* studies. Secondly, even though AN is capable of inhibiting platelet aggregation across species, they claimed it only decreased platelet levels in human subjects. This obstacle has hindered studies related to pharmacokinetics and toxicity in monkeys, dogs, and mice. Finally, the extensive biotransformation of AN suggested that the anti-platelet aggregation and thrombocytopenia effects may not be directly mediated by intact AN, but rather through active metabolites.

While investigating the effects of AN in other species, an AN metabolite, 2-amino-5,6-dichloro-3,4,-dihydroquinazoline (AM) produced only in human subjects was identified, characterized, and synthesized. The structure of AM, 2-amino-5,6-dichloro-3,4,-dihydroquinazoline is provided in Figure 1.7.

Lane et al (2001) demonstrated that administration of AM to BALB/c mice caused thrombocytopenia and altered Mk content in the BM compartment. However, no AN -parent compound controls were included. In support of the *in vivo* data, acute administration of AM blocked *in vitro* Mk migration. Moreover, chronic administration of AM blocked *in vitro* TPO-induced maturation of human and murine bone marrow stem cells into mature polyploid Mks. Lane et al (2001) proposed that these data indicated that biotransformation of AN into active metabolite was essential for its thrombocytopenic effect in mice. On the basis that only the human liver has the capacity to generate the active metabolite, AN itself can only induce thrombocytopenia in humans. Unlike AN, AM does not affect platelet levels and does not inhibit ADP-induced human platelet aggregations. However, AM seems to promote thrombocytopenia by inhibiting Mk migration and maturation.

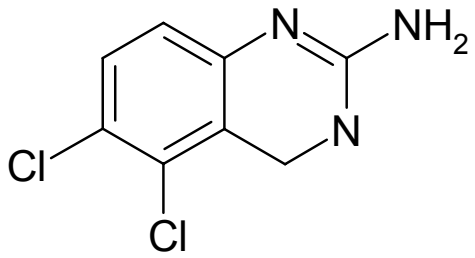


Figure 1. 7Structure of AM (2-amino-5,6-dichloro-3,4,-dihydroquinazoline), Metabolite of AN

In summary, Lane et al (2001) described an AN metabolite that selectively inhibits Mk maturation and migration, without influencing platelet aggregation. The availability of AM enabled, for the first time, parallel *in vitro* and *in vivo* animal studies centered on the cellular mechanisms by which AN decreases platelet levels.

Although experiments by Lane et al (2001) did not include the parent compound as a positive control, they further concluded that AM was 50 times more potent than AN itself. However, Erusalimsky et al (2002) and Wang et al (2005) carried out work where side-by-side comparisons of AN and AM for their ability to inhibit Mk maturation in cultures of human cord blood–derived $CD34^+$ cells. In these experiments AM at concentrations up to 2 μM (~432 ng/mL) showed no evidence of activity on a number of megakaryocyte maturation parameters, including expression of the differentiation marker CD61 (platelet glycoprotein 11b/111a), increase in cell size, and polyploidy. In sharp contrast, AN was active at doses as low as 10 nM, with half maximal inhibition (ID_{50}) observed at 100 nM, and a maximal effect seen at 1–2 μM. Erusalimsky et al (2002) also investigated the *in vivo* activity of AM in BALB/c mice. In contrast to Lane et al (2001) AM was given intraperitoneally at 7.5mg/kg to BALB/c mice for 7days (directly mimicking the design of Lane et al, 2001) failed to show any statistically significant difference in the mean platelet count between groups. BALB/c mice dosed with AN demonstrated a significant decrease in circulating platelet counts (reduction of 35-45% verses concurrent control values). Lane et al. (2001) also suggested that AM was a metabolite "produced only in human subjects," thereby offering an explanation for the lack of thrombocytopenic effects of AN in animal species. However, an extensive review of the pre-clinical data (preclinical studies were carried out on behalf of Roberts Pharmaceutics Co.) by

Erusalimsky et al (2002) showed that all animal species investigated (rats, dogs, and rabbits) produce significant amounts of AM. For example, in rats given AN at 5 mg/kg per day (dietary dosing), Cmax of AM was 38.6 nM and for AN 45nM. As previously mentioned the structure of AN metabolites had not been determined by Roberts pharmaceuticals. Therefore, the differences in the results obtained by the groups may be explained by structural differences in the AM utilized by Lane and Erusalimsky. Furthermore, the purity of AM used by the two groups varies significantly. Presence of AN in the batch of AM used by Lane et al may also have contributed to the difference in results.

Another explanation for the species difference in the activity of AN comes from the work of McCarty et al (2006). These investigators showed that TPO-stimulated growth of murine BaF3 megakaryocytes transfected with human c-mpl (TPO receptor) was markedly reduced (ranging from 40 to 80 %) in the presence of 16nM to 500μM of AN. In contrast, the untransfected BaF3 megakaryocyte progenitors demonstrated no change in growth (0 to 10%) with AN across the same concentration range. This suggests that AN has greater affinity towards the human c-mpl (TPO) receptor than the murine c-mpl (TPO) receptor. Clearly the debate on the mechanism of action of AN resulting in platelet count reduction is unresolved.

Toxicology

A full package of toxicology studies including acute and repeat dose studies, one year repeat dose studies in the rat and dog and safety pharmacology, reproductive toxicology, carcinogenicity and genotoxicity studies were completed with AN in the dog, rat, rabbit, mouse and monkey (SBA, 1997) . In all species evaluated a dose dependent inhibition of platelet aggregation was noted. A decrease in platelet count was noted in the rodent species (10% of control) at higher doses then that required to inhibit platelet aggregation. In ex vivo platelet aggregation studies in rats, dogs and monkeys, AN inhibited ADP or collagen induced platelet aggregation in a dose dependant manner. The dose at which collagen induced platelet aggregation was inhibited (ED 50) in the rat and monkey were 1.2 and 4mg/kg of AN, respectively.

Clinical Trial Findings

Repeat dose clinical studies with AN in healthy volunteers showed that doses sufficient to inhibit platelet aggregation were unfortunately also associated with a substantial increase in cardiovascular side effects. At that time, an unexpected finding was a dose dependent decrease in platelet count. The reduction in platelet count was found not to reduce platelet survival time or cause significant bleeding. In addition, no drug induced platelet antibodies were detected. Doses ranging between 1-3mg given every 8 hours for 6 to 9 days were shown to cause a profound reduction in platelet count with a nadir of 20 x10 ^{9}/ l occurring within 10 and 12 days of commencement of treatment. This reduction in platelet count opened a new avenue of clinical research suggesting AN may have a role in the treatment of ET as well as other chronic myeloproliferative disorders, including polycythemia vera, chronic myeloid leukemia, and agnogenic myeloid metaplasia (Finazzi et al 1999; Petrides, 2006). A study conducted in 8 patients, 5 of whom were diagnosed with ET demonstrated that no anti-aggregating effect was detectable at concentrations which significantly reduced platelet counts (Balduini, 1992; Petrides, 2006).

Clinical trials have shown that AN has a large volume of distribution and is extensively metabolised; less than 1% is recovered unchanged in the urine. Plasma half-life after a 2.0-mg dose is 1.3 hours, with a mean Cmax value of 734ng/mL. AN's efficacy and safety have been evaluated in open-label, non-comparative trials, in which the response rate (reduction in platelet) was 60-93% (Pescatore et al, 2000; Petrides, 2006). The recommended starting dosage is 0.5 mg four times a day or 1 mg twice a day, with dosage adjustment to the lowest effective amount required to reduce and maintain platelet count below 600 x 10^{9}/L. Approximately 25% of patients treated with AN develop fluid retention (considered to be due to the vascular dilating pharmacology of AN), and some develop frank congestive heart failure (Petrides, 2006). Changes in cardiac function were noted at doses $\geq$10mg/kg in the dog. These changes included reduction in blood pressure and heart rate as well as elevated T waves. The mechanism for the cardiac findings are not understood (SBA,1997). Side effects resulting in discontinuation of treatment with AN occur in 15% of the total number of patients given AN (Oertel et al, 1998; Petrides, 2006).

1.16 Taxol

Taxol is an anti-cancer therapeutic that inhibits cell cycling by anti-microtubule agent that promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization (Needleman et al, 2005). Taxol stabilizes microtubules by straightening the GDP protofilament and slowing down the transition of protofilaments from straight to a curved configuration. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions. In addition, taxol induces abnormal arrays of microtubules throughout the cell cycle and multiple asters of microtubules during mitosis (Geard et al, 1994). The haematotoxicity associated with Taxol treatment is discussed in chapter 5.

1.17 Background to the Project

The development of new pharmaceuticals requires a tremendous investment in time (typically 10 to 13 years) and resources (typically hundreds of millions of pounds). Part of drug development is preclinical and clinical safety evaluation, the assessment of potential toxicity of new drugs. For a variety of therapeutics, haematotoxicity is often the dose-limiting factor. Haematotoxicity is usually assessed as part of the preclinical studies in experimental animals. However, information on the mechanism of the haematotoxicity noted can not be elucidated from the toxicology studies. Over the last 10 years *in vitro* bone marrow models have been developed. The integration of *in vitro* bone marrow studies into the discovery process may facilitate early evaluation for potential haematotoxicity and facilitate identification of the potential mechanisms of the observed haematotoxicity and could reduce drug development time. The identification of the potential mechanism of the haematotoxicity would enable
a) The determination whether the haematotoxicity is animal species specific; b) potentially provide clinical investigators more specific biomarkers to identify the haematotoxicity in clinical trials. As with all toxicities noted in preclinical studies, potential mechanism (s) for the toxicities noted are required for regulatory submissions to enable the progression of clinical trials of new medicines and ultimately their approval.

1.18 Aims of the Project

Megakaryocytic lineage specific toxicity has been reported in clinical trials with anagrelide hydrochloride (AN). The goals of this project were to investigate the utility of *in vitro* bone marrow cultures to 1) assess the haematopoietic lineage specificity of vincristine sulphate, vinblastine sulphate, hydroxyurea and anagrelide hydrochloride using clonogenic cultures; 2) to elucidate the mechanism(s) of the megakaryocytic lineage specific inhibition of anagrelide hydrochloride using both clonogenic and LTBMC. The potential mechanism(s) and target specificity of AN action were elucidated by determining:

- The ability of pluripotent stem cells to maintain a consistent number of pluripotent cells, in LTBMC, whilst others continue to differentiate into lineage specific progenitors in the presence of AN.
- The effect of AN on the development and maturation of megakaryocytic specific lineage by assessing cell cycling, ploidy, cell surface receptor and tubulin expression

To achieve these aims the following initial steps were necessary**;** the establishment and validation of: (i) a robust murine haematopoietic clonogenic assay to assess the ability of haematopoietic stem cells to differentiate into Colony Forming Units of the megakaryocytic cell lineage, (ii) murine clonogenic assays to evaluate haematopoietic stem cell differentiation into the granulocytic/ monocytic and erythroid cell lineages were established prior to the commencement of this programme of work (assay validation data Chapter 2). (iii) human cord blood haematopoietic clonogenic cultures to evaluate haematopoietic stem cell differentiation into the granulocytic/ monocytic, erythroid and megakaryocytic cell lineages (iv) long-term murine and human cord blood liquid culture system and assessment of: a) The developing stromal layer by light microscopy; b) The morphological identification of cells present in the non-adherent layer; c) The assessment of the ability of a fully established LTBMC to produce lineage committed colonies (by plating non adherent cells into clonogenic cultures); d) Determination of the phenotype of the haematopoietic cells differentiating in the LTBMC using flow cytometry techniques and immunohistochemical techniques.

Chapter 2

2.0 Materials and General Methods

2.1 Materials

The details of the source of the murine and human cord blood haematopoietic cells used in the clonogenic and LTBMC are provided below. In addition, the ET reference compounds used in this thesis, the antibodies used to identify haematopoietic lineages in the non-adherent layers of LTBMC (murine and hCB) and the tublin –α and tubulin –β antibodies used are also provided. Details of all other reagents utilized and culture plates, flasks, culturing media, flow cytometry reagents (for surface and intracellular antigen detection and cell cycle analysis,) and APAAP staining (for the identification of CFU-Mk colonies) are provided in Appendix A (pages 194 to 198).

Haematopoietic Cell Source	Supplier	Catalogue Number
Human umbilical cord and placental blood	Poietic Technologies, USA	88364
Balb/C mouse BM	Charles River, UK	-

Essential Thrombocythemia Reference Compounds

The reference compounds used for the evaluation of clonogenic cultures is provided below

Essential Thrombocythemia reference compounds	Supplier	Catalogue Number
Vincristine Sulfate	Sigma, UK	V8388
Vinblastine Sulfate	Sigma, UK	V1377-10MG
Hydroxyurea	Sigma, UK	S647268
Anagrelide Hydrochloride	Shire Pharmaceuticals,UK	-
DMSO	Sigma, UK	D4540-100ML

Antibodies for Identification of Murine Haematopoietic Cells

Antibodies used for the identification on cell lineages of cells collected from murine LTBMC (non-adherent cells) is provided below

Antibody	Supplier	Catalogue Number
Rat-Anti mouse CD117 PE	Serotec, Oxford, UK	76599
Rat-Anti mouse Mono/Macro(F4/80)	Serotec, Oxford, UK	65321
Rat-Anti mouse Erythroid (ter119)	Serotec, Oxford, UK	97539
Rat-Anti mouse Granulocyte precursor	Serotec, Oxford, UK	63181

Human Mk-CFU Cell Surface and Intracellular Tubulin Markers

Antibodies used for the identification of Human Mk-CFU colonies and intracellular Tubulin are provided below

Antibody	Supplier	Catalogue Number
Mouse-Anti human CD34	Becton Dickinson, USA	555821
Mouse-Anti human CD61	Becton Dickinson, USA	553346
Mouse-Anti human CD42	Becton Dickinson, USA	559458
Mouse Anti human Tubulin α	MP Biomedicals, USA	69125
Mouse Anti- human Tubulin β	MP Biomedicals, USA	69126

2.2 General Methods

2.2.1 Murine and hCB BFU-E and CFU-GM Clonogenic cultures

The GM/E clonogenic assay was based upon the cultures described by Metcalf et al (1997). 0.3 ml of murine bone marrow cells (following positive selection of CKit positive cells) or hCB cells (10^4cells /ml) were added to 3 mL Methocult media (Metachem Diagnostic, UK). The cell count was adjusted for cell viability and the cell count was determined using the Advia 12 analyser (Bayer, USA). Details of the positive selection of murine CD117 positive cells, the thawing process of hCB cells and composition the murine and hCB clonogenic culture media are provided in Appendix A. The tubes were then vortexed and allowed to

stand (5 minutes), at 37°C in a water bath before 3.3 mL were dispensed into Falcon (Becton Dickinson, USA) cell culture six-well plates. The plates were incubated in a CO_2 incubator. Burst Forming Units -Erythroid (BFU-E) were counted after 10 days (murine BFU-E and CFU-GM colonies) and 12 days (hCB BFU-E and CFU-GM colonies) incubation and the Colony Forming Units-Granulocyte, Monocytic (CFU-GM) were counted after 12 days incubation. The basic murine and hCB clonogenic culture method is outlined in Figure 2.1. Descriptions of the murine and hCB BFU-E and CFU-GM colonies are provided in Appendix A.

The murine clonogenic cultures were established prior to the commencement of this thesis, the murine BFU-E and CFU-GM colony counts, with and without pre-selection of CKit positive murine HSC, obtained during the validation are provided in tables 2.1 to 2.4.

Experiment No	Culture 1	Culture 2	Culture 3	Mean colony count per well	Standard Error of Mean
1	43	38	47	43	5
2	51	37	40	43	6
3	33	39	47	40	6
4	30	51	40	40	9
5	32	39	47	39	6
6	27	56	34	39	12

Table 2.1Murine BFU-E Clonogenic Cultures, Without Pre Selection: Assay Validation

Mononuclear cells, prepared using lymphoprep^R were added to murine granulocytic/ monocytic and erythroid clonogenic culture media (Methocult M3434). The cultures were incubated in a CO_2 incubator at 37°C, for 10 days. The BFU-E colonies per well was determined using an inverted microscope. Murine BFU-E clonogenic culture validation was carried out in triplicate on 6 separate occasions.

Experiment No	Culture 1	Culture 2	Culture 3	Mean colony count per well	Standard Error of Mean
1	27	39	22	29	9
2	29	12	19	20	9
3	13	22	26	20	7
4	15	47	26	29	16
5	26	17	33	25	8
6	41	19	27	29	11

Table 2.2Murine CFU-GM Clonogenic Cultures, Without Pre Selection: Assay Validation

Mononuclear cells, prepared using lymphoprep^{R} were added to murine granulocytic/ monocytic and erythroid clonogenic culture media (Methocult M3434). The cultures were incubated in a CO_2 incubator at 37^{o}C, for 10 days. The CFU-GM colonies per well was determined using an inverted microscope. Murine CFU-GM clonogenic culture validation was carried out in triplicate on 6 separate occasions.

Experiment No	Culture 1	Culture 2	Culture 3	Mean colony count per well	Standard Error of Mean
1	80	77	91	83	7
2	82	71	87	80	8
3	67	90	88	82	13
4	71	68	79	73	6
5	62	77	84	74	11
6	85	77	72	78	7

Table 2.3Murine BFU-E Clonogenic Cultures, With CKit Pre-Selection: Assay Validation

C-Kit positive cells prepared using Lymphoprep followed by C-Kit positive selection using Dynal beads murine stem cells were added to murine granulocytic/ monocytic and erythroid clonogenic culture media (Methocult M3434) . The cultures were incubated in a CO_2 incubator at 37^{o}C, for 10 days. The BFU-E colonies per well was determined using an inverted microscope. Murine BFU-E clonogenic culture validation was carried out in triplicate on 6 separate occasions.

Experiment No	Culture 1	Culture 2	Culture 3	Mean colony count per well	Standard Error of Mean
1	41	53	57	50	7
2	47	55	60	48	6
3	48	57	62	56	6
4	45	59	63	56	8
5	46	43	50	46	3
6	48	59	51	51	5

Table 2.4Murine CFU-GM Clonogenic Cultures, With CKit Pre-Selection: Assay Validation
C-Kit positive cells prepared using Lymphoprep followed by C-Kit positive selection using Dynal beads were added to murine granulocytic/ monocytic and erythroid clonogenic culture media (Methocult M3434) . The cultures were incubated in a CO_2 incubator at 37^{o}C, for 10 days. The CFU-GM colonies per well was determined using an inverted microscope. Murine CFU-GM clonogenic culture validation was carried out in triplicate on 6 separate occasions.

2.2.2 Murine Megakaryocytic (Mk) Clonogenic Cultures - Standard Culture Conditions

1.7 mL of HCC-4900 media (Metachem Diagnostics, Northampton, UK) was placed in a sterile tube. The suppliers (Metachem Diagnostics, Northampton, UK) suggested the cytokine concentrations and other components used; 1.1mg/mL collagen, 0.9% Methylcellulose based media (HCC-4900), 1% bovine serum albumin, 10 μg/mL bovine pancreatic insulin, 200 μg/mL human transferring, 10 ng/mL rm. IL-3 and 50 ng/mL rm. thrombopoietin.

0.1 mL of CKit positive cells (1x10^{6}cells) were added to 1.7 mL Megacult-C media. Cell count was determined using an automated cell counter (Advia 120, Bayer, USA). The cell count was adjusted for cell viability. The tubes were then vortexed and allowed to stand for 5 minutes at 37^{o}C in a water bath. 1.2 mL of 1.1 mg/mL collagen, stored on ice prior to the procedure, was added to the tubes. The tubes were then vortexed and allowed to stand for 5 minutes at 37^{o}C in a water bath. 3.2 mL of the final culture mixture was dispensed into each well of a six well culture plate. The colonies were identified morphologically using an inverted microscope, following 10 days incubation as described by Gribaldo et al, 1996 and Dobo et al 1999. The basic CFU-Mk murine clonogenic method is outlined in Figure 2.1.

2.2.3 Murine Megakaryocytic (Mk) Clonogenic Cultures -Modified Culture Conditions

In a series of experiments 0.1mL of rm IL-6 (10ng/mL) (Metachem Diagnostics,

Northampton, UK) was added to the HCC-4900 media (Metachem Diagnostics, Northampton, UK) media mix. The remaining procedure was the same as described in section 2.2.2

2.2.4 hCB CFU-Mk Clonogenic Cultures

0.1mL of viable hCB cells ($1x10^5$cells/mL), were added to 2mL of warm MegaCultTM –C (Methachem Diagnostics, UK) and 1.2mL of collagen (details of the MegaCult-C media composition is provided in Appendix A). The cell count was adjusted for cell viability. The viability count was determined by flow cytometry. The tube was mixed well and plated at a plating concentration of 1 x 10^4cells/mL. The plates were then incubated at 5% CO2, 95% relative humidity at 35^{o}C. The colonies were identified morphologically using an inverted microscope, following 12 days incubation, as previously described by Gribaldo et al, 1996 and Dobo et al 1999 The basic method is outlined in Figure 2.1. The hCB clonogenic cultures were established by a year in industry student whilst this work was on-going. The hCB BFU-E and CFU-GM colony counts, obtained during the validation are provided in tables 2.5 to 2.7.

Experiment No	Culture 1	Culture 2	Culture 3	Mean colony count per well	Standard Error of Mean
1	145	157	169	157	12
2	148	139	185	157	20
3	164	157	184	168	11
4	173	163	195	177	13
5	173	167	194	178	12
6	146	153	170	156	10

Table 2.5 hCB BFU-E Clonogenic Cultures: Assay Validation

hCB cells were added to human granulocytic/ monocytic and erythroid clonogenic culture media (Methocult -GF) . The cultures were incubated in a CO_2 incubator at 37^{o}C, for 12 days. The BFU-E colonies per well was determined using an inverted microscope. hCB BFU-E clonogenic culture validation was carried out in triplicate on 6 separate occasions.

Experiment No	Culture 1	Culture 2	Culture 3	Mean colony count per well	Standard Error of Mean
1	62	74	77	71	8
2	81	50	63	65	13
3	59	68	75	67	7
4	71	55	78	68	10
5	63	68	71	67	3
6	72	77	63	71	6

Table 2.6 hCB CFU-GM Clonogenic Cultures: Assay Validation

hCB cells were added to human granulocytic/ monocytic and erythroid clonogenic culture media (Methocult -GF) . The cultures were incubated in a CO_2 incubator at 37^{o}C, for 12 days. The CFU-GM colonies per well was determined using an inverted microscope. hCB CFU-GM clonogenic culture validation was carried out in triplicate on 6 separate occasions.

Experiment No	Culture 1	Culture 2	Culture 3	Mean colony count per well	Standard Error of Mean
1	84	71	73	76	7
2	69	87	74	77	8
3	63	88	75	75	10
4	83	71	77	77	5
5	89	65	76	77	10
6	87	72	68	76	8

Table 2.7 hCB CFU-Mk Clonogenic Cultures: Assay Validation

hCB cells were added to human CFU-Mk clonogenic culture media (Megacult-C) . The cultures were incubated in a CO_2 incubator at 37^{o}C, for 12 days. The CFU-Mk colonies were stained and the number of CFU-Mk colonies per well determined using an inverted microscope. hCB CFU-Mk clonogenic culture validation was carried out in triplicate on 6 separate occasions.

Compound	Concentration	BFU-E colonies / well			CFU-GM colonies per well			CFU-Mk colonies /well		
		1	2	3	1	2	3	1	2	3
Vincristine Sulphate	0	186	185	195	82	77	78	82	88	87
	0.001	176	180	165	71	73	71	71	68	63
	0.1	147	135	133	51	48	44	28	31	36
	0.01	166	167	152	64	61	55	47	41	38
	1	83	96	77	21	27	31	20	18	14
	10	55	47	69	14	16	11	7	9	11
Chloramphenicol Succinate	0	186	172	185	79	88	71	82	80	73
	0.001	177	165	160	61	57	54	77	75	80
	0.01	127	111	124	45	41	52	59	61	66
	0.1	79	68	70	36	33	39	47	52	56
	1	35	41	36	21	18	26	31	29	35
	10	12	11	15	11	9	17	20	18	27
Diazipam	0	178	168	196	83	81	84	90	82	85
	0.001	167	180	173	71	88	79	88	81	74
	0.01	158	164	152	68	77	70	77	61	66
	0.1	138	133	141	62	71	74	58	63	65
	1	93	108	92	44	48	38	37	26	41
	10	54	46	66	32	48	31	15	35	22
	0	167	190	172	80	71	83	83	88	74
Methotrexate	0.001	128	151	168	66	59	61	78	75	66
	0.01	93	104	84	60	44	47	68	62	57
	0.1	63	72	69	41	41	46	56	50	48
	1	43	36	22	25	39	22	43	40	35
	10	17	23	11	15	4	16	30	25	19

Table 2.8 Evaluation of hCB Clonogenic Cultures with Known Haematotoxic and Non-haematotoxic Compounds

hCB cells were added to human CFU-GM/BFU-E and CFU-Mk clonogenic culture media. The cultures were incubated in a CO_2 incubator at 37^{o}C, for 10 and 12 days. The CFU-GM, BFU-E and CFU-Mk colonies per well was determined using an inverted microscope. A concentration related decrease in colony count was noted with all compounds.

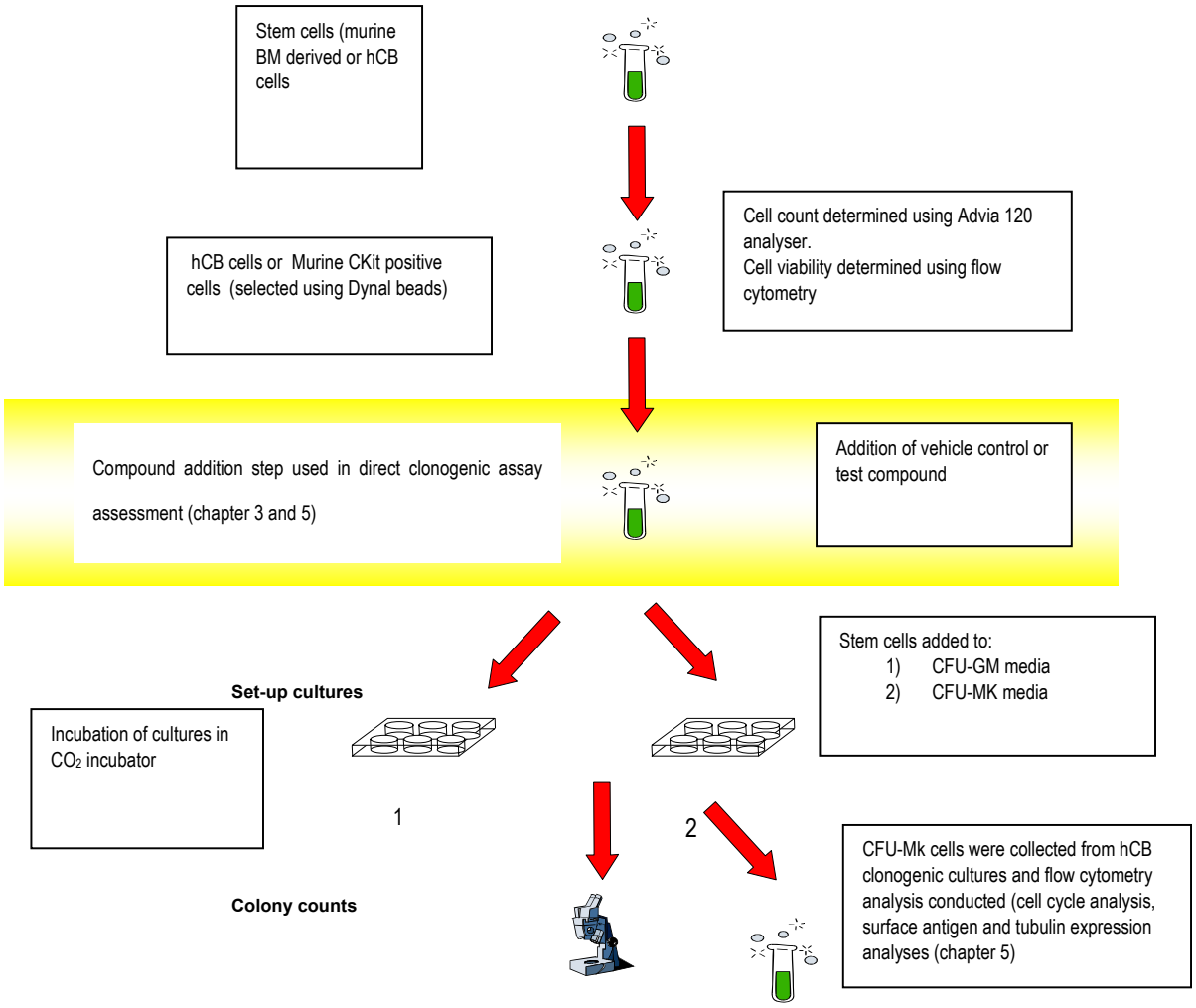


Figure 2. 1 Diagrammatic Representation of Clonogenic Cultures Using Murine or hCB Stem Cells

CKit positive murine or hCB stem cells (10^4cells/mL) were added to murine or hCB clonogenic cultures (Chapter 3 and 5). Test compound or vehicle control was added to the clonogenic cultures at clonogenic culture initiation, and remained within the cultures throughout the culturing period. The cultures were incubated in CO_2 incubator. At the end of the incubation time number colonies (BFU-E, CFU-GM and CFU-Mk) was determined using an inverted microscope and /or flow cytometry analysis (CFU-Mk colonies only) was conducted (chapters 3 and 5). Flow cytometry analysis included cell cycle analysis, surface antigen expression and tubulin expression, following AN treatment (chapter 5).

2.2.5 Murine and hCB LTBMC

The method initially used was based upon the cultures described by (Dexter et al 1978), a liquid culture system that contains an established layer of stromal cells (support cells) and

non-adherent cells. The stromal layer is developed from fresh bone marrow in tissue culture flasks or plates where the stromal cells attach to the culture surface. The cultures are given fresh medium weekly, and after 5 to 6 weeks the stromal layer is usually developed. Once the stromal layer is developed, freshly harvested, pre-selected progenitor cells from primary bone marrow can be added to the cultures. It is the proliferation and development of these freshly harvested cells that are supported by the already established stromal layer.

Stromal Layer Development: Culture Initiation: 100 µL of murine bone marrow or hCB cells (1 x10^6 cells/mL) was added to 9.90mL of Myelocult media (Methachem Diagnostics, UK) containing penicillin / Steptomycin (Sigma, UK) 833µl , 20µL hydrocortisone (Sigma, UK) (10^{-6}M) into a 25mL culture flask. The cell count was determined using an automated cell counter (Advia 120, Bayer, USA). The flasks were then placed into a 5% CO_2 incubator at 37^o C.

Stromal Layer Development: Weekly Feeding: Once a week the flasks containing the developing stromal layers were removed from the incubator, taking care not to shake the cultures, and as much of culture media as possible (9.5-9.7 mL), containing non- stromal cells (maturing granulocytes, monocytes and megakaryocytes) was removed. 9.5 mL of fresh media, containing antibiotics, was then added to the cultures. The stromal layer reached 95-97% confluency by the end of 5 weeks.

Inoculation of Ckit murine Positive Cells or hCB cells to Developed Stromal Layer: Once a confluent stromal layer was established, the stromal layer was inoculated with CKit positive murine cells (mLTBMC) or hCB cells (human LTBMC). At weekly intervals, 9.0 -9.5 mL of the media was removed and replaced with fresh culture medium. A diagrammatic representation of LTBMC is provided in Figure 2.2.

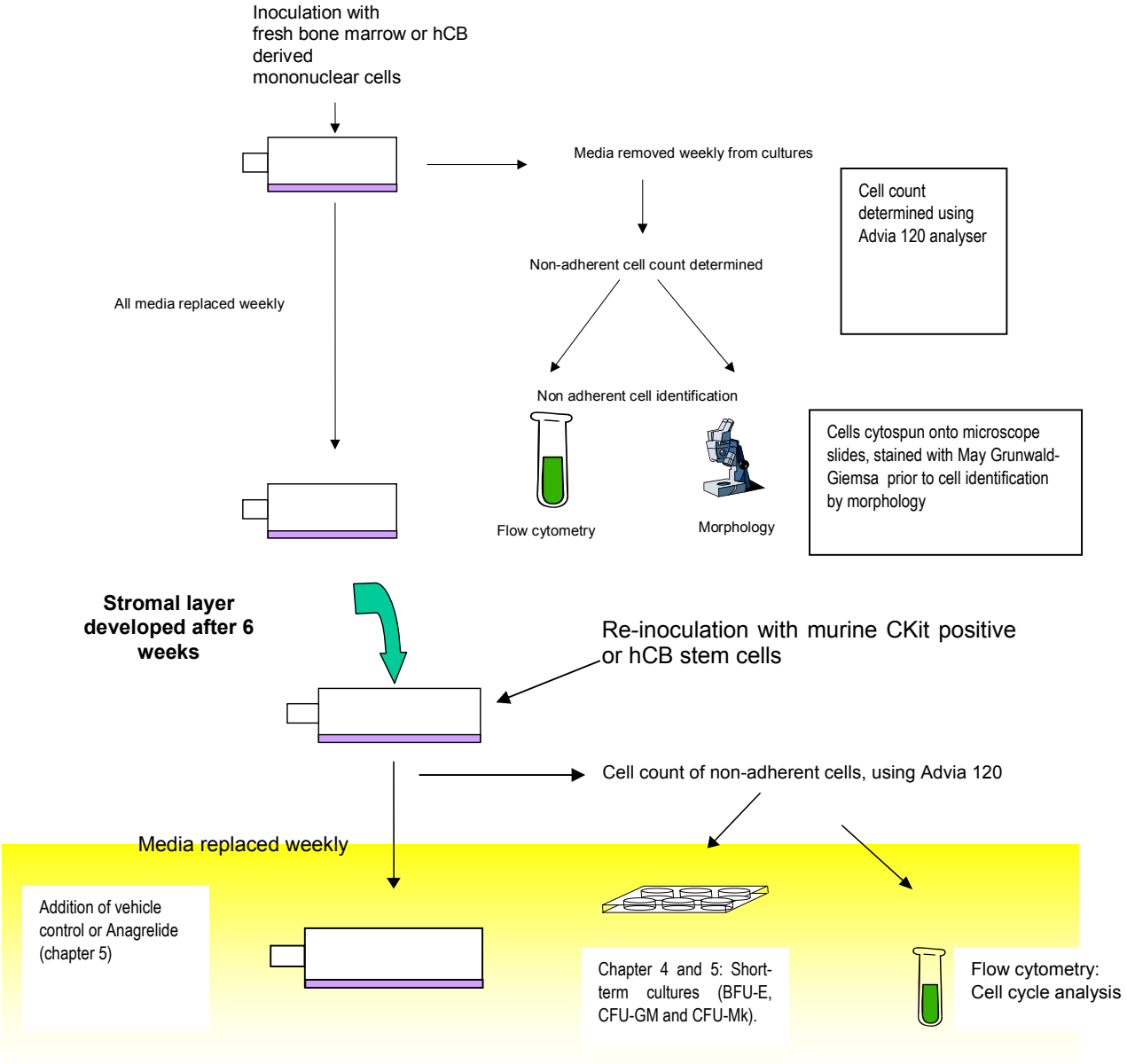


Figure 2. 2 Diagrammatic Representation of LTBMC, Using Murine or hCB Stem Cells

Murine mononuclear cells or hCB cells (10^6cells/mL) were added to the LTBMC media and the flasks incubated in a CO_2 incubator. Weekly collections of non-adherent cells were made when culturing media was replaced. Non adherent cells were identified by morphology and surface antigen expression by flow cytometry (chapter 4). When stromal layer was confluent fresh murine or hCB cells were added (10^6cells/mL). Anagrelide or vehicle control was added to the hCB LTBMC when confluent stromal layer was inoculated with fresh hCB cells (chapter 5). Non-adherent cells collected weekly from the established LTBMC were added to murine or hCB clonogenic assays and the number of CFU-GM, BFU-E and CFU-Mk colonies determined (chapter 4 and 5). In addition, following anagrelide treatment, cell cycle analysis, surface receptor expression and tubulin-α and tubulin- β expression and mean fluorescence intensity was determined by flow cytometry (chapter 5).

2.2.6 Flow Cytometry Analysis

Surface Membrane Staining

The recommended quantity of the Flurescein isothiocyanate (FITC)-conjugated and Phycoerythrin (PE)-conjugated monoclonal antibodies were added to a set of tubes (two tubes per sample to be analyzed). 100µL of non-adherent cells, from human or murine LTBMC or cells collected from clonogenic cultures were added to all the tubes. The tubes were gently mixed and incubated for 15 minutes at room temperature (18-23^{O}C) in the dark and then centrifuged for 3 minutes at 600g. Supernatant was decanted, leaving behind approx. 50µl of fluid and 2 mL of Cell Wash (Becton Dickinson, CA, USA) added to each tube and then lightly mixed to re-suspend the cells. The tubes were again centrifuged for 3 minutes at 600g. The supernatant was removed so that approx. 50µL of fluid is left behind. The cells ($1x10^4$ cells /mL) were re-suspended in 0.5 mL of Cellfix (Becton Dickinson, CA, USA).

10 000 cells were analyzed from each sample using a FACScan flow cytometer (Becton Dickinson, CA, USA) using CellQuest software. Two dimensional dot plots (TDDP) were used to distinguish the different cell phenotypes as well as the relative size and relative complexity of the cells. The four sub-populations of the FITDDP are shown in Appendix A.

Intracellular Staining

The method employed has previously been described by Jung et al, 1993. 50µL of cells collected from clonogenic cultures or non adherent layers from LTBMC suspension sample was placed into a falcon tube. 100µL of reagent A (IC-Fix^{TM} Cell Fixation Buffer) of the Fix and Perm cell permeabilsation kit was added to each tube. The tubes were incubated at room temperature (18-22°C) and then 2mL of Cell Wash (Becton Dickinson, CA, USA) was added to all the tubes. The tubes were centrifuged for 15 minutes at 400g. Supernatant was decanted, leaving behind approx. 50µL of fluid. 100µL of Reagent B (IC-Perm^{TM} Cell Permeabilization Buffer) of the Fix and Perm cell permeabilsation kit was added to each tube and the recommended quantity of the FITC conjugated monoclonal antibody was added. The antibodies to be used are specific for the intracellular antigen that is being measured and for the species being tested and incubated at room temperature (18-22°C) for fifteen minutes.

At the end of the incubation period 2mL of Cell Wash (Becton Dickinson, CA, USA) was added to all the tubes. The tubes were centrifuged for 15 minutes at 400g. Supernatant was decanted, leaving behind approx. 50µL of fluid. The cells ($1x10^4$ cells /mL) were re-suspended in 0.5mL of Cellfix (Becton Dickinson, CA, USA). The cells were analysed using the CellQuest program, using the FACSCalibur.

2.2.7 Cytospin Technique

In order to identify the non-adherent cells from LTBMC morphologically, the cells ($1x10^4$ cells /mL) were aspirated from the cultures and transferred to a centrifuge tube. Cell count was determined using an automated cell counter (Advia 120, Bayer, USA). 0.5 mL of PBS was added to the tubes, which were then centrifuged at 400g, 5 minutes. The supernatant was removed and cells resuspended in 400µL methanol, to fix the cells. 200µL of fixed cell suspension attached to duplicate microscope slides using a cytospin (100g, 2 minutes). The cytospin preparations were stained with May-Grunwald staining.

2.2.8 Viability Count

100 µl ($1x10^5$cells/mL) of stem cells was placed into falcon flow cytometry tubes to which 20 µl of Via-Probe (7-AAD) was added to the cells. Cell count was determined using an automated cell counter (Advia 120, Bayer, USA). The cells were incubated for 10 minutes before analysis. Analysis was conducted using the Cell Quest programme. 10 000 cells were analysed from each sample using a FACScan flow cytometer (Becton Dickinson, CA, USA) using CellQuest software. Histogram analysis was used to distinguish viable cells; the viable (7-AAD negative) cells were expressed as a percentage of the total population counted.

2.2.9 Cell Cycle Analysis

1 mL ($1x10^5$ cells/mL) of the non-adherent cells (collected from LTBMC) or cells collected from Clonogenic cultures were placed into a falcon flow cytometry tube and centrifuge for 5 minutes at 300*g* at room temperature (20 -25^o C); the cell count was determined using an

automated cell counter (Advia 120, Bayer, USA). The supernatant was aspirated off the cells, leaving approximately 50 µL of residual fluid in the tube. 1 mL of Buffer Solution (Becton Dickinson, CA, USA) was added to the cell pellet and the cells were vortexed to re-suspend and then centrifuged for 5 minutes at 300*g* at room temperature (20 -25^{o} C). The supernatant was aspirated off the cells, and 1 mL of 70 % ethanol was added to the cell pellet and the cells. The 70% ethanol was aspirated off the cells, taking care not to disturb the cells in the base of the test tube. The cells (approx.1 x10^{5} cells/mL) were resuspended in 1mL of the buffer solution (Becton Dickinson, CA, USA). The cells were centrifuged at 600g for 2 minutes. The buffer solution was poured off the pellet of cells. 250 µL of Solution A (trypsin in a spermine tetrahydrochloride detergent buffer) was added to each tube and gently mixed and incubated at room temperature for 10 minutes. 200 µL of Solution B (trypsin inhibitor and ribonuclease A in citrate stabilizing buffer with spermine) was added to each tube and gently mixed and incubated at room temperature for 10 minutes. 200 µL of cold Solution C (propidium iodide (PI) and spermine tetrahydrochloride in citrate stabilizing buffer) was added to each tube and gently mixed, incubated in the fridge for 10 minutes prior to analysis. Analysis was conducted on the Becton Dickinson FACScan using CellQuest programme. Data analysis conducted using Becton Dickinson ModFit (Becton Dickinson, CA, USA).

2.2.10 May Grunwald-Giemsa Staining for Light Microscopy

May-Grunwald stain (Dako, Cambridgeshire, UK) was diluted 1 in two using pH 6.4 buffered water. The cytospin preparations thermanox cover slips were placed in May-Grunwald staining solution for 3 minutes. The cover slips were then placed in the Giemsa staining solution for 3 minutes. The cover slips were rinsed, and the stain was developed in pH 6.4 buffered distilled water, then rinsed in distilled water and finally allowed to air dry.

2.2.11 Evaluation Essential Thrombocythemia Reference Compounds Using Colony Forming Cultures

Sigma-Aldrich Company Ltd (UK) supplied vincristine, vinblastine, hydroxyurea and chloramphenicol. The three compounds were bought in their dessicate state and were diluted to the required concentrations by reconstitution with sterile PBS on the day of culture.

Anagrelide was supplied by Shire Pharmaceutical. The compounds were dissolved using neat dimethylsulfoxide (DMSO), with a 2% (v/v) final concentration of DMSO (using PBS). 10µl of control or each compound concentration was added to the GM/E and Mk clonogenic culture mixtures (previously described) prior to dispensing into wells of six- well plates. Culture incubation conditions, incubation time and method of colony counting were the same as previously described.

2.2.12 Data Analysis

For each cell lineage the concentration, at which 50% inhibition of colony growth (IC50) occurred was calculated using ROBOSAGE software package (an automated curve fitting software package; version 7.30). Robosage is a Microsoft EXCEL (Redmond, Wash.) add-in program developed at GlaxoSmithKline (M. W. Lutz, personal communication). The nonlinear optimization function in Robosage is based on the Marquardt-Levenberg algorithm. The Marquardt-Levenberg algorithm enables the determination of the steepest descent of a non-linear curve. It dynamically mixes Gauss-Newton and gradient-descent iterations.
The formula used was:

y=(a-d)/(1+(x/c)^b)+d

This model describes a sigmoidal curve with an adjustable baseline, a. The equation can be used to best fit curves where response is either increasing or decreasing with respect to the independent variable, X. X is a concentration of drug under test (agonist or antagonist). Y is the response. A is the limiting response as X approaches zero. As X increases without bound, Y tends toward its lower limit, d. C is the inflection point (EC50 or IC50) for the curve that is, Y is halfway between the lower and upper asymptotes when X=C. B is the slope-factor or Hill coefficient. The sign of b is positive when the response increases with increasing dose, and is negative when the response decreases with increasing dose (inhibition).

Two Sided Students *t* analysis (testing the null hypothesis that means of treated and control groups are equal) and Dunnett's test (comparing each treatment group mean with the control group mean) was conducted to determine any statistical significance of the lineage specific toxicity noted.

Chapter 3

3.1 Experimental Part 1: Megakaryocytic Clonogenic Cultures

3.1.1 Megakaryopoiesis: Overview

Megakaryopoiesis concludes with the release of platelets. As with the other lineages it is a multistage process of cellular differentiation and maturation regulated by a variety of cytokines (George, 2004). As discussed in Chapter 1, the development and maturation of the committed megakaryocytic stem cells is unique in that they develop a highly polyploid DNA content and the formation of platelets results from the projection of megakaryocytic cytoplasm through the sinusoidal endothelium (Deutsch and Tomer, 2006).

3.1.2 Essential Thrombocythemia

As discussed in Chapter 1, ET is an acquired MPD characterized by a sustained elevation of platelet number with a tendency for thrombosis and hemorrhage. The prevalence in the general population is approximately 30/100,000. The median age at diagnosis is 55 to 70 years, but the disease may occur at any age. The clinical picture is dominated by a predisposition to vascular occlusive events and hemorrhages. Thromboses of large arteries represent a major cause of mortality associated with ET or can induce severe neurological, cardiac or peripheral artery manifestations. The molecular pathogenesis of ET, is associated with the JAK2 V617F mutation. Therapeutic interventions in ET are limited to decisions concerning the introduction of anti-aggregation therapy and/or starting platelet cytoreduction. The cytoreduction therapies used include hydroxyurea, vincristine, vinblastine and anagrelide.

3.1.3 Aims of the Investigation

For a number of decades, *in vitro* haemapoietic cultures have been available for the evaluation of the potential myelotoxicity of compounds. Although anti-cancer and anti-viral therapies are known to cause myelotoxicity, the first cell lineage most often affected *in vivo* is the megakaryocytopoietic. Until the availability of recombinant thrombopoietin, this aspect of haematopoiesis has not been possible to evaluate easily *in vitro*. The initial aim of this investigation was

- To establish a robust murine megakaryocytic culture system (a robust short-term culture

system for the erythroid and granulocytic/monocytic cell lineages had been established prior to the initiation of this work).

- The second aim of the investigation was to determine if murine short-term *in vitro* assays could identify lineage specific haematopoietic effects, using compounds that are used in the treatment of ET, and therefore target megakaryocytes specifically. The four reference compounds evaluated were Vincristine (VC), Vinblastine (VB), Hydroxyurea (HU), and Anagrelide (AN). Part of this work formed an industrial placement student project, where the student, D. Puvanendrampillai, conducted the analysis of megakaryocytic cultures only. The effect of the 4 compounds on granulocyte/ monocyte, erythrocyte and megakaryocyte maturation was assessed in short-term cultures, and data was expressed as IC50 values (the concentrations at which *in vitro* colony growth was inhibited by 50%). The molecular targets of the four reference compounds are varied: DNA damage, or inhibition of DNA synthesis or inhibition of the formation of microtubules. The characteristics of the reference compounds are shown in Table 3.1.

Compound	Class	Pharmacology	Reference
Vincristine	Vinca alkaloid	Inhibits formation of microtubules resulting in cell cycle arrest at metaphase	Gibbs and Sorensen 2000
Vinblastine	Vinca alkaloid	Inhibits formation of microtubules resulting in cell cycle arrest at metaphase	Moss et al 1999 and Stubbs and Swaney 1996)
Hydroxyurea	Alkylating agent	Quenches the tyrosyl free radical at the active site of the M2 protein subunit of ribonucleotide reductase, inactivating the enzyme. Causes cell death in S phase and synchronization of the fraction of cells that survive	Gibbs and Sorensen 2000: Tefferi, 2006
Anagrelide	Quinazolin derivative	Inhibits cyclic nucleotide phosphodiesterase and the release of arachindonic acid from phospholipase, possibly by inhibiting phospholipase A_2. Mechanism of platelet count reduction unknown	(Petrides, 2006)

Table 3. 1 Pharmacology of Reference Compounds

The pharmacology of the 4 ET treatment reference compounds to be used in the determination of the lineage specificity of each treatment using murine clonogenic cultures is provided.

3.2 Methods

3.2.1 Murine BM Preparation

The methods employed for the aspiration of bone marrow from Balb/C mice, pre-selection of mononuclear BM cells and the isolation of CKit positive murine BM cells is described in Appendix A.

3.2.2 Murine Megakaryocytic (Mk) Clonogenic Cultures - Standard Culture Conditions

Megakaryocytic clonogenic as described in Chapter 2 were utilized in these experiments (section 2.2).

For APAAP staining, 1.5 mL of the final culture mixture was dispensed into each well of a double chamber culture slide. The plates were incubated in a CO_2 incubator. Colony Forming Units-Megakaryocytic (CFU-Mk) were counted after 5, 6, 7 and 8 days incubation. The colonies were identified morphologically using an inverted microscope.

3.2.3 Murine Megakaryocytic (Mk) Clonogenic Cultures -Modified Culture Conditions

Modified megakaryocytic clonogenic as described in Chapter 2 were utilized in these experiments (section 2.3).

3.2.4 Murine Granulocytic /Monocytic and Erythroid Clonogenic Assay (GM/E)

GM/E clonogenic assays as described in Chapter 2 were utilized in these experiments (section 2.1).

3.2.5 Alkaline Phosphotase Anti- Alkaline Phosphotase (APAAP) Staining For the Identification of Murine CFU-Mk Colonies

The method was based up on the technique described by Mason (1985). The chamber slides

were removed from the incubator and the outer plastic was removed from the chamber. A pre-cut polypropylene separator was placed onto the chamber slide and a filter card placed on top of the slide to soak up the culture liquid. The filter card was removed, once excess liquid had been collected, leaving the separator in place. The slide was then placed into a container of acetone maintained on ice for 5 minutes. After the incubation time the slides were removed from the acetone and allowed to dry at room temperature. 0.5 ml (5µg/mL) of the Rat Anti-Mouse CD41 monoclonal antibody (diluted 1 in 500 in phosphate buffer prior to use) was placed onto the slide. The slides were incubated at room temperature for 30 minutes. At the end of the incubation period the anti-CD41 antibody was poured off the slides. The slides were rinsed with 1.0 ml of phosphate buffer.

0.5 ml (5µg/mL) of Rabbit anti-Rat immunoglobulin, diluted 1 in 100 in phosphate buffer) was placed on each slide. The slides were incubated at room temperature for 30 minutes. At the end of the incubation period the rabbit anti-rat immunoglobulin was poured off the slides. The slides were rinsed with 1.0 ml of phosphate buffer.

0.5 ml (10µg/mL) of Rat APAAP antibody (diluted 1:25 as recommended by the manufacturer) was placed on each slide. The slides were incubated for 30 minutes at room temperature. At the end of the incubation period the Rat APAAP antibody was poured off the slides. The slides were rinsed with 1.0 ml of phosphate buffer. 0.5 ml of 1% Fast Red solution was placed onto each slide. The slides were incubated for 20 minutes at room temperature. At the end of the incubation period the Fast Red solution was poured off the slides. The slides were rinsed with 1.0 ml of phosphate buffer. The positively stained colonies were identified and counted using a light microscope.

Controls for APAAP staining:

Two negative controls were used to determine the amount of background staining on every staining run.

Negative control 1: The addition of CD41 was omitted.

Negative control 2: 0.5 ml (5µg/mL) of an isotype control antibody was added in place of CD41.

3.2.6 Evaluation Essential Thrombocythemia Reference Compounds Using Colony Forming Cultures

The source of the reference compounds and preparation of the stock solutions of the compounds is described in Chapter 2 (section 2.2.11). The final concentrations used in this thesis ranged from 0.1 to 1000μM. However, for the evaluation of AN only this range was extended. Concentrations evaluated for AN ranged from 0.0001 to 10000μM. 10μl of control or each compound concentration was added to the GM/E and Mk clonogenic culture mixtures (previously described) prior to dispensing into wells of six- well plates. Culture incubation conditions, incubation time and method of colony counting were the same as previously described. All reference compound concentrations were evaluated in triplicate on three separate occasions.

3.2.7 Data Analysis

Determination of IC50 concentrations was carried out as described in Chapter 2 (section 2.2.12).

3.3 Results

3.3.1 Mk Clonogenic Cultures Using Standard Conditions

In this investigation, the preliminary short -term megakaryocytic cultures were established, using pooled bone marrow, from BALB/c mice, in three separate experiments, with the assays conducted in triplicate on each occasion. To determine the day at which the maximum colony counts were achieved counts were conducted on days 5, 6, 7 and 8 (Figure 3.2). The colony counts are shown in Table 3.2. CFU-Mk colony counts increased from day 5 and reach their peak on day 7 and then declined on day 8.

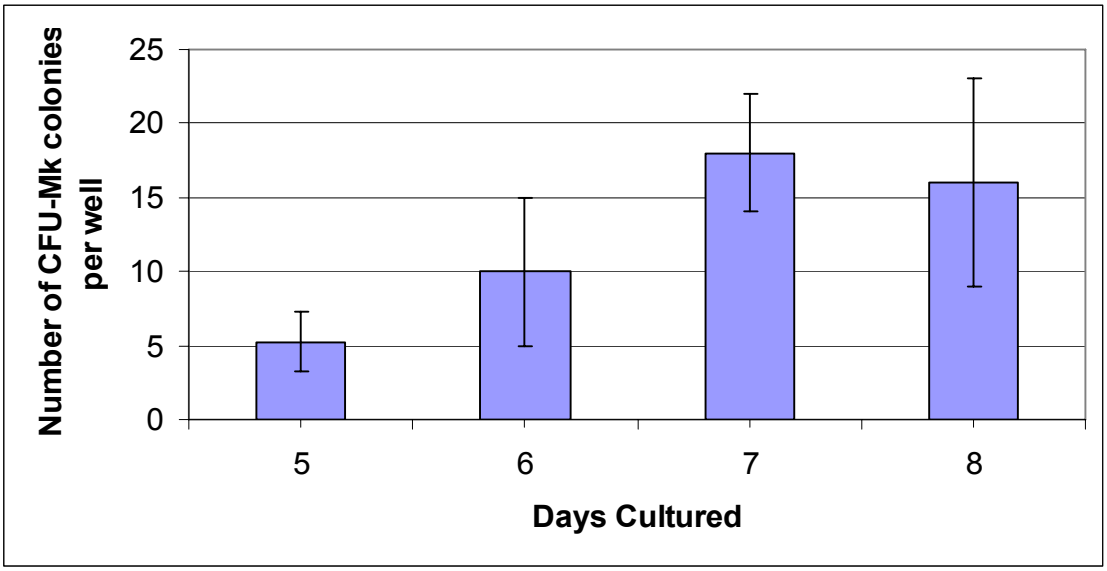


Figure 3. 1 Optimum Incubation time for Murine CFU-Mk Colonies Using Megacult-C Culture Medium with cytokines IL-3 and Tpo.

The CFU-Mk colony counts per well following 5, 6, 7 and 8 days incubation in a CO_2 incubator. CFU-Mk colony counts increased from day 5 and reach their peak on day 7 and then declined on day 8. Mean colony counts from 3 separate experiments, evaluated in triplicate in each experiment. Data shown is Mean +/- SEM.

Experiment No	Culture Plate Number											
	Day 5			Day 6			Day 7			Day 8		
Culture	1	2	3	1	2	3	1	2	3	1	2	3
1	3	6	4	10	7	4	17	24	27	18	15	20
2	5	3	6	13	14	8	17	19	18	14	20	15
3	4	8	9	10	12	16	22	27	29	14	18	16
Mean	5			10			18			16		
SEM	2			5			4			7		

Table 3. 2 Optimum Incubation time for Murine CFU-Mk Colonies Using Megacult-C Culture Medium with cytokines IL-3 and Tpo.

The CFU-Mk colony counts per well following 5, 6, 7 and 8 days incubation in a CO_2 incubator. CFU-Mk colony counts increased from day 5 and reach their peak on day 7 and then declined on day 8.

As shown in the figure 3.1, the optimal day for counting CFU-Mk colonies was Day 7. Furthermore the number of CFU-Mk colonies present on Day 7 ranged from 14 to 21, giving a

mean CFU-Mk colony count of 18. As the variation in colony counts between occasions was not (> +/- 2SD), the standard deviation of the triplicate counts on each occasion was considered acceptable. Additionally, the number of colonies that were grown in this culture system was quite low compared to the number of colonies observed in similar cultures by other researchers (Berthier, 1993). The assay system required further work to try to increase the number of colonies.

3.3.2 Alkaline Phosphatase Anti Alkaline Phosphatase (APAAP) Staining For the Identification of CFU-Mk Colonies

APAAP staining was conducted on CFU-Mk colonies to confirm their cell lineage, using CD41 as the lineage specific colony marker. The CFU-Mk colonies were identified by the presence of dark red reaction product at the CD41 (gpIIb/IIIa) antigen site (Figure 3.2). Additionally negative controls were set up on each occasion. No APAAP reaction products were seen when either anti-mouse CD41 antibody was omitted (Figure 3.3) or when an isotype control antibody was substituted for the anti-mouse CD41 antibody (Figure 3.4).

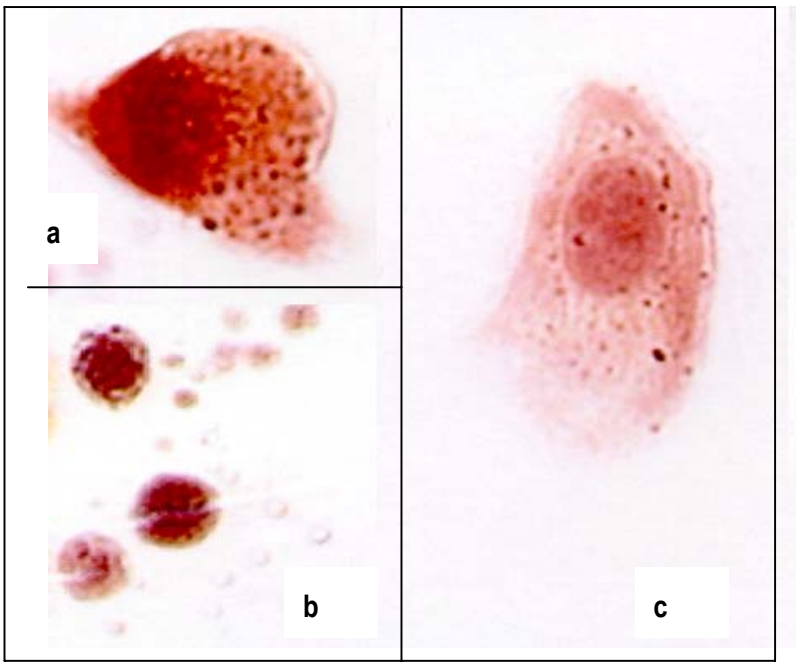


Figure 3. 2 (a, b and c) CFU-Mk Colonies Identified by the Presence of Dark Red Reaction Product at the CD41 (GpIIb/IIIa) Antigen Site.

APAAP dark red staining indicating CD41 expression on CFU-MK colonies (x 100 objective), following 7 days culturing in CFU-Mk clonogenic cultures.

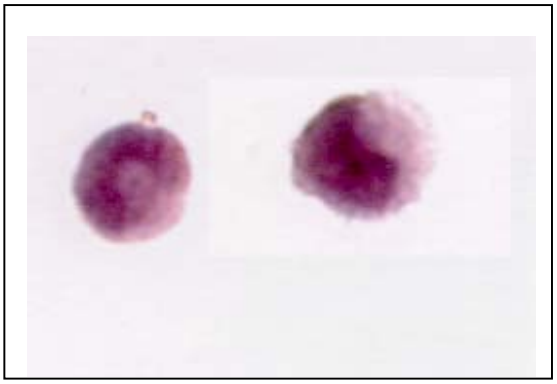


Figure 3. 3 Negative Control: Anti-CD41 Replaced with Isotype Control Antibody

APAAP staining of CFU-Mk colonies no dark red granules present (x100 objective), following 7 days culturing in CFU-Mk clonogenic cultures.

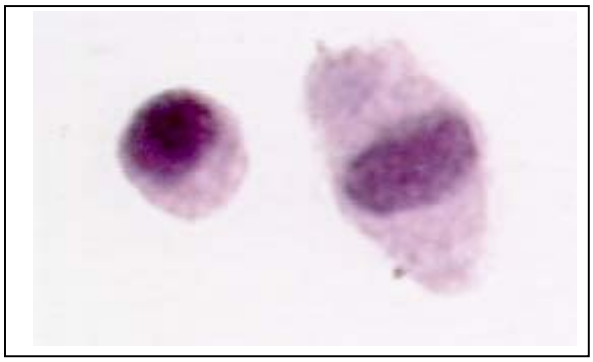


Figure 3. 4 Negative Control: Anti-CD41 Omitted

APAAP staining of CFU-Mk colonies no dark red granules present (x100 objective) following 7 days culturing in CFU-Mk clonogenic cultures.

3.3.3 Pre-Selection of Pluripotent Stem Cells

The mononuclear cell separation removes mature erythrocytes and granulocytes from the bone marrow sample. The mononuclear layer is a heterogeneous cell population of pluripotent progenitor cells; lineage committed cells as well as mature monocytes and macrophages. Pre-selection of pluripotent stem cells would ensure that all lineage-committed stem cells were not added to the culture system. The effect of pre- selection of pluripotent stem cells (identified by the cell surface phenotype Sca –1 +, CKit + and cell lineage specific –low), from the mononuclear cell population prior to addition to the CFU-Mk assay system was investigated.

The number of CFU-Mk colonies grown in this series of experiments ranged between 28 and 34 colonies (Figure 3.5) and the CFU-Mk colony counts are shown in table3.3. Paired t test statistical analysis was performed to elucidate the significance of any difference in number of megakaryocytic colonies resulting from the pre-selection. The statistical analysis indicated that the increase in colony numbers with the introduction of pre-selection of pluripotent progenitor cells was significant at $p<0.01$. Furthermore, the intra and inter assay variation was within +/-2SD.

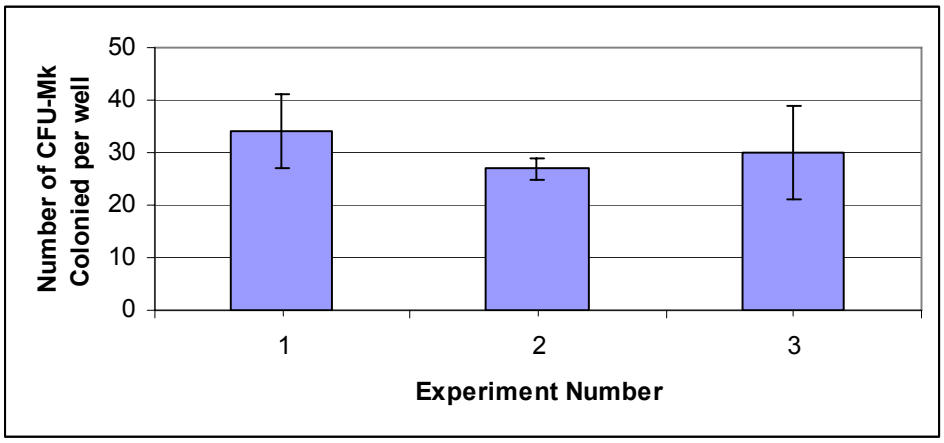


Figure 3. 5 Effect of Pre- Selection of Pluripotent Progenitor on CFU-Mk Colony Numbers

The CFU-Mk colony counts per well following pre- selection of pluripotent stem cells (identified by the cell surface phenotype Sca –1 +, CKit + and cell lineage specific –low), from the mononuclear cell population prior to addition to the CFU-Mk clonogenic media. The number of CFU-Mk colonies grown ranged between 28 and 34 colonies. Mean colony counts from 3 separate experiments, evaluated in triplicate in each experiment are provided. Data shown are mean +/- SEM

Experiment No	Culture Plate Number			
	1	2	3	Mean CFU-Mk Colony count / well for each experiment
1	30	36	39	35
2	25	34	26	28
3	33	30	27	30

Table 3. 3 Effect of Pre- Selection of Pluripotent Progenitor on CFU-Mk Colony Numbers

The CFU-Mk colony counts per well following pre- selection of pluripotent stem cells (identified by the cell surface phenotype Sca –1 +, CKit + and cell lineage specific –low), from the mononuclear cell population prior to addition to the CFU-Mk clonogenic media. The number of CFU-Mk colonies grown ranged between 28 and 34 colonies.

3.3.4 Effect of Recombinant Murine Interleukin-6 (rmIL-6) on Colony Numbers

In this investigation, an evaluation of the effect of adding 0.1mL of rm IL-6 (10ng/mL) on megakaryocytic colony numbers was determined. *In vitro* murine Megakaryocyte cultures were conducted with and without rmIL-6. Additionally, pre-selection of pluripotent stem cells and staining of CFU-Mk colonies, using APAAP, to aid in the morphological identification of CFU-Mk was conducted as previously described.

The addition of rmIL-6 resulted in a statistically significant increase in the number of CFU-Mk colonies from 29 (without IL-6) to 41 (Figure 3.6). The CFU-Mk colony counts with and without the addition of IL-6 are shown in Table 3.4.

	Mean CFU-Mk Colony count per well					
Culture I.D.	1	2	3	4	5	6
Without mIL-6 Addition	26	31	25	31	32	29
With mIL-6 Addition	40	45	41	43	38	39

Table 3. 4 Comparison of Colony Forming Units-Megakaryocytic Counts with and Without Murine IL-6 Addition

The CFU-Mk colony number achieved with and without the addition of rmIL-6. 0.1mL of rm IL-6 at a concentration of 10ng/mL was added. Addition of mIL-6 resulted in an increase in CFU-Mk colony counts.

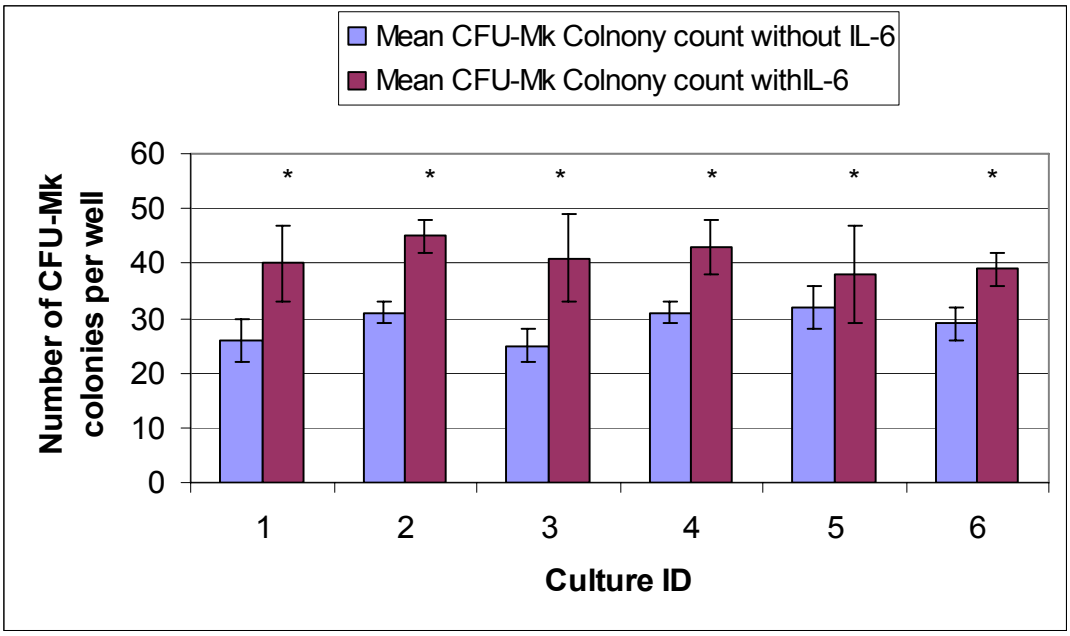


Figure 3. 6 Comparison of Colony Forming Units-Megakaryocytic Counts with and Without Murine IL-6 Addition

The effect on CFU-Mk colony number achieved with and without the addition of mLI-6. 0.1mL of rm IL-6 at a concentration of 10ng/mL was added. Addition of mIL-6 resulted in an increase in CFU-Mk colony counts. * Indicates $p<0.01$ of with IL-6 cultures compared with without IL-6 addition cultures. Mean colony counts from 3 separate experiments, evaluated in triplicate in each experiment are shown. Data shown are mean +/- SEM.

3.3.5 Identification of Lineage Specific Haematopoietic Effects, using Essential Thrombocythemia (ET) Treatment Compounds

The effect of the 4 compounds, (Vincristine (VC), Vinblastine (VB), Hydroxyurea (HU), and Anagrelide (A)) on granulocyte/ monocyte, erythrocyte and megakaryocyte maturation was assessed in short-term cultures, and data was expressed as IC50 values (the concentrations at which *in vitro* colony growth was inhibited by 50%). The change in colony counts for the granulocyte/ monocyte, erythrocyte and megakaryocyte cell lineages are shown in Figures 3.7 – 3.9, respectively. Moreover, the CFU-GM, BFU-E and CFU-MK colony counts at each concentration of each ET compound evaluated are shown in tables 3.6 to 3.10.

Experiment No.	Concentration (uM)	CFU-GM Colony counts /well following treatment with vincristine			CFU-GM Colony counts /well following treatment with vinblastine		
1	**Culture No.**	**1**	**2**	**3**	**1**	**2**	**3**
	0	53	48	55	58	48	52
	0.1	32	43	35	41	35	45
	1	39	27	29	29	21	28
	10	19	24	25	20	16	19
	100	11	17	9	10	5	7
	1000	5	2	1	2	0	3
2	0	52	55	50	60	48	51
	0.0001	N/T	N/T	N/T	N/T	N/T	N/T
	0.001	N/T	N/T	N/T	N/T	N/T	N/T
	0.1	45	30	37	49	32	37
	1	35	28	30	31	21	27
	10	20	19	26	24	14	18
	100	9	11	13	10	5	6
	1000	1	2	5	0	2	3
3	0	56	47	53	53	59	50
	0.1	43	36	31	37	38	42
	1	39	29	26	22	25	30
	10	28	19	16	13	18	22
	100	17	10	11	4	5	10
	1000	5	2	2	0	1	3

Table 3. 5 Change in Granulocytic/Monocytic Colony Number after Treatment with Vincristine and Vinblastine

The CFU-GM colony counts following treatment with 0.1 mL of vehicle control (2% (v/v) DMSO), or vincristine or vinblastine at concentrations 0.1 to 1000μM. The cultures were incubated for 10 days and the number of CFU-GM colonies determined using an inverted microscope. A reduction in the colony counts with an increase in compound concentration was noted

Experiment No.	Concentration (uM)	CFU-GM Colony counts /well following treatment with Hydroxyurea			CFU-GM Colony counts /well following treatment with Anagrelide		
1	**Culture No**	**1**	**2**	**3**	**1**	**2**	**3**
	0	48	52	55	50	56	47
	0.0001	N/T	N/T	N/T	43	45	38
	0.001	N/T	N/T	N/T	38	37	33
	0.1	40	38	43	41	36	35
	1	32	29	36	31	29	27
	10	25	22	27	27	22	22
	100	14	16	17	23	15	14
	1000	3	0	5	15	10	11
	10000	N/T	N/T	N/T	1	2	2
2	0	52	51	47	44	49	56
	0.0001	N/T	N/T	N/T	38	41	43
	0.001	N/T	N/T	N/T	38	40	40
	0.1	44	47	33	34	37	40
	1	37	35	27	29	30	33
	10	28	26	23	20	25	27
	100	15	13	14	19	22	22
	1000	1	5	4	11	13	15
	10000	N/T	N/T	N/T	1	2	3
3	0	50	53	48	50	47	56
	0.0001	N/T	N/T	N/T	47	40	40
	0.001	N/T	N/T	N/T	42	41	38
	0.1	43	39	40	37	36	35
	1	31	34	36	29	34	30
	10	24	21	28	22	26	25
	100	11	17	14	16	20	18
	1000	2	2	5	11	14	14
	10000	N/T	N/T	N/T	1	5	1

Table 3. 6 Change in Granulocytic/Monocytic Colony Number after Treatment with Hydroxyurea and Anagrelide

The CFU-GM colony counts following treatment with 0.1 mL of vehicle control (2% (v/v) DMSO), or anagrelide or hydroxyurea at concentrations 0.0001 to 1000μM. The cultures were incubated for 10 days and the number of CFU-GM colonies determined using an inverted microscope; Not tested (NT). A reduction in the colony counts with an increase in compound concentration was noted.

Experiment No.	Concentration (uM)	CFU-E Colony counts /well following treatment with Vincristine			CFU-E Colony counts /well following treatment with Vinblastine		
1	**Culture No**	**1**	**2**	**3**	**1**	**2**	**3**
	0	77	75	80	71	85	75
	0.1	80	85	83	65	61	60
	1	72	75	71	50	55	48
	10	57	55	61	36	33	39
	100	37	34	38	16	11	14
	1000	15	11	15	1	0	0
2	0	74	79	75	71	78	74
	0.0001	N/T	N/T	N/T	N/T	N/T	N/T
	0.001	N/T	N/T	N/T	N/T	N/T	N/T
	0.1	80	83	79	62	64	60
	1	72	76	69	51	55	50
	10	58	55	51	31	34	30
	100	38	33	37	15	12	14
	1000	15	18	12	0	0	0
3	0	76	81	72	75	70	73
	0.0001	N/T	N/T	N/T	N/T	N/T	N/T
	0.001	N/T	N/T	N/T	N/T	N/T	N/T
	0.1	81	85	79	62	63	60
	1	70	72	74	50	54	51
	10	53	59	56	29	36	34
	100	37	33	36	13	17	13
	1000	17	15	13	0	1	0

Table 3. 7 Change in Erythroid Colony Number after Treatment with Vincristine and Vinblastine

The BFU-E colony counts following treatment with 0.1 mL of vehicle control (2% (v/v) DMSO) or vincristine or vinblastine (0.1 to 1000µM). The cultures were incubated for 10 days and the number of BFU-E colonies determined using an inverted microscope. A reduction in the colony counts with an increase in compound concentration was noted.

Experiment No.	Concentration (uM)	CFU-E Colony counts /well following treatment with Hydroxyurea			CFU-E Colony counts /well following treatment with Anagrelide		
1	**Culture No**	**1**	**2**	**3**	**1**	**2**	**3**
	0	73	71	74	72	77	71
	0.0001	N/T	N/T	N/T	71	70	71
	0.001	N/T	N/T	N/T	71	71	73
	0.1	60	65	62	64	66	59
	1	47	41	44	58	57	51
	10	31	28	32	50	51	51
	100	7	30	7	44	44	43
	1000	0	0	0	35	36	39
	10000	N/T	N/T	N/T	16	18	18
2	0	73	75	71	72	77	69
	0.0001	N/T	N/T	N/T	71	71	70
	0.001	N/T	N/T	N/T	74	71	71
	0.1	62	61	65	63	61	64
	1	44	48	40	53	58	56
	10	29	31	32	50	49	53
	100	3	9	5	44	46	45
	1000	0	1	0	39	36	36
	10000	N/T	N/T	N/T	17	14	19
3	0	74	74	71	71	76	70
	0.0001	N/T	N/T	N/T	71	68	74
	0.001	N/T	N/T	N/T	71	70	71
	0.1	61	63	65	66	61	60
	1	47	40	45	56	55	53
	10	32	29	33	50	48	52
	100	5	4	9	44	48	45
	1000	0	0	0	38	38	37
	10000	N/T	N/T	N/T	18	15	19

Table 3. 8 Change in erythroid colony number after treatment with Hydroxyurea and Anagrelide

The BFU-E colony counts following murine clonogenic culture treatment with vehicle control (2% (v/v) DMSO) and with vehicle control (2% (v/v) DMSO), or hydroxyurea or anagrelide (0.0001 to 1000μM). . The cultures were incubated for 10 days and the number of BFU-E colonies determined using an inverted microscope. . A reduction in the colony counts with an increase in compound concentration was noted; Not tested (NT).

Experiment No.	Concentration (uM)	CFU-Mk Colony counts /well following treatment with Vincristine			CFU-Mk Colony counts /well following treatment with Vinblastine		
1	**Culture No**	**1**	**2**	**3**	**1**	**2**	**3**
	0	46	44	48	44	47	40
	0.1	45	47	46	46	43	48
	1	46	41	41	38	35	40
	10	31	28	27	30	26	33
	100	22	19	27	15	17	15
	1000	5	7	7	4	9	4
2	0	45	48	40	44	47	47
	0.1	44	43	47	44	49	41
	1	40	44	43	39	37	43
	10	29	27	31	28	32	29
	100	19	22	25	17	17	15
	1000	4	8	5	5	6	6
3	0	44	43	46	47	48	41
	0.1	46	43	44	44	48	39
	1	40	45	44	39	37	40
	10	31	29	29	26	30	29
	100	20	24	25	14	17	18
	1000	7	4	9	4	8	6

Table 3. 9 Change in Megakaryocytic Colony Number after Treatment with Vincristine and Vinblastine

The CFU-Mk colony counts following treatment with 0.1mL of vehicle control (2% (v/v) DMSO), or vincristine or vinblastine (0.1 to 1000μM). The cultures were incubated for 10 days and the number of CFU-Mk colonies determined using an inverted microscope. A reduction in the colony counts with an increase in compound concentration was noted

Experiment No.	Concentration (uM)	CFU-Mk Colony counts /well following treatment with Hydroxyurea			CFU-Mk Colony counts /well following treatment with Anagrelide		
1	Culture No.	1	2	3	1	2	3
	0	45	40	47	44	49	41
	0.0001	N/T	N/T	N/T	45	48	46
	0.001	N/T	N/T	N/T	45	47	49
	0.1	50	59	53	38	45	44
	1	47	49	44	30	31	18
	10	39	37	39	16	12	10
	100	27	33	32	11	8	19
	1000	17	14	17	9	7	11
	10000	N/T	N/T	N/T	0	2	0
2	0	41	49	46	44	48	48
	0.0001	N/T	N/T	N/T	40	48	45
	0.001	N/T	N/T	N/T	50	40	49
	0.1	51	59	52	40	44	41
	1	44	49	47	20	20	25
	10	36	39	39	13	18	16
	100	27	33	34	10	14	8
	1000	18	19	10	8	8	14
	10000	N/T	N/T	N/T	1	0	1
3	0	46	43	49	48	40	41
	0.0001	N/T	N/T	N/T	44	48	45
	0.001	N/T	N/T	N/T	46	46	42
	0.1	59	51	56	40	41	47
	1	50	44	49	26	22	20
	10	39	33	40	14	17	10
	100	35	26	25	10	11	14
	1000	19	14	12	9	10	11
	10000	N/T	N/T	N/T	1	1	1

Table 3. 10 Change in Megakaryocytic Colony Number after Treatment with Hydroxyurea and Anagrelide

The CFU-Mk colony counts following murine clonogenic culture treatment with 0.1 mL of vehicle control (2% (v/v) DMSO), or hydroxyurea or Anagrelide(0.1 to 1000µM). Not tested (NT). The cultures were incubated for 10 days and the number of CFU-Mk colonies determined using an inverted microscope; NT-not tested. A reduction in the colony counts with an increase in compound concentration was noted

Examples of granulocytic/ monocytic, erythroid and megakaryocytic colonies are shown in Figures 3.10 – 3.12. A reduction in the colony counts with an increase in compound concentration was noted in all the cell lineages with all the compounds evaluated. Interestingly, different IC50 values were obtained for the granulocyte/ monocyte, erythrocyte and megakaryocyte cell lineages with the same reference compound (Table 3.11). Differences in the sensitivities of the cell lineages towards the other reference compounds, evaluated based on their IC50 values is also shown in table 3.3. For example, 50% inhibition of megakaryocytic colonies was achieved at a concentration of 88.6µM with anagrelide, whereas

greater than 10-fold higher concentration of anagrelide was required to inhibit the granulocyte/ monocyte (1210µM) and erythrocyte (3540µM) colony counts by 50%. Furthermore, the data also shows that some compounds demonstrated no difference in sensitivity towards specific cell lineages. For example, IC50 concentrations for vincristine are similar for the granulocytic / monocytic and erythroid lineages.

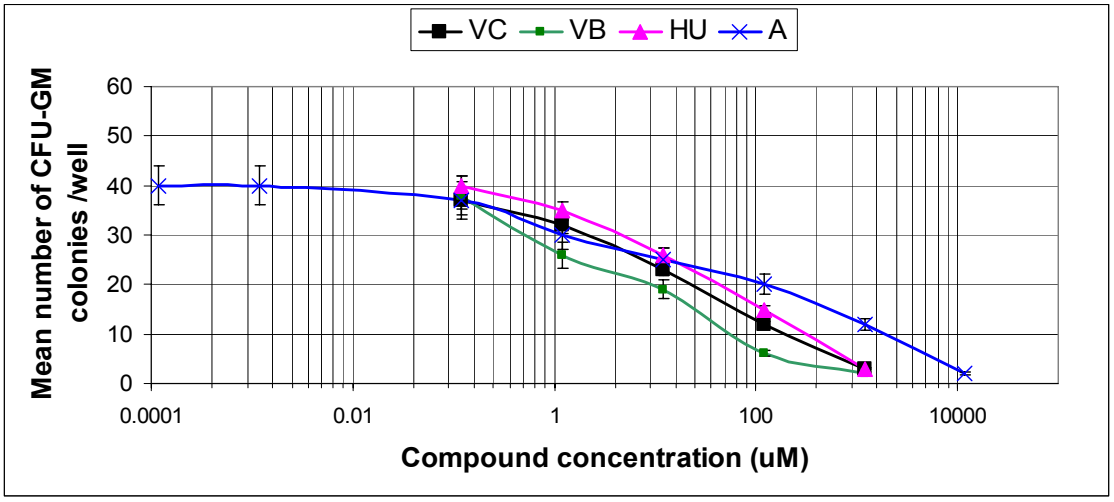


Figure 3. 7 Change in granulocytic/monocytic colony number after treatment with reference compounds

The change in the mean colony counts following CFU-GM culture treatment with vehicle control (2% (v/v) DMSO), Vincristine (VC), Vinblastine (VB), Hydroxyurea (HU), and Anagrelide (A). A concentration dependant decrease in CFU-GM colony count was noted. VB and VC demonstrated specificity towards the granulocytic/monocytic lineage with AN demonstrating the least specificity to the granulocytic/monocytic lineage. CFU-GM colony counts from 3 separate experiments, with each concentration evaluated in triplicate are shown. Data shown as Mean +/- SEM. Mean vehicle CFU-GM (for all reference compounds) control colony counts 51 (+/- 12).

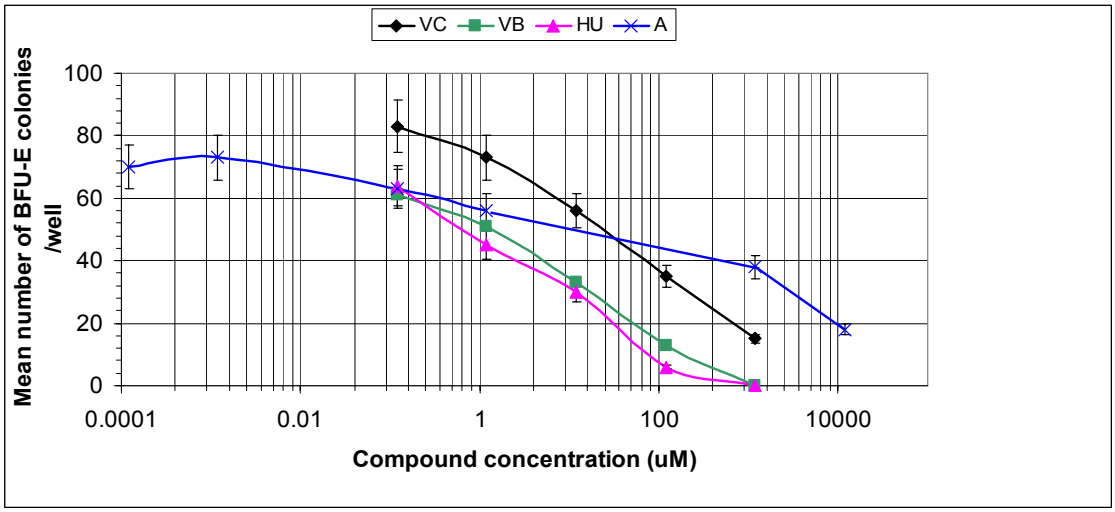


Figure 3. 8 Change in erythroid colony number after treatment with reference compounds

The change in the mean colony counts following BFU-E culture treatment with vehicle control (2% (v/v) DMSO), Vincristine (VC), Vinblastine (VB), Hydroxyurea (HU), and Anagrelide (A). A concentration dependant decrease in BFU-E colony count was noted. VB and VC demonstrated specificity towards the erythrocytic lineage with AN demonstrating the least specificity to the erythrocytic lineage. Mean colony counts from 3 separate experiments, with each concentration evaluated in triplicate. Data shown as Mean +/- SEM. Mean vehicle control (for all reference compounds) BFU-E colony counts 76 (+/- 18)

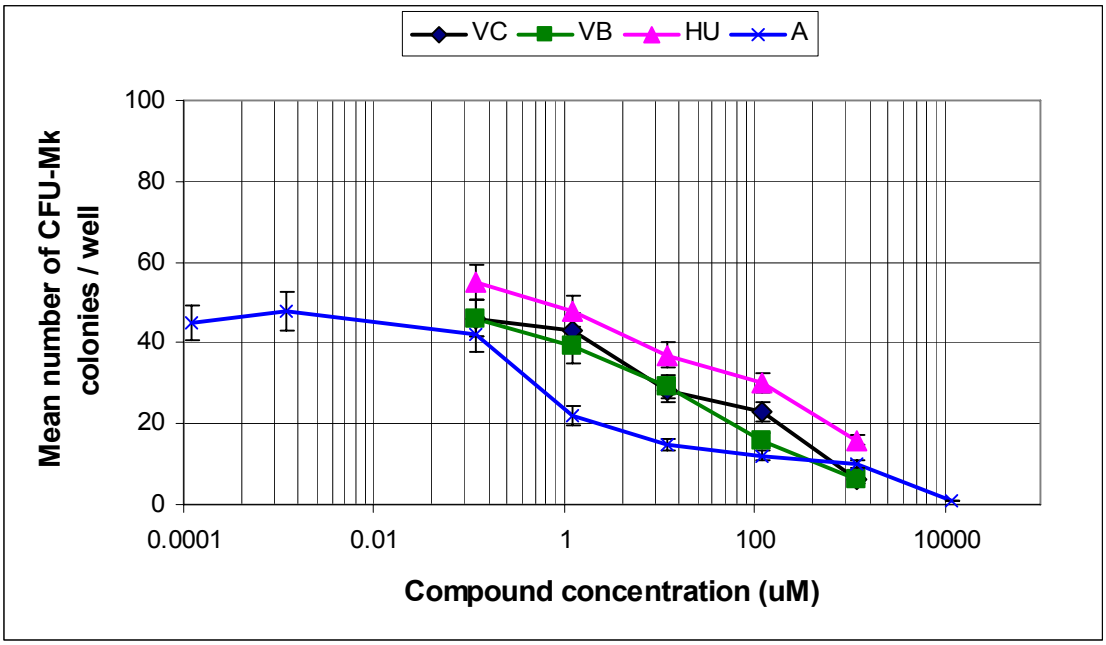


Figure 3. 9 Change in megakaryocytic colony number after treatment with reference compounds

The change in the mean colony counts following CFU-Mk culture treatment with vehicle control (2% (v/v) DMSO), Vincristine (VC), Vinblastine (VB), Hydroxyurea (HU), and Anagrelide (A) A concentration dependant decrease in CFU-Mk colony count was noted. AN, VB and VC demonstrated specificity towards the megakaryocytic lineage with HU demonstrating the least specificity to the megakaryocytic lineage. Mean colony counts from 3 separate experiments, with each concentration evaluated in triplicate. Data shown as Mean +/- SEM. Mean vehicle control (for all reference compounds) CFU-Mk colony count 44 (+/- 19).

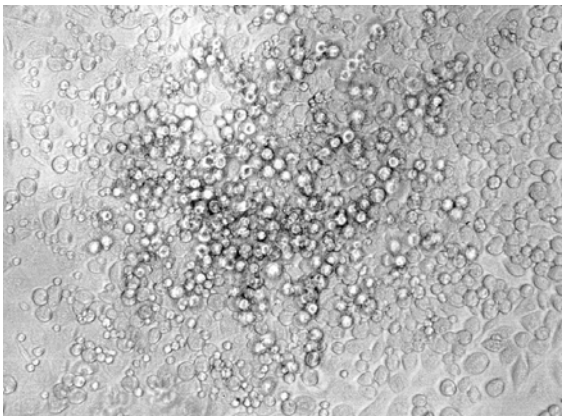


Figure 3. 10 Murine Granulocytic/ Monocytic Colony

A phase contrast of methylcellulose based culture-showing star shaped CFU-GM colony (objective x 100)

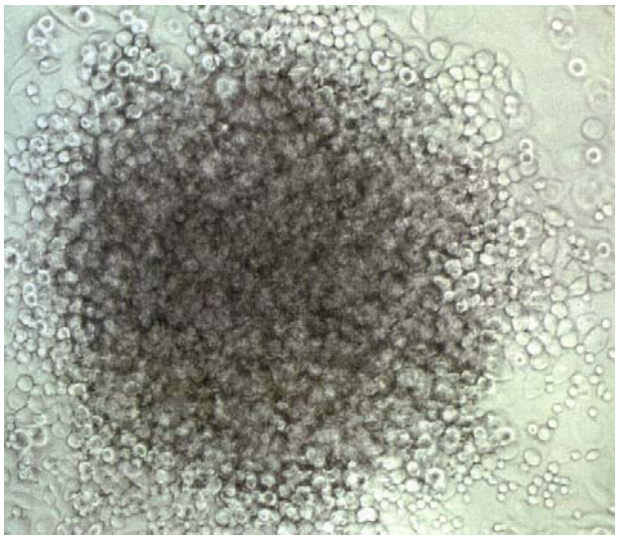


Figure 3. 11 Murine Erythroid Colony

A phase contrast of methylcellulose based culture showing spherical BFU-E colony of cells with slight red pigmentation (objective x 100)

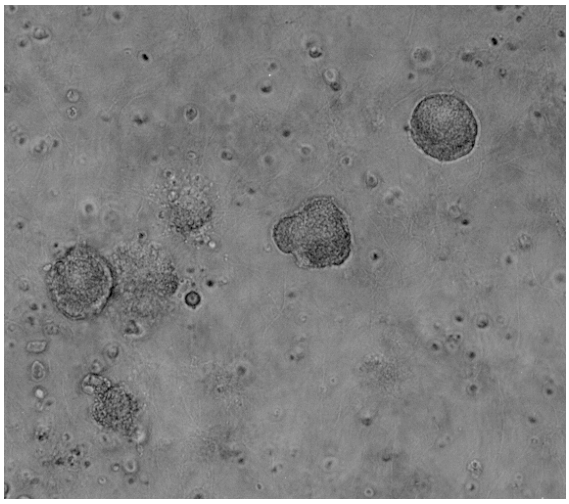


Figure 3. 12 Murine Megakaryocytic Colony

A phase contrast of collagen based culture showing CFU-Mk colony (objective x 100)

Compound	IC_{50} in µM Growth Inhibition of cell lineages
Vincristine	34.3 E = 28 GM < 3.05 Mk
Vinblastine	13.6 GM = 12.2 Mk < 4.34 E
Hydroxyurea	632 Mk < 489 E < 270 GM
Anagrelide	3540 E < 1210 GM < 88.6 Mk

Table 3. 11 Growth Inhibition of the Different Cell Lineages towards the 4 Reference Compounds

The IC50 growth inhibition concentrations of each compound tested and the relative haemopoietic cell lineage sensitivity towards each compound. Mk = Megakaryocytes, GM = Granulocytic/Monocytic E = Erythroid = similar lineage specificity to compound, based on IC 50 concentration < reduced lineage specificity to compound, based on IC 50 concentration. IC50 concentration determined using Robosage (automated curve fitting Microsoft add-in program).

3.4 Discussion

3.4.1 Use of Recombinant Human Thrombopoietin

Culture media containing recombinant human thrombopoietin had been described to support the development of murine BALB/c megakaryocytic colonies by several researchers. Indeed the initial investigations demonstrated that the combination of cytokines IL-3 and Tpo together with the addition of collagen encouraged maturation and differentiation of the haemapoietic stem cells into megakaryocytic lineage. The identity of the colonies seen was confirmed using APAAP staining, with an antibody raised against glycoproteins IIIa/ IIb. Also the use of APAAP negative controls ensure that the positive staining seen was not due to non-specific rather than specific binding to the target antigen. However, the number of colonies grown was

disappointing. Furthermore due to the low colony numbers and the high inter assay variability the evaluation of potential megakaryocytic toxicity would be impossible. Further work was required to increase the number of colonies grown.

3.4.2 The Effect of Purifying the Cell Population and Using Recombinant Murine Thrombopoietin

In this series of experiments a change to the culture protocol was made to try and improve the CFU-Mk colony growth. Additionally, pre-selection of haematopoietic stem cells was introduced to ensure that only totipotent stem cells were added to the culture system. This was achieved by the introduction of positive selection of haematopoietic stem cells (Ckit +) from the mononuclear cell population. More importantly the pre-selection of haematopoietic stem cells standardised the number of progenitor cells added to the cultures on each occasion. The introduction of this change increased the number of colonies from a maximum of 18 in the initial experiments to 31 CFU-Mk colonies, a 1.7-fold increase. Also the inter assay variation of colony counts per well was reduced to within +/- 2SD. Once again, the APAAP staining enabled confirmation of the identity of the cell lineage.

3.4.3 The Effect of Murine Interleukin-6

Murine interleukin-3 (mIL-3) and murine interleukin-6 (mIL-6) are reported to work in synergy to increase the number of megakaryocytic colonies (Drayer et al, 2000). This series of experiments showed that the addition of mIL-6 leads to a further statistically significant increase in the number of CFU-Mk colonies.

To achieve a robust culture system with optimal maturation and differentiation of the haematopoietic stem cell into the megakaryocytopoietic cell lineage, pre-selection of haematopoietic stem cells for addition to the cultures, use of murine thrombopoietin and murine IL-6 and IL-3 were demonstrated to be essential.

The work carried out on CFU-Mk clonogenic culture method prior to its use in the evaluation of ET reference compounds demonstrates the importance of ensuring that the method irrespective of the manufacturers' reassurances is fully validated prior to use. A similar

validation exercise was carried out on the murine CFU –GM and BFU-E clonogenic method, prior to the initiation of this thesis.

3.4.4 Lineage Specific Haematopoietic Effects, using Essential Thrombocythemia (ET) Treatment Compounds

The GM/E and Mk clonogenic haematopoietic cultures enabled the evaluation of the haematotoxicity of compounds towards the granulocytic/monocytic, erythroid and megakaryocytic cell lineages. The IC50 data and the ranking order demonstrated that some of the compounds evaluated had shown selective toxicity towards specific cell lineages. Anagrelide was most specific (in terms of inhibiting colony numbers) towards the megakaryocytic lineage and the least towards the granulocytic/monocytic and erythroid (myeloid) cell lineage. The reduction in colony count of CFU-GM and BFU-E was not very marked and in order to determine the IC50 concentrations for these lineages the concentration range used was extended. Vincristine also demonstrated a specificity (in terms of inhibiting colony numbers) towards the megakaryocytic lineage, whereas vinblastine and hydroxyurea showed specificity (in terms of inhibiting colony numbers) towards the granulocytic /monocytic and erythroid cell lineages, respectively. The difference in lineage specificity between vincristine and vinblastine is surprising, particularly as both are routinely used in the treatment of ET. Further work would be required to determine the reasons for this difference.

In addition, to fully validate the murine CFU-Mk clonogenic assay it would have been of value to include compounds that are not used in the treatment of ET, but have, in vivo, been shown to cause reduction in platelet counts as well as compounds that are generally known not to cause haematotoxicity. A further validation of the CFU-Mk clonogenic assay was carried out by a sandwich student. In this validation, a further 7 compounds were evaluated including AZT, chloramphenicol, methotrexate as well as vincristine and vinblastine (data not shown in the thesis). The fuller validation demonstrated chloramphenicol was the least specific towards the megakaryocytic lineage followed by methotrexate. Vincristine once again demonstrated the greatest sensitivity towards the megakaryocytic lineage.

Limited *in vitro* GM/E and MK clonogenic human culture data is available with any haematotoxic compounds let alone compounds used for the treatment of ET. A similar lineage specificity to that demonstrated in the *in vitro* short -term murine haematopoietic cultures is found, although the culture conditions and compound exposure times varied (Oertel MD 1998).

All of the compounds tested are known to decrease megakaryocyte and myeloid cell lineages *in vivo* in man (Gibbs and Sorensen 2000; Moss et al 1999; Stubbs and Swaney 1996; Tefferi, 2006; Petrides, 2006). Both anagrelide and vincristine have been shown to possess the most potent megakaryocyte inhibiting properties clinically. Clinical trial data demonstrated that AN doses ranging between 1-3mg given every 8 hours for 6 to 9 days was caused a profound reduction in platelet count with a nadir of 20 x10 ^{9}/ l occurring within 10 and 12 days of commencement of treatment (SBA, 1997). The mean Cmax at 2mg/kg (once daily) was 23.6nM (734ng/mL). The murine CFU-Mk colony IC50 concentration 88.6μM was 3,754 times higher than the human *in vivo* concentration at a dose of 1mg/kg. This disparity is indicative of AN having a greater affinity towards the human c-mpl (TPO) receptor than the murine c-mpl (TPO) receptor as suggested by McCarty et al (2006).

Hydroxyurea and vinblastine although used to treat ET, are known to show greater specificity to the myeloid cell lineages (Gibbs and Sorensen 2000: Tefferi, 2006). Therefore, the current results from *in vitro* assays agree with the lineage specificity noted in humans *in vivo*.

The mean Cmax concentration of HU at a clinical dose of 50mg (total human dose) is 8.42mM (3.1mg/mL) (Nicholas et al, 1998). This concentration is 13 fold greater than the murine megakaryocytic IC50 concentration. In this case greater sensitivity to inhibition of Mk colony growth was demonstrated by the murine haematopoietic cells, suggesting that murine haematopoietic cells are more sensitive to HU. Unfortunately, exposure data following in vivo dosing in ET patients with vinblastine and vincristine is not available for comparison with the in vitro data generated in this thesis.

The cultures may be capable of some metabolism primarily through the action of macrophages, although there number would be very low following positive selection for pluripotent stem cells prior to addition to clonogenic cultures. However, media from the treated cultures were not collected for the determination of concentration of the ET reference compounds at the end culturing period. Therefore a clear understanding the dynamics of the test compound in the cultures is not known. It is important to note that the semi-solid methyl cellulose based media used in the clonogenic cultures would also make treatment concentration checks at the end of the culturing period difficult as well as affect the homogeneity of the compound concentrations.

In summary, the current results indicate that *in vitro* murine GM/E clonogenic cultures could potentially aid in the ranking of new drug candidates and may facilitate compound safety evaluation.

Chapter 4

4.1 Experimental Part 2: Long Term Bone Marrow Cultures (LTBMC)

In LTBMC most components of haematopoiesis are reproduced *in vitro*. In fact the unique feature of these cultures is that sustained haematopoiesis occurs in the absence of added growth factors but is absolutely dependent upon the establishment of an adherent layer of bone marrow derived stromal cells. The stromal cells provide the appropriate environment to promote the survival, self-renewal, proliferation, and differentiation of the haematopoietic progenitor cells (Keil et al, 2000).

The formation of the multi- adherent cell layer takes place in four to six weeks of culture. Primitive haematopoietic cells added to the developed stromal layer migrate beneath and between the stromal cells where, in intimate association with the stromal cells, haematopoiesis is established (Daniel, 1989). Furthermore the most immature cells remain within the adherent layer and as these cells divide and mature some of the progeny are released into the growth medium. Once the stromal layer is developed, freshly harvested, pre-selected progenitor cells from primary bone marrow can be added to the cultures. It is the proliferation and development of these freshly harvested cells that are supported by the already established stromal layer. Weekly collection of non-stromal cells enables the determination of their cell lineage by GM/ E and Mk clonogenic cultures and / or flow cytometry analysis.

Thus, LTBMC provides a unique system with which to investigate effects on several aspects of haematopoiesis that can not be achieved using just clonogenic assays. For example, cell adhesion interactions that bind progenitor cells and their progeny to specific stromal elements as well as the synthesis, secretion, and presentation of growth-stimulatory and growth-inhibitory factors by stromal cells can be studied (Daniel et al, 1989).

4.1.1 In Vitro Characterization of Cord Blood Cells

Cord blood has been found to contain high concentrations of haematopoietic stem cells, higher than that found in adult bone marrow (Broxmeyer et al, 1992). It has also been demonstrated that both term and pre-term umbilical cord contain a significantly higher number of early and lineage committed progenitor cells than in adult peripheral blood (Broxmeyer et al, 1990). *In vitro* assays conducted with human cord blood derived haematopoietic stem cells give rise to all the cell lineages, when exposed to the appropriate cytokine cocktails (Huss, 1993). Comparison of cord blood and bone marrow *in vitro* cell lineages demonstrated that most colonies were monocyte/macrophage colonies (60.0 +/- 4.6%) in cord blood derived cultures, whereas those from bone marrow were largely granulocyte colonies (88.3 +/- 6.2%). The differences in growth curve, colony size, and turnover state of CFUs between cord blood and bone marrow seemed, in part, to result from the dominance of monocyte/macrophage colony-forming cells in cord blood. The cell cycling rate of CFUs in cord blood was remarkably lower than that in bone marrow (De Bruyn et al, 1995; Hofmeister et al, 2007). From these results, it seems clear that CFUs in cord blood may differ in some aspects from those of bone marrow derived haematopoietic cells. Overall, however, the cord blood derived stem cells are considered to be representative of bone marrow derived stem cells.

Comparison of the capacity to differentiate *in vitro* into various cell lineages derived from frozen (cryopreserved) and fresh cord blood cells have also been conducted. The results suggest that morphologically and phenotypically (surface cell antigen expression) the colonies from fresh and cryopreserved cord blood stem cells are identical (Kobylka, et al, 1998; Hofmeister et al, 2007).

4.1.2 Haematopoietic cell phenotype determination by flow cytometry

In the early 1980's surface antigens, were identified on the outer and inner surface of all leukocytes. Furthermore some of these surface antigens were specific for specific cell populations. Monoclonal antibodies to the surface antigens are commercially produced and assigned a so-called 'cluster of differentiation' (CD) specific for the cell type and /or function of the antigen (Bernard, 1984). The antibodies are conjugated to fluorescent molecules such

as Fluorescein isothiocyanate (FITC). These fluorescent molecules have the ability to absorb light of specific wavelengths and simultaneously emit light of longer wavelength. Furthermore the emission wavelengths are specific for the fluorescent probes. Flow cytometry exploits the light absorption and emission properties of the conjugated antibodies to enable rapid analysis of the surface characteristics of a large number of cells. Additionally the use of a cell fixative at the end of the procedure stabilizes the binding.

Flow cytometry enables the simultaneous measurement of several characteristics of cells in suspension, as they flow through a fluidic stream. Cells are aspirated into the cytometer at a set rate (60µl /minute) and hydrodynamically focused so that each single cell passes through an argon laser beam, sensing point (Delude, 2005). Light that is reflected and refracted by the cell enables determination of the relative size and relative complexity of the cell. Additionally light spontaneously emitted by the attached fluorochrome (conjugated antibody) is detected by wavelength band specific detectors. The data from these detectors enables the determination of the percentage of cells expressing the specific surface antigen.

4.1.3 Aims of the Investigation

The aim of this investigation was to characterize the cells in the human cord blood (hCB) and murine LTBMC during the stromal development phase and after the confluent stromal layer was inoculated with haematopoietic stem cells (CD117 positive cells). Furthermore, both the adherent (stromal) cells and the non-adherent cells from the LTBMC were characterized, using light microscopy and flow cytometry.

4.2 Methods

4.2.1 Murine Long Term Bone Marrow Cultures-Standard Conditions

The method employed is described in chapter 2 (section 2.2.5) and the aspiration of murine bone marrow, pre-selection of mononuclear BM cells and the selection of CKit positice cells are described in Appendix A.

4.2.3 Murine LTBMC

The method employed is described in chapter 2 (section 2.2.5)

4.2.4 Cytospin Technique

The non-adherent cells were cytospun onto microscope slides as described in Chapter 2 (section 2.7).

4.2.5 May Grunwald-Giemsa Staining For Light Microscopy

The cytospun slides were stained with May-Grunwald-Giemsa as described in Chapter 2 (section 2.2.10).

4.2.6 Murine Long Term Bone Marrow Cultures - Modified Conditions

In a series of experiments 10μL of IL-6 (5ng/mL) and 100μL of SCF (25ng/mL) were added to the Myelocult media. The remaining procedure was the same as described above. The addition of the cytokines was to reduce the variability of these cytokines present in the Myelocult media. Both these cytokines have been identified to promote healthy stromal cell development (Hauser et al, 1997). Additionally SCF has been shown to aid in the rapid establishment of the stromal layer, and reduce the decline in progenitor cell content (Dunlop et al, 1993).

4.2.7 Flow Cytometry Analysis

The antibodies (markers) were selected to enable identification of the lineage specificity of the non-adherent murine and hCB derived cells, as shown in Table 4.1. and 4.2, respectively. Two markers conjugated to different fluorescent tags were analyzed in the same preparation (Table 4.1). The method employed for the cell surface antigen staining is described in Chapter 2 (section 2.6)

Antibodies	Target	Fluorescent tag	Quantity Added (Conc)	Tube
Rat anti- mouse CD117 PE	Totipotent Stem cell	FITC/Green	10µL (5µg/mL)	1, 2 and 3
Rat anti- mouse Mono/Macro(F4/80) /FITC	Early mono/macro precursor	PE/Orange	10µL (5µg/mL)	1
Rat anti- mouse Erythroid (ter119)/FITC	Early erythroid precursor	FITC/Green	10µL (5µg/mL)	2
Rat anti- mouse Granulocyte precursor/FITC	Early granulocyte precursor	PE/Orange	10µL (5µg/mL)	3

Table 4. 1 Lineage specific markers

The antibodies used to identify the murine cell lineages present within the non-adherent layer of murine LTBMC are listed. In addition the lineage specificity, fluorescent tag, quantity of antibody added to each tube is shown.

Antibody	Tube	Concentration of Antibody (µg/mL)	Expression/Target
CD34/FITC	1 ,2 and 3	5	Stem cell
CD33/PE	2	5	Mk Glycoprotein IIIb Part of GPIIa/IIIb complex
CD Cmpl/PE	3	5	GP1 receptor
CD erythroid /PE	4	5	Erythroid receptor

Table 4. 2 Antibodies for Receptor Expression

The antibodies used to identify the hCB cell lineages present within the non-adherent layer of hCB LTBMC are listed. In addition the lineage specificity, fluorescent tag, quantity of antibody added to each tube is provided.

4.2.8 Murine GM/E and Mk clonogenic cultures

A series of murine GM/E/ Mk clonogenic assays were used to evaluate the clonogenic ability of the non-adherent cells collected weekly after the inoculation of the confluent stromal layers with C117 positive cells. The culture preparations and conditions employed are those previously outlined in chapter 2. (section 2.2.1). 0.3mL (1 x10^5/mL) of non-adherent cells was added to the Methocult medium (for the evaluation of erythroid and granulocytic cell lineages) and 0.3mL (1 x10^5/mL) of non-adherent cells was added to murine Megacult media

(for the evaluation of megakaryocytic cell lineage) as described in Chapter 2 (section 2.2.4). Cell count was determined using an automated cell counter (Advia 120, Bayer, USA).

4.2.9 Preparation of cryoperserved mononuclear hCB

The method employed is described in Appendix A.

4.2.10 Viability Cell Count

Cord cell viability was determined using the method described in Chapter 2 (section 2.2.8).

4.2.11 Human LTBMC Medium

Human cord blood cultures were utilised as described in chapter 2 (section 2.2.5).

4.2.12 Inoculation of Confluent Stromal Layer with Fresh hCB Stem Cells (CD34 Positive)

Once a confluent stromal layer was established, the stromal layer was 'seeded' with fresh hCB cells. The method employed is described in Chapter 2 (section 2.2.5).

4.2.13 hCB Clonogenic Cultures

hCB clonogenic cultures as described in Chapter 2 were utilized in these experiments (section 2.2.5).

4.2.14 Collection of hCB Derived CFU-Mk Colonies

Once the CFU-Mk cultures had been incubated for 12 days (culturing method and treatment with AN as described above), the culure medium was placed into BD 25mL culture tubes. 10 mL of fresh media was added and the tubes were centrifuged for 20 minutes at 400g. After centrifugation, the cells were removed washed once more as already described before carrying out cell counts, cell cycle analyses, ploidy analyses, cell surface receptor evaluation and microtubule analyses.

4.3 Results

4.3.1 Murine LTBMC Using Standard and Modified Culture Conditions (Falcon Flasks)

Freshly aspirated mouse bone marrow-derived mononuclear cells were cultured under standard and modified conditions. All the cultures were set-up in Falcon T25 culture flasks, as recommended in the original method published by Dexter et al (1978). Examination of the developing stromal layers using an inverted microscope at weekly interval revealed that the cells did not adhere to the Falcon flasks and by the end of six weeks a confluent stromal layer had not been achieved, regardless of whether standard or modified culture conditions had been followed. The cells in the Falcon flasks were spherical in shape, with an occasional cluster of cells floating in the liquid culture media (Figures 4.1 and 4.2). Additionally the spindle-shaped cells (fibroblast-like cells) and flattened cells (macrophages) described by Dexter (1974) as being predominant components of the developing stromal layer were not observed.

To ensure that the mononuclear bone marrow cells added to the LTBMC contained fibroblast-like cells and macrophages the standard GM/E clonogenic culture was set-up. Within four days of culturing spindle -shaped cells and flattened cells were observed (Figure 4.3).

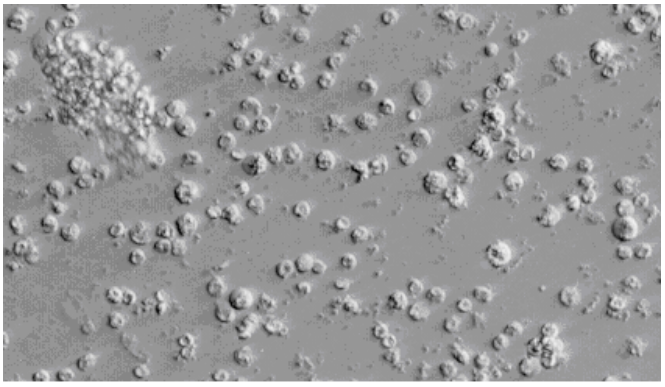


Figure 4. 1 Murine LTBMC

A Hofmann modulation contrast of a murine LTBMC taken on day 21 after the addition of BM derived mouse mononuclear cells, using the standard LTBMC conditions (objective x 400)

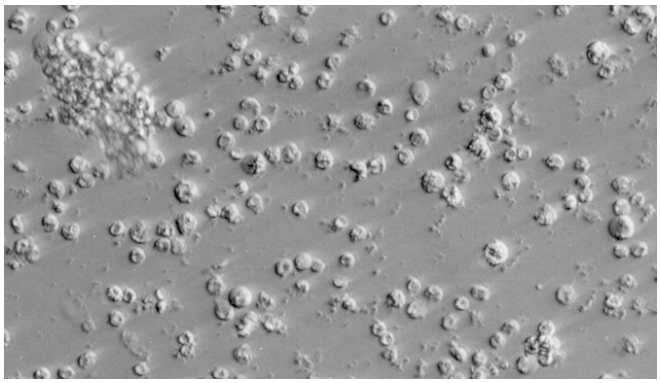


Figure 4. 2 Murine LTBMC

A Hofmann modulation contrast of a murine LTBMC taken on day 21 after the addition of BM derived mouse mononuclear cells using the modified LTBMC conditions (objective x 400)

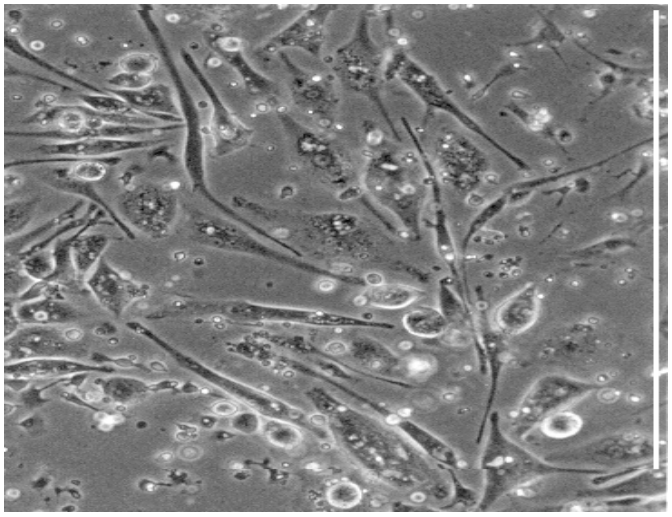


Figure 4. 3 Murine GM/E Clonogenic Assay

A phase contrast of Fibroblast-like and flattened cells were visible after 14 days of culturing (objective x200)

4.3.2 Visualization of Murine LTBMC (Standard Method), Using Nunc Culture Flasks

In this series of experiments, the stromal layer did not firmly attach to the Falcon flask surface using the culture conditions and identical flask manufacturer as described by Dexter et al (1976). Confluent stromal layer formation is dependent upon good adhesion between the adherent cells and the plastic culture vessel surface. In this series of experiments, several

components of LTBMC techniques were evaluated:

- Murine LTBMC were established using the standard culture conditions, but the media mixture was placed into Nunc T25 flasks. The change in flask manufacturer was to evaluate if stromal development could be achieved by changing the plastic source. Photographs were taken weekly of the developing stromal layer, until a confluent stromal layer was obtained.
- During the stromal layer development phase, between 9.0 and 9.5 mL of culture media was changed at weekly intervals. The cells present in the media (non-adherent cells) removed from the cultures were counted. The cells were also cytospun onto glass slides, fixed and stained with May Grunwald Giemsa, in order to morphologically identify the cells. Photographs of the stained cells were also taken.

- Once a confluent murine stromal layer was established, the stromal layer was 'seeded' with fresh enriched pluripotent stem cells (CD117 positive) and maintained for a period of 4 weeks. At weekly intervals, 9.0 -9.5 mL of the media was removed and replaced with fresh culture medium. The non -adherent cells, present in the media removed from the cultures, were counted and plated into semisolid culture medium (cell concentration 1x 10^4 / ml per plate), to evaluate differentiation into the granulocytic / monocytic and erythroid (myeloid) and megakaryocytic colonies after 10-12 days (short-term cultures).
  On each occasion 3 murine LTBMC were set up and the evaluation conducted on three separate occasions.

4.3.3 Examination of Developing Murine Stromal Layer Using an Inverted Microscope

The developing murine stromal layers were examined weekly, by phase contrast, using an inverted microscope. By the end of the first week, all the flasks were found to contain islands of cells. Also, spindle-shaped cells (probably fibroblast-like cells) and flattened cells (probably macrophages) were observed in addition to round cells (probably progenitor cells), which were closely associated with the adherent cells.

Small and well-haemoglobinized clusters of erythroid cells that were loosely attached to adherent cells were also seen. However, after the first two weeks in culture, these erythroid clusters were no longer found.

At the end of week two, the number of cells attached to the bottom of the flask seemed to have increased. Moreover, by the end of the fifth week, a confluent adherent layer with persistence of islands of small round cells, resembling 'cobble stones', were found in association with adherent cells. Also, by the fourth week, a small number of the spindle-shaped cells began to accumulate small droplets of lipid and were located in different regions of the culture flasks.

The stromal layers achieved between 90 to 95% confluency in all cultures studied, and areas of active haematopoiesis and fat cells were also found irregularly distributed in the culture flasks. Figures 4.4 to 4.9 show the weekly development of the stromal layer.

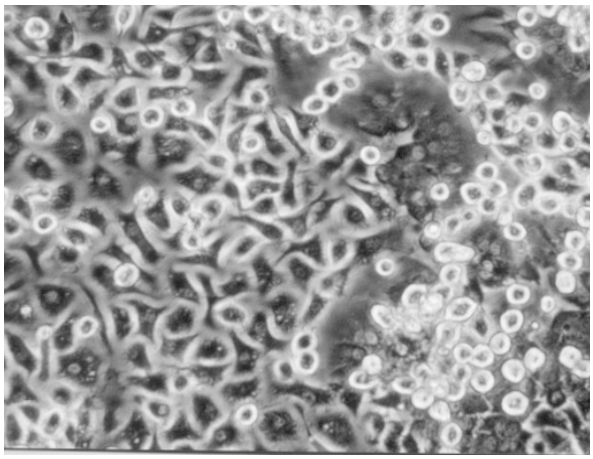


Figure 4. 4 Murine LTBMC: Week 1 of Stromal Layer Development

A phase contrast of murine LTBMC stromal layer fibroblast-like and flattened cells, 1 week after initiating the culture, using the standard murine LTBMC method (objective x200)

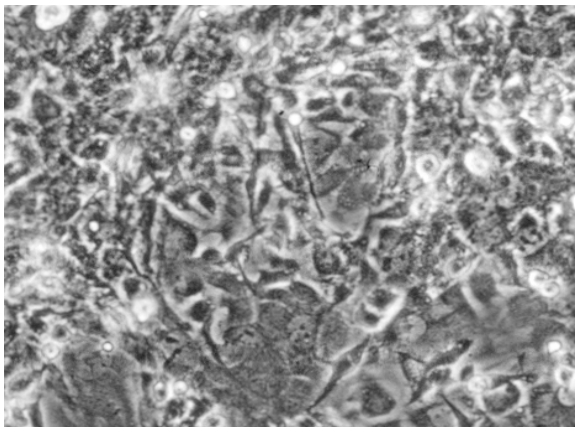


Figure 4. 5 Murine LTBMC: Week 2 of Stromal Layer Development

A phase contrast of murine LTBMC stromal layer fibroblast-like and flattened cells, on week 2, using the standard murine LTBMC method

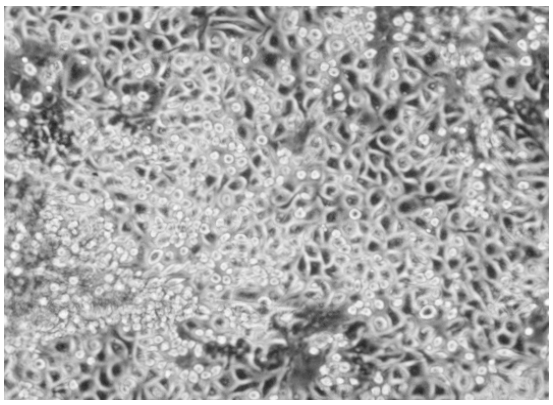


Figure 4. 6 Murine LTBMC: Week 3 of Stromal Layer Development

A phase contrast of murine LTBMC stromal layer showing an increase in stromal layer confluency on week 3, using the standard murine LTBMC method (objective x100)

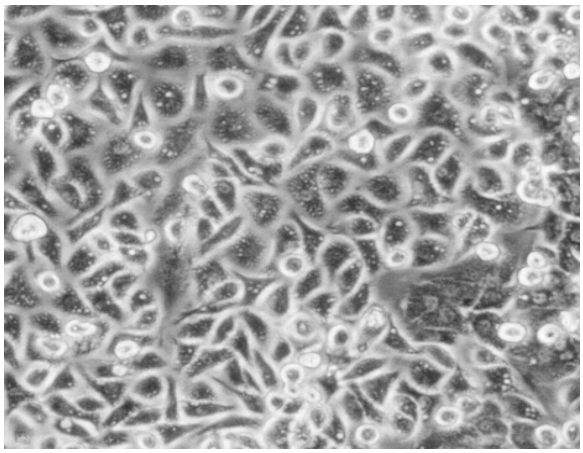


Figure 4. 7 Murine LTBMC: Week 4 of Stromal Layer Development

A phase contrast of murine LTBMC stromal development at week 4; Islands of progenitor cells can be noted in the stromal layer (objective x400)

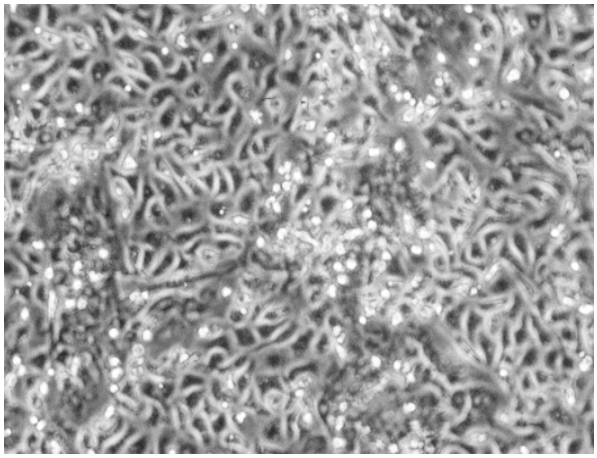


Figure 4. 8 Murine LTBMC: Week 5 of Layer Stromal Development

A phase contrast of a murine LTBMC stromal development, following 5 weeks of culturing. A confluent stromal layer is shown. (objective x200)

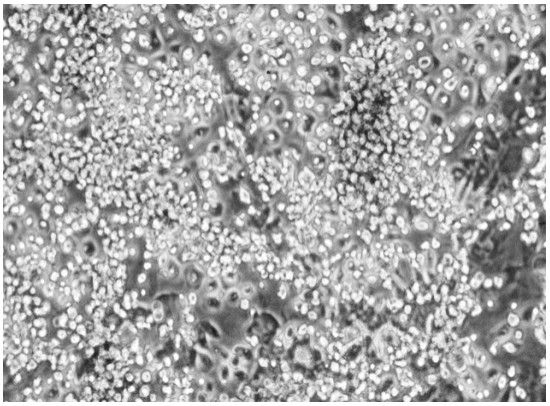


Figure 4. 9 Murine LTBMC: Week 6 of Stromal Layer Development

A phase contrast of murine LTBMC stromal development at week 6 of culturing. Large 'cobblestone' area care clearly visible (objective x100)

4.3.4 Characterization of the Murine Non-Adherent Layer During Stromal Layer Development

The weekly total cell counts of non -adherent murine cells declined steadily during the stromal layer development phase (Figure 4.10). As almost all non-adherent cells were removed each week, the data show that the cultures still produce non-adherent cells, but at a declining rate, reaching a plateau of $2.6x10^4$ cells per week. The weekly non-adherent cell counts are shown in Table 4.3.

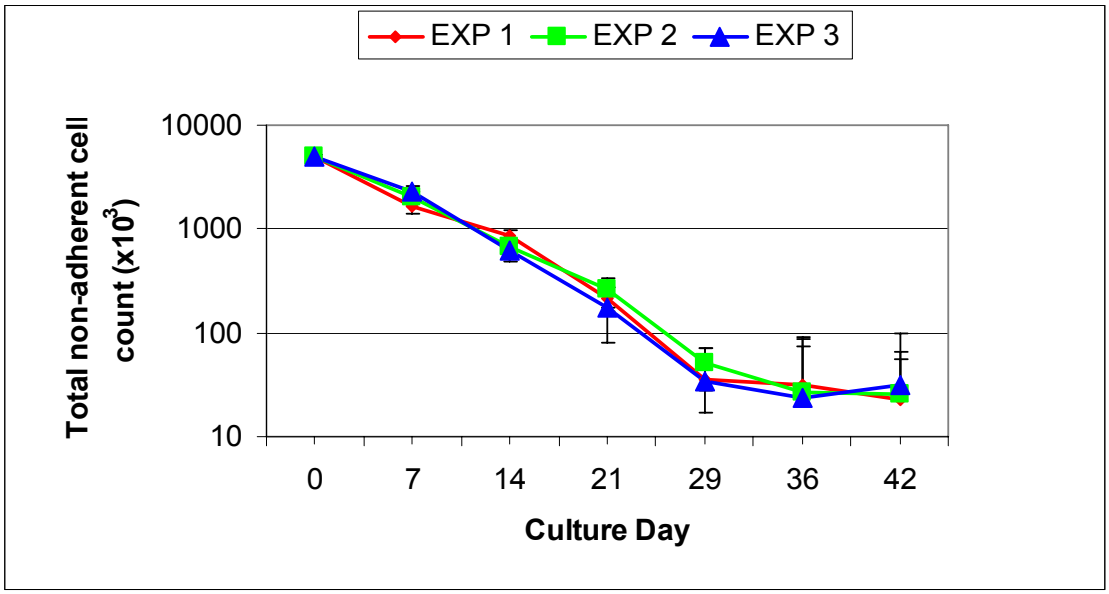


Figure 4. 10 Murine Stromal Layer Development: Mean Total Cell Counts Of Non-Adherent Cells Collected

The weekly total cell counts of non -adherent cells, during murine LTBM culture stromal cell development. A steady decline in non-adherent cell numbers is noted over time. Mean data from three separate experiments with each concentration evaluated in triplicate. Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure

Exp. No	Culture No.	Cell count of non-adherent Cells in murine LTBMC (x10³/L)						
		Day 0	Day 7	Day 14	Day 21	Day29	Day36	Day 42
1	1	500	173	85	31.4	1.0	3.0	3.1
	2	500	141	63	21.4	4.7	2.1	1.1
	3	500	192	51	12.5	4.9	4.4	2.3
2	1	500	201	63.7	35.4	6.2	3.1	3.1
	2	500	147	49.8	24.9	4.9	2.1	1.2
	3	500	271	88.7	17.4	3.9	2.5	3.1
3	1	500	264	68.2	14.6	2.9	3.2	2.6
	2	500	198	51.9	11.3	3.6	2.2	3.1
	3	500	213	65.3	26.3	3.6	1.6	3.4

Table 4. 3 Murine Stromal Layer Development: Mean Total Cell Counts Of Non-Adherent Cells Collected

The weekly total cell counts of non -adherent cells, during murine LTBM culture stromal cell development. A steady decline in non-adherent cell numbers is noted over the 42 day period.

4.3.5 Morphological Identification of Murine Non-Adherent Cells During Stromal Layer Development

The non-adherent cells collected during the stromal layer development, were cyto-spun, fixed and then stained with May Grunwald Giemsa, in order to morphologically identify the cells.

The numbers of each of the individual cell types identified declined with time in culture, but at different rates (Figure 4.11). The percentage and absolute cell counts of the different lineages collected are shown in tables 4.4 and 4.5. The steepest decline of the nucleated cells was observed for the mononuclear cell population. This term is used to describe cells that were difficult to distinguish morphologically into any of the specific cell types. Also the shallowest decline was noted for the monocyte/macrophage cell populations. Erythrocytes were observed at day 7 only. The numbers of all the other cell types (promyelocytes, pro-monocytes, metamyelocytes and mono/macrophages) plateaued by day 35. This differential decline leads to a change in percentages of the different cell types, as shown in Figure 4.12, with the monocytes/macrophages being by far the most prevalent cell type after Day 28 in culture. Photographs taken of the cyto- spin preparations confirm the cell types counted and examples are shown in Figures 4.13 and 4.14.

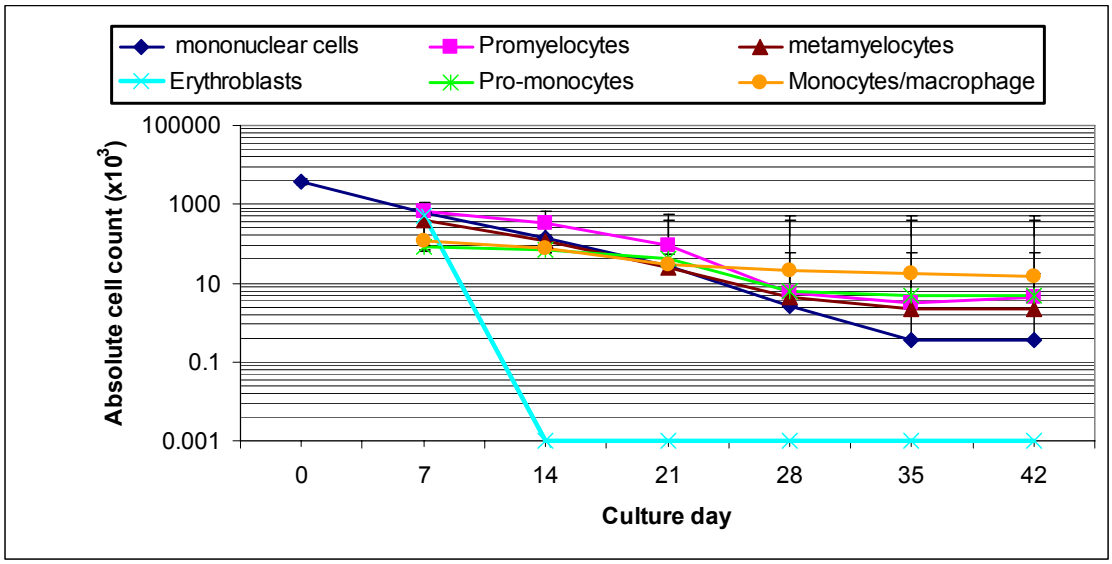


Figure 4. 11 Murine Stromal Layer Development: The Absolute Count of Different Cell Types Collected in the Non-Adherent Layer

The mean absolute counts of the different cell types within the non-adherent cells identified by cytospinning the cells onto microscope slides and staining with May Grunwald Giemsa are shown. The numbers of each of the individual cell types identified declined with time in culture, but at different rates. The steepest decline of the nucleated cells was observed for the mononuclear cell population. The shallowest decline was noted for the monocyte/macrophage cell populations. Erythrocytes were observed at day 7 only. The numbers of all the other cell types (promyelocytes, pro-monocytes, metamyelocytes and mono/macrophages) plateaued by day 35. Mean data from three separate experiments. Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure

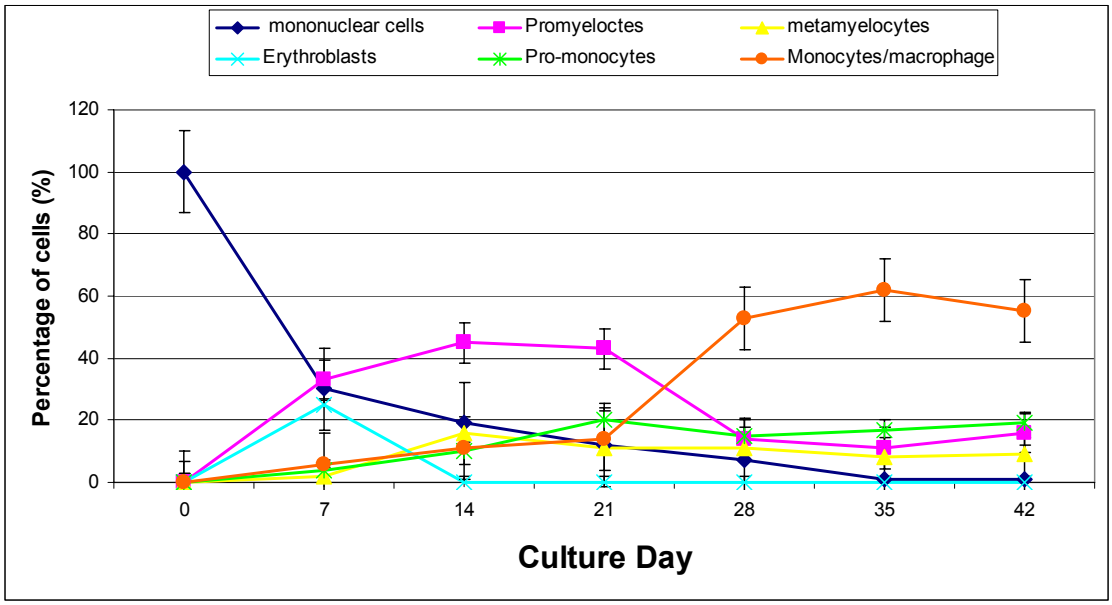


Figure 4. 12 Murine Stromal Layer Development: The Percentage of Different Cell Types Collected in the Non-Adherent Layer, Stained with May Grunwald Giemsa

The mean percentage counts of the different cell types within the non-adherent cells identified by cyto-spinning the cells onto microscope slides and staining with May Grunwald Giemsa are shown. The numbers of each of the individual cell types identified declined with time in culture, but at different rates. The steepest decline of the nucleated cells was observed for the mononuclear cell population. The shallowest decline was noted for the monocyte/macrophage cell populations. Erythrocytes were observed at day 7 only. The numbers of all the other cell types (promyelocytes, pro-monocytes, metamyelocytes and mono/macrophages) plateaued by day 35. Mean data from three separate experiments. Data shown as Mean +/- SEM.

Exp. No	Culture day non-adherent cells collected	Mononuclear cells (%)			Promyelocytes (%)			Metamyelocytes (%)			Erythrocytes (%)			Promonocytes (%)			Monocytes /macrophages (%)		
Culture ID		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	0	92	83	86	0	0	0	0	0	0	0	0	0	0	0	0	8	17	14
2		86	95	77	0	0	0	0	0	0	0	0	0	0	0	0	14	5	23
3		88	81	74	0	0	0	0	0	0	0	0	0	0	0	0	12	19	26
1	7	22	26	24	28	40	47	2	2	2	32	22	17	1	5	2	15	5	8
2		27	30	29	31	44	43	2	4	0	27	17	15	4	2	3	9	3	10
3		33	27	24	33	29	42	3	4	2	24	23	17	7	4	9	0	13	6
1	14	18	31	21	41	45	37	10	13	21	19	0	0	1	0	0	11	11	21
2		22	14	19	36	46	44	14	18	19	16	7	2	0	2	0	12	13	16
3		16	24	18	50	38	48	16	15	15	4	18	12	0	1	0	14	4	7
1	21	22	26	18	48	40	37	18	15	25	0	1	6	0	0	0	12	18	14
2		20	14	13	44	50	49	15	12	17	0	2	0	0	0	0	21	22	21
3		15	20	10	49	43	43	11	19	24	0	0	0	0	0	0	25	18	23
1	28	3	9	11	18	10	21	11	9	17	0	1	0	0	0	0	68	71	51
2		11	4	8	16	13	24	7	15	21	2	0	0	1	0	0	63	56	47
3		13	6	4	11	11	15	11	15	16	0	1	0	0	0	0	65	67	65
1	35	1	2	0	7	10	16	7	11	5	0	1	0	0	0	0	85	76	79
2		1	1	0	12	18	13	6	10	6	0	0	0	0	0	0	81	71	81
3		0	1	1	13	5	10	7	10	9	0	2	0	0	0	0	80	82	80
1	42	1	1	0	16	20	2	9	3	7	0	0	0	0	0	0	74	76	91
2		1	2	0	14	14	11	5	3	6	1	0	0	0	0	0	79	81	83
3		1	1	0	14	7	7	8	4	3	0	0	0	0	0	0	77	88	90

Table 4. 4 Murine Stromal Layer Development: The Percentage of Different Cell Types Collected in the Non-Adherent Layer, Stained with May Grunwald Giemsa

The percentage counts of the different cell types within the non-adherent cells identified by cytospinning the cells onto microscope slides and staining with May Grunwald Giemsa are provided. The numbers of each of the individual cell types identified declined with time in culture, but at different rates. The steepest decline of the nucleated cells was observed for the mononuclear cell population. The shallowest decline was noted for the monocyte/macrophage cell populations. Erythrocytes were observed at day 7 and only very occasionally thereafter. The numbers of all the other cell types (promyelocytes, pro-monocytes, metamyelocytes and mono/macrophages) plateaued by day 35.

Exp. No	Culture day non-adherent cells collected	Mononuclear cells ($x10^3$/l)	Promyelocytes ($x10^3$/l)	Metamyelocytes ($x10^3$/l)	Erythrocytes ($x10^3$/l)	Promonocytes ($x10^3$/l)	Monocytes /macrophages ($x10^3$/l)
1	0	3750	0	0	0	0	0
2		3619	0	0	0	0	0
3		3851	0	0	0	0	0
1	7	593	660	400	507	80	120
2		632	713	415	415	61	143
3		551	632	392	616	102	95
1	14	134	321	115	0	72	77
2		129	410	127	0	51	85
3		145	356	116	0	89	67
1	21	31	124	33	0	68	22
2		27	93	25	0	43	30
3		25	87	23	0	38	44
1	28	1.7	5.1	4.8	0	3.1	15.1
2		2.4	3.8	2.5	0	8.0	34.1
3		3.1	6.0	5.5	0	6.1	21.7
1	35	0.4	2.7	1.3	0	3.1	12.9
2		0.3	3.9	2.0	0	5.3	17.8
3		0.2	4.8	3.5	0	0.1	20.0
1	42	0.3	3.7	1.1	0	8.1	18.4
2		0.3	8.0	3.9	0	3.9	11.4
3		0.3	4.4	2.6	0	5.0	14.8

Table 4. 5 Murine Stromal Layer Development: The Absolute Count of Different Cell Types Collected in the Non-Adherent Layer
The mean absolute counts of the different cell types within the non-adherent cells identified by cytospinning the cells onto microscope slides and staining with May Grunwald Giemsa are provided. The numbers of each of the individual cell types identified declined with time in culture, but at different rates. The steepest decline of the nucleated cells was observed for the mononuclear cell population. The shallowest decline was noted for the monocyte/macrophage cell populations. Erythrocytes were observed at day 7 only. The numbers of all the other cell types (promyelocytes, pro-monocytes, metamyelocytes and mono/macrophages) plateaued by day 3.

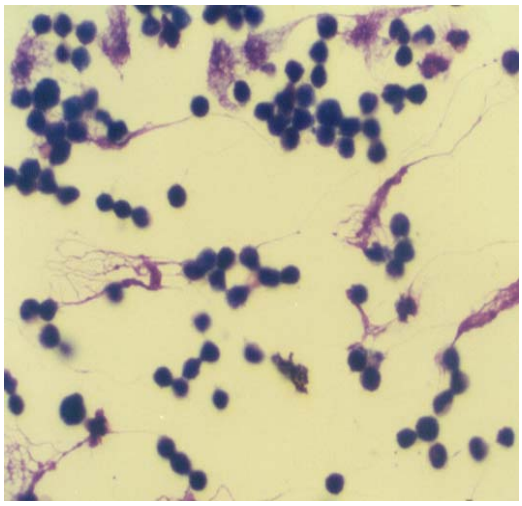


Figure 4. 13 Non-Adherent Cells From Murine Nunc LTBMC: Week 0

May Grunwald Giemsa stained murine bone marrow mononuclear cells on the day of harvest, prior to addition to LTBMC flasks. (Objective x100)

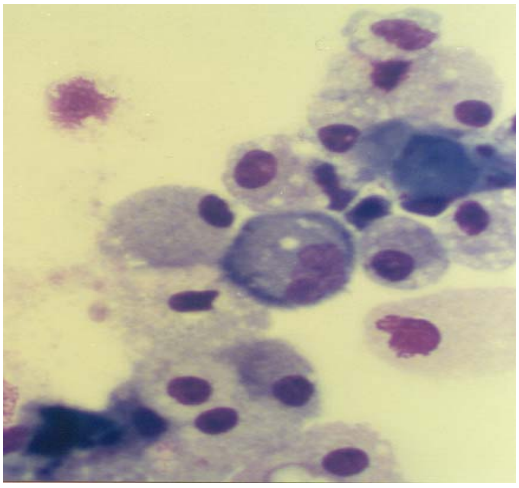


Figure 4. 14 Non-Adherent Cells From Nunc Murine LTBMC: Week 4

May Grunwald Giemsa stained marrow mononuclear cells and monocytes non-adherent cells identified within the non adherent layer at week 4 during stromal layer development (objective x400)

4.3.6 Confluent Murine Stromal Layer Inoculated with Mouse CD117 Positive Cells

At weekly intervals, murine non- adherent cells were collected, counted and plated into *in vitro* short- term cultures to evaluate differentiation into the myeloid and megakaryocytic cell lineages. In all three experiments carried out, the cell count increased from the initial inoculation density of 4.13x 10^4 to about 7.28x 10^6 cells (Figure 4.15). The cell counts are shown in table 4.6. As almost all murine non-adherent cells were removed in the weekly collection process, the data show that the stromal layer is now capable of consistent production of non-adherent cells at a slightly increasing rate. Figure 4.16 shows the cell density in the non-adherent compartment throughout the entire culturing period in the murine LTBMC described here.

Exp. No	Culture No.	Cell count of non-adherent Cells in murine LTBMC (x10^3/L)			
		Day 49	Day 56	Day 63	Day 70
1	1	5.8	6.8	55.1	751
	2	4.6	7.2	66.2	629
	3	3.6	5.1	71.0	471
2	1	2.7	8.2	47.9	458
	2	4.8	4.9	66.8	761
	3	5.9	5.1	71.6	515
3	1	2.1	7.8	50.2	546
	2	5.1	9.1	55.3	457
	3	4.7	43.9	80.3	813

Table 4. 6 The Non-Adherent Weekly Cell Count from Murine LTBMC after Established Stromal Layer was Inoculated with $CD117^+$ Cells

The total non-adherent cell count from murine LTBMC throughout the culturing period is shown. The non adherent cell count decrease during stromal cell development (Day 7 to day 42). Following inoculation with fresh CD117 positive cells the mean total non-adherent cell count increased on day 49 and the number of non-adherent cells was maintained for the rest of the culturing period.

4.3.7 Inverted Microscope Examination of Murine LTBMC

Haemapoietic cells actively involved in maturation and differentiation were seen on top of the stromal cells and appeared as small round cells with a phase-bright appearance and often occurred as large tightly packed foci.

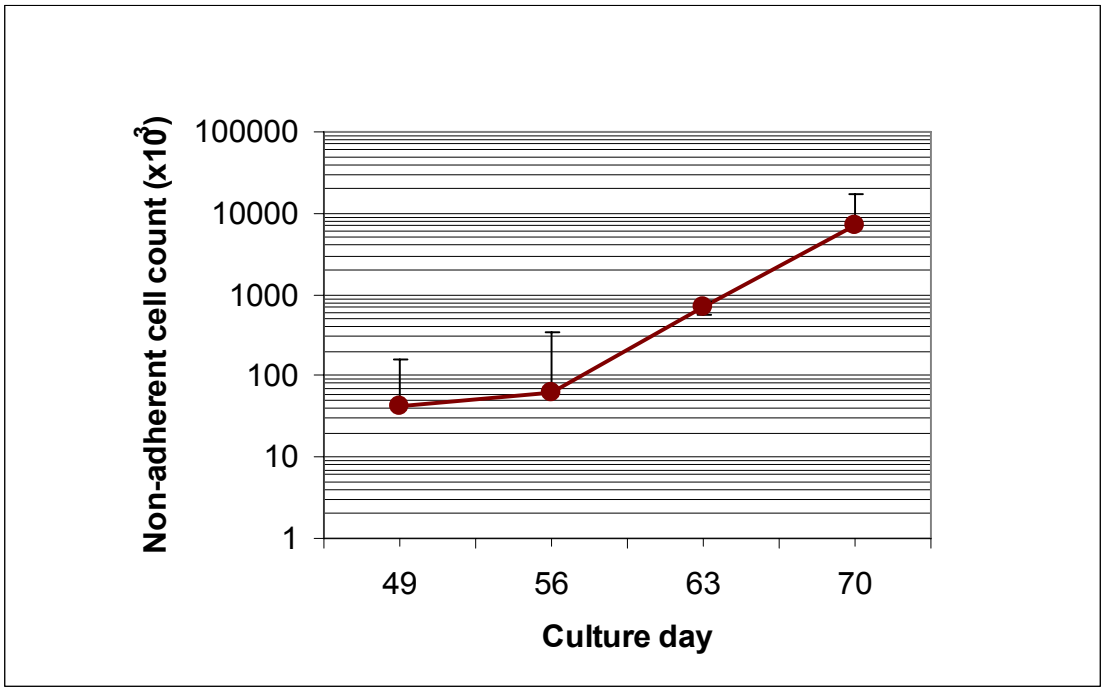


Figure 4. 15 The Non-Adherent Weekly Cell Count From Murine LTBMC After Established Stromal Layer was Inoculated with CD117$^+$ Cells

The total cell count following inoculation with CD117 positive cells onto a confluent stromal layer. The total cell count increase over the 4 week period. Mean data from three separate experiments. Each experiment was carried out in triplicate. Data shown as Mean +/- SEM.

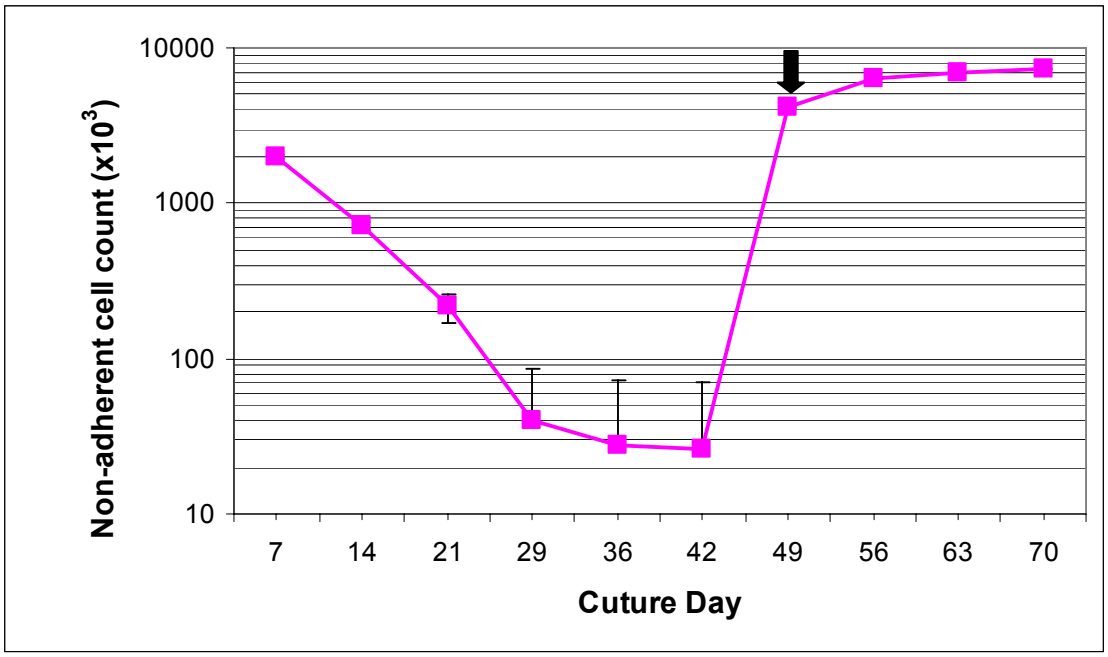


Figure 4. 16 The Mean Non-Adherent Cell Count Of The Murine LTBMC, Throughout The Entire Culturing Period (Day 7-70)

The total non-adherent cell count from murine LTBMC throughout the culturing period is shown. The non adherent cell count decrease during stromal cell development (Day 7 to day 42). Following inoculation with fresh CD117 positive cells the mean total non-adherent cell count increased on day 49 and the number of non-adherent cells was maintained for the rest of the culturing period. Mean data from three separate experiments. Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure

Inoculation with CD117 positive cells ⬇

4.3.8 Murine GM/E and Mk Clonogenic Cultures of Non -Adherent Cells after Inoculation with CD117 Positive Cells

On a weekly basis, the murine non -adherent cells, present in the LTBMC media removed from cultures inoculated with CD117 positive cells, were counted and plated into short- term cultures, and the ability of these cells to differentiate into the granulocytic / monocytic and erythroid and megakaryocytic cell lineages determined. Figures 4.17 to 4.19, show the number of colonies of each lineage grown.

The cells were added to GM/E and Mk clonogenic cultures differentiated into colonies of the erythroid, mono/granulocytic and megakaryocytic lineages, (Figures 4.17 to 4.19). The colony counts are shown in table 4.7. Also all cell lineages showed an increase in colony number with

time with colony numbers reaching a plateau at weeks three and four, with the exception of one megakaryocytic culture at week 3.

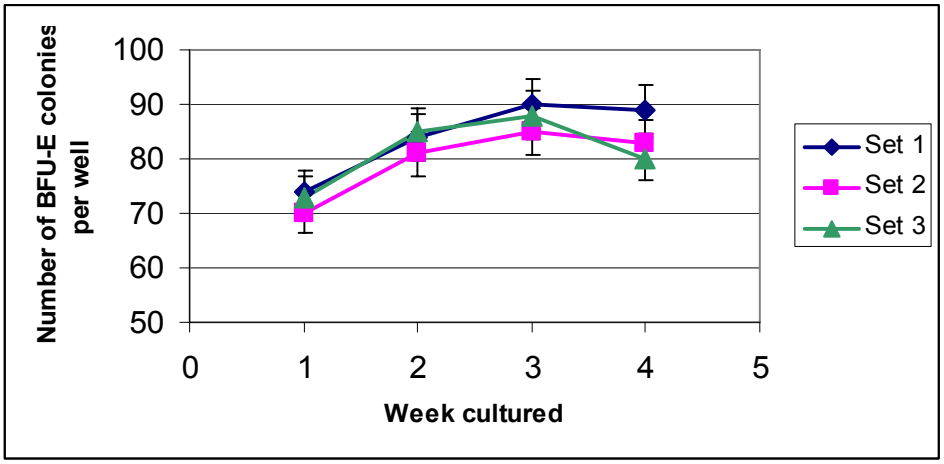


Figure 4. 17 Number of Burst Forming Units-Erythroid (BFU-E) Colonies Grown From Murine Non-Adherent Cells Collected Weekly; After Established Stromal Layer Was Inoculated With CD117^{+} Cells

The mean number of BFU-E colonies from weekly collections of non-adherent cells from murine LTBMC. An increase in BFU-E colony counts with time is noted, with the colony counts reaching a plateau at week 3. Mean data from three separate experiments (sets 1, 2 and 3) are shown. Data shown as Mean +/- SEM.

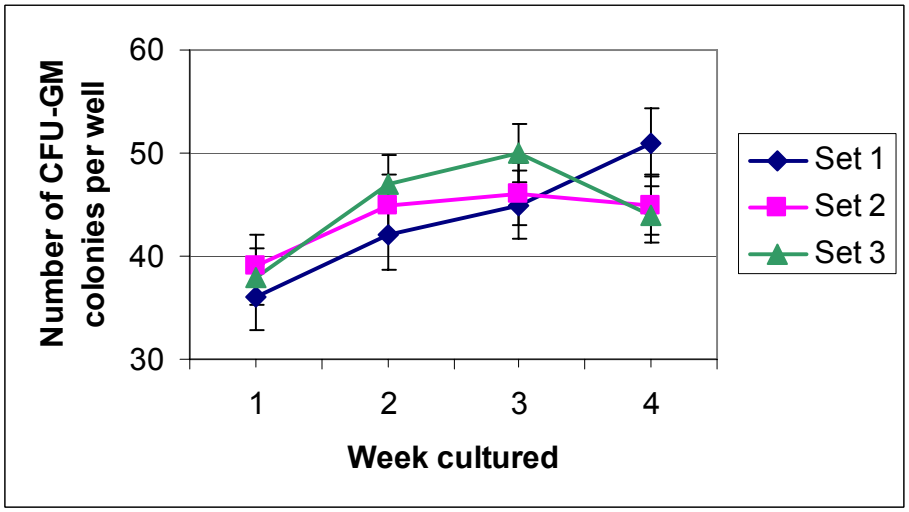


Figure 4. 18 Number of Colony Forming Units-Granulocytic/ Monocytic (CFU-GM) Colonies Grown From Murine Non-Adherent Cells Collected Weekly, After Established Stromal Layer as Inoculated With CD117^{+} Cells

The mean number of CFU-GM colonies from weekly collections of non-adherent cells from murine LTBMC. An increase in CFU-GM colony counts with time is noted, with the colony counts reaching a plateau at week 3. Mean data from three separate experiments (sets 1, 2 and 3) are shown. Data shown as Mean +/- SEM.

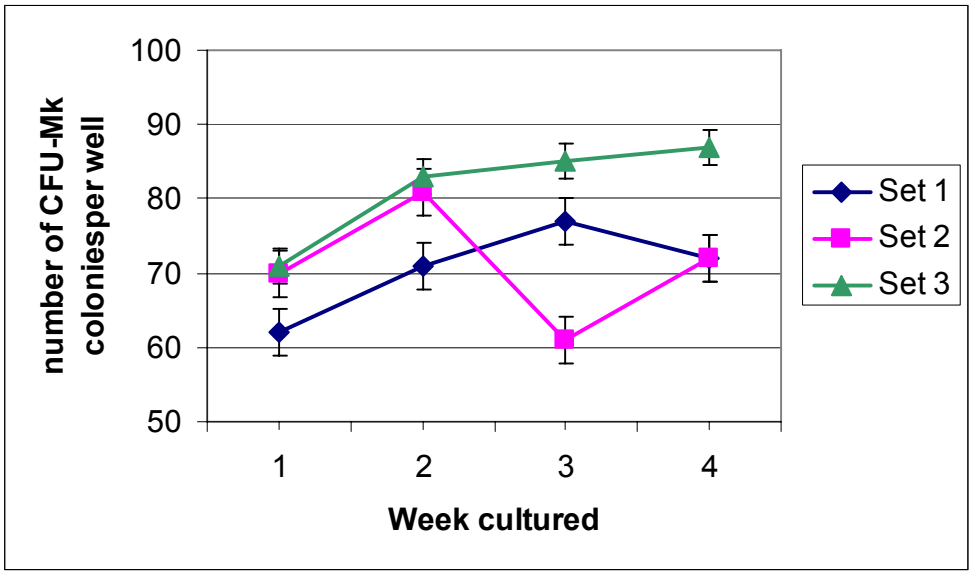


Figure 4. 19 Number of Colony Forming Units-Megakaryocyte (CFU-Mk) Colonies Grown From Murine Non-Adherent Cells Collected Weekly, After Established Stromal Layer Was Inoculated with CD117$^+$ Cells

The mean number of CFU-Mk colonies from weekly collections of non-adherent cells from murine LTBMC. An increase in CFU-Mk colony counts with time is noted, with the colony counts reaching a plateau at week 3 in two out of the 3 experiments. In experiment 2 a reduction in CFU-Mk colony counts is noted on week 3. At week 4 the colony count increases. Mean data from three separate experiments (sets 1, 2 and 3) are shown. Data shown as Mean +/- SEM.

Week of collection	Mean No BFU-E colonies /well			Mean No CFU-GM colonies /well			Mean No CFU-Mk colonies /well		
	Plate No			Plate No			Plate No		
	1	2	3	1	2	3	1	2	3
1	74	70	73	36	39	38	62	70	71
2	84	81	85	42	45	47	71	81	83
3	90	85	88	45	46	50	77	61	85
4	89	83	80	51	45	44	72	72	87

Table 4. 7 Number of BFU-E, CFU-GM and CFU-Mk Colonies Grown From Murine Non-Adherent Cells Collected Weekly; After Established Stromal Layer Was Inoculated With CD117$^+$ Cells

The BFU-E, CFU-GM and CFU-Mk colony counts achieved following harvesting non-adherent cells from murine LTBMC that had been inoculated with fresh CD117 positive cells on week 0. All cell lineages showed an increase in colony number with time with colony numbers reaching a plateau at weeks three and four, with the exception of one megakaryocytic culture at week 3.

4.3.9 Murine LTBMC: Adherence of Stromal Cells During the Stromal Layer Development Period, Using T25 Culture Flasks From Several Manufacturers

As discussed previously, the original LTBMC described by Dexter et al (1976), recommended the use of Falcon flasks. However, one of the problems encountered during the initial part of this project, was the stromal layer did not firmly attached to the Falcon flask surface. This made changing the medium and the separation of the non-adherent cells from the stromal layer impossible. Light microscopy examination of the developing stromal layers, from these initial studies, showed the cultures to be devoid of any fibroblasts. Fibroblasts are an important component of the stromal layer, helping the layer to adhere to the culture flask thus their absence would affect the quality of the resultant stromal layer.

In this current investigation, tissue culture plastic from different suppliers was used in a series of murine LTBMC to elucidate whether different properties of the plastics, both in materials used in the manufacture and method employed to sterilize the flasks, results in differences in the adherence of the developing stromal layer.

Murine LTBMC cultures were initiated in T25 plastic culture flasks from Nunc, Falcon and Costar. The development of stromal layer in the T25 flasks was monitored using light microscopy. Photographs were taken of the developing stromal layer. The non-adherent cells collected during the weekly feeds during the stromal development period from Nunc flasks only, were counted and the cell lineage phenotype of the cells determined, by flow cytometry. On week 6, the confluent stromal layers, in the Nunc flasks, were inoculated with fresh bone marrow. Thereafter for 4 weeks, the cell lineage phenotype of the non-adherent cells collected at weekly intervals was determined by flow cytometry.

4.3.10 Examination of Murine LTBMC Stromal Layer Development in T25 Flasks using an Inverted Microscope.

The developing stromal layers were examined weekly, by phase contrast, using an inverted microscope.

Nunc flasks

The development of the murine stromal layer was similar to that described previously. By the end of the first week, all the flasks were found to contain islands of cells. Also, spindle-shaped cells (probably fibroblast-like cells) and flattened cells (probably macrophages) were observed in addition to round cells (probably progenitor cells), which were closely associated with the adherent cells.

Small and well-haemoglobinized clusters of erythroid cells that were loosely attached to adherent cells were also seen. However, after the first two weeks in culture, these erythroid clusters were no longer found.

At the end of week two, the number of cells attached to the bottom of the flask seemed to have increased. Moreover, by the end of the fifth week, a confluent adherent layer with persistence of islands of small round cells, resembling 'cobble stones', by light microscopy, were found in association with adherent cells. Also, by the fourth week, a small number of the spindle-shaped cells began to accumulate small droplets of lipid and were located in different regions of the culture flasks.

The stromal layers achieved between 90 to 95% confluency in all cultures studied, and areas of active haematopoiesis and fat cells were also found irregularly distributed in the culture flasks. Figures 4.20 to 4.24 show the cell types described, using Nunc flasks.

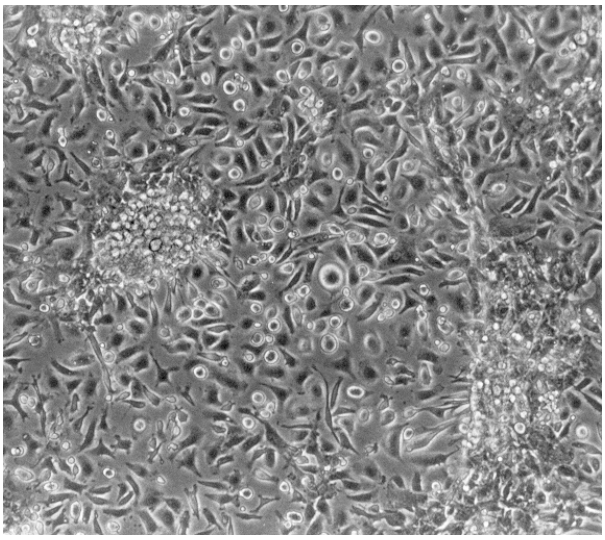


Figure 4. 20 Nunc Flask - Murine Stromal Layer Development: Week 1

A Phase contrast of murine stromal layer development at the end of one week incubation, in a Nunc T25 flask. Large number of cells can be seen adhering to the flask bottom surface. (Objective x100)

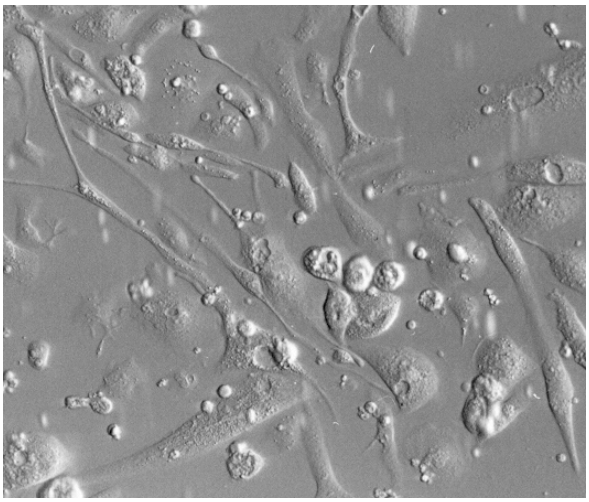


Figure 4. 21 Nunc Flask Murine Stromal Layer Development: Week 3

Hofmann Modulation contrast of murine stromal cell layer development following 3 weeks incubation in a Nunc T25 flask. Fibroblast-like cells can be noted forming a stromal net work. (Objective x200)

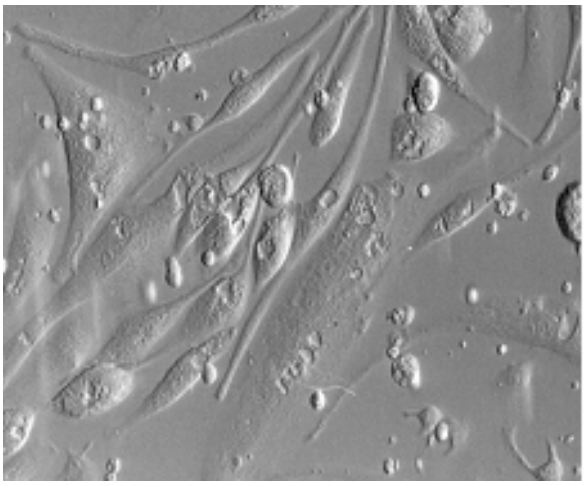


Figure 4. 22 Nunc Flask- Murine Stromal Layer Development: Week 4

Hofmann Modulation contrast of murine stromal layer development following 4 weeks incubation in a Nunc T25 flask. Cells are shown adhering to the surface of the flask. (Objective x200)

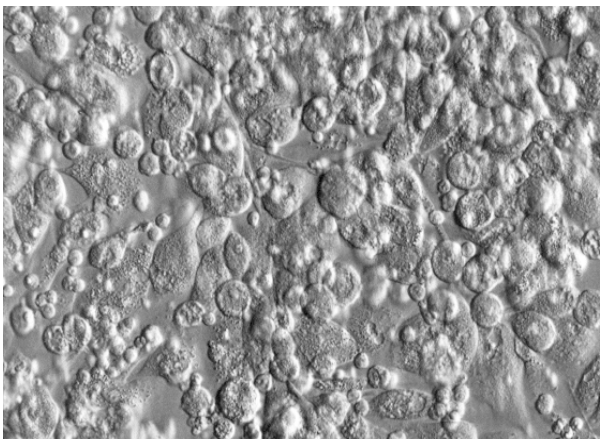


Figure 4. 23 Nunc Flask - Murine Stromal Layer Development: Week 6

Hofmann Modulation contrast of murine stromal layer development following 4 weeks incubation in a Nunc T25 flask. A confluent stromal layer is noted. (Objective x200)

Falcon flasks

Very few cells were found to adhere to the Falcon flask. The cells appeared round and flat with very few fibroblastic cells present. Also the number of cells and their appearance did not change throughout the stromal development period. A confluent layer did not develop by the end of the sixth week. Figures 4.24 to 4.28 show the cells described, using Falcon flasks.

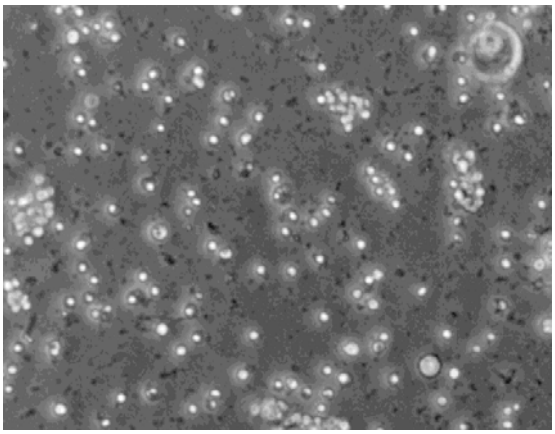


Figure 4. 24 Falcon Flask- Murine Stromal Layer Development: Week 1

A Phase contrast of murine stromal layer development following one week's incubation, in a Falcon T25 flask. The cells are round and flat and appear to be floating within the culture medium. (Objective x100)

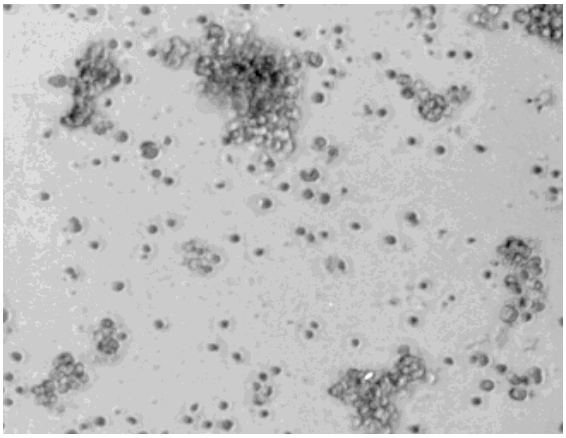


Figure 4. 25 Falcon Flask - Murine Stromal Layer Development: Week 2

A Phase contrast of murine stromal layer development following 2 week of incubation, in a Falcon T25 flask. The cells appear are round and have not formed a fibroblast-like net work. (Objective x100)

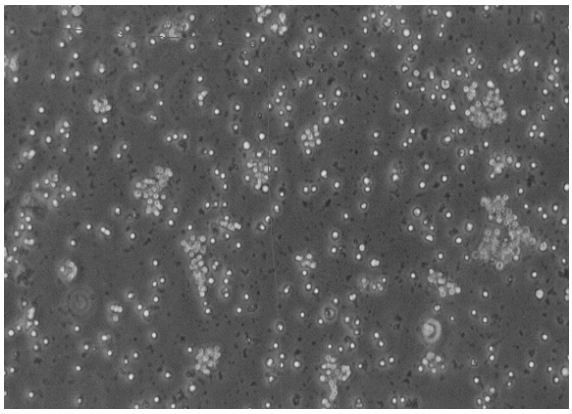


Figure 4. 26 Falcon Flask- Murine Stromal Layer Development: Week 3

A Phase contrast of murine stromal layer development following 3 week incubation, in a Falcon T25 flask. The cells appear to be as round flat cells. No network of cells is noted. (Objective x100)

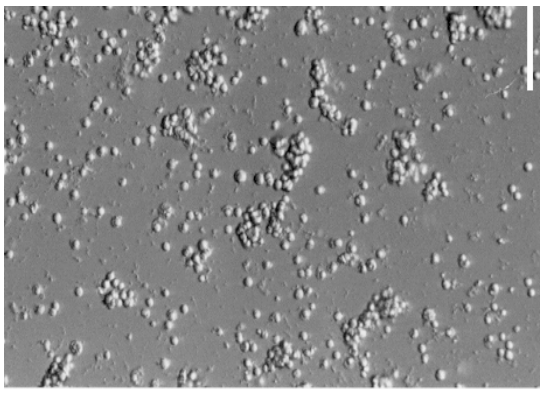


Figure 4. 27 Falcon Flask- Murine Stromal Layer Development: Week 4

A Hofmann Modulation contrast of murine stromal layer development following 4 week incubation, in a Falcon T25 flask. The cells appear to be as round flat cells. No network of cells is noted (Objective x100)

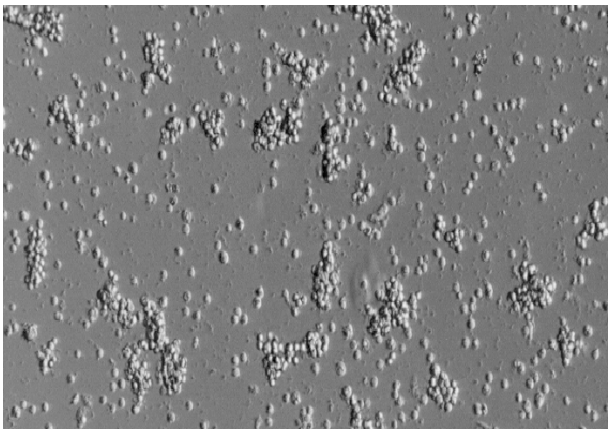


Figure 4. 28 Falcon Flask- Murine Stromal Layer Development: Week 6

A Hofmann Modulation contrast of murine stromal layer development following 6 week incubation, in a Falcon T25 flask. The cells appear to be as round flat cells. No network of cells is noted (Objective x100)

Costar flasks

By the end of the first week, all the flasks were found to contain small round cells with a few fibroblasts adhering to the flask. The number of cells adhering to the flask appeared to be greater than that observed in the Falcon flasks but not as high as that observed in the Nunc flasks. By the end of the second week, all the flasks were found to contain islands of cells. Also, spindle-shaped cells and flattened cells (as described above) were observed in addition to round cells (probably progenitor cells), which were closely associated with the adherent cells.

At the end of week three, the number of cells attached to the bottom of the flask seemed to have increased. Additionally, by the end of the sixth week, a near confluent adherent layer with persistence of islands of small round cells, resembling 'cobble stones', by light microscopy, were found in association with adherent cells. Also, a few spindle-shaped cells began to accumulate small droplets of lipid and were located in different regions of the culture flasks, by the sixth week. The stromal layers achieved between 60-65% confluency in all cultures studied, and areas of active haematopoiesis and fat cells were also found irregularly distributed in the culture flasks. Figures 4.29 to 4.32 show the cells described, using Costar flasks.

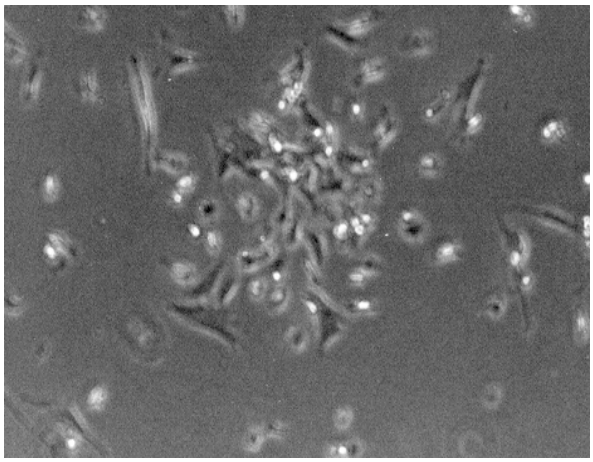


Figure 4. 29 Costar Flask- Murine Stromal Layer Development: Week 2

A Hofmann Modulation contrast of murine stromal layer development following 1 week incubation, in a Costar T25 flask. The cells appear to be as round flat cells (Objective x100)

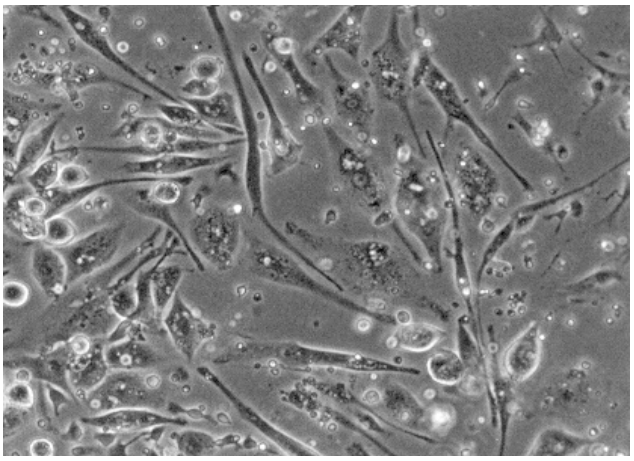


Figure 4. 30 Costar Flask- Murine Stromal Layer Development: Week 3

A Phase contrast Hofmann Modulation contrast of murine stromal layer development following 3 week incubation, in a costar T25 flask. The cells appear to be adhering to the bottom of the flask. Fibroblast-like cells can be seen. (Objective x100)

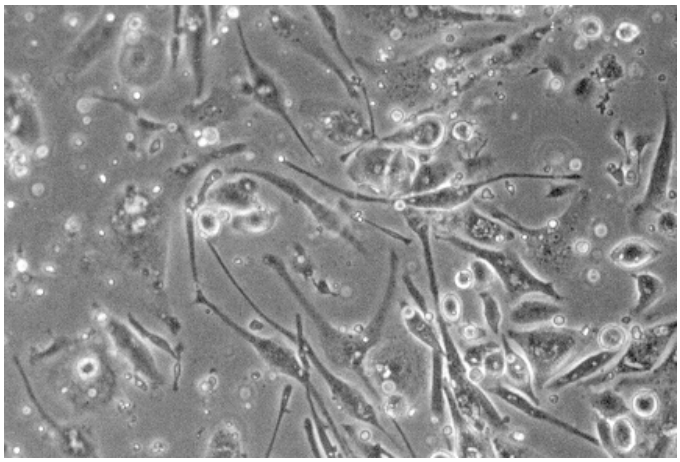


Figure 4. 31 Costar Flask- Murine Stromal Layer Development: Week 4

A Phase contrast of murine stromal layer development after 4 week incubation, in a costar T25 flask. The cells appear to be adhering to the bottom of the flask. Fibroblast-like cells can be seen (Objective x100)

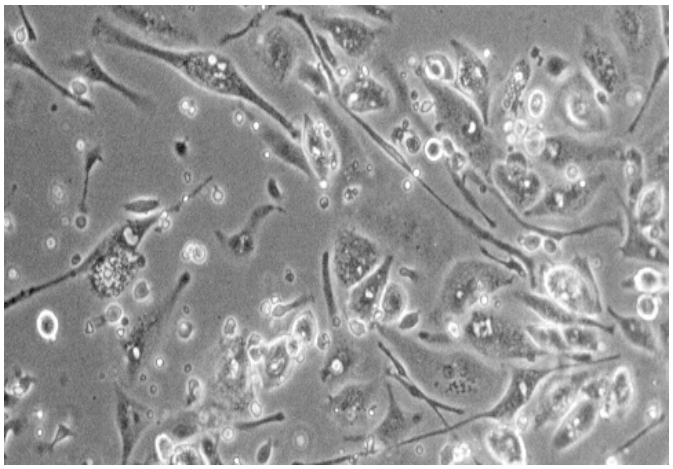


Figure 4. 32 Costar Flask- Murine Stromal Layer Development: Week 6

A Phase contrast of murine stromal layer development after 6 weeks incubation, in a costar T25 flask. The cells appear to be adhering to the bottom of the flask. Fibroblast-like cells can be seen forming a network. (Objective x100)

4.3.11 Examination of Developed Murine Stromal Layer on Thermanox Coverslips

The Thermanox coverslips were examined at the end of the six-week period, using an inverted microscope. A 95-98% confluent stromal layer was observed with persistence of islands of small round cells, resembling 'cobble stones' at the end of the stromal development period. Also areas of active haematopoiesis and fat cells were also found irregularly distributed in the

culture. Subsequently the same coverslips were fixed and stained with May Grunwald Giemsa. The May Grunwald Giemsa staining revealed greater detail of the fibroblast -like cells forming an interconnecting network. Associated within and between the cells were numerous round cells probably those described as 'cobble stones' by inverted microscope. Also interspersed within the fibroblast- like cells were large mononuclear cells with vast cytoplasm, the identity of these cells in unknown (Figures 4.33 and 4.34). The areas of active haematopoiesis appeared as clusters of tightly packed cells.

Light Microscopy of Thermanox Cover Slips

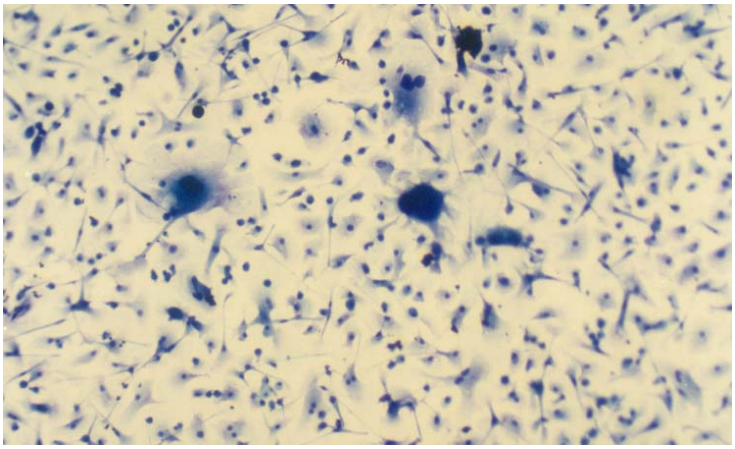


Figure 4. 33 Week 3 Murine Stromal Layer Development

May Grunwald Giemsa Staining shows fibroblast-like cells and flattened cells after 3 weeks of stromal layer development (Objective x100)

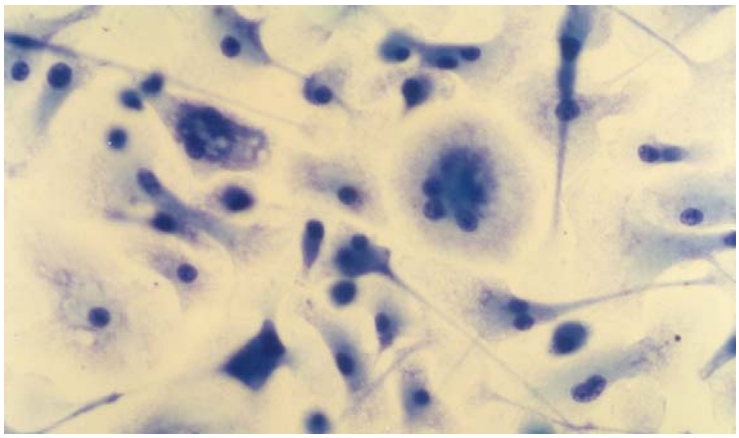


Figure 4. 34 Week 3 Murine Stromal Layer Development

May Grunwald Giemsa staining shows fibroblast-like cells and flattened cells after 3 weeks of murine stromal layer development. (Objective x400)

4.3.12 Cell Count of the Murine Non-Adherent Layer During Stromal Layer Development

The weekly total cell counts of murine non -adherent cells declined steadily during the stromal layer development phase, from $5x10^6$ to 2.97 $x10^4$, (Figure 4.35). Furthermore, between days 35 and 43 the non-adherent cell numbers plateaued.The total cell counts of non-adherent cells collected weekly during stromal layer development are shown in table 4.8.

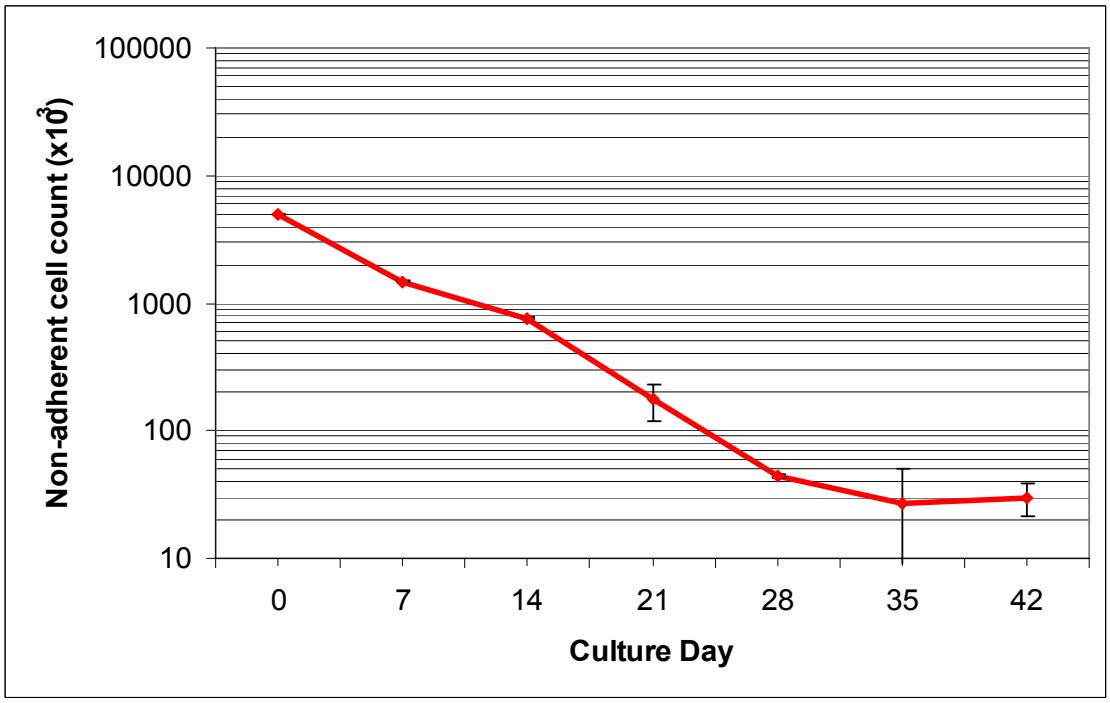


Figure 4. 35 Murine Stromal Layer Development: Mean Total Cell Counts Of Non-Adherent Cells

The weekly total cell counts of murine non -adherent cells declined steadily during the stromal layer development phase, from $5x10^6$ to 2.97 $x10^4$. Between days 35 and 43 the non-adherent cell numbers plateaued. Mean data from three separate experiments. Each experiment was carried out in triplicate. Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure

Exp. No	Culture No.	Cell count of non-adherent Cells in murine LTBMC (x10^3/L)						
		Day 0	Day 7	Day 14	Day 21	Day29	Day35	Day 42
1	1	500	153	82.1	38.2	5.2	2.9	2.0
	2	500	147	85.1	40.1	4.6	3.0	1.9
	3	500	169	76.2	44.0	3.2	2.9	2.6
2	1	500	214	68.2	30.2	4.9	2.9	2.9
	2	500	225	56.8	34.2	4.0	3.1	2.5
	3	500	153	84.2	27.3	5.1	2.9	2.4
3	1	500	236	65.3	29.6	6.2	2.2	2.1
	2	500	241	89.2	33.6	4.2	1.9	1.0
	3	500	189	77.0	35.0	4.9	3.0	1.9

Table 4. 8 Murine Stromal Layer Development: Mean Total Cell Counts Of Non-Adherent Cells

The non-adherent cell counts collected weekly during murine stromal layer development. The weekly total cell counts of murine non -adherent cells declined steadily during the stromal layer development phase, from 5x10^6 to 2.97 x10^4. Furthermore, between days 35 and 43 the non-adherent cell numbers plateaued.

4.3.13 Identification of Murine Non-Adherent Cells, By Cell Surface Antigens (Phenotype) During Stromal Layer Development

Flow cytometric analysis was conducted on murine non -adherent cells collected weekly, during the stromal development period, in order to identify the populations of cells present. By using antibodies to cell lineage -specific cell surface antigens it was possible to identify cells of the erythroid, granulocytic /monocytic, lineages as well as the primitive pluripotent progenitor cells. The cell counts of all four populations of cells (Table 4.9) decrease during the first four weeks and then plateau during the last two weeks of the stromal development period. Additionally, a dramatic reduction in erythroid numbers was noted during the development period (Figure 4.36). The monocytic/ macrophage cell population was found to be the predominant cell lineage in the non-adherent layer. Furthermore, the granulocyte precursors and progenitor cells were more prominent than erythroid precursors. Also the four populations identified using the lineage specific markers were found to identify 98.93% of all the cells present.

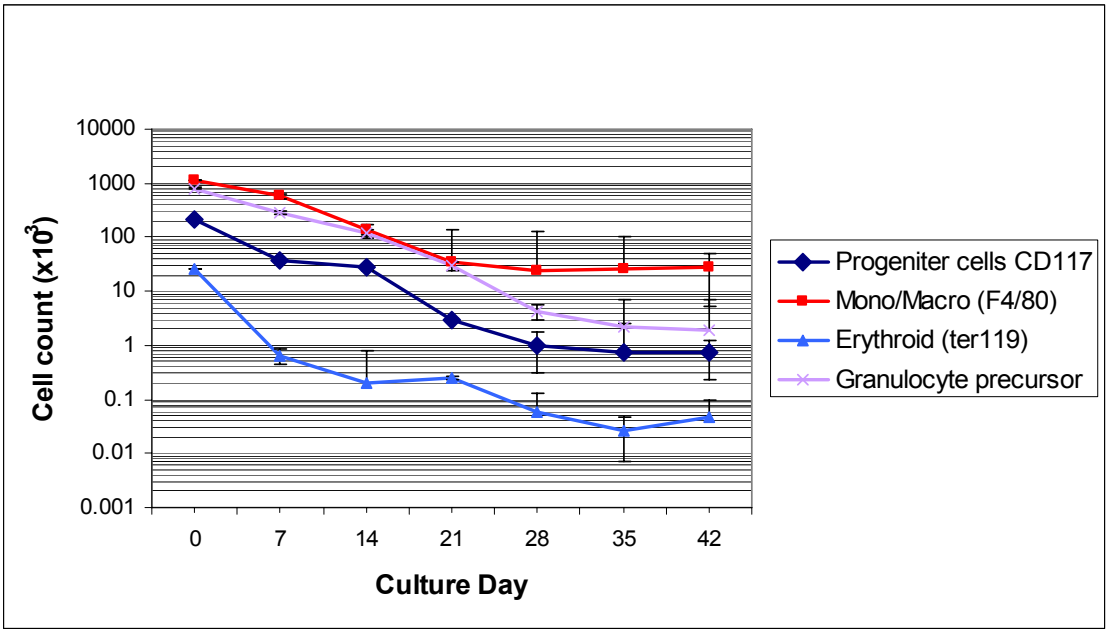


Figure 4. 36 Murine Stromal Development: Absolute Count of the Non-Adherent Cell Populations, Identified By Surface Antigen Detection per Culture

The mean absolute count of the different cell populations the non-adherent cells collected weekly for 6 weeks is shown. The different cell populations were identified using flow cytometry to identify lineage specific surface antigens. The cell counts of all four populations of cells decrease during the first four weeks and then plateau during the last two weeks of the stromal development period. A reduction in erythroid numbers was noted during the development period. The monocytic/ macrophage cell population was found to be the predominant cell lineage in the non-adherent layer. Data from three separate experiments Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure

Exp No	Day of collection of non-adherent cells	Progeniter cells CD117 ($x10^3$/L)	Mono/Macro (F4/80) ($x10^3$/L)	Erythroid (ter119) ($x10^3$/L)	Granulocyte precursor ($x10^3$/L)
1	0	4.9	73.2	4.1	17.8
	7	2.2	76.1	0.2	16.5
	14	1.3	87.2	0.8	12.3
	21	1.1	88.2	0.2	10.0
	28	1.9	88.6	0.1	9.3
	35	1.0	89.0	0.1	10.0
	42	0.8	88.3	0.1	10.4
2	0	2.2	79.2	2.1	18.2
	7	2.6	72.5	0.7	22.6
	14	4.7	80.9	0.3	13.5
	21	1.9	79.9	0.2	17.3
	28	2.4	89.1	0.1	8.2
	35	3.4	86.4	0.2	10.1
	42	4.1	89.3	0.2	7.2
3	0	5.6	78.1	4.1	12.6
	7	3.1	78.4	0.2	19.3
	14	4.7	74.6	0.7	20.1
	21	2.2	75.1	0.1	23.2
	28	2.7	86.1	0.2	11.8
	35	3.9	92.7	0	5.1
	42	2.5	96.2	0.2	2.1

Table 4. 9 Murine Stromal Development: Mean Absolute Count of the Non-Adherent Cell Populations, Identified By Surface Antigen Detection

The mean absolute count of the different cell populations the non-adherent cells collected weekly for 6 weeks during stromal layer development is shown. The different cell populations were identified using flow cytometry to identify lineage specific surface antigens. The absolute counts of all four populations of cells decrease during the first four weeks and then plateau during the last two weeks of the stromal development period. Additionally, a reduction in erythroid count was noted during the development period. The monocytic/ macrophage cell population was found to be the predominant cell lineage in the non-adherent layer. The granulocyte precursors and progenitor cells were more prominent than erythroid precursors.

4.3.14 Confluent Murine Stromal Layer Inoculated with CD117 Positive Cells

At weekly intervals, murine non- adherent cells were collected, counted and the cell lineage determined using flow cytometry. In all three experiments carried out, the cell count increased from the initial inoculation density (mean count of 4.6 $x10^4$) to a mean of $1.7x10^6$ by day 70 (Figure 4.37); the cell counts are provided in table 4.10. Figure 4.38 shows the cell density in the non-adherent compartment throughout the entire culturing period in the murine LTBMC described here. As can be seen the inoculation with fresh pluripotent cells increased the cell density by approx. two orders of magnitude.

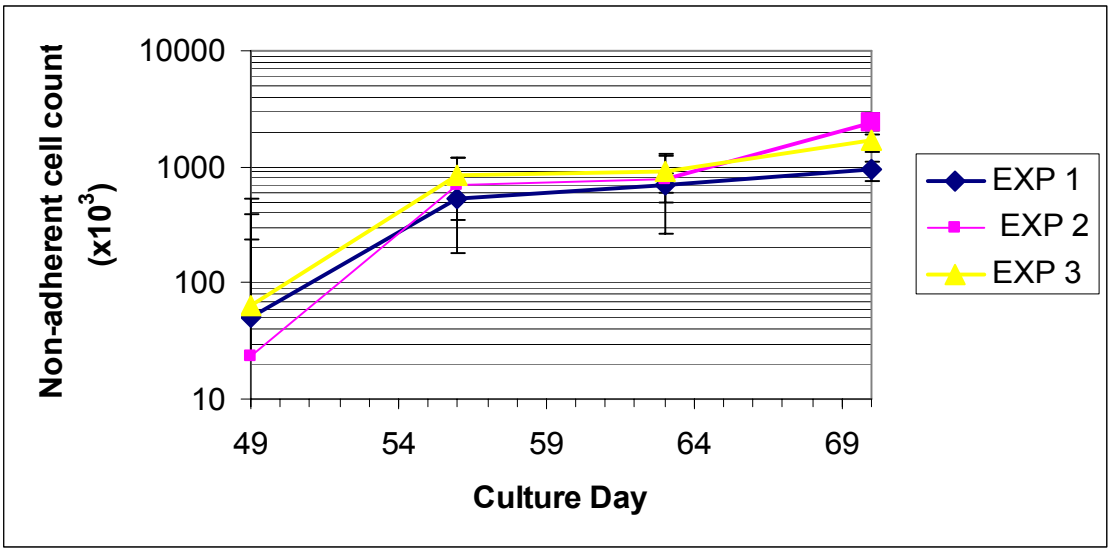


Figure 4. 37 The Non-Adherent Weekly Cell Count from Murine LTBMC After Established Stromal Layer was Inoculated With CD117$^+$ Cells

The total counts of non-adherent cells collected weekly from murine LTBMC after the confluent stromal layer was inoculated with fresh CD117 positive cells. In all three experiments carried out, the cell count increased from the initial inoculation density (mean count of 4.6 x10^4) to a mean of 1.7x10^6 by day 70. Data is from three separate experiments Data shown as Mean +/- SEM

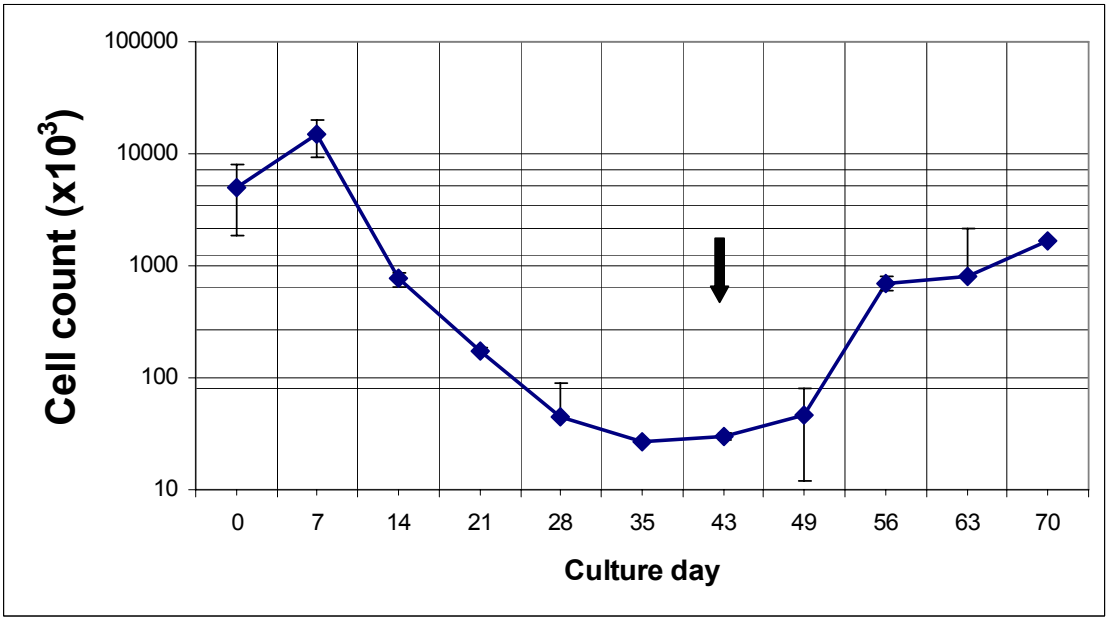


Figure 4. 38 The Mean Non-Adherent Cell Count of The Murine LTBMC, Throughout The Entire Culturing Period (Day 0-70)

The absolute count of non-adherent cells collected from murine LTBMC throughout the culturing period (stromal cell development and 4 weeks after the confluent stromal layer was inoculated with fresh murine CE117 positive cells). During stromal layer development the absolute count declined (day 43). Following inoculation with CD117 positive cells the absolute count increased. Data from three separate experiments Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure

Inoculation with CD117 positive cells ⬇

Exp. No	Culture No.	Cell count of non-adherent Cells in murine LTBMC ($x10^3$/L)			
		Day 49	Day 56	Day 63	Day 70
1	1	4.6	11.1	15.2	110
	2	5.2	12.3	15.7	119
	3	4.1	9.1	16.5	135
2	1	5.0	10.7	17.1	135
	2	4.4	14.2	14.7	120
	3	4.8	10.6	16.9	142
3	1	4.3	14.7	18.1	142
	2	5.4	12.0	15.6	176
	3	4.1	9.3	17.3	162

Table 4. 10 The Non-Adherent Weekly Cell Count From Murine LTBMC After Established Stromal Layer was Inoculated with $CD117^+$ Cells

The cell counts of non-adherent cells collected weekly from murine LTBMC after the stromal layer was inoculated with fresh CD117 positive cells is shown. The cell counts increased weekly. Data from three separate experiments is shown

4.3.15 Identification of Non-Adherent Cells, By Cell Surface Antigens (Phenotype) after Murine Stromal Layer was Inoculated With CD117 Positive Cells

Flow cytometric analysis was conducted on murine non -adherent cells collected weekly, after inoculation with CD117 positive cells, in order to identify the populations of cells present. Using antibodies to mouse cell lineage specific cell surface antigens of the erythroid, granulocytic /monocytic, lineages as well as the primitive pluripotent progenitor cells were identified (Figure 4.39). The absolute counts of the cell lineages identified by flow cytometry are shown in Table 4.11. The number of non-adherent cells of monocytic /macrophage, progenitor cells and granulocytic precursors was maintained during the first three weeks with a slight increase in the fourth week. The numbers of erythroid precursors were maintained at a steady level throughout the culturing period. The monocytic/ macrophage cells and granulocytic precursors appeared to be the dominant cell populations of the non -adherent cells, with equal amounts of each cell population present. The progenitor cells and unstained cells represented a smaller proportion of the non-adherent layer cell populations and the erythroid precursors the smallest population (Figure 4.39).

During stromal development the monocytic/macrophage, granulocytic precursor, erythroid and progenitor cells declined dramatically and then plateaued (Figure 4.40). Higher cell counts

were noted for all four-cell populations after the inoculation of the cultures with CD117 positive progenitor cells, (Figure 4.40).

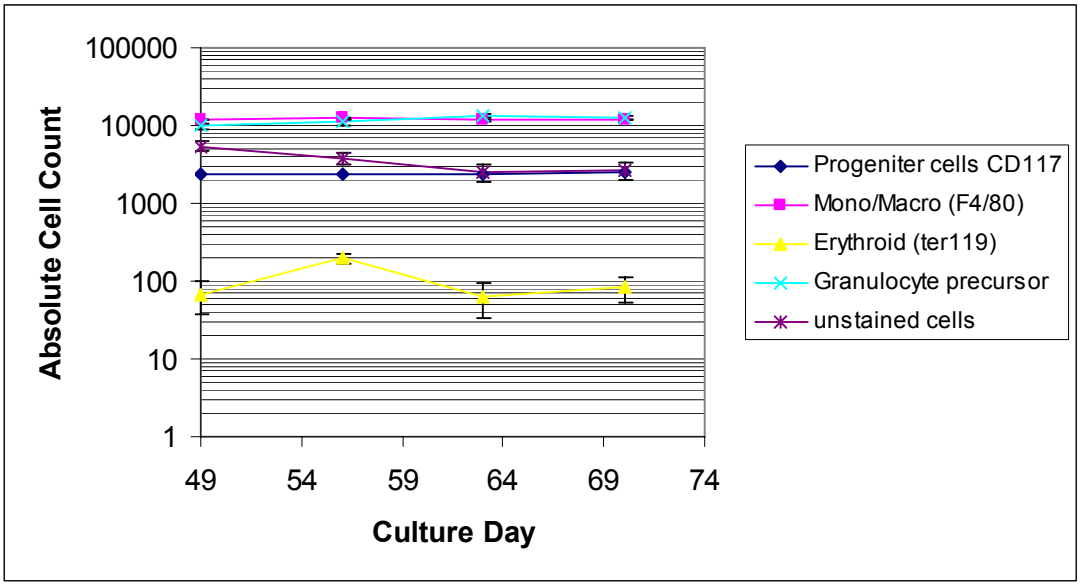


Figure 4. 39 Phenotype of Murine Non-Adherent Cells After Stromal Layer Inoculated With CD117 Positive Cells

The cell populations identified by flow cytometry analyses of surface antigen expression of murine non-adherent cells after the confluent stromal layer was inoculated with fresh murine CD117 positive cells. The number of non-adherent cells of monocytic /macrophage, progenitor cells and granulocytic precursors was maintained during the first three weeks with a slight increase in the fourth week. The numbers of erythroid precursors were maintained at a steady level throughout the culturing period. The monocytic/ macrophage cells and granulocytic precursors appeared to be the dominant cell populations of the non -adherent cells, with equal amounts of each cell population present. The progenitor cells and unstained cells represented a smaller proportion of the non-adherent layer cell populations and the erythroid precursors the smallest population Data from three separate experiments Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure

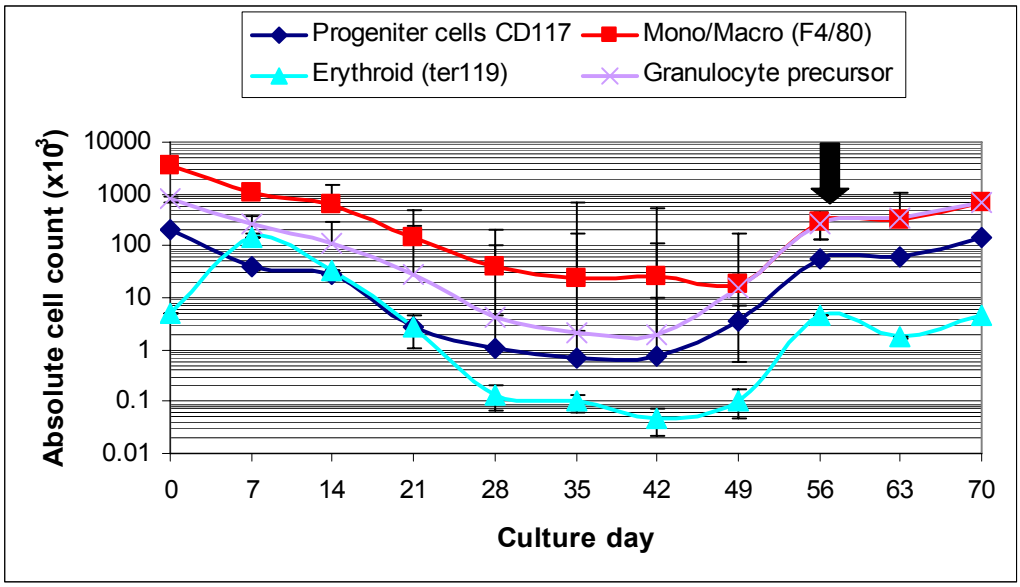


Figure 4. 40 Phenotype of Murine Non-Adherent Cells Throughout the Culturing Period

The murine cell lineages identified using lineage specific surface cell antigens throughout the LTBMC period (stromal layer development and four weeks following inoculation with murine CD117 cells. During stromal development the monocytic/macrophage, granulocytic precursor, erythroid and progenitor cells declined dramatically and then plateaued. Higher cell counts were noted for all four-cell populations after the inoculation of the cultures with CD117 positive progenitor cells. Data from three separate experiments Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure

Inoculation with CD117 positive cells ⬇

Exp No	Day of collection of non-adherent cells	Progeniter cells CD117 ($x10^3$/L)	Mono/Macro (F4/80) ($x10^3$/L)	Erythroid (ter119) ($x10^3$/L)	Granulocyte precursor ($x10^3$/L)
1	49	4.4	38.2	0.2	32.6
	54	5.2	42.2	0.2	35.1
	59	3.7	43.1	0.4	46.6
	64	6.2	41.2	0.6	42.7
	69	4.4	38.2	0.2	32.6
	74	5.2	42.2	0.2	35.1
2	49	8.1	46.3	0.1	31.1
	54	9.3	42.0	1.1	42.4
	59	13.7	35.6	0.2	45.7
	64	9.6	38.0	0.1	46.7
	69	8.1	46.3	0.1	31.1
	74	9.3	42.0	1.1	42.4
3	49	11.7	35.6	0.4	36.2
	54	9.3	40.0	0.6	33.7
	59	6.3	38.9	0.1	40.1
	64	9.3	41.4	0.2	37.1
	69	11.7	35.6	0.4	36.2
	74	9.3	40.0	0.6	33.7

Table 4. 11 Phenotype of Murine Non-Adherent Cells After Stromal Layer Inoculated With CD117 Positive Cells

The cell populations identified by flow cytometry analyses of surface antigen expression of murine non-adherent cells after the confluent stromal layer was inoculated with fresh murine CD117 positive cells. The number of non-adherent cells of monocytic /macrophage, progenitor cells and granulocytic precursors was maintained during the first three weeks with a slight increase in the fourth week. The numbers of erythroid precursors were maintained at a steady level throughout the culturing period. The monocytic/ macrophage cells and granulocytic precursors appeared to be the dominant cell populations of the non -adherent cells, with equal amounts of each cell population present. The progenitor cells and unstained cells represented a smaller proportion of the non-adherent layer cell populations and the erythroid precursors the smallest population Data from three separate experiments.

4.3.16 Visualization of Stromal Cell Development of hCB Cell LTBMC

Examination of developing stromal layer of hCB LTBMC, by inverted microscope.

The developing stromal layers were examined weekly, by phase contrast, using an inverted microscope. The findings were as follows:

First week: all the flasks were found to contain islands of cells that appeared as flattened cells. Spindle-shaped cells (probably fibroblast-like cells) and flattened cells (probably macrophages)

were observed in addition to round cells (probably progenitor cells), which were closely associated with the adherent cells. As noted in the murine LTBMC, small and well-haemoglobinized clusters of erythroid cells that were loosely attached to adherent cells were also seen. However, after the first week in culture, these erythroid clusters were no longer found.

End of week two: the number of cells attached to the bottom of the flask increased.

Week 4: A small number of the spindle-shaped cells began to accumulate small droplets of lipid and were located in several regions of the culture flasks.

Week 5: A confluent adherent layer composed of numerous cell types (macrophages, fibroblasts fat cells) with islands of small round cells, resembling 'cobble stones', by light microscopy. The stromal layers achieved between 90 to 95% confluency in all cultures studied, and areas of active haematopoiesis and fat cells were also found irregularly distributed in the culture flasks. Photographs taken at weeks 2, 4 and 5 show the cell types described (Figures 4.41 to 4.43).

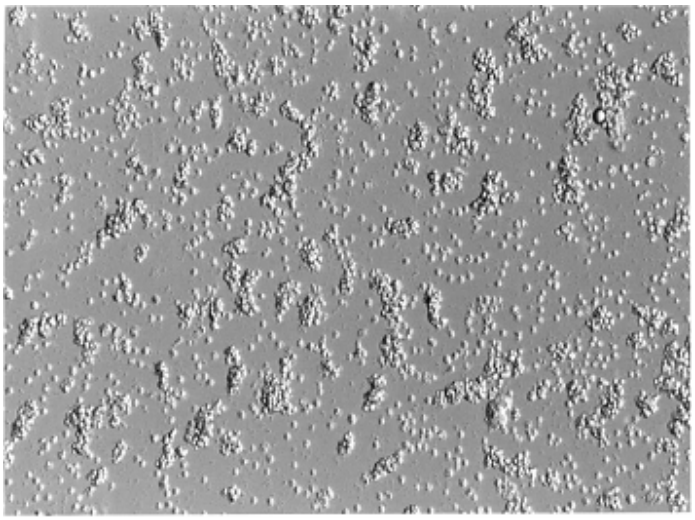


Figure 4. 41 hCB LTBMC

A phase contrast of human LTBMC stromal layer development 2 weeks after culture initiation is shown. The cells can be seen adhering to the flask bottom. (objective x 100)

Figure 4. 42 hCB LTBMC

A Hofmann Modulation contrast of human LTBMC stromal layer development 4 weeks after culture initiation is shown A small number of the spindle-shaped cells began to accumulate small droplets of lipid and were located in several regions. (objective x200)

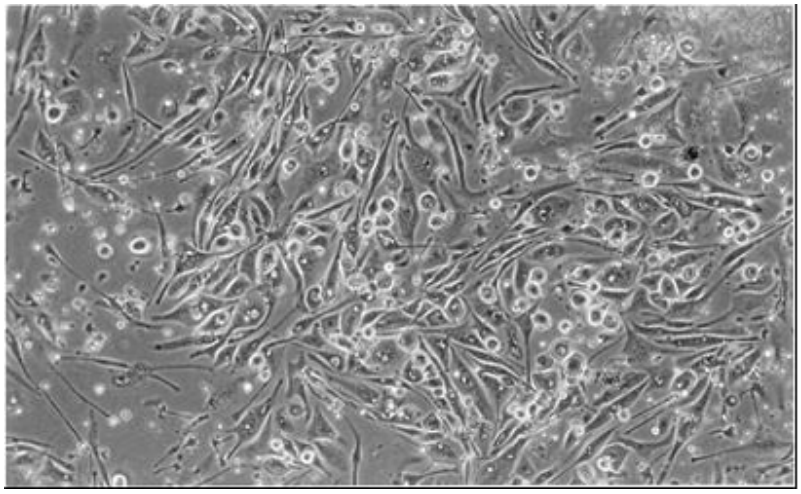


Figure 4. 43 hCB LTBMC

A phase contrast of human LTBMC stromal layer development 5 weeks after culture initiation is shown. A confluent adherent layer composed of numerous cell types (macrophages, fibroblasts fat cells) with islands of small round cells, resembling 'cobble stones are shown. (objective x200)

4.3.17 Characterization of the Non-Adherent Layer during hCB LTBMC Stromal Layer Development

The weekly total cell counts of hCB non -adherent cells declined steadily during the stromal

layer development phase (Figure 4.44). The cell counts are provided in Table 4.12.

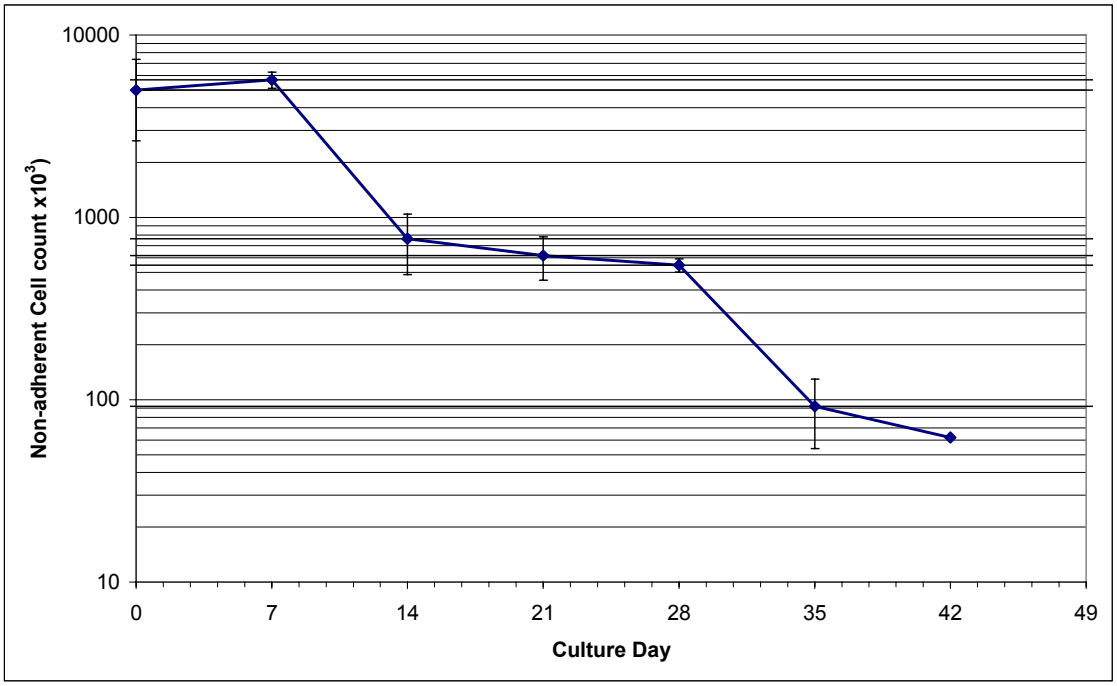


Figure 4. 44 hCB Stromal layer development: Mean total cell counts of non-adherent cells

The total human non-adherent cell count during hCB LTBMC stromal layer development; the weekly total cell counts of hCB non -adherent cells declined steadily during the stromal layer development phase. Data from three separate experiments Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure.

Exp. No	Culture No.	Cell count of non-adherent Cells in hCB LTBMC (x10^3/L)						
		Day 0	Day 7	Day 14	Day 21	Day28	Day35	Day 42
1	1	500	489	149	11.1	9.7	1.8	0.8
	2	500	414	208	14.8	10.9	2.5	0.5
	3	500	399	160	10.7	8.6	3.3	0.7
2	1	500	518	259	14.5	11.2	4.1	0.8
	2	500	539	148	12.6	10.7	1.9	0.9
	3	500	489	102	18.9	13.6	2.7	0.9
3	1	500	543	161	11.3	8.2	1.5	0.7
	2	500	531	170	12.0	9.2	1.4	0.9
	3	500	510	149	10.7	8.6	1.9	0.9

Table 4. 12 Total Cell Counts of Non-Adherent Cells hCB during Stromal Layer Development

The total human non-adherent cell count during hCB LTBMC stromal layer development; the weekly total cell counts of hCB non -adherent cells declined steadily during the stromal layer development phase.

4.3.18 Morphological Identification of hCB Non-Adherent Cells during Stromal Layer Development

The hCB non-adherent cells collected, during the stromal layer development, were cyto-spun, fixed and then stained with May Grunwald Giemsa, in order to morphologically identify the cells. The cell types identified morphologically (percentage and absolute counts) are shown in Tables 4.13 and 4.14.

The steepest decline was observed for the mononuclear cell population. The monocyte/macrophage cell population initially declined (day 7) and the steadily increased weekly reaching a peak at day 35 and then declined on day 42. Erythrocytes were observed at day 7 only. The numbers of the remaining cell types (promyelocytes, pro-monocytes, metamyelocytes and mono/macrophages) plateaued by day 42 (figure 4.45).

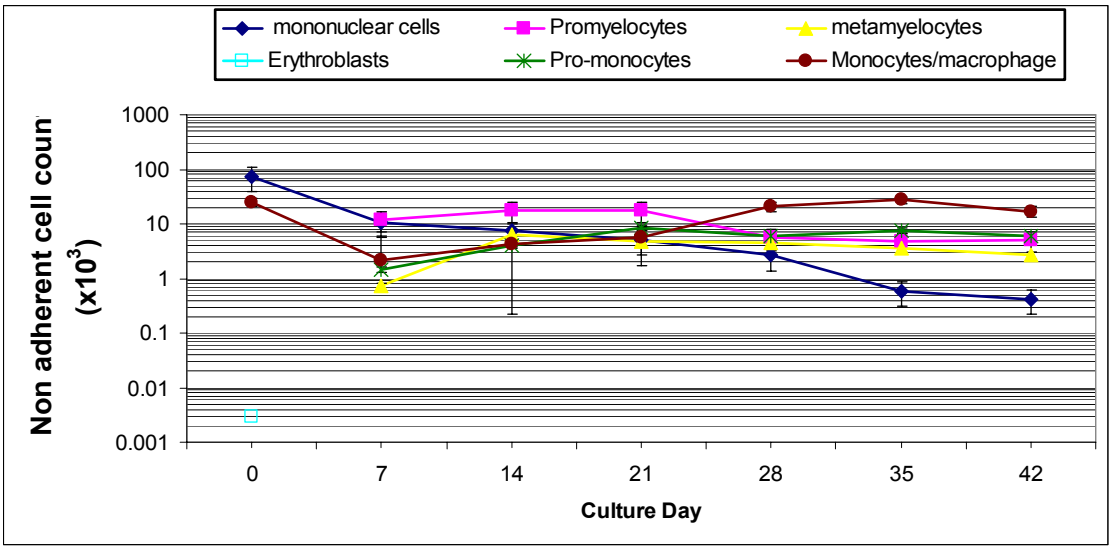


Figure 4. 45 hCB Stromal Layer Development: The Absolute Count of Different Cell Types Collected in the Non-Adherent Layer, Identified by May Grunwald Giemsa Staining Morphology

The cell types present in the non-adherent cells collected throughout the hCB stromal cell development, identified by morphology. The steepest decline was observed for the mononuclear cell population. The monocyte/macrophage cell population initially declined (day 7) and the steadily increased weekly reaching a peak at day 35 and then declined on day 42. Erythrocytes were observed at day 7 only. The numbers of the remaining cell types (promyelocytes, pro-monocytes, metamyelocytes and mono/macrophages) plateaued by day 42. Data from three separate experiments is show. Data shown as Mean +/- SEM. SEM bars shown for each experiment, however, the bars are too small to be shown clearly in figure

Exp. No	Culture day non-adherent cells collected	Mononuclear cells (%)			Promyelocytes (%)			Metamyelocytes (%)			Erythrocytes (%)			Promonocytes (%)			Monocytes /macrophages (%)		
Culture ID		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	0	95	83	95	0	0	0	0	0	0	0	0	0	0	0	0	5	17	5
2		98	95	91	0	0	0	0	0	0	0	0	0	0	0	0	2	5	9
3		90	94	93	0	0	0	0	0	0	0	0	0	0	0	0	10	6	7
1	7	31	35	38	32	36	41	1	1	1	26	27	15	5	1	3	5	0	2
2		40	38	28	27	33	33	1	2	0	26	22	28	3	5	7	3	0	4
3		33	34	27	28	33	43	2	1	1	31	22	24	2	4	3	4	6	2
1	14	22	28	27	44	34	46	7	11	15	1	0	0	24	24	10	2	3	2
2		33	24	25	46	47	40	16	10	12	0	1	0	2	16	22	3	2	1
3		25	28	24	49	38	42	13	15	19	1	0	0	7	11	15	5	8	0
1	21	7	9	5	11	16	9	9	12	11	4	2	2	11	8	21	58	53	52
2		8	3	6	13	12	19	15	13	16	1	1	2	10	12	8	53	59	49
3		4	9	4	17	12	11	14	14	13	0	0	3	14	12	18	51	53	51
1	28	0	1	1	12	14	9	3	7	4	0	0	0	17	14	18	68	64	68
2		0	0	0	15	12	10	7	5	6	0	1	0	11	16	12	67	66	72
3		0	0	0	11	14	15	5	8	8	0	1	0	13	17	16	71	60	61
1	35	1	0	0	9	11	14	6	3	6	0	0	0	10	18	13	74	68	67
2		0	2	0	17	8	15	5	9	4	0	0	0	11	12	16	67	69	65
3		0	0	0	12	11	13	8	10	8	0	0	0	17	13	14	63	66	65
1	42	0	0	2	4	15	16	5	8	6	0	0	0	12	17	16	79	60	60
2		1	2	1	15	9	11	7	4	9	0	0	0	13	10	11	64	75	68
3		1	1	0	16	11	14	8	5	5	0	0	0	12	13	13	63	70	68

Table 4. 13 hCB Stromal Layer Development: The Percentage of Different Cell Types Collected in the Non-Adherent Layer, Identified by May Grunwald Giemsa Staining Morphology The cell types present in the non-adherent cells collected throughout the hCB stromal cell development, identified by morphology. The steepest decline was observed for the mononuclear cell population. The monocyte/macrophage cell population initially declined (day 7) and the steadily increased weekly reaching a peak at day 35 and then declined on day 42. Erythrocytes were observed at day 7 only. The numbers of the remaining cell types (promyelocytes, pro-monocytes, metamyelocytes and mono/macrophages) plateaued by day 42. Data from three separate experiments is shown.

Exp. No	Culture day non-adherent cells collected	Mononuclear cells ($x10^3$/l)	Promyelocytes ($x10^3$/l)	Metamyelocytes ($x10^3$/l)	Erythrocytes ($x10^3$/l)	Promonocytes ($x10^3$/l)	Monocytes /macrophages ($x10^3$/l)
1	0	75.3	0	0	0	0	22
2		68.4	0	0	0	0	15
3		79.4	0	0	0	0	25
1	7	17.3	12.7	0.7	0	1.3	1.8
2		11.0	11.5	0.4	0	1.7	2.1
3		15.8	10.3	0.6	0	1.9	2.0
1	14	7.9	19.3	3.2	0	3.2	2.7
2		8.9	15.0	6.9	0	1.8	3.8
3		6.3	14.9	5.4	0	2.5	4.1
1	21	5.3	14.8	5.4	0	8.3	6.3
2		6.6	19.2	7.4	0	7.4	5.6
3		4.4	14.7	9.3	0	9.4	8.3
1	28	3.6	5.7	3.2	0	6.3	19.5
2		4.3	9.4	5.4	0	5.1	22.6
3		7.3	5.7	4.1	0	7.0	21.0
1	35	1.4	4.8	3.6	0	7.4	32.9
2		1.9	5.1	4.9	0	8.2	24.7
3		0.6	5.9	4.2	0	8.8	26.7
1	42	0.3	5.3	3.2	0	6.0	17.6
2		0.6	5.9	3.9	0	5.8	16.1
3		0.5	5.4	2.3	0	6.7	18.1

Table 4. 14 hCB Stromal Layer Development: The Absolute Count of Different Cell Types Collected in the Non-Adherent Layer, Identified by May Grunwald Giemsa Staining Morphology

The cell types present in the non-adherent cells collected throughout the hCB stromal cell development, identified by morphology. The steepest decline was observed for the mononuclear cell population. The monocyte/macrophage cell population initially declined (day 7) and the steadily increased weekly reaching a peak at day 35 and then declined on day 42. Erythrocytes were observed at day 7 only. The numbers of the remaining cell types (promyelocytes, pro-monocytes, metamyelocytes and mono/macrophages) plateaued by day 42. Data from three separate experiments is shown.

4.3.19 Identification of hCB Non-Adherent Cells, By Cell Surface Antigens (Phenotype) Throughout Culturing Period

Flow cytometric analysis was conducted on hCB non -adherent cells collected weekly, throughout the culturing period, in order to identify the populations of cells present. The use of human cell lineage -specific cell surface the identification of cells of the erythroid, granulocytic /monocytic, lineages as well as the primitive pluripotent progenitor cells (Figure 4.46). The percentage of the different populations of haematopoietic cells identified in the non-adherent layer throughout the hCB LTBMC culturing period is shown in Table 4.15. The monocytic/ macrophage cell population (CD14 expression cells) was found to be the predominant cell lineage in the non-adherent layer and represented between 50 and 63% of the total non-adherent cell population throughout the culturing period. The percentage of granulocytic/ monocytic cell precursors (CD33 expressing cells) was maintained at the same level, between 20 and 28% throughout the culturing period. Additionally, a slight reduction in the percentage of granulocytic precursor cells was noted at day 49. The percentage of megakaryocytic precursors (c-mpl expressing cells) decreased from days 7 through to day 35. After inoculation with fresh hCB (once stromal layer confluency had been achieved), the percentage of c-mpl expressing cells was maintained at 8% of the total non-adherent cell population. The percentage of pluripotent cells (CD34 expressing cells) was maintained at levels between 4 and 8% throughout the culturing period. Erythrocytic precursors (CD35) represented less than 1% of the total non-adherent cell population throughout the culturing period.

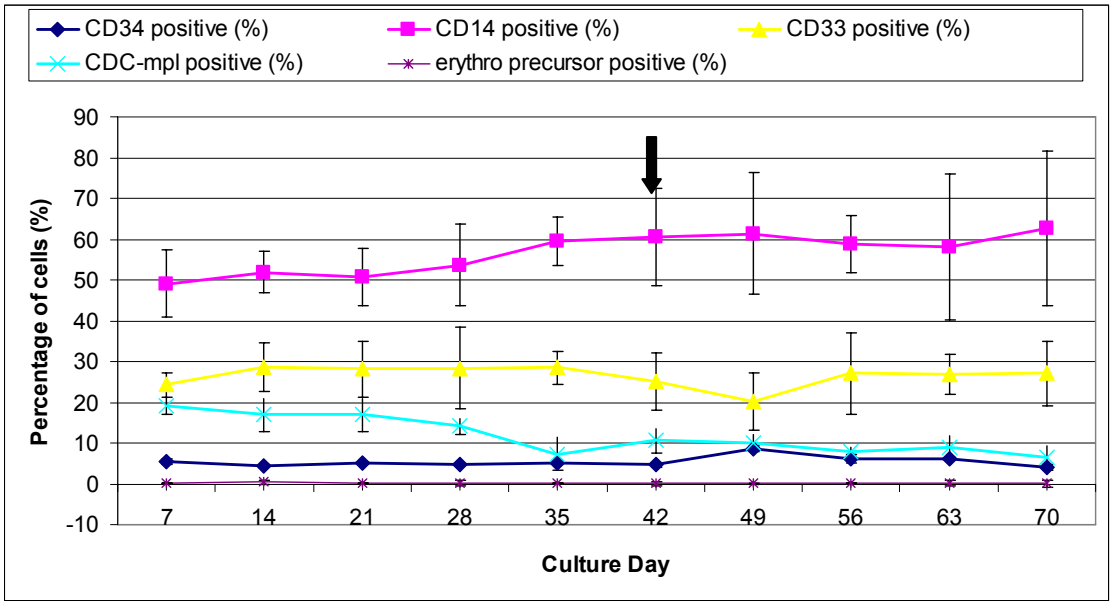


Figure 4. 46 Percentage of the Non-Adherent Cell Populations, Identified By Surface Antigen Detection per Culture

The percentage of different cell types present in the non adherent cells during hCB LTBM stromal layer development. Cell types were identified by flow cytometry of lineage specific surface antigen expression. The monocytic/ macrophage cell population (CD14 expression cells) was found to be the predominant cell lineage in the non-adherent layer throughout the culturing period. The percentage of granulocytic/ monocytic cell precursors (CD33 expressing cells) was maintained at the same level throughout the culturing period. Additionally, a slight reduction in the percentage of granulocytic precursor cells was noted at day 49. The percentage of megakaryocytic precursors (c-mpl expressing cells) decreased from days 7 through to day 35. Data from three separate experiments Data shown as Mean +/- SEM.

↓ Inoculation with fresh hCB cells

Exp No	Day of collection of non-adherent cells	CD34 positive (%)	CD14 positive (%)	CD33 positive (%)	CDC-mpl positive (%)	erythro precursor positive (%)
1	0	98	2	0	0	0
	7	5.3	49.1	24.3	20.2	0.2
	14	4.5	51.9	28.5	16.9	0.4
	21	5.0	50.7	28.1	16.9	0.3
	28	5.1	60.2	27.0	8.2	0
	35	4.4	57.6	30.0	8.0	0
	42	3.3	67.1	19.0	11.1	0.1
	49	8.3	62.8	18.1	10.3	0
	56	6.1	59.3	11.4	15.4	0
	63	7.3	60.4	14.7	9.5	0
	70	5.4	62.3	7.5	3.3	0
2	0	94	5	1	0	0
	7	5.2	60.1	19.2	17.4	0.1
	14	1.0	40.7	33.2	25.0	0.2
	21	7.1	49.1	30.2	14.0	0
	28	4.0	66.9	24.7	6.1	0
	35	7.1	63.6	21.7	8.0	0
	42	3.6	52.9	31.0	13.0	0
	49	9.2	66.1	12.7	10.3	0
	56	5.7	63.1	16.3	9.3	0
	63	4.7	64.5	9.5	14.0	0
	70	4.0	66.6	13.8	5.4	0
3	0	99	1	0	0	0
	7	1.0	40.7	33.2	25.0	0.2
	14	5.1	51	33.9	10.2	0.4
	21	5.1	52	27.2	15.7	0.7
	28	5.6	51.5	33.7	9.2	0.2
	35	7.0	59.1	27.7	6.5	0
	42	3.4	59.1	23.0	16.0	3.4
	49	7.1	64.7	18.5	11.5	0
	56	6.3	58.3	14.3	6.2	0
	63	7.5	64.0	10.5	7.4	0
	70	5.3	68.4	11.2	5.8	0

Table 4. 15 Percentage of the Non-Adherent Cell Populations, Identified By Surface Antigen Detection throughout the Culturing Period

The percentage of different cell types present in the non adherent cells during hCB LTBM stromal layer development. Cell types were identified by flow cytometry of lineage specific surface antigen expression. The monocytic/ macrophage cell population (CD14 expression cells) was found to be the predominant cell lineage in the non-adherent layer throughout the culturing period. The percentage of granulocytic/ monocytic cell precursors (CD33 expressing cells) was maintained at the same level throughout the culturing period. Additionally, a slight reduction in the percentage of granulocytic precursor cells was noted at day 49. The percentage of megakaryocytic precursors (c-mpl expressing cells) decreased from days 7 through to day 35. Data from three separate experiments are shown.

4.3.20 Confluent Stromal Layer Inoculated with hCB Cells

After the confluent layer of stromal cells was inoculated with fresh hCB cells, non- adherent cells were collected at weekly intervals, counted and plated into *in vitro* short- term cultures to evaluate differentiation into the myeloid and megakaryocytic cell lineages. The cell count increased from the initial count of 2.4 $x10^6$ cells on day 7 to 6.0 $x10^6$ cells (day 49). Figure 4.47 shows the cell density in the non-adherent compartment throughout the entire culturing period in the hCB LTBMC described here. As can be seen the inoculation with fresh hCB cells increased the cell numbers by approx. two orders of magnitude.

Inverted Microscope Examination

Haematopoietic cells actively involved in maturation and differentiation were seen on top of the stromal cells and appeared as small round cells with a phase-bright appearance and often occurred as large tightly packed foci.

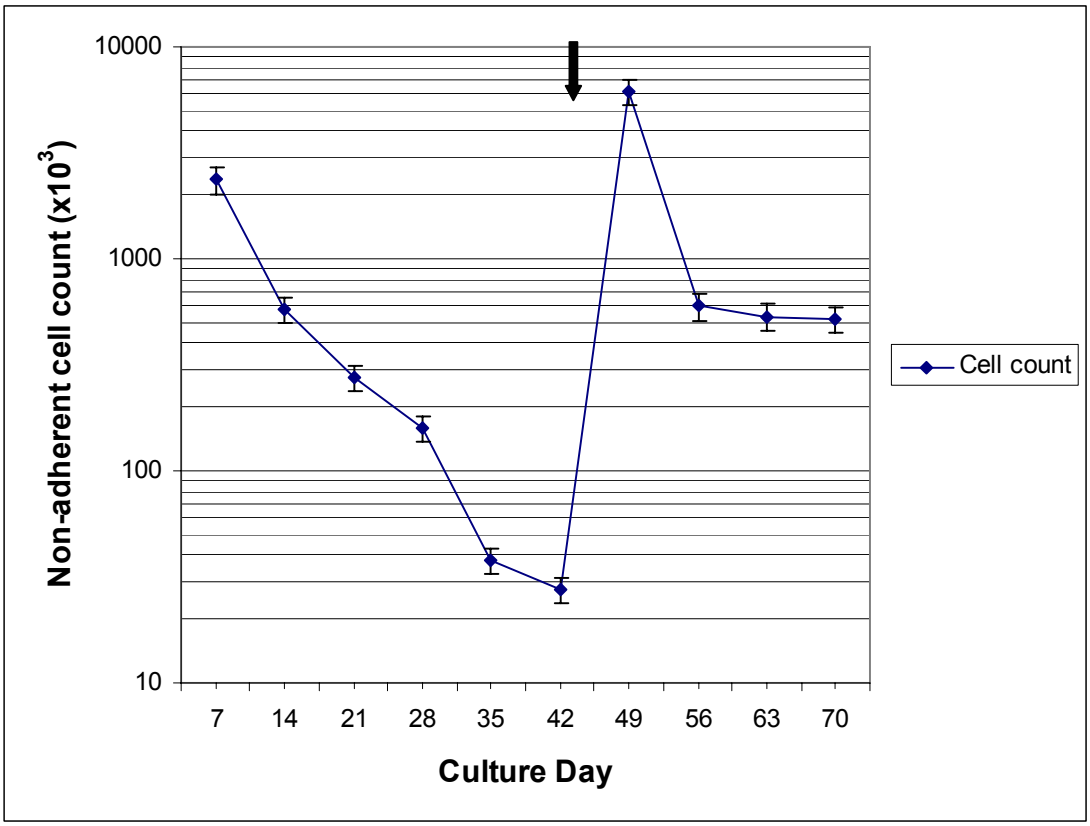


Figure 4. 47 Mean Non-Adherent Cell Count of the hCB LTBMC, Throughout The Entire Culturing Period

The mean non-adherent cell count throughout the hCB LTBMC period(stromal cell development and 4 weeks after the confluent stromal cell was inoculated with fresh CD34 positive cells. The non adherent cell count decreases during stromal layer development, but is then maintained after inoculation with fresh CD36 positive cells. Each concentration was evaluated in triplicate on three separate occasions. Data shown are Mean +/- SEM

↓ Inoculation with fresh hCB cells

Figure 4.48 shows the number of colonies of each lineage grown. The cells plated into the short -term cultures differentiated into colonies of the erythroid, mono/granulocytic and megakaryocytic lineages. Also the number of each specific colony per well remained consistent throughout the 4 weeks. Table 4.16 shows the individual BFU-E, CFU-GM and CFU-Mk colony counts achieved from non-adherent cells collected from the hCB LTBMC.

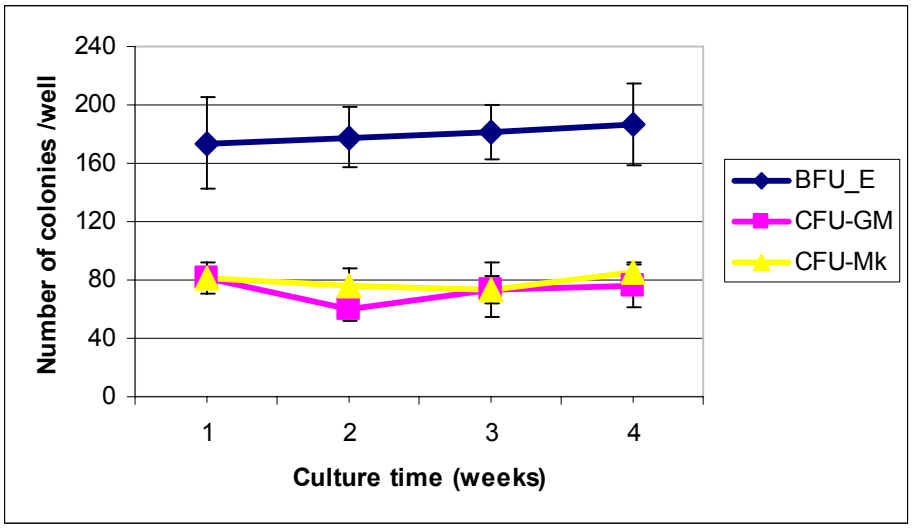


Figure 4. 48 Number Of Burst Forming Units-Erythroid (BFU-E), Colony Forming Units – Granulocytic/Monocytic (CFU-GM) And Colony Forming Units –Megakaryocytic (CFU-Mk) Colonies Per Well Grown From Non-Adherent Cells Collected Weekly; After Established Stromal Layer Was Inoculated With hCB Cells

The mean number of BFU-E, CFU-GM and CFU-Mk colonies grown weekly from non-adherent cells collected weekly from hCB LTBMC after inoculation with fresh CD36 positive cells. The number of CFU-GM, BFU-E and CFU-Mk colonies was maintained throughout the 4 week period. Each concentration evaluated in triplicate on three separate occasions. Data shown are Mean +/- SEM. SEM error bars for each cell lineage are shown, however, some are too small to been seen clearly in figure

Week of collection	Mean No BFU-E colonies /well			Mean No CFU-GM colonies /well			Mean No CFU-Mk colonies /well		
	Plate No			Plate No			Plate No		
	1	2	3	1	2	3	1	2	3
1	188	193	141	66	51	48	67	85	93
2	201	165	168	74	56	49	66	87	77
3	195	154	196	77	62	81	73	64	84
4	173	161	226	83	62	84	91	87	80

Table 4. 16 Number Of Burst Forming Units-Erythroid (BFU-E), Colony Forming Units – Granulocytic/Monocytic (CFU-GM) And Colony Forming Units –Megakaryocytic (CFU-Mk) Colonies Per Well Grown From Non-Adherent Cells Collected Weekly; After Established Stromal Layer Was Inoculated With hCB Cells

The number of BFU-E, CFU-GM and CFU-Mk colonies grown weekly from non-adherent cells collected weekly from hCB LTBMC after inoculation with fresh CD36 positive cells. The number of CFU-GM, BFU-E and CFU-Mk colonies was maintained throughout the 4 week period. Each concentration evaluated in triplicate on three separate occasions.

4.4 Discussion

4.4.1 Murine LTBMC Using Standard and Modified Culture Conditions (Falcon Flasks)

The literature describes that in murine LTBMC a confluent stromal layer is developed within 5-6 weeks of culture, consisting of macrophages /fibroblasts, blanket cells and fat cells interspersed with 'cobblestone' areas, which are compact groups of primitive haemapoietic cells which are beneath blanket cells (Dexter et al 1976). Furthermore Falcon manufactured the culture flasks described in the literature. However, this was not seen in this experiment series. Only rounded cells of various sizes were observed even after 28 days in culture. The absence of macrophages/ fibroblasts in the stromal layer probably hindered the development of a confluent adherent layer. Furthermore both phase contrast and Hofmann Modulation Contrast microscopy of the murine LTBMC did not reveal the presence of macrophages or fibroblast -like cells. However, macrophages and fibroblasts were easily visible in short-term GM/E clonogenic cultures using the same source of bone marrow as in the current experiments. Therefore, the inability to establish an adherent layer in the LTBMC was not due to the absence of fibroblast- like cells and macrophages in the mononuclear bone marrow cells preparation.

4.4.2 Visualization of Murine LTBMC (Standard Method), Using Nunc Culture Flasks

Development Phase of Stromal Layer Of Murine LTBMC

In this series of experiments, stromal layer confluency appears to require five to six weeks after culture initiation. The weekly morphological examination of the developing stromal layer showed small islands of spindle-like cells - fibroblastic cells and flat cells- macrophages by the end of the first week. Indeed by the end of the development phase, the fibroblastic cells and the macrophages are found to be the predominant cell types present within the adherent layer.

The presence of the fat cells was only evident from the fourth week suggesting that these cells only appear when the cultures are close to achieving confluency. In fact others have shown

that there is a correlation between the long-term survival of stem cells and the presence of fat cells in the stromal layer (Dexter, et al 1984). Thus, their presence in cultures seems to provide an indication of a successful stromal layer for supporting haematopoiesis. Also haematopoietic foci of small round cells are prominent once the cultures are close to achieving confluency.

Well-haemoglobinized clusters of erythroid cells seen loosely attached to adherent cells noted in the first few weeks of stromal development only. These are most probably the progeny of erythroid progenitors present in the mononuclear cell population added initially to the culture flasks. Erythropoietin required for the development of erythroid precursors to Burst Forming Cells -Erythroid is not produced by stromal cells, but by the kidney, therefore the absence of these cells in the LTBMC is not surprising. Also the presence and quick loss of the erythroid cells did not appear to inhibit stromal cell development, confirming that they play no role in the establishment or maintenance of the stromal layer. The stromal layer development took 5 to 6 weeks to reach confluency and is similar to that described by Dexter et al (1984) for murine cultures.

Whilst stromal layer was developing the production of murine non -adherent cells declined weekly, and then finally reached a plateau at weeks five and six - once stromal cell confluency was attained. This is similar to that described by Goliaei et al (1995).

Morphology of The Murine Non-Adherent Cells During LTBMC Stromal Layer Development

The total murine non-adherent cell count and the cell counts for each cell population identified by morphology, decreased during the stromal development period and reached a plateau between day 28 and day 42.

The most dramatic change in the cell populations monitored was the proportional increase over the first four weeks in the relative numbers of monocytes and macrophages in the non-adherent cells collected, which by week 6, represented 90% of the non -adherent cell population. Thus the monocytes and macrophages appear to be the most predominant cell type in both the adherent (discussed in the previous section) and non-adherent layer. This

concurs with work conducted by Kriegler (1990) where macrophages were shown to play an important role in the regulation of haematopoiesis *in vitro*. Subsequent work conducted by Goliaei et al, (1995) demonstrated that there was a delicate balance between the number of macrophages present and the maintenance of progenitor cell proliferation and differentiation. When macrophages were added to confluent murine LTBMC a modification of haematopoiesis was noted. *In vivo*, macrophages have been shown to play a similar role to that seen in *vitro*; an integral component of the microenvironment important in supporting haematopoiesis, by the production of cytokines to initiate and drive cell differentiation and maturation.

A relative increase in the granulocyte population was noted in the first two weeks, with the promyelocytes and the metamyelocytes representing 40% and 18% of the total non-adherent population, respectively. The relative proportion of the promyelocytes and metamyelocytes continued to decline during the stromal development period and also reached a plateau by the sixth week of culturing. Also the erythroblasts (nucleated red blood cells) lineage declined the quickest with no cells of that lineage observed after week 2.

Daniel et al (1989) evaluated the proliferative capacity of established LTBMC for producing Colony Forming Units- GM (CFC-GM) and Burst forming units - Erythroid (BFU-E), without the addition of fresh inoculum of pluripotent stem cells. The number of both types of colonies varied from culture to culture. The BFU-E numbers decline after only three weeks post stromal layer confluency. The CFU-GM colony numbers declined after 6-8 weeks. The complete removal of non- adherent cells from a confluent stromal layer produces an *in vitro* culture system with growth -promoting potential. Therefore, the growth and differentiation of the freshly inoculated pluripotent progenitor cells is evaluated and not that of any pre- existing progenitor cells (Reimann and Burger, 1979)

Inoculated Murine LTBMC with Confluent Stromal Layer

After the murine stromal layer reached confluency and freshly isolated mouse bone marrow mononuclear cells were re -inoculated, there was a continuous production of non-adherent cells throughout the four-week period that the cultures were maintained. Also, the cell count of the murine non-adherent layer was maintained despite the total removal of non-adherent

cells at the weekly cell collections. This suggests that an active murine LTBMC system had been established enabling the continuous proliferation and differentiation of haematopoietic progenitors.

Murine Clonogenic Cultures Of Non- Adherent Cells After Inoculation With CD117 Positive Cells

Cells present within the murine non-adherent cell population harvested at weekly intervals from the CD117 inoculated cultures were able to proliferate and differentiate into colonies of the erythroid, granulocytic/ monocytic and megakaryocytic cell lineages. Also colony numbers for all cell lineages were maintained throughout the four-week period over which the cells were collected. Although a slight drop in megakaryocytic colony numbers was seen in some cultures at week three, a return to previous levels was noted by week four. As this was only seen in one study of three, it may imply some experimental error at week 3 in that study. Proliferation of freshly aspirated bone marrow in established murine LTBMC has been described by Reimann et al (1979) and Talts et al (1998), respectively. Reimann et al (1979) reported total Colony Forming Units $_C$ (total colony numbers) not distinguishing between CFU-GM or BFU-E's, whereas Talts et al (1998) counted the CFU-GM colonies only. Megakaryocytes have been observed in LTBMC culture and quantified by counting the number present in the LTBMC cultures by Briddel et al (1992).

4.4.3 Murine Stromal Layer Development in T25 Flasks from Different Manufacturers

In the studies described here, the ability to establish confluent murine stromal layers was examined using T25 flasks from three different manufacturers. As seen in the previous study a confluent stromal layer developed after five to six weeks using Nunc flasks. Indeed by the end of the development phase, the fibroblastic cells and the macrophages were found to be the predominant cell types present within the adherent layer. Furthermore, fat cells were evident as well as islands of small round cells, resembling 'cobble stones', in association with adherent cells.

Only 65% confluency was achieved using Costar flasks and less than 40% confluency using Falcon flasks. This suggests that properties in the plastic used in the manufacture of the flask influence directly the ability of the cells to adhere. Furthermore, differences in the ability of macrophage/fibroblast like cells to adhere to plastic have previously noted (Gordon, 1993) where adherence of some fibroblasts was restricted by the properties of the plastic used. Also changes to the procedures employed to sterilize the flasks may alter the properties of the plastic. This may help explain the difference in stromal layer development found by Dexter (1976) and this study. Regrettably no information on the differences in the flasks is available due to the reluctance of the manufacturers to discuss their respective plastic formulation or sterilization procedures.

The ability to grow the stromal layer on Thermanox coverslips will enable further characterisation of the stromal layer using immunohistochemistry and electron microscopy. For example, immunohistochemistry could be used to identify the different cell populations in the stromal layer using cell specific surface markers and electron microscopy could be used to elucidate the intricate stromal structure.

4.4.4 Murine non-adherent cell counts (Nunc flasks only)

The total cell counts of murine non-adherent cells throughout the culturing period (stromal development and after inoculation with CD117 positive cells) was similar to that reported earlier. Also the rapid decline in non-adherent cell numbers during stromal development and the maintenance of non-adherent cell numbers, after inoculation with CD117 positive cells the same as noted previously and at the same time points. These findings substantiate the robustness of the LTBMC developed.

4.4.5 Development Phase of Stromal Layer of hCB LTBMC

In the work described here, hCB stromal layer confluency appears to require five weeks after culture initiation. The weekly morphological examination of the developing stromal layer showed small islands of spindle-like cells (fibroblastic cells) and flat cells (macrophages) by

the end of the first week. By the end of the development phase, the fibroblastic cells and the macrophages were found to be the predominant cell types present within the adherent layer.

The presence of the fat cells was only evident from the fourth week suggesting that these cells only appear when the cultures are close to achieving confluency. In fact others have shown that there is a correlation between the long-term survival of stem cells and the presence of fat cells in the stromal layer (Allen and Dexter, 1984). Thus, their presence in cultures seems to provide an indication of a successful stromal layer for supporting haemapoiesis. Also haemapoietic foci of small round cells are prominent once the cultures are close to achieving confluency.

Well-haemoglobinized clusters of erythroid cells seen loosely attached to adherent cells were noted in the first week of stromal development only. These are most probably the progeny of erythroid progenitors present in the mononuclear cell population added initially to the culture flasks. Erythropoietin required for the development of erythroid precursors to Burst Forming Cells -Erythroid is not produced by stromal cells, but by the kidney, therefore the absence of these cells in the LTBMC is not surprising. Also the presence and quick loss of the erythroid cells did not appear to inhibit stromal cell development, confirming that they play no role in the establishment or maintenance of the stromal layer. The stromal layer development took 5 weeks to reach confluency and is similar to that described by Dexter et al (1976) for human bone marrow cultures. Whilst the stromal layer was developing the number of non -adherent cells remained consistent.

4.4.6 Characterization of the hCB Non-Adherent Cells during LTBMC Stromal Layer Development

The total hCB non-adherent cell count and the cell counts for each cell population identified by morphology remained slightly increased, when compared to the number of cells initially added to the cultures, during the stromal development period.

The greatest change in the cell populations monitored was the proportional increase over the first five weeks in the relative numbers of monocytes, pro-monocytes and macrophages in the

non-adherent cells collected, which by week 6, represented 80% of the non -adherent cell population. Thus the monocytes, its precursors and macrophages appear to be the most predominant cell type in both the adherent (discussed in the previous section) and non-adherent layer. *In vivo*, macrophages have been shown to play a similar role to that seen in *vitro*; an integral component of the microenvironment important in supporting haematopoiesis, by the production of cytokines to initiate and drive cell differentiation and maturation.

A relative increase in the granulocyte population was noted in the first three weeks, with the promyelocytes and the metamyelocytes representing 40% and 18% of the total non-adherent population, respectively. The relative proportion of the promyelocytes and metamyelocytes continued to decline during the stromal development period and also reached a plateau by the fifth week of culturing.
Also the erythroblasts (nucleated red blood cells) lineage declined the quickest with no cells of that lineage observed after week 1.

4.4.7 Inoculated LTBMC with Confluent Stromal Layer

There was a continuous production of non-adherent cells throughout the four-week period that the cultures were maintained. Also, the cell count of the non-adherent layer was maintained despite the nearly total depletion of non-adherent cells at weekly intervals. This suggests that an active LTBMC system had been established enabling the continuous proliferation and differentiation of haemapoietic progenitors.

4.4.8 Clonogenic Cultures of Non Adherent Cells after Inoculation with Fresh hCB Cells

Cells present within the hCB non-adherent cell population harvested at weekly intervals from the inoculated cultures were able to proliferate and differentiate into colonies of the erythroid, granulocytic/ monocytic and megakaryocytic cell lineages. Also colony numbers for all cell lineages were maintained at constant levels throughout the four-week period that the cells were collected.

4.4.9 Identification of Cell Lineages within the Murine and hCB Non-Adherent Cells, Using Flow Cytometry

The morphological identification by light microscopy, of the cell lineage of some of the cells was difficult, and cells that could not be identified were classified as mononuclear cells. Cell lineage identification was easier using lineage specific markers. Also a larger number of cells could be analysed; 10, 000 cells by flow cytometry as opposed to 100 cells by light microscopy, improving the accuracy of the analysis. Additionally comparisons of the cell lineages identified by morphology and flow cytometry demonstrated similarities in the proportions of the different cell lineages (analysed in the stromal development phase only). For example, the mono/macro precursors and the granulocytic precursors are the predominant precursor cell lineages, as determined by flow cytometry and cell morphology. Additionally, both methods of lineage identification identified similar proportions of progenitor cells and erythroid cells. The use of flow cytometry and light microscopy for the identification of cell populations, have both advantages and disadvantages. Flow cytometric analysis enables large numbers of cells to be analyzed, and identifies cells that are difficult to distinguish morphologically, whereas light microscopy can aid visualization of different developmental stages of cells that are not accompanied with a change in surface markers. The utility of both flow cytometry and morphology in combination was demonstrated by this work. In order to confirm the cell populations identified by flow cytometry, immunohistochemical staining techniques could have been employed on cytospins of the non adherent cells. This additional confirmation step was not carried out in this work. Both flow cytometry and immunohistochemistry are recognized as diagnostic tools in the diagnosis of leukemia, lymphomas and a number of other tumors (Solomon and Kossev, 2002). Therefore the additional confirmation of the flow cytometric analysis, by immunohistochemistry could be considered unnecessary.

4.4.10 Comparison of Murine LTBMC with hCB LTBMC

The development of the hCB stromal layer morphologically appears to be similar to that described for murine stromal layer development. hCB stromal layers appear to reach confluency by the end of 5 weeks whereas the murine stromal layers appear to require 6

weeks.

Comparison of the hCB non-adherent cells with murine non-adherent cells identified using flow cytometry shows similarities. For example, the monocytic and granulocytic cell populations were the most prominent cell lineage identified in both human and murine cultures. Additionally, for both species a fast decline of erythrocytic precursors was seen. Both murine and hCB LTBMC were able to proliferate and differentiate into colonies of the erythroid, granulocytic/ monocytic and megakaryocytic cell lineages. Also colony numbers for all cell lineages were maintained throughout the four-week period that the cells were collected.

Chapter 5

5.1 Experimental Part 3: Elucidation of mechanism of action of Anagrelide Hydrochloride

5.1.1 Anagrelide Hydrochloride (AN)

Anagrelide (6-7- dichloro-1, 5 dihydroimidazo (2, 1-b)-quinazoline-2 (H3) –one monohydrochloride monohydrate), is currently licensed for the treatment of Essential Thrombocythemia (ET). However, AN was originally developed by Roberts laboratories Inc (Eatontown, USA) as a potential anti-thrombotic therapy. The anti-thrombotic pharmacology of AN results from the inhibition of platelet aggregation. Single dose administration up to and including a dose of 25mg, resulted in the desired anti-thrombotic activity with no reduction in peripheral blood platelet count during the two week follow- up period. However, initial repeat dose studies in healthy volunteers highlighted the ability of anagrelide to reduce platelet counts. Doses greater than or equal to 1mg were noted to cause a reduction in peripheral blood platelet count (SBA. 1997). A detailed review of the structure, pharmacology, preclinical data on AN is provided in Chapter 1.

Bone marrow aspirates from patients with ET pre and post treatment with anagrelide demonstrated no alteration in the number of megakaryocytes (SBA, 1997). Additionally, doses that resulted in the reduction of platelet counts in the pre-clinical toxicology studies caused no significant reduction in megakaryocytes. Therefore the reduction in circulating platelets was not a direct result of a reduction in the number of megakaryocytes within the bone marrow. No reduction in erythrocytic or granulocytic or monocytic cell counts was noted, highlighting the specificity of anagrelide (AN) to the megakaryocytic lineage alone. The mechanism (s) involved in this unique megakaryocytic lineage specific inhibition is currently unknown. Several hypotheses of the potential mechanism have been put forward by a number of researchers; the current understanding of the potential mechanism is discussed on Chapter 1. AN has been in clinical use for the treatment of ET (second line treatment) for at least 8 years and although it has been shown to reduce platelet counts effectively in ET patients, there is some concern that it may cause myelofibrosis.

Mazur et al (1992) demonstrated that AN at concentrations of 32 μM to 161μM, resulted in an increase (65%) in the percentage of megakaryocytes in developmental stages I and II when compared with concurrent controls (41.5% in developmental stages I and II). This shift in the developmental stage of cultured human megakaryocyte is a morphological change only, and in terms of megakaryocyte function is unclear.

Lane et al, (2001) hypothesized that all *in vitro* and *in vivo* studies carried out with AN's thrombocytopenic effects have been complicated, for a number of reasons. Firstly, the water-insoluble nature of AN (solubility of AN in water was 2μg/mL) has hindered *in vitro* studies, secondly, AN only decreases platelet levels in human subjects. This obstacle has hindered studies related to pharmacokinetics and toxicity in monkeys, dogs, and mice. Finally, the extensive biotransformation of AN has suggested that the anti-platelet aggregation and thrombocytopenia effects may not be directly mediated by intact AN, but rather through active metabolite AM. Lane et al (2001) proposed that biotransformation of AN into active metabolite was essential for its thrombocytopenic effect in mice. On the basis that only the human liver has the capacity to generate the active metabolite, AN compound can only induce thrombocytopenia in humans.

Erusalimsky et al (2002) and Wang et al (2005) showed that all animal species investigated (rats, dogs, and rabbits) produce significant amounts of AM. For example, in rats given AN at 5 mg/kg per day (dietary dosing), Cmax of AM was 38.6 nM and for AN 45nM.
Another explanation for the species difference in the activity of AN comes from the work of McCarty et al (2006). These investigators suggest that AN has greater affinity towards the human c-mpl (TPO) receptor than the murine c-mpl (TPO) receptor. Clearly the debate on the mechanism of action of AN resulting in platelet count reduction is unresolved.

5.1.2 Taxol

As previously discussed, taxol is a known anti-microtubule agent that promotes the assembly of microtubules from tubulin dimers and stabilizes microtubules by preventing depolymerization (Needleman et al, 2005). This stability results in the inhibition of the normal dynamic reorganization of the microtubule network that is essential for vital interphase and mitotic cellular functions. Bone marrow suppression is the major dose-limiting toxicity noted with Taxol. Neutropenia is the most common finding in clinical trials with Taxol with up to

30% decrease in neutrophil counts (Breton et al, 2001). Thrombocytopenia has been noted in 20% of the patients (platelet counts <100,000 cells/mm3) (White and de Alarcon, 2002. In this work taxol was used as a positive control (compound known to cause tubulin changes in megakaryocytes) for tubulin flow cytometry analyses.

5.1.3 Human Cord Blood Haematopoietic Stem Cells

Over the last decade, a series of studies have revealed human cord blood as an abundant source of haematopoietic stem cells (Broxmeyer et al, 1989; Hofmeister et al, 2007).
However, it is only in the last few years that human cord blood derived haematopoietic stems cells have become commercially available for *in vitro* use.
Cord blood contains high concentrations of haematopoietic stem cells, higher than that found in adult bone marrow (Broxmeyer et al, 1992; Hofmeister et al, 2007). *In vitro* assays conducted with human cord blood derived haematopoietic stem cells give rise to all the cell lineages, when exposed to the appropriate cytokine cocktails[3]. Comparison of cord blood and bone marrow *in vitro* cell lineages demonstrated that most colonies were monocyte/macrophage colonies (60.0 +/- 4.6%), whereas those from bone marrow were largely granulocyte colonies (88.3 +/- 6.2%). The differences of growth curve, colony size, and turnover state of CFUc between cord blood and bone marrow seemed, in part, to result from the dominancy of monocyte/macrophage colony-forming cells in cord blood. The cell cycling rate of CFUc in cord blood was remarkably lower than that in bone marrow. From these results, it seems clear that CFUc in cord blood may differ in some aspects from those of bone marrow derived haemopoietic cells (Hofmeister et al, 2007).

5.1.4 Aims

The work described here aims to investigate the mechanism(s) of the inhibition of megakaryocytic maturation, *in vitro*, by an agent used in the treatment of ET, AN.
Although several advances to define the action of AN have been reported the elucidation of the mechanism(s) of megakaryocytic inhibition is currently unclear. AN appears to be selectively suppress megakaryocytes. Although a similar inhibition of murine megakaryocytes has been demonstrated both *in vivo* and in vitro, human megakaryocytes clearly appear to be more sensitive to the action of AN (chapter 3). Therefore in order to elucidate the mechanism

of the inhibition of megakaryopoiesis human cord blood cells will be used as a source of human haematopoietic stem cells.

To elucidate the mechanism of the selective inhibition of human Mk progenitors a number of approaches were used:

1. Characterization of the effect of AN on cell cycling of hCB derived pluripotent stem cells in LTBMC. This enables the evaluation of the effects of AN on the early haemapoietic precursors (pluripotent and multipotent stem cells. In addition, the ability of the AN treated pluripotent and multipotent stem cells to give rise to lineage specific progenitors of the erythroid, monocytic/granulocytic and megakaryocytic cell lineages was evaluated.

2. Establishment of the AN concentration at which 50 percent of the CFU-E, GM-CFU and Mk colonies growth inhibition is achieved (IC50) using hCB derived stem cells in clonogenic cultures
3. Characterization of the effect of AN on cell cycling and megakaryocytic ploidy using hCB derived stem cells in clonogenic cultures.
4. Determination of the effects of AN on the expression of megakaryocytic cell surface antigens using hCB derived stem cells in clonogenic cultures.

Characterization of the effect of AN on Mk-CFU colony microtubule organisation using hCB derived stem cells in clonogenic cultures. In this work taxol was used as a positive control (compound known to cause tubulin changes in megakaryocytes) for tubulin flow cytometry analyses.

5.2 Methods

5.2.1 Procedure for Thawing Cryoperserved Mononuclear hCB

The method employed is described in Appendix A

5.2.2 Viability Count

Cord cell viability was determined using the method described in Chapter 2 (section 2.2.8).

5.2.3 Human LTBMC Medium

Human cord blood cultures were utilized as described in chapter 2 (section 2.2.5).

5.2.4 Inoculation of Confluent Stromal Layer with Fresh hCB Stem Cells (CD34 positive cells)

The method employed is described in chapter 2 (section 2.2.12). Within 1 hour of the fresh hCB stem cells being added to the established stromal layers, AN was added to the culture systems. Concentrations of AN evaluated ranged from 0.01 to 10µM**.** Each concentration was evaluated on 5 separate occasions, in triplicate on each occasion. The preparation of the AN was carried out as described in section Chapter 2 (section 2.2.18)

5.2.7 hCB Clonogenic Cultures

Non-Adherent Cells Collected From hCB LTBMC Treated with AN

For the assessment of non-adherent cells collected from the hCB LTBMC, 0.1mL of non-adherent cells ($1x10^5$cells/mL) were added to the clonogenic culture media as described in Chapter 2 (sections 2.2.7 and 2.2.8). Cell count was determined using an automated cell counter (Advia 120, Bayer, USA).

Assessment of Affects of AN on Cell Lineages

hCB clonogenic cultures as described in Chapter 2 were utilized in these experiments (sections 2.2.7 and 2.2.8). 0.1ml of viable hCB cells ($1x10^5$cells/mL) were added to the cultures. Cell count was determined using an automated cell counter (Advia 120, Bayer, USA). Concentrations of AN evaluated ranged from 0.01 to 10µM**.** Each concentration was evaluated on 3 separate occasions, in triplicate on each occasion. The preparation of the AN was carried out as described in section Chapter 2 (section 2.2.11).

5.2.6 Collection of CFU-Mk Colonies

The method employed is described in chapter 4 (section 4.2.14)

5.2.7 Cell Cycle Analysis

Cell cycle analysis as described in chapter 2, section 2.2.9 was employed.

5.2.8 Flow cytometry of Cell Surface Receptors

Cell preparation and flow cytometry analyses previously described in Chapter 2 (section 2.2.6) were employed. Table 5.1 details the specific cell surface target for each antibody employed.

Antibody	Tube	Concentration of Antibody (µg/mL)	Expression/Target
CD34/FITC	1 ,2 and 3	5	Stem cell
CD61/PE	1, 2 and 4	5	Mk Glycoprotein IIIb Part of GPIIa/IIIb complex
CD42/FITC	4	5	GP1 receptor
CD42/PE	3	5	GP1 receptor

Table 5. 1 Antibodies for receptor expression

The human CFU-Mk lineage specific antibodies used in this investigation are shown, together with their respective antigen target, volume and concentration used as well as the tube combination employed.

5.2.9 Intracellular Staining

Intracellular staining technique as described in Chapter 2, (section 2.2.6) was employed. 10µL of anti-Tubulin α or anti-Tubulin –β was added to the cells. Table 5.2 details the specific cell surface target for each antibody employed.

Antibody	Tube	Concentration of Antibody (µg/mL)	Expression/Target
Mouse Anti-Human Tubulin α	1	5	Tubulin α
Mouse Anti-Human Tubulin β	2	5	Tubulin –β

Table 5. 2 Antibodies for Intracellular staining

The anti- human tubulin-α and tubulin –β antibodies used are shown

5.3 Results

5.3.1 Evaluation of the Effect of AN on Cell Cycling of hCB Derived Pluripotent Stem Cells in LTBMC

Effects of AN on Cell Cycling

Cell cycle analyses were carried out on vehicle and AN treated hCB LTBMC. Non-adherent cells were collected at weekly intervals and cell cycle analysis performed using flow cytometry. Figures 5.1 to 5.3 show the percentage of non-adherent haemopoietic cells in G0/G1, G2/M (mitotic) and synthesis phase (S). Non adherent cells from each AN treated culture was collected and pooled prior to analyses. Mean data from 3 separate occasions are shown. In addition, table 5.3 shows the cell cycle analyses from each culture.

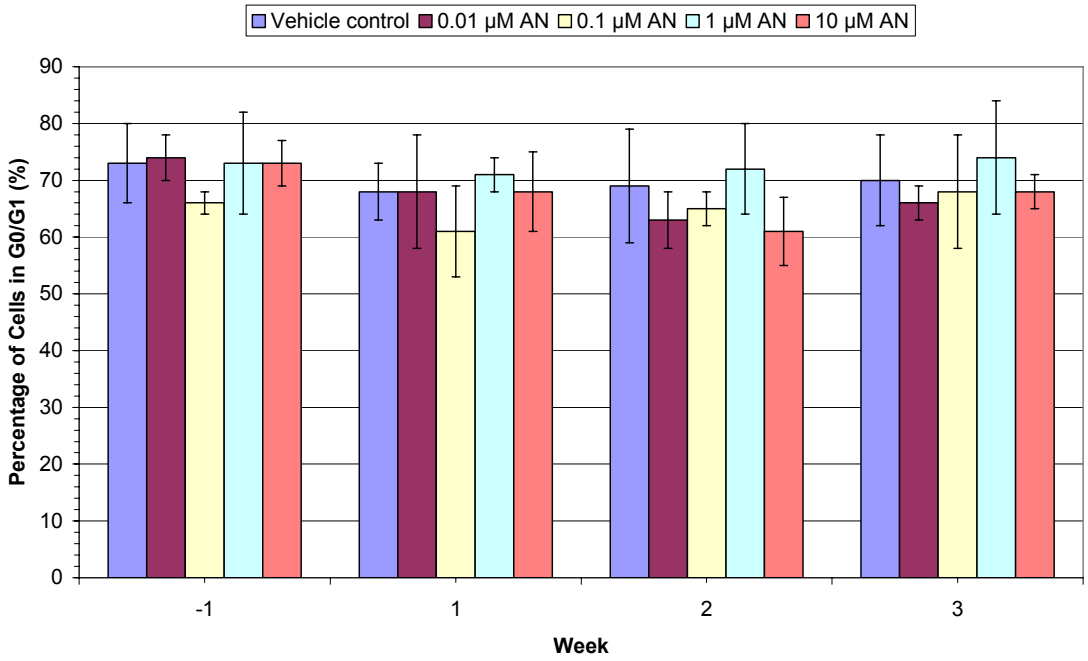


Figure 5. 1Cell Cycle Analyses: Percentage of Non-Adherent cells Collected from hCB LTBMC in G0/G1

The mean percentage of Mk culture cells in the G0/G1 of cell cycling is provided. Vehicle control and AN at concentrations from 0.1 to 10µM were evaluated in triplicate on 3 separate occasions. No change in the percentage of cells in G0/G1 was noted in vehicle control or any AN treated cultures. T-test and Dunnetts test performed comparing AN treated cultures concurrent weekly vehicle control. No statistical difference in the percentage of cells in G0/G1 was noted in vehicle control or any AN treated cultures.

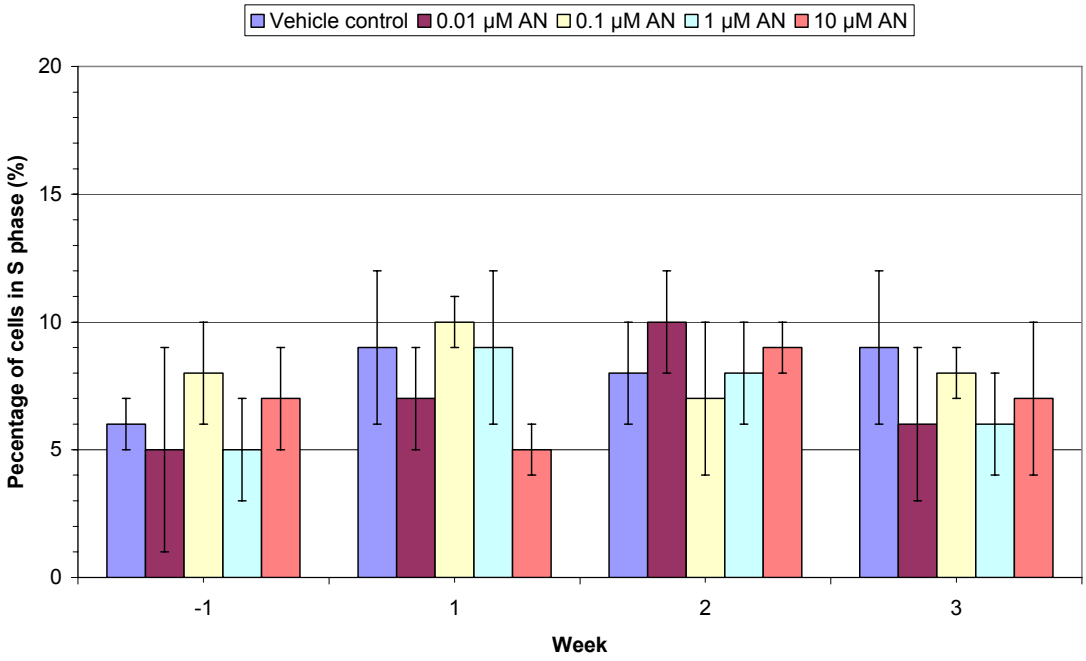


Figure 5. 2 Cell Cycle Analyses: Percentage of Non-Adherent cells Collected from hCB LTBMC in S Phase

The mean percentage of Mk culture cells in the S phase of cell cycling is provided. Vehicle and AN at concentrations from 0.1 to 10µM were evaluated in triplicate on 3 separate occasions. No change in the percentage of cells in S phase was noted in vehicle control or any AN treated cultures. T- test and Dunnetts test performed comparing AN treated cultures with concurrent weekly vehicle control. No statistical difference in the percentage of cells in S phase was noted in vehicle control or any AN treated cultures.

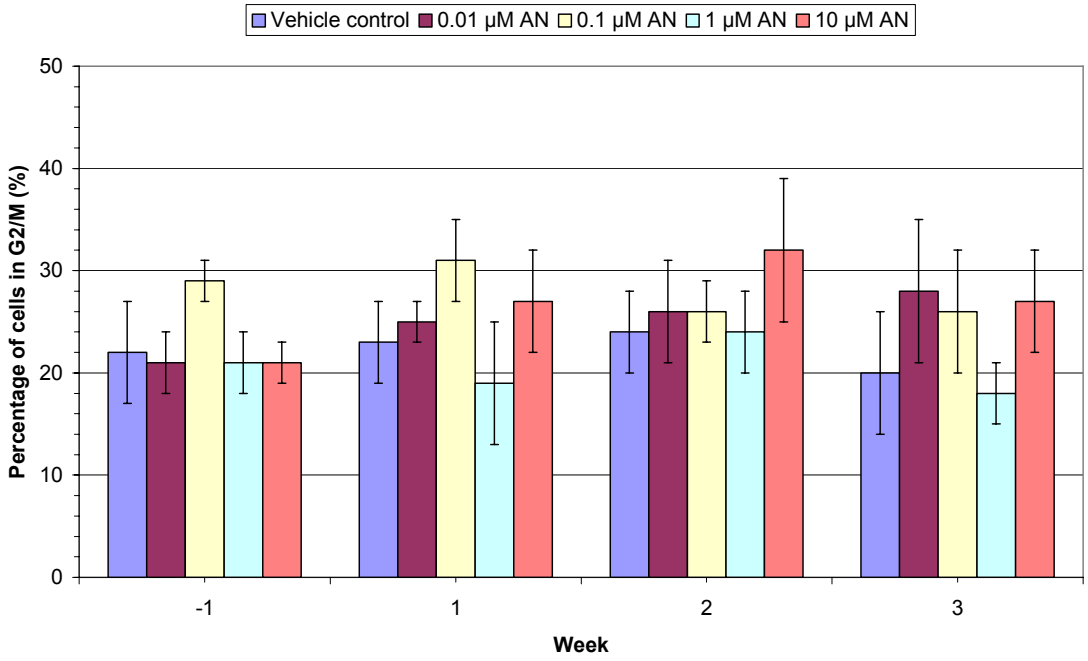


Figure 5. 3 Cell Cycle Analyses: Percentage of Non-Adherent cells Collected from hCB LTBMC in G2/M

The mean percentage of Mk culture cells in the G2/M of cell cycling is provided. Vehicle and AN at concentrations from 0.1 to 10µM were evaluated in triplicate on 3 separate occasions. No change in the percentage of cells in G2/M was noted in vehicle control or any AN treated cultures. T- test and Dunnetts test performed comparing AN treated cultures with concurrent weekly vehicle control. No statistical difference in the percentage of cells in G2/M was noted in vehicle control or any AN treated cultures.

The cell cycle analyses carried out weekly on vehicle control treated cutlures (Figure 5.1 to 5.3) shows no alteration in cell cycling, with 70% of the cells in G0/G1, 8% in S and 22% in G2/M phase. No statistically significant changes in the percentage of G0/G1, S or G2/M were noted in the weekly analyses.

Cell cycle analyses carried out on cultures treated with AN at concentrations ranging from 0.01 to 10 µM (Figure 5.1 to 5.3). No statistically significant alteration in cell cycling when compared with vehicle control. At 0.1 µM AN concentration, all three weekly experiments show the same slight numerical (statistically insignificant) increase in the percentage of cells in G2/M phase when compared with vehicle control levels (for each corresponding weekly cvehicle control), but not when compared with pre-treatment levels. A similar numerical (statistically insignificant) increase in the percentage G2/M cells was noted at 10 µM AN concentration in weeks 2 and 3 when compared with pre-treatment and vehicle control levels.

Treatment	Sampling Occasion	Percentage of colonies in G0/G1 (%)			Percentage of colonies in S (%)			Percentage of colonies in G2/M (%)		
	EXP. No	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Vehicle	Pre-Dose	73	70	71	10	7	11	17	23	18
	Post Dose Week 1	71	68	73	7	11	9	22	21	18
	Post Dose Week 2	81	70	64	4	8	8	15	22	28
	Post Dose Week 3	84	73	70	6	3	6	10	24	24
	Post Dose Week 4	68	70	61	4	8	10	28	22	29
0.01µM AN	Pre-Dose	67	72	66	5	9	7	28	19	27
	Post Dose Week 1	64	78	70	11	7	8	25	15	22
	Post Dose Week 2	69	73	67	4	10	7	27	17	26
	Post Dose Week 3	61	70	66	13	4	10	26	26	24
	Post Dose Week 4	74	70	74	3	9	8	23	21	18
0.1µM AN	Pre-Dose	70	64	61	5	6	9	24	30	30
	Post Dose Week 1	68	75	80	4	4	2	28	21	18
	Post Dose Week 2	78	74	71	4	4	4	18	22	25
	Post Dose Week 3	77	72	70	7	9	9	16	19	21
	Post Dose Week 4	70	74	74	4	4	8	26	22	18
1µM AN	Pre-Dose	72	74	77	12	1	5	16	25	18
	Post Dose Week 1	66	73	81	6	9	4	28	18	15
	Post Dose Week 2	77	70	72	9	12	6	14	18	22
	Post Dose Week 3	73	75	77	8	6	8	19	19	15
	Post Dose Week 4	74	71	77	10	4	5	16	25	18
10µM AN	Pre-Dose	71	74	69	13	6	4	16	20	27
	Post Dose Week 1	69	74	70	12	11	5	19	15	25
	Post Dose Week 2	71	73	74	16	4	5	13	23	21
	Post Dose Week 3	75	73	79	12	8	4	13	19	17
	Post Dose Week 4	72	74	78	3	9	6	25	17	16

Table 5. 3 Cell Cycle Analyses of hCB Derived LTBMC Treated with Vehicle or AN Weekly for 4 Weeks

The percentage of non-adherent cells, collected from hCB LTBMC, in the G0/G1, S and G2/M of cell cycling is shown. Vehicle and AN at concentrations of 0.01 to 10µM were evaluated in triplicate on five separate occasions. No change in the percentage of colonies in G0/G1, S and G2/M was noted in vehicle control or any AN treated cultures.

5.3.2 Ability of AN Treated LTBMC Non-Adherent Cells to Differentiate into Lineage Specific Progenitors

Non adherent cells were collected at weekly intervals from hCB LTBMC treated weekly with either vehicle or AN for 3 weeks were collected, washed and added to hCB clonogenic cell cultures. The mean colony counts per well for the granulocyte/ monocyte; erythrocyte and

megakaryocyte cell lineages are shown in Figures 5.4 to 5.6, respectively and the granulocyte/ monocyte; erythrocyte and megakaryocyte colony counts are shown in table 5.4. No statistically significant change in colony counts per well was noted irrespective of AN concentration evaluated in the erythroid or granulocytic/monocytic or megakaryocytic cell lineages.

Treatment	Sampling Occasion	Colony Counts /Well								
		CFU-GM			BFU-E			CFU-Mk		
Exp. No		1	2	3	1	2	3	1	2	3
Vehicle	Post Dose Week 1	70	68	72	184	198	180	85	88	72
	Post Dose Week 2	64	62	77	179	178	190	81	79	81
	Post Dose Week 3	72	64	71	191	181	188	77	70	79
0.01µM AN	Post Dose Week 1	66	61	68	177	168	167	72	75	78
	Post Dose Week 2	62	63	64	164	184	181	74	71	77
	Post Dose Week 3	70	62	62	170	172	190	69	70	82
0.1µM AN	Post Dose Week 1	58	61	60	178	182	181	72	76	71
	Post Dose Week 2	70	55	57	181	174	183	71	68	77
	Post Dose Week 3	68	60	61	180	167	185	77	72	70
1µM AN	Post Dose Week 1	60	78	66	175	190	181	70	73	77
	Post Dose Week 2	66	64	62	170	181	188	68	74	72
	Post Dose Week 3	64	70	61	190	173	184	77	79	73
10µM AN	Post Dose Week 1	66	73	79	189	195	178	63	77	68
	Post Dose Week 2	61	65	61	179	193	176	80	71	65
	Post Dose Week 3	63	69	60	181	190	180	72	76	74

Table 5. 4 The Mean CFU-GM, BFU-E and CFU-Mk Colony Counts Following Treatment with Vehicle or AN in hCB LTBMC

The mean number of colonies per well after treatment with AN treatment (0.01 to 10µM), weekly for 4 weeks. No statistically significant change in BFU-E, CFU-GM or CFU-Mk colony counts per well was noted irrespective of AN concentrations evaluated.

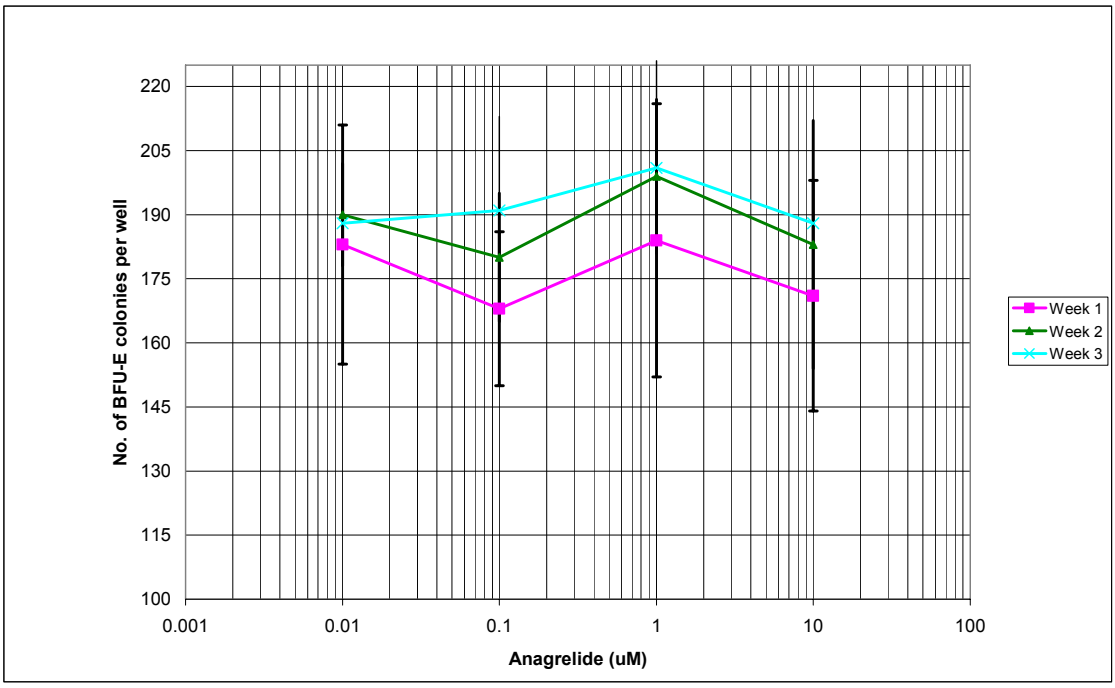


Figure 5. 4 Change in Erythroid Colony Number after Treatment with AN in hCB LTBMC

The mean number of colonies per well after treatment with AN treatment (at concentrations from 0.1 to 10μM), weekly for 4 weeks. No statistically significant change in BFU-E colony counts per well was noted at any of the AN concentrations evaluated. Each concentration evaluated in triplicate on three separate occasions. Mean vehicle control BFU-E colony count 188 (+/- 28).

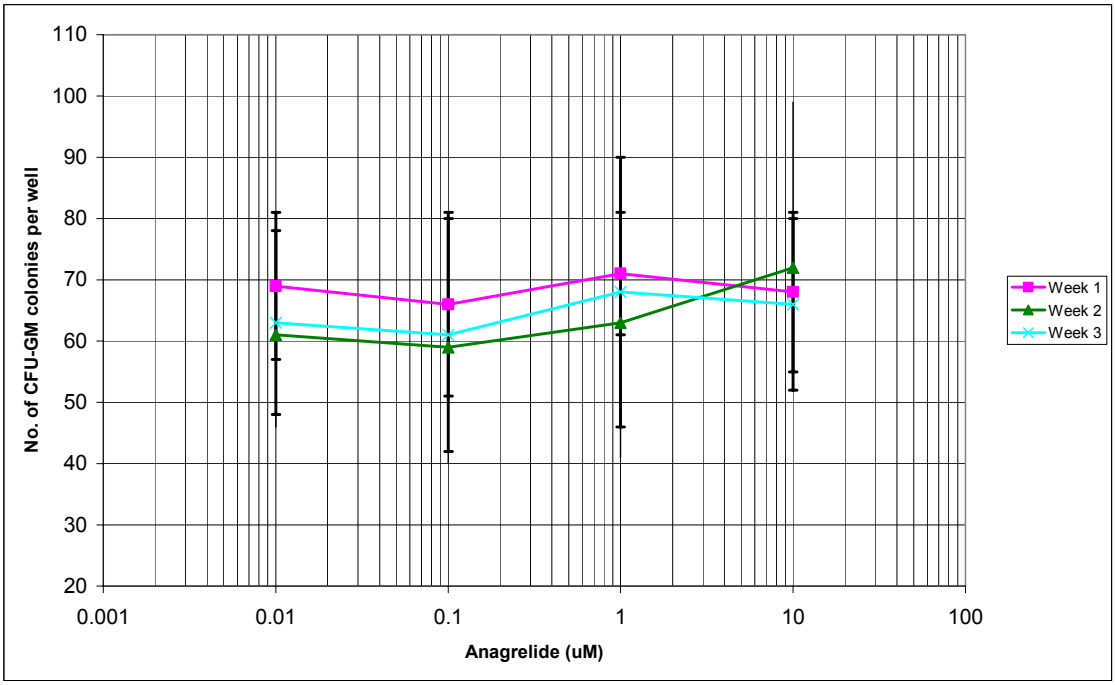


Figure 5. 5 Change in Granulocytic/Monocytic Colony Number after Treatment with AN in hCB LTBMC

Figure shows the mean number of colonies per well after treatment with AN at concentrations from 0.1 to 10µM. No statistically significant change in CFU-GM colony counts per well was noted at any of the AN concentrations evaluated. Each concentration evaluated in triplicate on three separate occasions. Mean vehicle control CFU-GM colony count 69 (+/- 15).

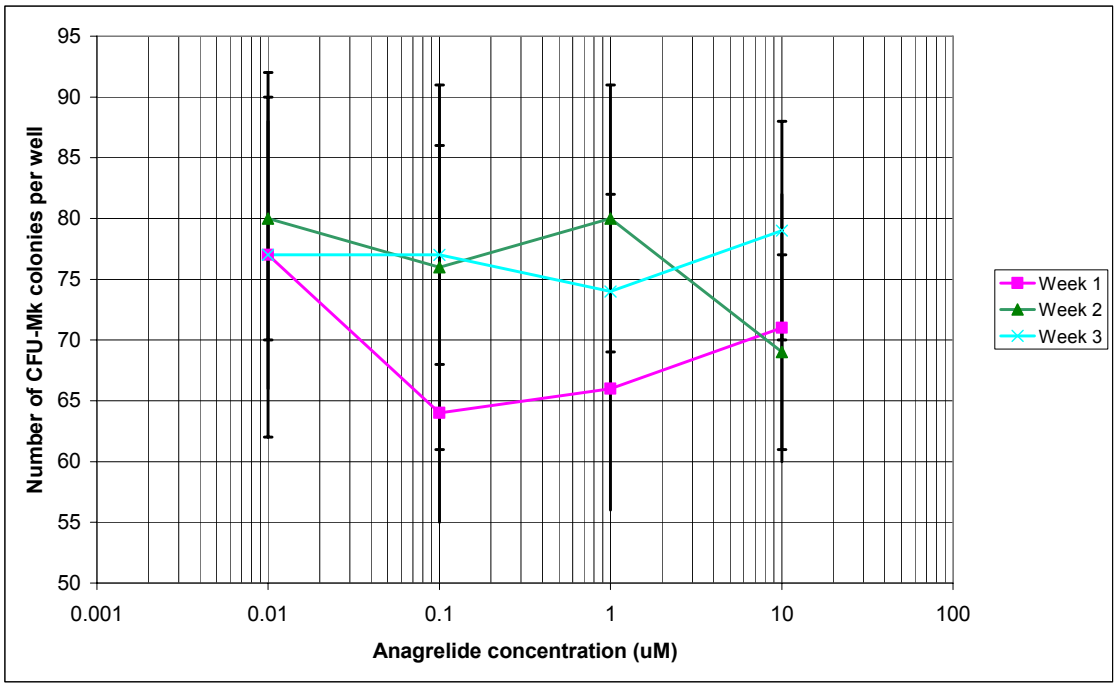


Figure 5. 6 Change in Megakaryocytic Colony Number after Treatment with AN in hCB LTBMC

Figure shows the mean number of colonies per well after treatment with AN concentrations from 0.01 to 10μM. No statistically significant change in CFU-Mk colony counts per well was noted irrespective of AN concentration evaluated. Each concentration evaluated in triplicate on three separate occasions. Mean vehicle control CFU-Mk colony count 85 (+/- 11).

5.3.3 Establishment of the AN IC50 Concentration for the CFU-E, CFU-GM and Mk Colonies, using hCB Derived Stem Cells in Clonogenic Cultures

The effect of AN on granulocyte/ monocyte, erythrocyte and megakaryocyte maturation was assessed in short-term cultures, and data was expressed as IC50 values (the concentrations at which *in vitro* colony growth was inhibited by 50%). The change in colony counts per well for the granulocyte/ monocyte, erythrocyte and megakaryocyte cell lineages are shown in Figures 5.7 to 5.9, respectively. The individual colony counts are shown in Table 5.5. Figure 5.10 shows the mean number of colonies per well for the granulocyte/ monocyte, erythrocyte and megakaryocyte cell lineages (of three experiments).

A reduction in the colony counts per well with an increase in AN concentration was noted in the megakaryocytic cell lineage only. The IC_{50} concentration of AN for the megakaryocytic cell lineage was 1.92µM. No biologically relevant decrease in colony number was noted in either the granulocytic/monocytic lineage or the erythrocytic cell lineage. Consequently the IC_{50} concentrations of AN for the granulocytic/monocytic lineage or the erythrocytic cell lineages could not be determined.

Treatment	Exp. No	Colony Counts /Well								
		CFU-GM			BFU-E			CFU-Mk		
	Replicate No.	1	2	3	1	2	3	1	2	3
Vehicle	1	54	73	79	192	173	205	80	83	80
	2	77	81	80	161	164	169	82	86	86
	3	72	71	81	166	151	116	83	84	83
0.01µM AN	1	83	74	70	193	160	206	73	74	77
	2	73	63	83	145	188	164	86	79	81
	3	68	56	59	160	186	185	69	65	71
0.1µM AN	1	75	54	76	215	196	188	61	66	60
	2	68	70	70	189	153	204	77	68	76
	3	59	73	76	139	154	172	61	62	63
1µM AN	1	59	73	76	215	196	188	52	57	49
	2	68	70	70	189	153	204	61	57	65
	3	75	54	76	157	181	169	55	55	52
10µM AN	1	61	69	61	139	154	172	47	42	44
	2	81	83	71	165	164	182	52	41	43
	3	63	75	77	206	200	216	46	41	46
100µM AN	1	41	68	77	185	196	159	40	38	32
	2	67	69	73	193	173	149	44	30	33
	3	84	93	72	165	183	176	38	35	32

Table 5. 5 Change in CF-GM, BFU-E and CFU-Mk Colony Number after Treatment with AN in hCB Clonogenic Cultures

The number of colonies per well after treatment with AN at concentrations of 0.01 to 10µM is shown. Each concentration evaluated in triplicate on three separate occasions. No biologically or statistically relevant decrease in colony number was noted in the CFU-GM or BFU-E colony numbers at any AN concentration evaluated. The IC50 concentrations of AN for the CFU-GM could not be determined. A reduction in the CFU-Mk colony counts per well with an increase in AN concentration was noted. The IC50 concentration of AN for the CFU-Mk was 1.92µM.

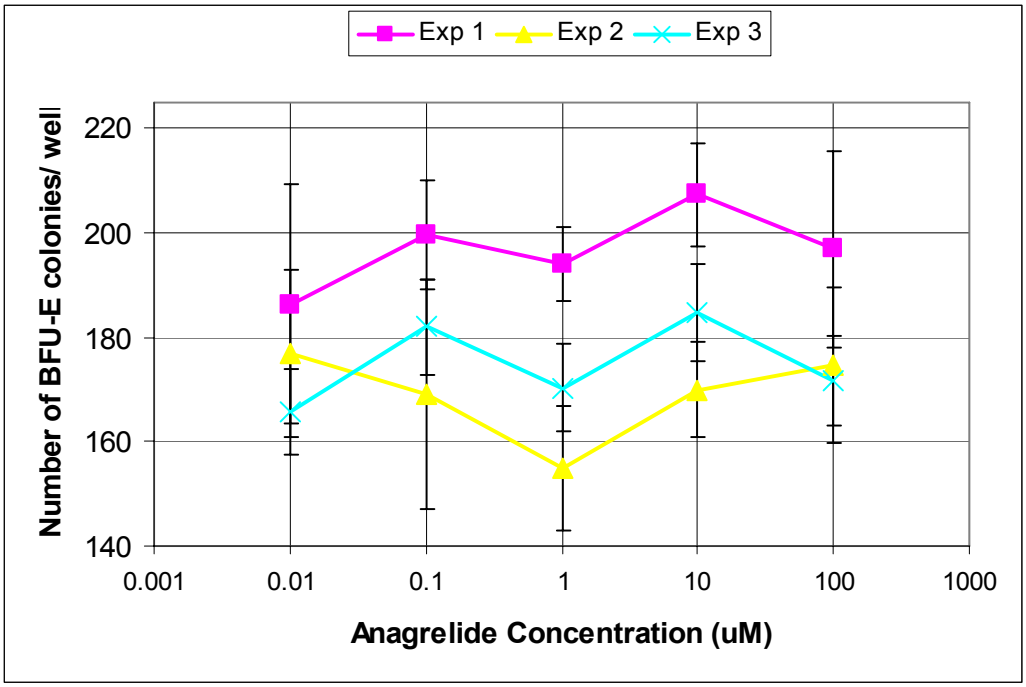


Figure 5. 7 Change in Erythroid Colony Number after Treatment with AN in hCB Clonogenic Cultures

The mean number of colonies per well after treatment with AN at concentrations from 0.01 to 100μM is shown. No biologically or statistically significant decrease in colony number was noted in the BFU-E colony numbers at any AN concentration evaluated. The IC50 concentrations of AN for the BFU-E could not be determined. Each concentration evaluated in triplicate on three separate occasions. Mean vehicle control colony count 188 /well (+/- 17 BFU-E colonies).

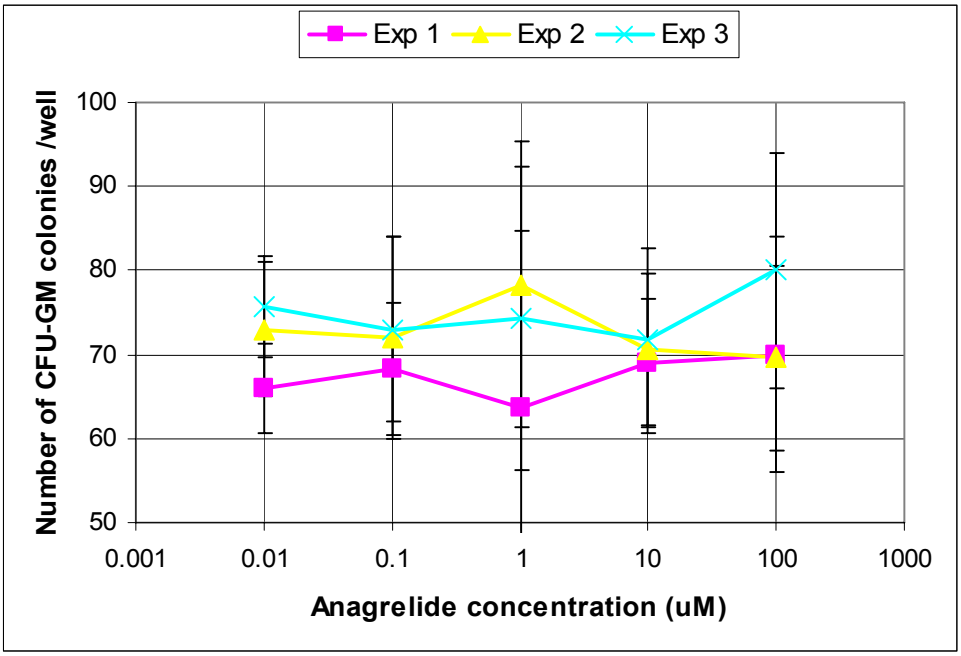


Figure 5. 8 Change in Granulocytic/Monocytic Colony Number after Treatment with AN in hCB Clonogenic Cultures

The mean number of CFU-GM colonies per well after treatment with AN at concentrations from 0.01 to 100μM is shown. No biologically or statistically relevant decrease in colony number was noted in the CFU-GM colony numbers at any AN concentration evaluated. The IC50 concentrations of AN for the CFU-GM could not be determined. Each concentration evaluated in triplicate on three separate occasions. Mean vehicle control colony count 83 colonies /well (+/- 24 CFU-GM colonies).

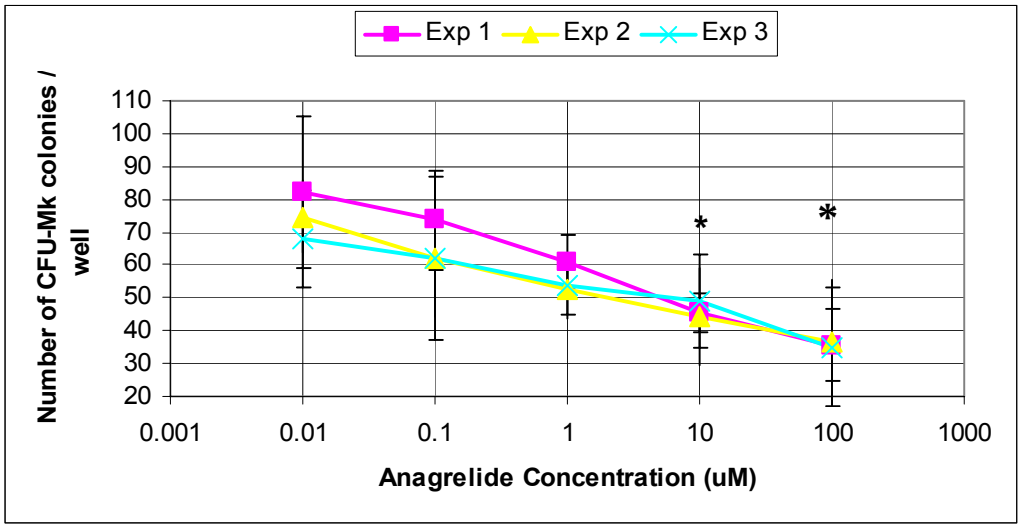


Figure 5. 9 Change in Megakaryocytic Colony Number after Treatment with AN In hCB Clonogenic Cultures

The mean number of colonies per plate after treatment with AN at concentrations from 0.01 to 100μM is shown. A reduction in the CFU-Mk colony counts per well with an increase in AN concentration was. The IC50 concentration of AN for the CFU-Mk was 1.92μM. Each concentration evaluated in triplicate on three separate occasions. Mean vehicle control colony count 76 colonies /well (+/- 31 CFU-Mk colonies). * P<0.05 compared AN treated cultures with concurrent no AN treatment control

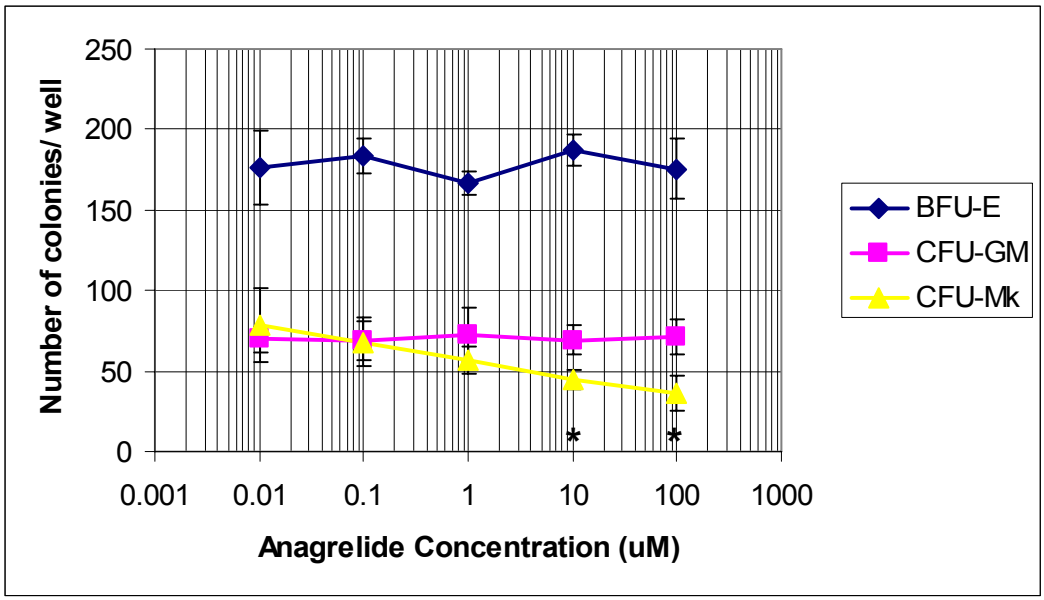


Figure 5. 10 Mean Change in Colony Number after Treatment with AN in hCB Clonogenic Cultures

The mean number of colonies per well after treatment with AN at concentrations from 0.01 to 100μM is shown. No biologically / statistically relevant decrease in colony number was noted in the CFU-GM or BFU-E colony numbers at any AN concentration evaluated. The IC50 concentrations of AN for the CFU-GM could not be determined. A reduction in the CFU-Mk colony counts per well with an increase in AN concentration was noted. The IC50 concentration of AN for the CFU-Mk was 1.92μM. Each concentration evaluated in triplicate on three separate occasions. Data shown are Mean +/- SEM. * P<0.05 compared AN treated cultures with concurrent no AN treatment control. Mean vehicle control BFU-E colony count 188 (+/- 17 BFU-E colonies). Mean vehicle control colony count 83 colonies /well (+/- 24 CFU-GM colonies). Mean vehicle control colony count 76 colonies /well (+/- 31 CFU-Mk colonies).

5.3.4 Evaluation of the effect of AN on cell cycling and megakaryocytic ploidy using hCB derived stem cells in clonogenic cultures

Cell Cycle Analyses

The previous cell cycle analysis was carried out on non-adherent cells collected from hCB LTBMC. In this series of experiments cell cycle analyses were carried out on vehicle and anagrelide treated cells in triplicate Mk-CFU clonogenic cultures on three separate occasions. Table 5.6 shows the cell cycle analyses. Figure 5.11 shows the percentage of cells in G0/G1, synthetic (S) and G2/mitotic phase (G2+M).

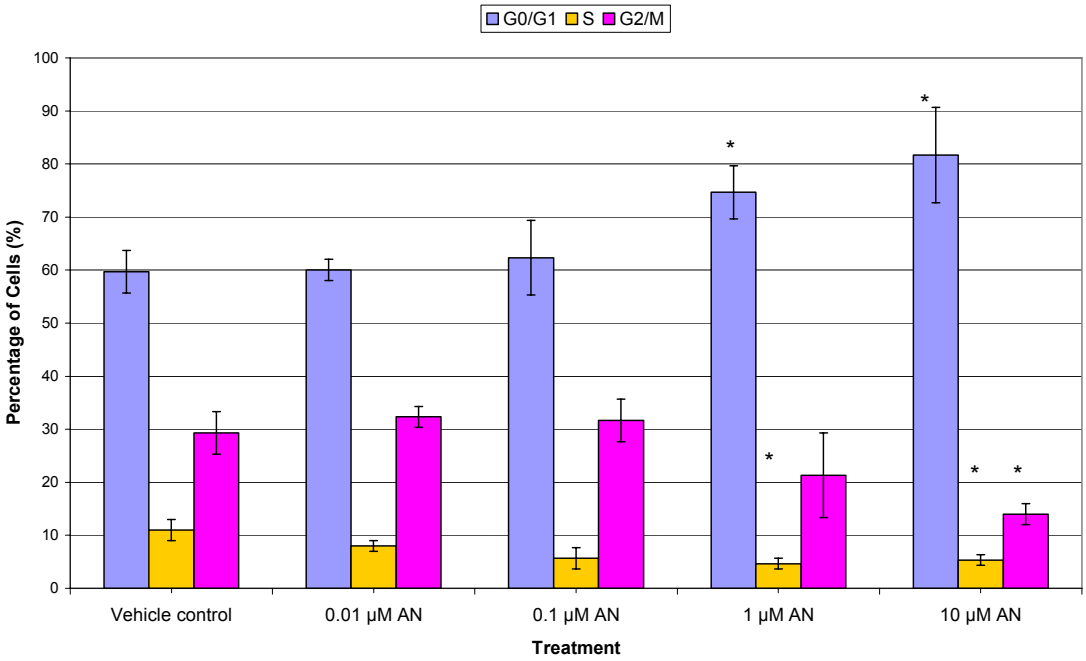


Figure 5. 11 Cell cycle analyses of AN Treated hCB Clonogenic Cultures

The mean percentage of Mk cells at different phases of cell cycling after treatment with vehicle or AN (0.01 to 10µM) shows a AN treatment related increase in CFU-Mk colonies in Go/G1 at concentrations of 1 and 10µM. A corresponding decrease in CFU-Mk colonies S and G2/M is also noted. Vehicle control or AN was evaluated in triplicate on three separate occasions. Data shown are Mean data from three separate experiments. Data shown are mean +/- SEM. *P<0.05 compared AN treated cultures with concurrent no AN treatment control.

The cell cycle analyses carried on vehicle control treated cutlures (Figure 5.11) shows no alteration in cell cycling, with 60% of the cells in Go/G1, 11% in S and 29% in G2/M phase.

Cell cycle analyses were carried out on Mk-CFU colonies collected from clonogenic cultures treated with AN at concentrations ranging from 0.01 to 10 µM (Figure 5.11). No treatment related change in the percentage of G0/G1, S and G2/M populations was noted at concentrations of 0.01 or 0.1µM AN. A treatment related increase in the precentage of colonies in G0/G1 and corresponding decrease in the percentage of colonies in S phase was noted at 1 and 10 µM AN. In addition at 10 µM AN concentration a decrease on the percentage of colonies in G2/M was also noted.

Treatment	Exp. No	Cell Cycle Data								
		Colonies in G0/G1 (%)			Colonies in S (%)			Colonies in G2/M (%)		
	Replicate No.	1	2	3	1	2	3	1	2	3
Vehicle	1	58	61	63	14	17	12	28	22	25
	2	57	60	62	15	14	11	28	26	27
	3	55	67	63	15	6	12	30	27	25
0.01µM AN	1	66	64	53	3	7	15	31	29	32
	2	57	63	62	10	4	13	33	33	25
	3	63	56	66	6	14	4	31	30	30
0.1µM AN	1	62	63	60	5	11	3	33	26	27
	2	64	62	61	6	10	13	30	28	26
	3	60	64	62	9	10	5	31	26	33
1µM AN	1	77	75	76	6	7	3	17	18	21
	2	70	79	77	11	3	3	19	18	20
	3	71	70	68	6	8	7	23	22	25
10µM AN	1	80	84	78	4	3	3	16	13	19
	2	81	83	77	1	3	7	18	14	16
	3	83	80	76	1	5	7	16	15	17

Table 5. 6 Cell cycle analyses of AN Treated hCB Clonogenic Cultures

The percentage of Mk colonies in G0/G1, G2/M and S phase, after treatment with vehicle or AN shows a AN treatment related increase in CFU-Mk colonies in Go/G1 at concentrations of 1 and 10µM. A corresponding decrease in CFU-Mk colonies S and G2/M is also noted. Vehicle or AN was evaluated in triplicate on three separate occasions.

The ploidy number of CFU-Mk colonies was determined of colonies collected from the cultures (Figure 5.12 and Table 5.7). The majority of colonies have a DNA content of 2N (60%), 25% 4N with 8N, 16N, 32N and 64N each representing <5% of the total CFU-Mk colony population. A statistically significant increase in the number of 2N colonies and a statistically significant decrease 4N and 8N ploidy were noted in CFU-Mk colonies treated with 1 and 10 µM AN, respectively. No changes were noted in the number of CFU-Mk colonies with DNA content > 8N.

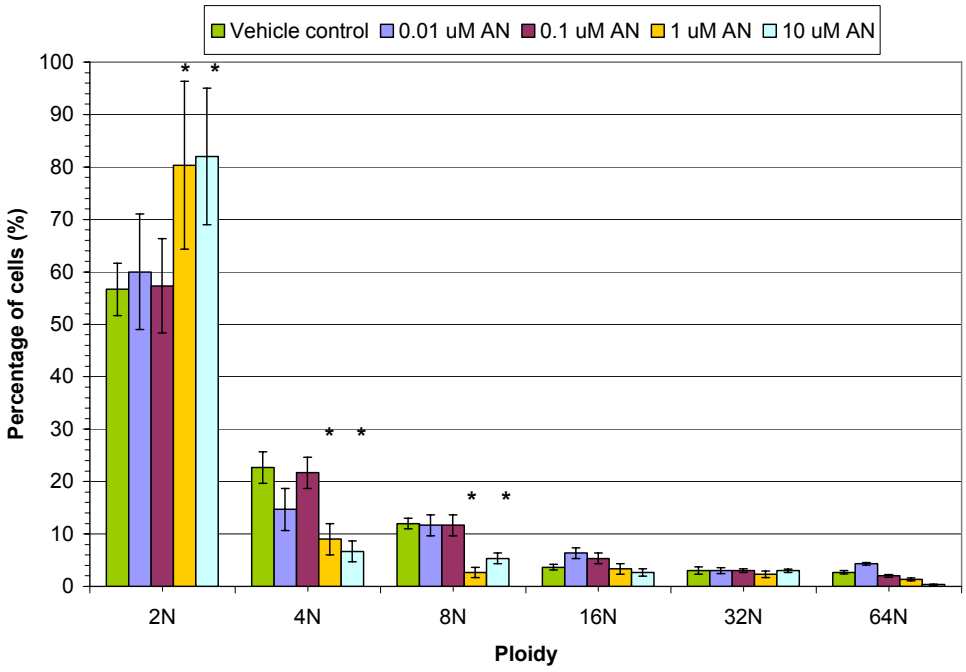


Figure 5. 12 Ploidy Determination of hCB CFU-Mk Colonies following AN Treatment

The mean percentage of Mk colonies with different ploidy levels after treatment with vehicle control or AN (0.01 to 10μM) shows an increase in the percentage of colonies with 2N DNA content at AN concentrations of 1 and 10μM. A decease in colonies with 4N and 8N DNA content is also noted at 1 and 10μM. Each concentration was evaluated in triplicate on three separate occasions. Data shown are mean +/- SEM. *P<0.05 compared AN treated cultures with concurrent no AN treatment control.

Treatment	Exp. No	Ploidy (N Number)Data					
		2N	4N	8N	16N	32N	64N
Vehicle	1	55	20	15	3	3	4
	2	60	22	11	5	3	1
	3	55	26	10	3	3	3
0.01μM AN	1	61	15	16	5	2	1
	2	66	12	8	6	3	5
	3	53	17	11	8	4	7
0.1μM AN	1	55	24	12	6	3	3
	2	61	17	16	4	2	0
	3	56	24	7	6	4	3
1μM AN	1	80	7	2	5	3	0
	2	80	9	4	3	3	1
	3	81	11	2	2	1	3
10μM AN	1	84	5	7	2	1	1
	2	82	7	5	2	4	0
	3	80	8	4	4	4	0

Table 5. 7 Ploidy Determination of hCB CFU-Mk Colonies following AN Treatment

The mean percentage of Mk colonies with different ploidy levels after treatment with vehicle control or AN (0.01 to 10μM) shows an increase in the percentage of colonies with 2N DNA content at AN concentrations of 1 and 10μM. A decease in colonies with 4N and 8N DNA content is also noted at 1 and 10μM. Vehicle or AN was evaluated in triplicate on three separate occasions.

5.3.5 Determination of the Effects of AN on the Expression of Megakaryocytic Cell Surface Antigens Using hCB Derived Stem Cells in Clonogenic Cultures

Treatment with AN resulted in a numerical decrease in the percentage of CD34 positive /CD 61 negative colonies and, although not statistically significant, at 1 and 10 μM anagrelide (Figure 5.13). Table 5.8 shows the percentage of cells with each of the surface antigen expression evaluated. The percentage of CD34 negative /CD61 positive colonies show a statistically significant decrease at 1 and 10 μM AN concentrations. The percentage of CD34 positive/CD61 negative, CD34 / CD61 dual positive and CD61 / CD42 dual positive colonies demonstrated no statistically significant changes at all AN concentrations evaluated.

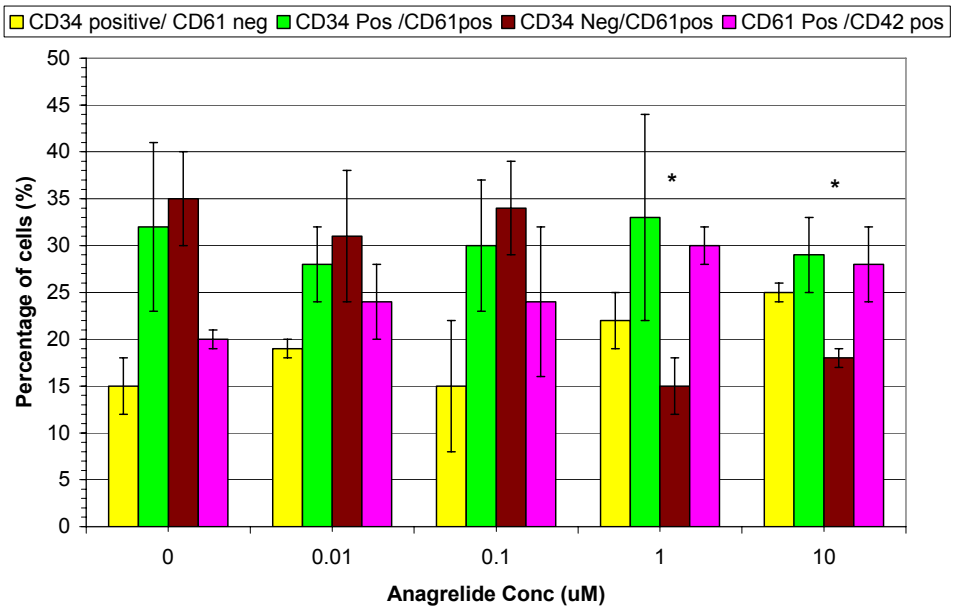


Figure 5. 13 Surface Receptor Expression Determination of CFU-Mk Colonies Treated with AN

The mean percentage of Mk colonies expressing lineage specific surface receptors after treatment with AN at concentrations from 0.01 to 10μM is shown. Treatment with AN resulted in a numerical decrease in the percentage of CD34 positive /CD 61 negative colonies and, although not statistically significant, at 1 and 10 μM AN. The percentage of CD34 negative /CD61 positive colonies show a statistically significant decrease at 1 and 10 μM AN concentrations. The percentage of CD34 positive/CD61 negative, CD34 / CD61 dual positive and CD61 / CD42 dual positive colonies demonstrated no statistically significant changes at all AN concentrations evaluated Each concentration was evaluated in triplicate on three separate occasions. Data shown are Mean +/- SEM. * $P<0.05$ compared AN treated cultures with concurrent no AN treatment control

Treatment	Exp. No	Percentage of Surface Antigen Expression (%)											
		CD34 positive/ CD61 negative			CD34 Positive /CD61 positive			CD34 Negative/ CD61positive			CD61 Positive /CD42 positive		
	Replicate No.	1	2	3	1	2	3	1	2	3	1	2	3
Vehicle	1	18	15	14	33	35	39	36	37	30	13	13	17
	2	14	16	17	27	33	30	33	35	36	26	16	17
	3	17	16	15	34	27	29	35	34	35	14	23	21
0.01µM AN	1	15	13	15	22	31	34	33	30	35	30	26	16
	2	15	16	14	34	33	30	34	27	31	17	24	25
	3	14	18	12	24	23	25	34	30	29	28	29	34
0.1µM AN	1	13	18	15	34	26	33	34	39	33	19	17	19
	2	14	11	16	31	28	35	34	37	31	21	24	18
	3	16	11	19	33	34	31	33	30	32	18	25	18
1µM AN	1	23	25	22	33	35	39	12	15	18	32	25	21
	2	21	19	27	41	30	35	18	18	12	20	33	26
	3	23	24	22	36	38	37	17	16	17	24	22	24
10µM AN	1	26	24	25	34	30	28	12	18	17	28	28	30
	2	28	22	29	27	35	31	15	16	14	30	27	26
	3	25	24	23	33	29	30	14	17	15	30	30	32

Table 5. 8 Surface Receptor Expression Determination of CFU-Mk Colonies Treated with AN

The percentage of Mk colonies expressing lineage specific surface receptors after treatment with AN is shown. Treatment with AN resulted in a numerical decrease in the percentage of CD34 positive /CD 61 negative colonies and, although not statistically significant, at 1 and 10 µM AN. The percentage of CD34 negative /CD61 positive colonies show a statistically significant decrease at 1 and 10 µM AN concentrations. The percentage of CD34 positive/CD61 negative, CD34 / CD61 dual positive and CD61 / CD42 dual positive colonies demonstrated no statistically significant changes at all AN concentrations evaluated

5.3.6 Evaluation of the Effect of AN on Mk-CFU Colony Microtubule Organization using hCB Derived Stem Colonies in Clonogenic Cultures

Two AN concentrations (1 and 10 µM) that have previously been shown to cause alteration in Mk precursor ploidy, cell cycling, Mk precursor cell surface receptor alteration and inhibit Mk –CFU colony growth were selected. Taxol was selected as a positive control compound. The defined pharmacology of taxol is as a microtubule disrupter, as well as clinical data demonstrating the ability of taxol to induce thrombocytopenia in 20% of patients. The concentration of Taxol used in this experiment (1µM) was selected based upon hCB cell viability (concentration at which 75 to 81% cell viability was achieved), Figure 5.14. The hCB viability percentages are shown in Table 5.8.

The percentage of tubulin –α and tubulin-β1 in Mk colonies after treatment with AN (1 and 10 μM) or Taxol (1μM) was determined, using intracellular flow cytometric staining techniques. Figure 5.15 shows the mean percentage of tubulin –α and tubulin-β1 in Mk colonies post anagrelide and Taxol treatment. Table 5.10 shows the percentage of tubulin –α and tubulin-β1 in Mk colonies post anagrelide and Taxol treatment. Although the number of tubulin –α and tubulin-β1 positive colonies tended to be lower, no statistically significant decrease in the expression levels of either tubulin –α and tubulin-β1 was noted.

The mean fluorescence intensity of the tubulin –α and tubulin-β1 staining of Mk colonies treated with AN or Taxol was compared with the mean fluorescence intensity of the untreated Mk colonies (Table 5.11). A statistically significant decrease in the mean fluorescence intensity was noted with tubulin-β1 staining (Figure 5.16) of Mk colonies treated with AN at a concentration of 10 μM and with Taxol at a concentration of 1 μM. No statistically significant change in mean fluorescence intensity was noted in tubulin –α staining of Mk colonies treated with AN or Taxol.

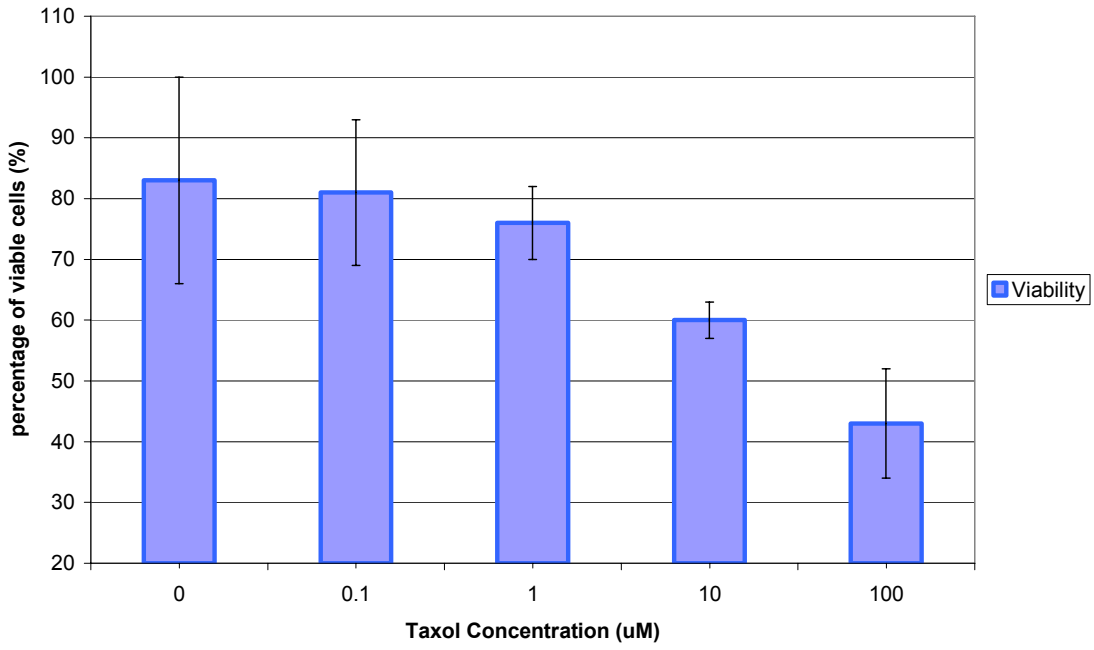


Figure 5. 14 Mean Percentage of Viable hCB after Treatment with Taxol.

The percentage of viable colonies (determined using Annexin V/ PI staining) is shown, following incubation with vehicle control and Taxol at concentrations from 0.1 and 100μM. The percentage of viable colonies decreases with Taxol concentration. Minimal cell viability is lost at Taxol concentrations of 0.1 and 1 μM. Each concentration evaluated in triplicate on three separate occasions. Data shown are Mean +/- SEM

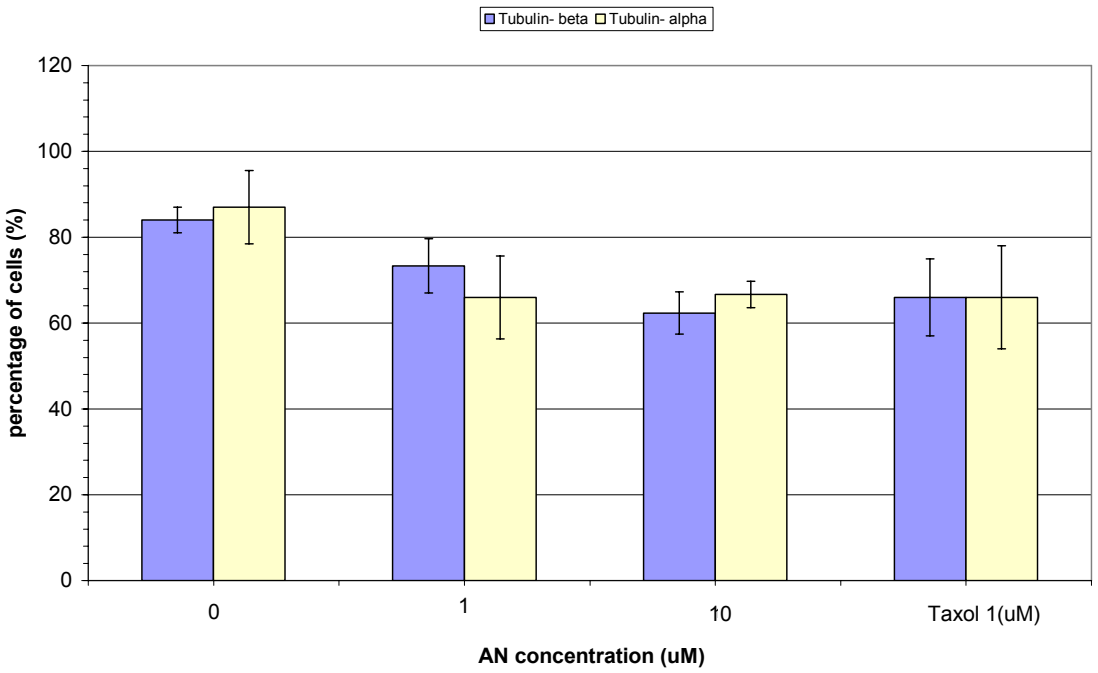


Figure 5. 15 Mean Percentage of Alpha and Beta Tubulin in Mk Colonies after Treatment with AN or Taxol.

The mean percentage of tubulin –α and tubulin-β1 expression in Mk colonies after treatment with AN (1 and 10 μM) or Taxol (1μM) is shown. The number of tubulin –α and tubulin-β1 positive colonies tended to be lower (compared with concurrent control) no statistically significant decrease in the expression levels of either tubulin –α and tubulin-β1 was noted. Each concentration evaluated in triplicate on three separate occasions. Data shown with +/- SEM

Treatment	Exp. No	Viability Counts (%)		
	Replicate No.	1	2	3
Vehicle	1	88	80	74
	2	81	84	85
	3	78	73	76
0.1μM Taxol	1	80	87	94
	2	88	75	89
	3	83	89	88
1μM Taxol	1	70	78	79
	2	67	71	89
	3	71	85	72
10μM Taxol	1	65	61	51
	2	58	68	50
	3	51	68	56
100μM Taxol	1	46	42	38
	2	40	41	45
	3	39	49	38

Table 5. 9 Percentage of Viable Mk-CFU Colonies after Treatment with Taxol

The percentage of viable colonies (determined using Annexin V/ PI staining) is shown, following incubation with vehicle control and Taxol at concentrations from 0.1 and 100μM. The percentage of viable colonies decreases with Taxol concentration. Minimal cell viability is lost at Taxol concentrations of 0.1 and 1 μM. Each concentration evaluated in triplicate on three separate occasions.

Treatment	Tubulin	Percentage of Tubulin expression (%)								
	Exp. No	1			2			3		
	Replicate No.	1	2	3	1	2	3	1	2	3
Vehicle	Tubulin-Alpha	80	79	73	80	83	78	77	80	84
	Tubulin-Beta	85	87	75	78	74	87	83	81	80
1μM AN	Tubulin-Alpha	75	77	80	79	81	76	79	80	77
	Tubulin-Beta	76	73	84	76	77	79	74	75	78
10μM AN	Tubulin-Alpha	63	67	63	64	68	61	60	63	61
	Tubulin-Beta	65	67	68	63	67	64	58	67	57
10μM AN	Tubulin-Alpha	63	60	63	57	59	64	61	60	59
	Tubulin-Beta	58	63	64	66	65	63	61	60	63

Table 5. 10 Percentage of Alpha and Beta Tubulin in Mk Colonies after Treatment with AN or Taxol

The percentage of tubulin –α and tubulin-β1 expression in Mk colonies after treatment with AN (1 and 10 μM) or Taxol (1μM) is shown. The number of tubulin –α and tubulin-β1 positive colonies tended to be lower (compared with concurrent control) no decrease in the expression levels of either tubulin –α and tubulin-β1 was noted. Each concentration evaluated in triplicate on three separate occasions.

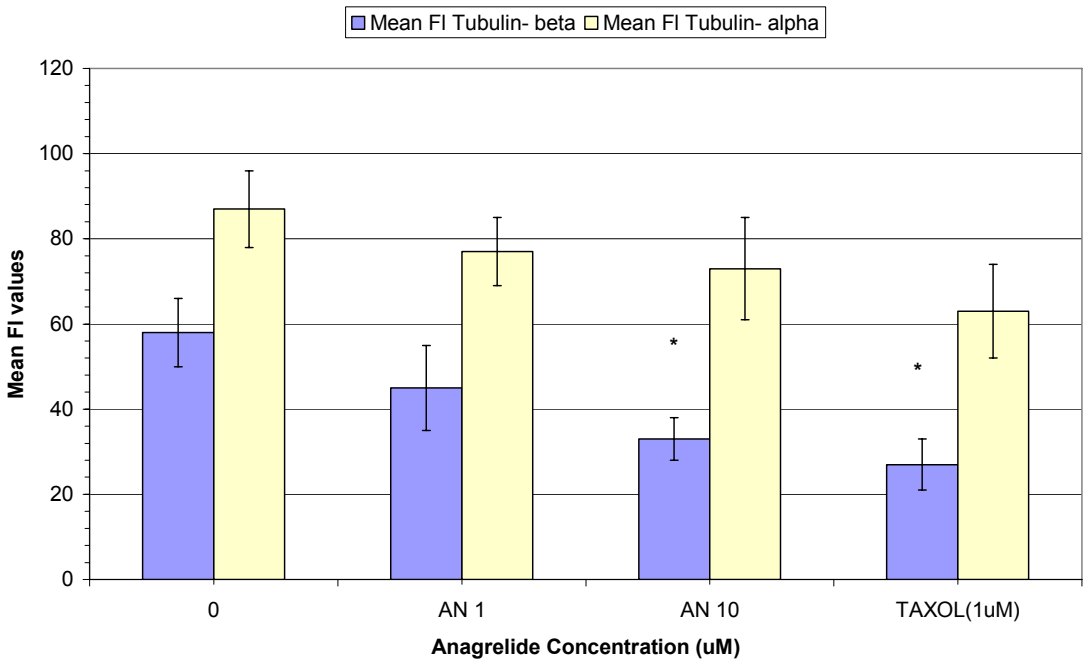


Figure 5. 16 Mean Fluorescence Intensity of Alpha and Beta Tubulin Staining in Mk Colonies After Treatment with AN or Taxol.

The mean fluorescence intensity of the tubulin –α and tubulin-β1 staining of Mk colonies treated with AN or Taxol was compared with the mean fluorescence intensity of the untreated Mk colonies. A statistically significant decrease in the mean fluorescence intensity was noted with tubulin-β1 staining of Mk colonies treated with AN at a concentration of 10 μM and with Taxol at a concentration of 1 μM. No statistically significant change in mean fluorescence intensity was noted in tubulin –α staining of Mk colonies treated with AN or Taxol. Each concentration evaluated in triplicate on three separate occasions. Data shown with +/- SEM * $p < 0.01$ compared AN treated cultures with no AN treatment group

Treatment	Tubulin	Mean Fluorescence Intensity Value								
	Exp. No	1			2			3		
	Replicate No.	1	2	3	1	2	3	1	2	3
Vehicle	Tubulin-Alpha	63	55	52	53	55	58	54	49	59
	Tubulin-Beta	80	83	87	84	88	86	79	86	74
1μM AN	Tubulin-Alpha	43	40	41	45	43	43	46	43	47
	Tubulin-Beta	73	70	75	71	77	74	70	70	71
10μM AN	Tubulin-Alpha	33	37	38	31	33	36	38	32	38
	Tubulin-Beta	74	75	78	71	73	71	72	70	75
10μM AN	Tubulin-Alpha	21	24	25	20	19	28	22	26	24
	Tubulin-Beta	54	51	50	53	57	63	64	61	57

Table 5. 11 Fluorescence Intensity of Alpha and Beta Tubulin Staining in Mk Colonies after Treatment with AN or Taxol

The fluorescence intensity of the tubulin –α and tubulin-β1 staining of Mk colonies treated with AN or Taxol was compared with the mean fluorescence intensity of the untreated Mk colonies. A statistically significant decrease in the mean fluorescence intensity was noted with tubulin-β1 staining of Mk colonies treated with AN at a concentration of 10 μM and with Taxol at a concentration of 1 μM. No statistically significant change in mean fluorescence intensity was noted in tubulin –α staining of Mk colonies treated with AN or Taxol. Each concentration evaluated in triplicate on three separate occasions.

5.4. Discussion

5.4.1 Evaluation of the Effect of AN on Cell Cycling of hCB Derived Pluripotent Stem Cells in LTBMC.

LTBMC enable the evaluation of the very early maturation and differentiation events of pluripotent haematopoietic stem cells (HSC) within a supporting microenviroment (stromal layer). The microenviroment layer is considered to be similar to that found *in vivo* (Dexter et al,1984). The microenviroment not only provides the cell to cell interactions that are essential for HSC differentiation and maturation but also the cytokines such as IL-3 and stem cell factor required to drive the initial maturation events. The concentrations of AN used were the same as those used in the clonogenic cultures where a reduction in megakaryocytic colony counts per well was noted. In addition the concentration range utilised encompasses the plasma concentration 23.6nM (734 ng/mL) of AN at the therapeutic human dose 2.0mg daily. AN was added to the LTBMC at weekly intervals for a total of three weeks.

No alteration in cell cycling or colony counts of the erythroid, granulocytic/monocytic or megakaryocytic cell lineages was observed. AN, at the concentrations evaluated, did not affect cell cycling of HSC. Additionally, the microenviroment was maintained enabling haemapoietic stem cells to maintain their ability to differentiate and mature further to give rise to multipotent precursors (non adherent cells) that were still capable of giving rise to erythroid, granulocytic/monocytic or megakaryocytic colonies, after repeated administration of AN. Finally, the number of colonies of each cell lineage was comparable to vehicle control levels demonstrating that the lineage specific precursors can differentiate. The evaluation of AN in hCB LTBMC suggests that the reduction in platelet counts noted after *in vivo* dosing in humans does not result from action of AN on the most primative haematopoietic cells that are found in the LTBMC.

5.4.2 Establishment of the AN IC50 Concentration for the CFU-E, GM-CFU and Mk Colonies, Using hCB Derived Stem Cells in Clonogenic Cultures

Clonogenic cultures enabled the evaluation of the haematotoxicity of AN towards the multipotent and lineage specific granulocytic/monocytic, erythroid and megakaryocytic precursors, events further down stream from those in the LTBMC. The findings from the clonogenic cultures demonstrated that the culture conditions respond in a lineage specific way to AN, replicating clinical responses. The IC50 established using hCB derived haematopoietic cells was 1.92μM. *In vitro* clonogenic cultures using human bone marrow-derived stem cells showed that concentrations between 0.32nM - 161μM inhibited megakaryocytic colony formation with an IC50 concentration of 32 μM (SBA, 1997). The 16 fold greater sensitivity noted with the bone marrow derived may be due to differences in the experimental procedures employed. The bone marrow derived stem cells were pre-incubated in AN for 6 hours washed and then added to culture media. The additional washing process employed to remove AN following pre-treatment may have damaged the haematopoietic cells prior to culturing. Alternatively, incubation with AN may have caused indirect cell toxicity. The procedure employed for this work ensured that the haematopoietic cells were exposed to AN throughout the culturing period by adding the cells to the culture media containing AN.

Recently, Wang et al, (2005) using hCB derived cells in clonogenic cultures similar to those

employed in this work, reported an IC50 concentration for AN of 0.26μM a concentration 123 and 7 fold higher than that reported by the manufacturer's of AN and reported herein, respectively. Most likely the difference in IC50 concentrations reflects differences in the culturing techniques, the genetic differences in the donors and the source of the bone marrow of the haematopoietic cells (Ortel MD, 1998).

5.4.3 The Effect of AN on Cell Cycling and Megakaryocytic Ploidy Using hCB Derived Stem Cells in Clonogenic Cultures

In contrast to the cell cycle analyses data from non-adherent cells collected from AN treated hCB LTBMC, a statistically significant decrease in the percentage of G2/M phase and a statistically significant increase in the percentage of G0/G1 phase cells was noted at AN concentrations of 1 and 10 μM, respectively. These data is indicative of cell cycle arrest at G0/G1. The development and maturation of the committed megakaryocytic stem cells is unique in that they develop a highly polyploid DNA content, associated with giant multi-lobed nuclei (Matsumura and Kanakura, 2002).

The majority of cells in the vehicle treated cultures had 2N (60%) DNA content, 25% with 4N DNA content and 8N, 16N, 32N and 64N each representing less than 5% of the total CFU-Mk colony population. These findings are similar to published data of Minamiguchi et al (2001), where 70% of the cord blood derived CFU-Mk colonies were shown to have 2N DNA content, using a similar method for cell cycling analyses.

A statistically significant increase in 2N CFU-Mk colonies was noted in CFU-Mk colonies treated with 1 and 10 μM AN. In addition, a statistically significant decrease, in the number of CFU-Mk colonies with DNA content at 4N and 8N was also noted. The cell cycle and ploidy data when considered together is suggestive that AN treatment resulted in cell cycle arrest predominantly of cells at the 2N ploidy stage of development, preventing the maturation of Mk cells of 4N ploidy and greater. Mk cells of greater than 4N are considered to predominantly release platelets into circulation. These findings concur with those of Mazur et al (1992), where concentrations of 32μM to 161μM, resulted in an increase (65%) in the percentage of megakaryocytes in developmental stages I and II (Chapter 3) when compared with concurrent controls (41.5% in developmental stages I and II). In addition, these data also

concur with the findings of Wang et al (2005) where AN and its metabolite AM were shown to inhibit the ploidy of Mk cells.

5.4.4 The Effects of AN on the Expression of Megakaryocytic Cell Surface Antigens using hCB Derived Stem Cells in Clonogenic Cultures

The expression of different receptors on the surface of haematopoietic progenitors permits the interaction with various regulatory elements present in their environment, which includes stromal cells, extracellular matrix molecules (EMM) and soluble regulatory factors (cytokines and growth/differentiation factors). The expression of these surface antigens is known to change during the maturation event (Civin and Locken, 1987). As the totipotent haematopoietic cells mature the surface antigen specific for the totipotent potential of the cells is lost, and instead lineage specific surface antigens develop.

Megakaryocytes progressively express lineage restricted surface antigens, some of which play essential roles in platelet physiology (Erickson et al, 1993; Wang et al 2005; Hong et al 2006). Two such antigens are CD61, part of a complex receptor GPIIb/ GPIIIa, and CD42 part of the GPIb –V-IX complex. The expression of these two antigens occurs at different stages of maturation. CD61 is expressed early in megakaryocytic development. In contrast CD42 was expressed later in megakaryocytic development. Only the CD34 negative /CD61 positive population demonstrated a statistically significant decreases at the two highest AN concentrations evaluated.

The loss / inhibition of cells expressing CD34 negative /CD61 positive receptor profile was indicative of an alteration of maturation and differential events that lead to the reduction in Mk cells and ultimately reduce the number of circulating platelets. Alteration in the maturation and differentiation events is further supported by the statistically significant reduction in the G2/M cell population again at AN concentrations of 1 and 10 μM. Recently, a statistically significant reduction in CD61 expression has been reported following treatment of hCB clonogenic cultures with AN at a concentration of 1μM (Wang et al, 2005). The reduction in the CD61 receptor expression was noted at the same concentration at which a reduction in CD34 negative/CD61 positive receptor expression was noted in this work. Interestingly, Wang et al (2005) evaluated only CD61 expression and not the combination of receptor

expressions as carried out in this thesis. Therefore this work may have identified a specific sub population of Mk cells affected by AN treatment.

5.4.5 The Effect of AN on Mk-CFU Colony Microtubule Organisation using hCB Derived Stem Cells in Clonogenic Cultures

Treatment with AN concentrations of 1 and 10 μM resulted in no changes in the percentage of cells expressing tubulin –α and tubulin-β1 levels. However, the decrease in mean fluorescence intensity noted with tubulin-β1 staining of Mk cells treated Taxol suggests that AN treatment results in the reduction tubulin - β1 levels per cell that in turn results in the inhibition of the normal reorganization of the microtubule network.

5.4.6 Drug- induced Immune thrombocytopenia

Neither Clonogenic culture nor LTBMC can be utilized to evaluate the potential of therapeutic agents to promote drug induced thrombocytopenia. Although the LTBMC contain antigen presenting cells (macrophages), no B-cells are present within the cultures to produce immunoglobulins. Plasma samples from patients treated with AN as well as anti-platelet antibody assays would be required to determine if an immunological mechanism may be involved in the reduction of platelets. No antibodies have been detected in 26 healthy volunteers treated with AN in phase I clinical studies or 48 ET patient samples (SBA, 1997). Therefore it is unlikely that the platelet count reduction noted in essential thrombocythemia patients is due to a drug induced immunological response.

5.4.7 Hypothesis of the action of AN on Mk-CFU colonies

Megakaryopoiesis results in the production and release of platelets. As with the other lineages it is a multistage process of cellular differentiation and maturation regulated by a variety of

cytokines. Mk cells originate from pluripotent stem cell. In fact the development and maturation of the committed megakaryocytic stem cells is unique in that they develop a highly polyploidy DNA content which is associated with giant multi-lobed nuclei. The formation of platelets results from the projection of megakaryocytic cytoplasm through the sinusoidal endothelium of the megakaryocytes (Figure 5.19).

Mk progenitors express the CD34 antigen, the earliest surface antigen identifying pluripotent stem cells, but mature Mk cells have lost the CD34 receptor (Erickson et al, 1993). Additionally the precise stage along the Mk differentiation at which the CD34 is lost is not known. By flow cytometry, CD34+ cells bearing platelet glycoproteins (GP) could be detected, but at a low frequency (less than 2% of the marrow CD34 positive (+) cells. These cells gave rise within another 4 days in culture to numerous Mk (up to 50%), showing that these CD34+ cells were greatly enriched in Mk precursors. Erickson et al, (1993) confirmed the presence of typical megakaryoblasts, by ultra-structural studies. Megakaryoblasts are large cells with a small nucleus and a vast cytoplasm. In some cells the cytoplasm is divided into smaller areas by microtubules. By flow cytometry, three populations of small cell size can be defined: CD34+ CD61-, CD34+ CD61+, and CD34- CD61+ cells (Chapter 3). The two CD61+ populations are almost pure immature Mk. Alpha-Granules, organelles containing alkaline phosphotase, are rare in the CD34+ / CD61+ cells, whereas they are more developed in the CD34- / CD61+ cells, which also exhibited demarcation membranes (Erickson et al, 1993).

In vitro data, reported in this thesis, has demonstrated the effects of AN on ploidy, cell cycle and receptor expression indicate that the action of AN may be at a late stage in Mk colony development. Alterations in ploidy and CD61 receptor expression following *in vitro* treatment of hCB clonogenic cultures with AN have also been reported recently by Wang et al (2005), corroborating the findings reported herein. The results from the experiments on tubulin –α and tubulin-β1 has identified another potential mechanism by which AN reduces platelet counts in patients with ET, via disruption of the microtubule organization. It is currently unclear whether an alteration in cell cycling or the inability to organize the microtubule network is the primary mechanism of AN mode of action. Two alternative therapies used in the treatment ET, vinblastine and vincristine, are know to inhibit cell mitosis by binding to the protein tubulin in

the mitotic spindle and preventing polymerization into the MTs (Islam et al, 2004). This mode of action is also shared with other natural agents such as colchicine and podophyllotoxin (Islam et al, 2004).

Table 5.3 shows the concentrations at which each of the changes in colony growth, ploidy, receptor expression, cell cycling and tubulin levels occurred. Based on the lowest concentration at which the changes were noted the inability to organize the microtubules appears to be secondary to alteration in cell cycling, surface receptor expression and inhibition in achieving high (greater than 8N) ploidy number. However, identification of the primary mechanism based solely on concentration appears to be very crude and most probably reflects a limitation of *in vitro* systems. Most likely a combination of mechanisms including inhibition of cell cycling and the inability to organize the microtubules contributes to the reduction in platelet release.

Clinical trial data demonstrated that AN doses ranging between 1-3mg given every 8 hours for 6 to 9 days caused a profound reduction in platelet count with a nadir of 20 $x10^{9}$/ L occurring within 10 and 12 days of commencement of treatment (SBA, 1997). The mean Cmax at 2mg/kg (once daily) was 23.6nM (734ng/mL), 10 fold below the lowest *in vitro* AN concentration at which alterations in cell cycle, ploidy, cell surface receptor expression and tubulin- β1 were noted. It is important to note that *in vivo* AN concentrations in the bone marrow are not known and may be lower than the plasma concentrations. Furthermore the distinction between *in vivo* and *in vitro* inhibitory concentrations may reflect genetic variability in humans, but most likely reflects differences between clonogenic cultures and *in vivo* bone marrow.

Statistically Significant Effect of AN Noted	Lowest Concentration of AN change noted (µM)
Colony growth inhibition	1
DNA cell cycle	1
Cell Ploidy	1
Surface receptors	1
Change in MFI of Tubulin -β1 expression	10

Table 5. 12 Alterations in Mk-CFU Cell Growth Noted following AN Treatment Throughout the Culturing Period (12 Days)

The lowest concentration at which a statistically significant change in hCB derived CFU-Mk clonogenic cultures of colony growth inhibition, cell cycling, cell ploidy and surface receptor expression was 1µM. A reduction in the MFI of tubulin –β1 expression was noted at 10 µM. However, each effect is possibly interrelated so it is difficult to distinguish a single mechanism of AN action.

6.0 General Discussion

6.1 Haematotoxicity

Dose limiting haematotoxicity has been associated with a range of therapeutic agents used for the treatment of a number of different conditions are known to block cell cycling (Botta et al, 1999). As a consequence of the blockade of cell cycling, haematopoietic cell differentiation and maturation is disrupted, resulting in clinically significant decrease in the number of mature cells released into blood circulation. Also self- renewal of the totipotent stem cells can be disrupted (Botta et al, 1999).

The potential for haematotoxicity is typically assessed as part of the preclinical toxicology studies in experimental animals where changes in peripheral blood cell numbers and bone marrow cellularity are determined usually at the end of the study; blood samples are routinely collected prior to the start of dosing, during dosing and at the end of the study. However, information on the potential mechanism of the haematotoxicity is rarely revealed. The identification of the mechanism (s) of the haematotoxicity may provide in sight whether haematotoxicity is likely to be specific to that toxicology species or likely to be of clinical relevance. For example, reductions in red cell counts, haemoglobin and heamatocrit were noted in rodents with p38 kinase inhibitors at doses evaluate including the predicted clinical therapeutic dose range (Tamura et al, 2000). However, no reduction in red cell parameters has been noted in clinical trails which strongly suggest that the changes are specific to rodents. In addition, information on the potential mechanism (s) of the toxicities noted in preclinical toxicology studies is required to enable the progression of the development of the new therapeutic agent, for regulatory submissions and eventual drug registration. The information is in turn used by the medicines approval agencies to review the risk: benefit of the product. Finally, the mechanism (s) elucidated may identify specific biomarkers of the toxicity noted that can be incorporated into the clinical protocols for safety monitoring. *In vitro* bone marrow cultures could be utilized in two different approaches:

Data from in vitro bone marrow screens into the discovery optimisation process could facilitate early detection of potential haematotoxicity and identify the most sensitive cell lineage. Compounds for a given therapeutic target could be ranked according to their haematotoxicity and those compounds with the desired haematotoxicity profile would be

progressed. However, the necessity to identify the different haemopoietic cell lineages by morphology (particularly in the case of clonogenic cultures) is time consuming and therefore not suitable for use as a high throughput screen. Interestingly, a multifunctional Biocheminescence Colony Forming Assay has recently been described that utilizes a 96 well format (Rich and Hall, 2005). The biocheminescence system may negate the need for manual colony identification stream lining the screening significantly.

Secondly, the results from *in vitro* assays can provide a means of elucidating the potential mechanism (s) for the haematotoxicity noted *in vivo*. These assays require small quantities of the therapeutic molecule to investigate the haematotoxicity and often only small quantities are available during the early phase of drug development. *In vivo* mechanistic studies can take significant time to conduct and clinical trails may be halted until the potential mechanism has been identified. Delays in the development process are costly.

6.2 Megakaryocytic Lineage Specific Haematotoxicity

Anti-cancer and anti-viral therapies are known to cause myelotoxicity, however the first cell lineage most often affected *in vivo* is the megakaryocytopoietic. The availability of recombinant thrombopoietin, has now made it possible to evaluate this lineage easily *in vitro*. In these studies, the murine CFU-Mk clonogenic assay was established and optimised and the murine GM/E and Mk clonogenic haematopoietic cultures were used to evaluate the lineage specific haematotoxicity of compounds used in the treatment of ET (Chapter 3). The IC50 data and the ranking order demonstrated that some of the ET compounds evaluated had shown selective toxicity towards specific cell lineages. Anagrelide was most toxic towards the murine megakaryocytic lineage and the least towards the murine granulocytic/monocytic, erythroid (myeloid) cell lineage. Vincristine sulfate also demonstrated a specificity towards the megakaryocytic lineage, whereas vinblastine and hydroxyurea showed specificity towards the granulocytic /monocytic and erythroid cell lineages, respectively (Chapter 3). The difference in the lineage sensitivity may reflect differences in the uptake and consequently intracellular concentrations of the compounds by the different lineage progenitors and is particularly demonstrated by AN. AN was developed as an anti-thrombotic agent targeting megakaryocytes, therefore higher intracellular uptake of AN by megakaryocytic progenitors

would not be surprising. However, high concentrations of all the compounds evaluated produced haematotoxicity in all cell lineages, it was most likely due to non-specific toxicity such as direct cytotoxicity.

To fully validate the murine CFU-Mk clonogenic assay it would have been of value to include compounds that are not used in the treatment of ET, such as 3C-azido-3C-deoxythymidine (AZT), that have been shown in vivo to cause reduction in platelet counts as well as compounds that are generally known not to cause haematotoxicity. A further validation of the CFU-Mk clonogenic cultures was completed using methotrexate, chloramphenicol succinate, vincristine sulfate and diazepam (data shown in Chapter 2). These studies demonstrated that chloramphenicol succinate and diazepam were the least specific towards the megakaryocytic lineage. Vincristine sulfate once again demonstrated the greatest toxicity towards the megakaryocytic lineage. Methotrexate demonstrated toxicity to the myeloid/monocytic and megakaryocytic cell lineages and chloramphenicol succinate was the most toxic towards the erythrocytic cell lineage. Methotrexate and chloramphenicol succinate block cell cycling, in the case of chloramphenicol succinate an accumulation of CFU-E colonies in G2M phase was noted (Chen, 2005). Interestingly, both methotrexate and chloramphenicol succinate also effect the bone marrow microenvironment. In contrast, vincristine sulphate inhibits the formation of microtubules. No haematotoxicity has been reported for diazepam in clinical use and demonstrated the haematotoxicity *in vitro* only at high concentrations.

Based on limited *in vitro* GM/E and MK clonogenic human culture data on compounds used for the treatment of ET, a similar lineage specificity to that demonstrated in the in vitro short - term murine haematopoietic cultures was found, although the culture conditions and compound exposure times varied (Oertel 1998).

It is important to note that the IC50 concentrations were determined following single in vitro exposures to the ET compounds, whereas the haematotoxicity in vivo is often noted after repeated administration. This raises a potential uncertainty of whether the mechanism of haematotoxicity *in vitro* and *in vivo* is the same. Interestingly, the IC50 concentrations for AN were noted to be markedly different when the murine IC50 concentration 88.6µM (Chapter 3) was compared with hCB IC50 concentration 1.92µM (Chapter 5). This 46 fold disparity may be indicative of differences in species sensitivity or may be indicative of AN having a greater affinity towards the human c-mpl (TPO) receptor than the murine c-mpl (TPO) receptor as

suggested by McCarty et al (2006). The sequence homology between mouse and human c-mpl (TPO) receptor is approximately 64%, which suggests that the difference in mouse and human AN megakaryocytic lineage toxicity is due to species sensitivity (Jandrot-Perrus et al, 2000).

The clonogenic cultures may be capable of some metabolism primarily through the action of macrophages, however, their number would be very low following positive selection for pluripotent stem cells prior to addition to clonogenic cultures. However, media from the treated cultures were not collected for the determination of concentration of the ET reference compounds at the end culturing period. Therefore a clear understanding of the dynamics of the test compound in the cultures is not known. It is important to note that the semi-solid methyl cellulose based media used in the clonogenic cultures would also make treatment concentration checks at the end of the culturing period difficult and serve to prevent equilibration of the concentration in the culture media of any metabolites of the test compound if produced.

6.3 Hypothesis of Mechanism of Action of Anagrelide (AN)

Using hCB derived haematopoietic stem cells, in both clonogenic and LTBMC the potential mechanism (s) of Mk –CFU reduction was assessed in this study (chapter 5). In hCB LTBMC repeated weekly exposure to a range of AN concentrations for a total of 3 weeks showed no significant changes in cell cycling, or in the number of non adherent cells produced by the cultures, suggesting that the reduction in platelet counts following repeated dosing in humans may not result from alterations to these parameters of multipotent stem cells (chapter 5). The action of AN did not result in an alteration of the morphology or confluency of the stromal layer. In addition, the action of AN did not inhibit the ability of the multipotent stem cells to differentiate into cells of the erythroid, granulocytic, monocytic or megakaryocytic cell lineages, again following repeated adminsitration (chapter 5).

Consequently, the investigation of the potential mechanism of action of AN was focused directly on Mk CFU cells, using hCB clonogenic cultures. *In vitro* hCB clonogenic indicate that the action of AN may be during Mk development (Chapter 5). Interestingly, these data also concur with the findings of Wang et al (2005) where AN was shown to inhibit the ploidy of Mk cells and result in a reduction in the expression of CD61 receptors on Mk cells collected from hCB clonogenic cultures. Wang et al (2005) evaluated only CD61 expression and not the

combination of receptor expressions as carried out in this thesis. Therefore this work may have identified a specific sub population of Mk cells affected by AN treatment (chapter 5). The decrease CD34 negative / CD 61 positive sub population of Mk cells most likely reflects the cell maturation arrest that leads to reduced/ inhibition of CD 42 receptor.

The results from the experiments on tubulin –α and tubulin-β1 identified another potential mechanism by which AN might reduce platelet counts in patients with ET, via disruption of the microtubule organization (chapter 5). It is, however, unclear whether an alteration in cell cycling or the inability to organize the microtubule network is the primary mechanism of AN's mode of action. Based on the lowest concentration at which changes were noted (Chapter 5), the inability to organize the microtubules (10μM) appears to be secondary to alteration in cell cycling, surface receptor expression and inhibition of increase in ploidy (1μM). This work suggests that the action of AN on CFU-Mk colonies is most likely a result of a combination of mechanisms 1) inhibition of cell cycling, 2) a disruption in the expression of cell surface receptor expression and 3) inhibition in the ability of the cells to increase in ploidy number associated with the inability to organize the microtubules leading to a reduction in platelet release (Figure 6.1). AN prevented the CFU-Mk cells achieving greater than 2N ploidy. This is particularly significant as only Mk cells with greater than 4N ploidy can form proplatelets and release platelets. Furthermore, cell cycling and tubulin activity in Mk progenitors is different from that in other haemopoietic cells; M phase is normally incomplete and Tubulin-β is pivotal in platelet production. Tubulin-β1 KO mice have been found to produce up to 60% less platelets when compared with wild type (Levin et al 1993). These differences may render the Mk lineage more sensitive to compounds that inhibit cell cycle and microtubulin formation.

Other therapies are known to cause platelet count reduction. For example, nucleoside reverse transcriptase inhibitors such as 3'-azido-2', 3'-dideoxythymidine (Gynn et al, 1972) and folate antagonists such as methotrexate (Storb et al, 1986) by cell cycle disruption. Interestingly, the mechanism of action of two other therapies used in the treatment of ET, Vinblastine and Vincristine is via inhibition of the formation of microtubules: predominatly resulting in cell cycle arrest at metaphase (chapter 3). These compounds inhibit mitosis by binding to the protein tubulin in the mitotic spindle, thereby preventing polymerization into the microtubules (Islam et al 2004). Therefore it would not be surprising that the mechanism of action of AN is

similar.

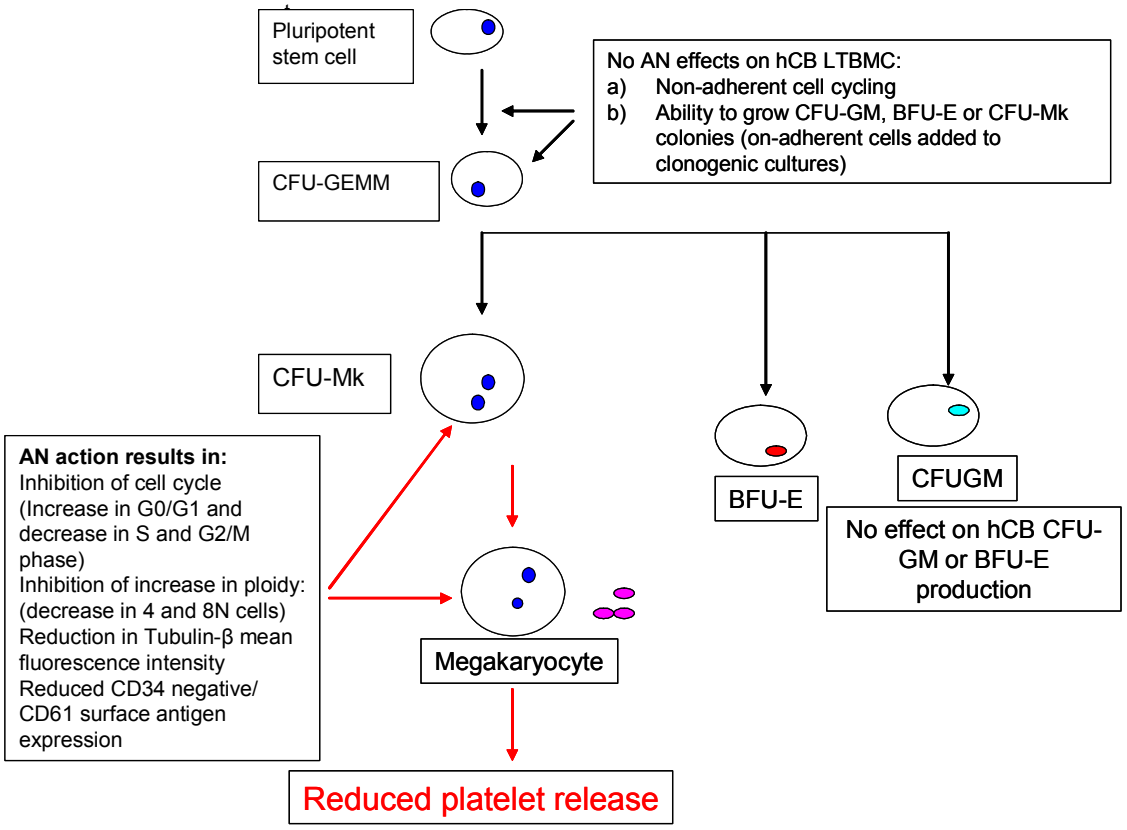


Figure 6. 1 Hypothesised Mechanism of Action of AN on Megakaryopoiesis.

AN does not effect primitive multipotent stem cells in hCB LTBMC (stromal cell confluency, cell cycling, or ability of the non adherent derived stem cells to differentiate into BFU-E, CFU-GM or CFU-Mk colonies). AN effect on CFU-Mk colonies are the result of a combination of mechanisms: inhibition of cell cycling, disruption of the expression of cell surface receptors and inhibition in the ability of the cells to increase in ploidy - associated with the inability to organize microtubules -ultimately leading to a reduction in platelet release. No effect on CFU-GM or BFU-E colonies noted.

Clinical trial data demonstrate that AN can cause profound platelet count reduction (nadir of 20 x10^9 /L) at a mean systemic exposure level (Cmax) of 23.6nM which is 81 fold below the lowest *in vitro* AN concentration at which alterations in cell cycle, ploidy, cell surface receptor expression and tubulin- β1 were noted, in the studies reported herein. It is important to note that *in vivo* AN concentrations in the bone marrow are not known and may be lower or higher than the plasma concentrations. There is currently no data to demonstrate a direct causality between effects on cell cycle inhibition, alteration in cell surface receptor expression,

inhibition in the ability of cells to increase in ploidy number and the inability to organize the microtubules leading consequently to a reduction in circulating platelets. Recently, however, Wang et al (2005) reported similar changes in ploidy and CD61 receptor expression following *in vitro* treatment of hCB cultures. It is also recognized, that synchronicity between nuclear and surface receptor expression and high ploidy are required for platelet release (Petrides, 2006). Therefore the direct comparison of the *in vitro* changes in Mk-CFU and *in vivo* platelet count reduction may be appropriate.

Although the concentration of AN added to the cultures is known, supernatants from LTBMC and clonogenic cultures were not collected and evaluated to confirm the concentrations of AN during the culture period. *In vivo* AN is rapidly cleared from plasma with a terminal half life of 2.3 hours +/-0.26 hrs. In the absence of clearance, *in vitro,* it is likely that some AN was present throughout the culturing period of 7 (LTBMC) to 10 (clonogenic cultures) days.

6.4 Source of Haematopoietic Cells

The Balb/C mouse has been shown to be a good source of bone marrow for use in the in vitro haematopoietic cultures. The quantity, quality and consistency in stem cell numbers collected, does make it an ideal source for bone marrow to facilitate haematotoxicity evaluations. Furthermore, *in vitro* murine haematopoietic cultures have been shown to be predictive for human haematotoxicity associated with anti- cancer and anti-viral therapeutics (Scheding et al 1994). However, the CD1 mouse rather than the Balb/C mouse is routinely used for *in vivo* toxicology studies. The difference in strain utilised in the *in vitro* verses *in vivo* studies may contribute to the different sensitivities to the haematotoxicity noted. Differences, however, in species sensitivity to haematotoxicity have been described (Van De Heuvel et al, 1999, Masubuchi N et al 2004, and Anderson et al 2000). Unfortunately obtaining human bone marrow for haematotoxicity assessments within the UK was very limited. Human cord blood has been shown to be the next best source of human haematopoietic stem cells (Chapter 5). Although it was possible to obtain human cord blood derived stem cells from commercial sources, the supply was often haphazard (often delivery hCB cells was unreliable) as well as costly (over £460 for 1x 10^{18} cells). Moreover, the supply limitations together with the regulatory restrictions on the use of human cells in research make it unlikely that human cord blood or bone marrow can be used routinely for haematotoxicity evaluations. In the future, non-human primates, such as the cynomolgus monkey (*Macaca fascicularis*), could be

explored as a potential source of bone marrow and cord blood cells. The cynomolgus monkey is sometimes used as an alternative toxicology species to the dog and background haematology data in this species demonstrates similarities to human haematopoiesis. For example, red cell lifespan is the same as in man - 120 days. The cynomolgus monkeys used in toxicology studies are bred at specialised breeding farms rather than wild caught; therefore cord blood collections from the breeding colonies may be possible.

6.5 LTBMC

The unique feature of LTBMC cultures is that sustained haematopoiesis occurs in the absence of added growth factors but is absolutely dependent upon the establishment of an adherent layer of bone marrow derived stromal cells. The latter provide the appropriate environment to promote the survival, self-renewal, proliferation, and differentiation of the haematopoietic progenitor cells (Daniel et al, 1989). LTBMC provide a unique system with which to investigate effects on several aspects of haematopoiesis that can not be achieved by simply using clonogenic assays. For example, cell adhesion interactions that bind progenitor cells and their progeny to specific stromal elements as well as the synthesis, secretion, and presentation of growth-stimulatory and growth-inhibitory factors by stromal cells can be studied.

In addition, the effects on haematopoiesis following repeated administration of test compounds on the stromal layer and on the early stem cell maturation can be evaluated, potentially mimicking *in vivo* haematotoxicity.

Whilst establishing and optimizing the LTBMC the importance of the type of culture flasks was highlighted; and the ability to establish confluent stromal layers was examined using T25 flasks from three different manufacturers. The properties of the plastic used in the manufacture of the flask are known to affect the surface charge of the plates which in turn can alter the ability of the cells to adhere. In addition, an alteration in the sterilization process can result in a change in the charge. A change in the plastic used in the manufacture of the flask was found to influence directly the ability of the cells to adhere (Chapter 4). Furthermore, differences in the ability of macrophage/fibroblast like cells to adhere to plastic have previously noted (Gordon, 1993) where adherence of some fibroblasts was restricted by the properties (charge) of the plastic used. Unfortunately no information on the differences in the

flasks is available due to the reluctance of the manufacturers to discuss their respective plastic formulation or sterilization procedures. It is most likely that a change in polycarbonates used possibly resulted in a change in the flask charge. Furthermore, the ability to grow confluent stromal layers in Nunc rather than Falcon flasks using the same batch of culture media and murine bone marrow confirmed that the inability to achieve confluent stromal layers in the Falcon flasks was not due to issues with the reagents, bone marrow collection or due to operator differences. Without clear communication between suppliers and their customers on changes made in their materials, not only time consuming but misleading data could be generated. The inability to establish confluent stromal layer in LTBMC resulted in a 4 month delay and was only resolved after collaborating with Addenbrookes Hospital Haematology department.

The combination of cell identification techniques flow cytometry and light microscopy enabled a more detailed understanding of the different cell populations within the non-adherent layer (Chapter 4). Each technique had its own advantages when compared to the other; flow cytometric analysis enabled the identification of cells that were difficult to distinguish morphologically whereas, light microscopy identified the later stages of cell development (past committed lineage specific progenitor) for which no specific antibodies were available.

The time taken to achieve confluent stromal layers was similar irrespective of whether human or mouse was the source of the haematopoietic stem cells. In addition similar numbers of non-adherent cells were generated.

There are, however, limitations to the LTBMC, irrespective of the source of haematopoietic stem cells. Cytokines that drive the differentiation to give rise to erythrocytic and megakaryocytic cell lineages (Epo and Tpo) are not present within the cultures; Epo (Eckardt et al, 2005) and Tpo (Wolber et al, 2002) are produced by the kidney and liver respectively. Indeed, the presence of erythroid cells within LTBMC can result in the demise of the cultures. Thus, effects on stem cell differentiation and maturation relating to Epo or Tpo production can not be evaluated in LTBMC; a limitation of the culture system. Finally, effects on the development and differentiation of lymphocytic cell lineage are not possible in LTBMC.

The assessment of any potential toxicity for a new chemical entity can not be made solely

using a series of *in vitro* cultures, each representing a potential target organ. When used in isolation no information on the potential interrelationships between toxicities can be elucidated. *In vitro* assays provide a means of elucidating the potential mechanism (s) for the toxicity noted *in vivo.* In addition the *in vitro* assays provide a means of ranking compounds for the same therapeutic target for which the primary toxicity or toxicity of concern is known.

6.6 Additional Avenues of Investigation

As mentioned earlier, the biocheminescence system may negate the need for manual colony identification stream lining the screening significantly. The preliminary validation report of eleven reference compound (Rich and Hall, 2005) suggests that this system may have potential as a screening tool. Future work could include the assessment of the biocheminescence system, using known haematotoxic compounds as well as compounds not reported to cause haematotoxicity.

Alterations in gene expression and transcriptional effects would be expected to occur as a consequence of drug therapy. Therefore, future work to elucidate the mechanism of AN's mechanism of megakaryocytic specific haematotoxicity could include a comparison of selected gene expression using quantitative reverse polymerase chain reaction (PCR), with and without anagrelide treatment. The genes proposed would include:

- Genes involved in DNA cell cycle, the Cyclins and the CDKs. The principal CDKs responsible for G1 progression and entrance into S phase CDK2, CDK3, CDK4, and CDK6.
- The D-type cyclins (D1, D2, and D3); the regulatory subunits for CDK4/6.
- Cyclin E, which activates CDK2 and possibly CDK3 and is expressed in late G1 and early S phase. Cyclin A which can also activate CDK2, but essential later, at the G1/S boundary and throughout S phase (Ewen, 2000).
- Tubulin- α and tubulin β genes
- c-mpl, JAK2 and TYK2 genes

The analysis of the data would provide an insight in to which signaling pathways are affected and their lineage sensitivity. Time course studies would need to be carried out to ensure that changes in gene expression were monitored throughout the culturing period. Concentrations of AN that would be evaluated would include IC10 as well as IC50 concentrations, as changes

in gene expression levels can be noted at concentrations lower than those associated with the observed toxicity. Haematotoxicity may occur at the transcriptional level therefore analysis of proteins such as GATA-1, FOG-1, TAL-1 that have recently been identified to play a role in the regulation of megakaryopoiesis would be a further avenue of investigation.

6.7 Summary

These studies have evaluated the utility of both clonogenic and LTBMC culture systems as a tool for assessing the lineage sensitivity to VC, VB, H and AN and the mechanisms of the megakaryocytic specific haematotoxicity of AN. Murine and human Mk-CFU clonogenic cultures and LTBMC were established and characterized

Compounds commonly used in the treatment of ET, a disease state resulting in an over production and release of platelets, were evaluated in murine and hCB clonogenic cultures. By ranking the ET compounds according to the lineage specific IC50 concentrations, anagrelide was most toxic towards the murine megakaryocytic lineage and the least towards the murine granulocytic/monocytic, erythroid (myeloid) cell lineage. Although the lineage specificity between human and murine cultures was the same, megakaryocytic, the IC50 concentrations differed. This species difference might be related to a difference in the uptake of the compounds by the haemopoietic progenitors. The most marked difference was noted with AN, indicative of differences in species sensitivity and /or is indicative of AN having a greater affinity towards the human c-mpl (TPO) receptor than the murine c-mpl (TPO) receptor as suggested by McCarty et al (2006).

Using hCB derived haematopoietic stem cells, in both clonogenic and LTBMC the potential mechanism (s) of Mk –CFU reduction was assessed. The action of AN did not inhibit the ability of the multipotent stem cells in hCB LTBMC to differentiate into cells of the erythroid, granulocytic, monocytic or megakaryocytic cell lineages. Focussing on the Mk committed progenitor cells revealed that AN treatment specifically caused an inhibition of cell cycling, disrupted the expression of cell surface receptors, inhibited an increase in cell ploidy and a disrupted microtubule organisation. AN was shown to prevent the CFU-Mk cells achieving greater than 2N ploidy. This is particularly significant as only Mk cells with greater than 4N ploidy have been shown to form proplatelets and release platelets. Furthermore, cell cycling

and tubulin activity during cell cycling in Mk progenitors is different from that compared with other haemopoietic cells; M phase is normally incomplete and Tubulin-β is more concentrated in mature Mk cells. These differences may render this lineage particularly sensitive to cell cycle and tubulin acting agents. This work confirms the potential utility of *in vitro* bone marrow cultures as a tool for evaluating compounds for their lineage specificity and as a tool to assess mechanism (s) of haematotoxicity as demonstrated herein providing an insight to mechanism of platelet count reduction by AN.

7. APPENDIX A

Materials

Murine selection and purification

The reagents used for the selection and purification of murine stem cells are shown below.

Antibodies for selection of murine haematopoietic cells	Supplier	Catalogue Number
Anti-C kit antibody coated Dynabeads	Dynal (UK) Ltd	113-35D
Dynabeads M-450 Sheep Anti-Rat (IgG) coated beads	Dynal (UK) Ltd	140-11
Rat -Anti mouse Ckit (CD117) monoclonal antibody (IgG)	Serotec, UK	073403

Murine bone marrow aspiration and Purification	Supplier	Catalogue Number
Murine bone marrow Purification	Life Technologies, UK	647881
Lymphoprep^{R}	Robbins Scientific Corporation, West Midlands, UK	442151

Murine Clonogenic Culture Reagents

Details of the murine clonogenic culture media, including their source employed in this project are provided below

Murine Clonogenic assay	Supplier	Catalogue Number
Granulocytic /Monocytic and Erythroid clonogenic assay Methocult GF M3434	Metachem Diagnostics, Northampton, UK	14425
Megakaryocytic clonogenic assay media HCC-4900 media	Metachem Diagnostics, Northampton, UK	23514
Recombinant Mouse (rm) IL-6 for CFU-MK cultures	Metachem Diagnostics, Northampton, UK	25547
Collagen for CFU-MK cultures	StemCell Technologies Inc	4902

hCB Clonogenic Culture Reagents

Details of the murine clonogenic culture media, including their source employed in this project are provided below

hCB Clonogenic assay	Supplier	Catalogue Number
MegaCult™ –C	StemCell Technologies Inc, UK	132248
MethoCult™ –GF	StemCell Technologies Inc, UK	128862

Murine LTBMC

Details of the manufacturer of the murine LTBMC media provided below

Murine LTBMC –Standard Mathod	Supplier	Catalogue Number
MyeloCult M	StemCell Technologies, UK	17753

Murine LTBMC –Modified method	Supplier	Catalogue Number
MyeloCult M	StemCell Technologies, UK	17753
IL-6 (5ng/ml)	StemCell Technologies, UK	84226

SCF (25ng/ml)	StemCell Technologies, UK	54719

hCB LTBMC

Details of the manufacturer of the hCB LTBMC media provided below

Human cord blood LTBMC	Supplier	Catalogue Number
MyeloCult H5100	StemCell Technologies, UK	65543

Megakaryocytic Culture Plates

Details of the type and manufacturer of the plates used for the human and murine megakaryocytic culture clonogenic cultures is provided below

Culture plates	Supplier	Catalogue Number
Double Chamber Slides	StemCell Technologies Inc	4813
6 –well plates	Becton Dickinson, UK	351146
Double Chamber Slides	StemCell Technologies Inc	4813

Plates Used For Murine And Human Granulocytic/Monocytic And Erythroid Clonogenic Assays

Details of the plates employed for the clonogenic (murine and hCB) cultures is provided below

Culture plates	Supplier	Catalogue Number
6 –well plates	Becton Dickinson, UK	351146

Flasks Used For Murine And Hcb LTBMC

Details of the plates employed for the LTBMC (murine and hCB) is provided below

Flasks	Supplier	Catalogue Number
T25 flasks (Costar)	Costar, UK	7543A
T25 flasks (Falcon)	Falcon, Becton Dickinson, UK	1134-1664
T25 flasks (Nunc)	StemCell Technologies Inc	166865

CFU-Mk: Identification of murine CFU-Mk Colonies

Reagents employed for the identification of murine CFU-Mk colonies are provided below

APAP staining antibodies and APAP staining reagents	Supplier	Catalogue Number
APAAP Rat monoclonal	Dako, UK	D0651
Rabbit Anti-Rat immunoglobulins	Dako, UK	M0737
Rat -Anti mouse Ckit (CD117) monoclonal antibody (IgG)	Serotec, UK	LS301
Rat Anti-Mouse CD41 (GpIIb/IIa)	Dako, UK	M7088
200 ml Acetone optimal	Sigma, UK	323969
Filter cards	Metachem Diagnostics, Northampton, UK	62138
Polypropylene separators	Metachem Diagnostics, UK	384125

Flow Cytometry Reagents

The source of the flow cytometry reagents employed for the surface cell receptor identification, cell cycle analysis, cell viability and intracellular markers are provided below

Flow cytometry Reagents	Supplier	Catalogue Number
FACSFlow	Becton Dickinson, UK	342003
CellFix	Becton Dickinson, UK	258102
Cell cycle Test-plus	Becton Dickinson, UK	855312
Via-Probe (7-amin-actinomycin D)	PharMingen San Diego USA	764992
Intracellular staining Kit	Becton Dickinson, UK	548319

LTBMC: Morphological Identification of Non-Adherent Cells

Reagents employed for the morphological identification of non-adherent cells from hCB and murine LTBMC is provided below

May Grunwald-Giemsa staining	Supplier	Catalogue Number
May-Grunwald stain	Dako, UK	AR164
pH 6.4 buffered water	-	-

General Methods

Murine Bone Marrow Source and Aspiration Procedure

Bone Marrow was harvested from male Balb/C mice from Charles River Laboratories Inc. (age: 4 - 6 weeks). The animals were killed by inhalation of carbon dioxide and cervical dislocation. The femora were dissected and the bone marrow aspirated into 1mL of Iscoves modified Dulbecco's culture medium IMDM (Life Technologies, UK), using a syringe with a 20-gauge needle.

Pre-Selection of Murine Mononuclear Bone Marrow Cell Population

Pluripotent stem cells, found in the mononuclear bone marrow cell population, were separated from the red blood cells using Lymphoprep^{R} (Robbins Scientific Corporation, West Midlands, UK). 0.5-ml peripheral blood was added to 6 ml of Lymphoprep^{R}. This preparation was centrifuged at 400g for 30 minutes at room temperature. The mononuclear cells were aspirated from the Lymphoprep^{R} interface and resuspended in IMDM. The cell count of the suspension was determined using an automated cell counter (Advia 120, Bayer, USA) and adjusted to 10^{5}cells /ml with IMDM.

Isolation of Murine Pluripotent Progenitor Cells (Ckit Positive Cells)

25µl of M-450 sheep Anti-Rat (IgG) coated Dynabeads (concentration of antibody 5µg/mL) were washed prior to use in IMDM. The tubes containing the beads were placed in a particle collector (MPC) to magnetically hold the beads to facilitate aspiration of the media the media. The washing procedure was repeated once more before the Dynabeads were re-suspended in 10µl of IMDM.

The magnetic beads were coated with 0.2mL of Anti-mouse CKit antibody (at a concentration of 5µg/mL) prior to positive selection. 20 µL of washed Anti-C kit antibody coated Dynabeads were added to 0.5 mL of 10^{5} mononuclear cells. The tube was incubated for 20 minutes at 4^{o} C for 20 minutes. At the end of the incubation time the cells were gently mixed

and placed in a Dynal MPC for two minutes. Before removing the tube from the Dynal MPC, the media and cells were aspirated off. The tube was removed from the Dynal MPC and the Dynabeads / C kit positive cells were re-suspended in 0.5 mL of IMDM. This procedure is termed positive selection.

Murine Granulocytic /Monocytic and Erythroid Clonogenic Assay (GM/E) - Standard Culture Conditions

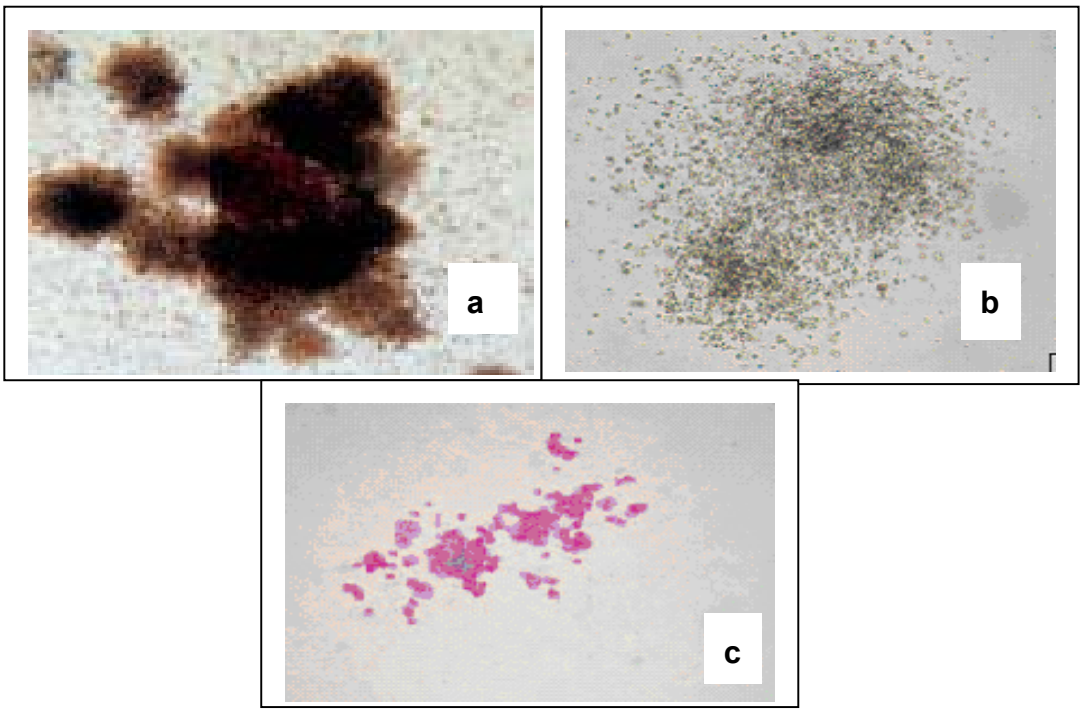
Composition Methocult GF M3434 was as follows:

0.9%	Methylcellulose in Iscoves MDM
15%	Fetal calf serum.
10^{-4}	2-mercaptoethanol
2 nM	L- glutamine
1%	Bovine serum albumin
10 μg/mL	Bovine pancreatic Insulin
200 μg/mL	Human transferrin
10 ng/mL	rm IL-3
10 ng/mL	rh IL-6
50 ng/mL	rm SCF
3 units /mL	rm Erythropoietin

hCB Megakaryocytic (Mk) Clonogenic Cultures

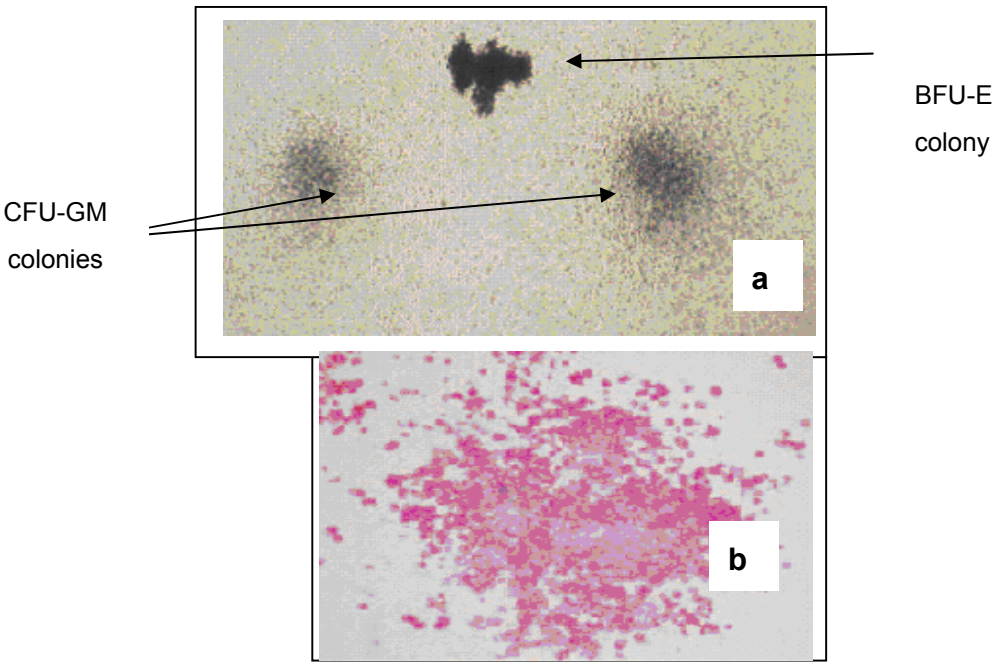
Composition MegaCult-C media was as follows:

1.1mg/mL	Collagen
1%	Bovine Serum Albumin
10μg/mL	Bovine Pancreatic Insulin
200 /mL	Human Transferrin (Iron Saturated)
10-4M	2-Mercaptoethanol
2mM	L-glutamine
1%	Methylcellulose in Iscove's Modified Dulbecco's Medium (IMDM)



Murine Clonogenic Colonies. a) BFU-E Colony; b) CFU-GM Colony; c) CFU-Mk Colony

Murine BFU-E colonies with a globular appearance and a red hue- objective x 200; Murine CFU-GM colonies that are clear (no staining) star shaped colonies- objective x100; Murine CFU-Mk colony stained with Giemsa stain (objective x 20)



hCB Clonogenic Colonies. a) BFU-E Colony and CFU-GM Colony; b) CFU -Mk Colony

a)hCB derived CFU-GM and BFU-E colonies grown in methylcellulose clonogenic cultures b) Giemsa stained CFU-Mk colonies.(Objective x 20 for all colonies shown)

Procedure for thawing cryoperserved mononuclear hCB

Original vial of cells

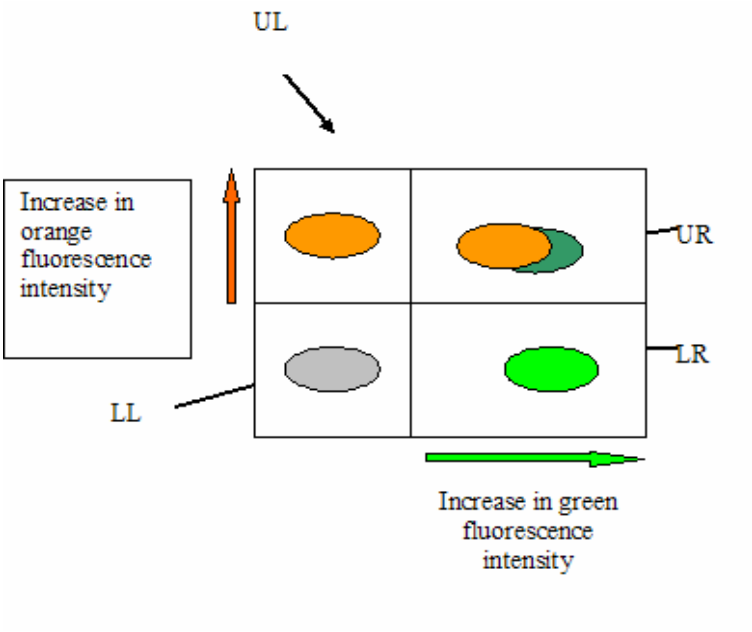
The cells (supplied at a concentration ranging from 1 x10^{12} to 1 x 10^{14} cells per vial) were thawed in a water bath at 35^{o}C by dipping and gently swirling intermittently and were aliquot into three equal sterile flow tubes once in the liquid state. This was done with extreme swiftness (within 20 seconds), so that the cells were in cryopreservative agents for as little time as possible.

Procedure for thawing hCB Cells (35^{o}C standard *temperature*)

IMDM was warmed containing 10% FBS (Sigma, UK), and 1mL of PBS (Phosphate Buffered Saline) was added to the powdered DNase I (2,000units/mL). Then 1mL of stock DNase I was added to 100 mL of conditioned medium to give a 20 U/mL of DNase I. DNase I was added to prevent the cells from clumping resulting from the release of DNA from any cells lysed following the freeze/ thaw process. Finally, 1 mL of Penicillin / Streptomycin (added to prevent bacterial growth) was added to the conditioned media. The vials of frozen cells were quickly thawed in a 35^{o}C water-bath by swirling in the water. The outside of the vial was wiped with 70% ethanol when thawed and the mononuclear cell suspension aseptically transferred to a 50ml conical tube. The vial containing the hCB was rinsed with 1mL of medium with the rinse added drop-wise to the cells while gently swirling the tube. 4mL of medium was slowly added, drop-wise, to the cells while gently swirling after each addition of several drops of medium. Before resting, the cells were gently mixed by pipetting up and down to ensure an even suspension. The cells were then rested for 10 minutes at 35^{o}C and 5% CO_2. 2.5mL of cells in media were placed into two 15mL tubes. Subsequently, the tube containing the cells was rinsed with 2mL of media and distributed evenly between the two tubes. The total volume of each tube was then made up to 10mL using the media. Before resting again, the cells were gently mixed to ensure an even suspension. The cells were then rested for 10 minutes at 35^{o}C and 5% CO_2. The tubes were then centrifuged at 400g at 35^{o}C for 15 minutes. After centrifugation, 8.5 mL of media was removed and discarded from both tubes. The cells were then gently re-suspended in the remaining media and pooled together. The cells were rested again for one hour at 35^{o}C and 5% CO_2. The cells were centrifuged for 5 min at 400g after this resting period. 2mL of media was removed and discarded, and the

remaining cells re-suspended in the remaining 1mL of media by gently pipetting up and down.

Flow Cytometry



Four Sub-Populations of Fluorescence Intensity Two- Dimensional Dot Plots (FITDDP)

Two dimensional dot plots (TDDP) were used to distinguish the different cell phenotypes as well as the relative size and relative complexity of the cells. The fluorescence intensity two dimensional dot plots (FITDDP), positively stained 'events' were expressed as percentages of the total population counted. An 'event' is the fluorescence signal produced by one cell. Four sub populations are described on FITDDP. These are green fluorescence and orange fluorescence negative (LL), green fluorescence negative/PE positive (UL), green fluorescence positive/ orange fluorescence negative (LR), and both green fluorescence / orange fluorescence positive (UR). The bracketed letters indicate the representation of events as quadrants for each population on a TDDP.

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