A STUDY OF THE CIRCULATING MYELOID PROGENITOR CELL IN MAN

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CONTENTS

SUMMARY

This thesis examines the circulating myeloid progenitor cell (PB CFU-GM) in normal subjects and in patients with Acute Non-lymphoblastic Leukaemia (ANLL). The aims are, firstly, to develop an accurate assay for PB CFU-GM; secondly, to establish the normal range for PB CFU-GM; thirdly, to study the changes in the levels of PB CFU-GM in normal subjects under various physiological conditions; fourthly, to study the changes in the levels of PB CFU-GM in patients with ANLL; fifthly, to study the collection and cryopreservation of peripheral blood mononuclear cells (PB MNC) from these patients during very early remission and sixthly, to use these cells for autologous stem cell rescue at relapse to test whether they possess haemopoietic reconstitutive capacity and whether longer lasting second remissions may result.

PB CFU-GM were assayed by culturing PB MNC in alpha-modified Eagle's medium with 15% foetal calf serum in 0.3% agar using human placental conditioned medium (HPCM) as a source of Colony Stimulating Activity (CSA). Colonies of >40 cells after 14 days' incubation were scored as CFU-GM. Using this system, studies in normal subjects demonstrated that no linear relationship exists between the number of PB MNC cultured and the number of CFU-GM detected. Further

assays performed with monocyte-depleted PB MNC alone and with the monocytes added back showed that there is a critical number of monocytes in this assay system above and below which CFU-GM growth decreases. When PB MNC were cultured at 0.625 to 10 x 10^5 cells per plate, the highest CFU-GM growth occurred in most subjects at either 1.25 or 2.5 x 10^5 cells per plate but in particular subjects it might occur at any of the five plating numbers used and the optimal plating number varies even in the same individual studied at different times. Thus it is important to perform this assay with several plating numbers in order to measure PB CFU-GM accurately. Because of the larger number of monocytes present, this monocyte effect is much more important in the PB CFU-GM assay system than in the bone marrow CFU-GM assay system where such an effect has already been demonstrated by others. The same monocyte effect was observed when PB MNC from patients with ANLL, acute lymphoblastic leukaemia, myelofibrosis, Hodgkin's Disease and drug induced agranulocytosis were cultured.

The monocyte effect when cryopreserved PB MNC were cultured was different from that when fresh cells were cultured. The highest CFU-GM growth usually occurred at a higher plating number (either 5 or 10 x 10^5 cells per plate) and this was shown to be due to changes in monocytes after the freeze-thaw process thereby affecting the CFU-GM/monocyte interaction. This study also shows that leucocyte feeder layers are a better source of CSA than HPCM when cryopreserved PB MNC are cultured.

In the present study, the normal range of PB CFU-GM was found to be wide and the levels were higher in males

than in females, as found in previous reports. The levels from normal subjects fitted a log-normal distribution. No significant difference was found between levels measured at 9 am and 2 pm or on consecutive days. Repeated measurements in individuals over a two year period showed moderate variation around each individual's own mean. Physical exercise led to a threefold increase in the levels of PB CFU-GM, reflecting the presence of a mobilisable pool. Such a readily mobilisable pool may contribute to the fluctuations noticed in individuals studied at different times. Previously reported lower normal ranges may be explained by a failure to take into account the monocyte effect.

Measurements of PB CFU-GM in 15 ANLL patients showed that a mean increase of 25 times the mean normal level occurred during very early remission, 15 to 29 days after the completion of induction chemotherapy. No such increase was found in patients not entering complete remission. Thus these high levels most probably reflect the intense recovery by normal haemopoietic cells while the patient is entering remission. The high levels lasted for several days while the platelet count rose rapidly. Such findings had not been reported before and raised the possibility of harvesting PB MNC during this phase for later autologous stem cell rescue.

Three or four continuous flow leukaphereses were performed on each of five ANLL patients during very early remission and yielded cells containing a mean of 37×10^4 CFU-GM/kg body weight for each patient. This represents five times the average yield of CFU-GM obtained by bone

marrow aspiration under general anaesthesia. There were no significant side-effects. These cells were cryopreserved and the PB CFU-GM remained viable after more than two years storage.

Two of five the patients who have had peripheral blood cells cryopreserved have relapsed. In the first patient, re-induction was attempted with high dose melphalan chemotherapy followed by infusion of his stored cells. Haemopoietic recovery started 11 days after the melphalan infusion, much earlier than the three to four weeks observed in other patients treated with high dose melphalan but not receiving stem cell rescue. However, leukaemic cells regrew quickly so that no definite conclusion could be drawn. The second patient received 1200 rads total body irradiation followed by autologous stem cell infusion containing 23 x 10^4 CFU-GM/kg body weight. Early recovery was again observed but recovery was incomplete. Eight weeks after infusion, the absolute neutrophil count was above 1,000/µl, the lymphocyte count was normal but the platelet count was only 19,000/µl and the patient required red cell transfusion. By thirteen weeks, there was evidence that the stem cell graft function was improving but the leukaemia had also relapsed. This case demonstrated that the harvested cells possess haemopoietic reconstitutive capacity but the incomplete recovery suggests that there are considerably fewer pluripotent stem cells per CFU-GM compared to bone marrow cells collected at stable remission. Whether these cells harvested in very early remission contain fewer leukaemic cells and will thus give rise to longer lasting second remissions requires further clinical investigation.

The use of such cells for haemopoietic reconstitution following supralethal chemoradiotherapy may represent a new therapeutic option for ANLL patients who are ineligible for allogeneic bone marrow transplantation because of age or lack of suitable donors.

ACKNOWLEDGEMENTS

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

ABBREVIATIONS

aMEM	Alpha-Modified Eagle's Medium
AIF	Acidic Iso-ferritins
ANLL	Acute Non-lympboblastic Leukaemia
BMT	Bone Marrow Transplantation
CC	Corrected Colony Count, CFU-GM/10^6 lymphocytes
CFU-s	Colony-Forming Unit-spleen
CFU-GEMM	Colony-Forming Unit, Granulocyte-Erythroid-Mega-karyocyte-Macrophage
CFU-GM	Colony-Forming Unit, Granulocyte-Macrophage or Myeloid Progenitor Cell
CSA	Colony Stimulating Activity
DAT	Induction chemotherapy for ANLL, consisting of Daunorubicin, cytosine-Arabinoside and Thioguanine.
DMSO	Dimethyl Sulphoxide
DPBS	Dulbecco's Phosphate Buffered Saline
FCS	Foetal Calf Serum
GvHD	Graft versus Host Disease
Hb	Haemoglobin
HBSS	Hank's Balanced Salt Solution
HPCM	Human Placental Conditioned Medium
PB CFU-GM	Peripheral Blood Myeloid Progenitor Cell
PGE	Prostaglandins of the E series
PB MNC	Peripheral Blood Mononuclear Cell
SE	Standard Error
SD	Standard Deviation
WCC	White Cell Count

CHAPTER 1   INTRODUCTION

PART 1 : HISTORICAL REVIEW

The importance of blood to the human body was recognised in ancient times. The Hippocratic school of medicine taught blood was the 'Paramount Humour' closely connected to life and vitality. Galen in the first Century supported the concept of the four 'humours' : blood, lymph (phlegm), yellow bile (choler) and black bile (melancholy). Galen taught that blood was made in the liver from digested food and distributed to the body through arteries and veins carrying various 'vital spirits'. He believed blood passed from the right to the left side of the heart through invisible pores in the wall between the ventricles to enter the arterial system.

Galen's teaching went unchallenged through the dark ages and not until the Renaissance did critical studies of anatomy and physiology lead to further understanding of blood and circulation. In 1555, Vesalius questioned Galen's view that pores existed between the two sides of the heart. In 1628, Harvey described the circulation of blood as we

know it now. Harvey's systematic study heralded the birth
of modern medicine based on objective observation and
stringent testing of hypothesis rather than tradition.

The invention of the light microscope by ~~Galileo~~ in
the early seventeenth century greatly extended the power of
human observation. "Small round globules" were described in
human blood by Leeuwenhoek in 1673, but the "ruddy globules"
were probably first observed by Swammerdam 15 years earlier.
The function of red blood cells became evident with the
discovery of haemoglobin (Funke 1851) and it's oxygen
carrying capacity (Hoppe-Seyler 1867). White blood cells
were first studied by Addison and they were named
'leucocyte' in 1855. Staining methods devised by Ehrlich in
1875 enabled different leucocytes to be identified. In 1842
Donne announced his discovery of 'globules' (platelets) in
the blood and they were given their present name by Giulio
Bizzozero in 1882 who also described their role in blood
coagulation. The origin of red blood cells was traced back
to the bone marrow by Neumann in 1868. The derivation of
platelets from megakaryocytes in the bone marrow was
described in 1902 by Wright. Vierordt was the first to
accurately determine the number of corpuscles in a cubic
millimeter of blood (1852) while haemoglobin concentration
was first measured with the haemoglobinometer designed by
Gowers in 1875.

It was realised that changes in bodily functions
were often accompanied by changes in blood. 'Chlorosis',
the name used since the 1500s to describe girls with a
greenish-yellow complexion, was recognised as a disease of
blood by Ashweil, and Sydenham recommended iron as a form of

treatment in the 1830s. Craigie, Bennett and Virchow, independently, described leukaemia in 1845 but the name 'leukaemia' was proposed by Virchow who recognised that leucocytes were involved. Pernicious Anaemia was described by Addison in 1856 but the value of liver therapy was not established until seventy years later by Minot and Murphy.

Advances in biochemistry also contributed to the study of blood and blood-forming organs, such as the unravelling of the structure and function of haemoglobin and discovering glucose-6-dehydrogenase deficiency as an important cause of non-spherocytic haemolytic anaemia in the 1950s. The increased understanding of normal cellular metabolism laid the ground work for the use of radioactive tracer studies used since the 1940s to study cell kinetics.

More recently, the haemopoietic system has been studied using haemopoietic stem cell culture systems. The existence of haemopoietic pluripotent stem cells capable of self-renewal and development into erythrocytes, leucocytes and platelets can be inferred from embryology. Each organism, however complex, is derived from the proliferation and differentiation of a single fertilised ovum. Thus such stem cells must exist, at least in the first weeks of in-utero existence. Similarly, the existence of oligo- and uni- potent progenitor cells which represent an intermediate stage of development between the pluripotent stem cells and the end cells can be inferred. However, traditional morphological or kinetic methods have been unable to identify these cells with certainty.

Till and McCulloch (1961) described the formation of splenic colonies 8-10 days after infusion of syngeneic bone

marrow cells into mice which had received a lethal dose of irradiation (hence the name CFU-s : colony forming unit in spleen). This provided the first in vivo model of pluripotent haemopoietic stem cells. Bradley and Metcalf (1966) described the first in vitro culture system of murine myeloid progenitor cells (Hence the name CFU-c : colony forming unit in culture). In vitro culture systems for the unipotent erythroid (Stephenson et al, 1971) and megakaryocytic progenitor cells (Mcleod et al, 1976) have since been described. A pluripotent haemopoietic progenitor cell (CFU-GEMM) has also been identified by in vitro culture (Fauser and Messner, 1978). 'CFU-GM', denoting colony forming unit — granulocyte-macrophage, is used in preference to 'CFU-c' as it is more informative as well as being in line with the nomenclature of the erythroid and megakaryocytic progenitor cells (BFU-E, CFU-E, CFU-M).

These pluripotent stem cell and progenitor cell culture systems have made it possible, for the first time, to study directly the hierarchy and the kinetics of these cells as well as the perturbations in disease states. These areas are reviewed in the rest of this Chapter.

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CHAPTER 1   INTRODUCTION

PART 2 : THE HAEMOPOIETIC STEM CELLS - A REVIEW

1. Pluripotent Haemopoietic Stem Cell

The first experimental evidence of a pluripotent
haemopoietic stem cell was described in the 1950's in
studies of haemopoietic reconstitution of lethally
irradiated mice by syngeneic bone marrow cells (Lorenz et
al, 1951; Ford et al, 1956; Mitchinson, 1956; Nowell et al,
1956). Subsequently Till and McCulloch showed that mouse
marrow cells, when injected into lethally irradiated
syngeneic mice, were capable of forming discrete nodules of
haemopoietic cells in the spleens of these animals eight to
ten days after injection (Till and McCulloch, 1961). These
nodules consisted of erythroid, granulocytic, megakaryocytic
and undifferentiated cells, either as pure populations or in
varying mixtures. These nodules were called "spleen
colonies", and the progenitor cells that formed them were
called "colony-forming unit - spleen" (CFU-s).

The single cell origin, or clonality, of these
nodules ("colonies") and the self-renewal capacity of CFU-s

within splenic colonies have been well established (Quesenberry and Levitt, 1979a). These features qualify the CFU-s as a pluripotent haemopoietic stem cell. There is experimental evidence to indicate that CFU-s consists of a family of pluripotent stem cells with different age structures (Johnson 1980; Ogawa et al, 1983; McCulloch 1983; Metcalf et al, 1983). There is also evidence that an even more primitive haemopoietic stem cell exists, which gives rise to the lymphoid as well as the myeloid cell lines (CFU-L-M) (Abramson et al, 1977).

In man the existence of pluripotent haemopoietic stem cells can be inferred from studies on the clonality of haemopoietic stem cells in chronic myeloid leukaemia (Whang et al, 1963) and polycythemia rubra vera (Adamson et al, 1976). In chronic myeloid leukaemia the specific Philadelphia chromosome was found in all the three lineages, myeloid, erythroid and megakaryocytic. In polycythaemia rubra vera, the same Glucose-6-Phosphate Dehydrogenase isoenzyme type was found in all the three lineages, indicating that they are from the same ancestor. Haemopoietic reconstitution by allogeneic or autologous bone marrow cells after supralethal chemo- radiotherapy in the treatment of patients with aplastic anaemia and haematological malignancies (Thomas et al 1975, 1977; Spitzer et al 1980) demonstrates clearly the existence of the pluripotent haemopoietic stem cell in man.

Dexter and Moore pioneered the development of in vitro long-term liquid culture systems for pluripotent stem cells (Dexter and Lajtha 1974; Dexter et al, 1977; Moore and Sheridan, 1979). Murine CFU-s can be maintained for several

months and prosimian (Tuqaia glis, the tree shrew) CFU-s can be maintained for up to one year. An adherent layer with fat-containing cells seems to be an essential component of these systems, providing the necessary haemopoietic inductive microenvironment besides being the actual source of the CFU-s. However, human CFU-s in culture have not survived beyond three months and this is probably due to the failure to establish a supportive adherent layer as in the murine and prosimian systems. Diffusion chambers have been implanted in the peritoneal cavities of mice to culture haemopoietic stem cells (Boyum and Borgstrom, 1970). The technique is used in only a few centres and will not be discussed further here.

Attempts have also been made to grow the pluripotent stem cell in a semi-solid medium. Mixed granulo-erythropoietic colonies grown in semi-solid medium from human bone marrow, peripheral blood, and cord blood were first described by Fauser and Messner (1978). Growth of these mixed colonies is dependent on erythropoietin and media conditioned by leucocytes in the presence of phytohemagglutinin (PHA-LCM) (Fauser and Messner 1979). Megakaryocytes, macrophages, eosinophils and lymphocytes have also been identified in these colonies. The name CFU-GEMM describes this pluripotent haemopoietic stem cell, with G, E, M, M standing for Granulocyte, Erythroid, Megakaryocyte and Macrophage/monocyte respectively. This name does not represent the full pluripotency of the cell but a name like CFU-GEMMoBT, with Mo, B and T standing for Macrophage/monocyte, B-Lymphocyte and T-Lymphocyte, becomes quite unwieldy so CFU-GEMM is more commonly used. CFU-Mix

has also been used, with Mix standing for Mixed Colonies as it is not always possible to detect all the cell types in these colonies. The exact relationship between CFU-GEMM and CFU-s in the hierarchy of haempoietic differentiation has not been fully defined. In mouse, there is considerable overlap between the two populations (Johnson, 1980) but it is likely that CFU-GEMM has lower self-renewal capacity (Nakahata and Ogawa, 1982). The assay system for human CFU-GEMM is, however, not fully standardised and the normal range described by different workers may vary up to ten-folds (Ash et al, 1981; Lu et al, 1983; Fabian et al, 1983; McCarthy and Gordon-Smith, 1984). One important technical consideration is to avoid overcrowded cultures leading to overlap of separate myeloid and erythroid colonies thereby producing false 'mixed colonies'.

Nevertheless, considerable knowledge has accrued from such studies. The CFU-s is morphologically similar to a lymphocyte (Moffat et al, 1967). No unique surface marker has been ascribed to the CFU-s which resides in the light density, non-T, non-B fraction of the 'lymphocyte' population in bone marrow and peripheral blood (Barr and Whang-Peng, 1975). Anti-serum raised against mouse-brain was found to have anti-CFU-s activity but it had effect against only a proportion of CFU-s (Golub, 1972; Monette and Stockel, 1980). Other monoclonal antibodies that have been reported are still being evaluated (Bodger et al, 1983). There is general agreement that self-renewal and committment to differentiation of the primitive haemopoietic stem cells appear to be governed by a stochastic rule, while the committed cells follow a complex multistep process of

differentiation from multipotential to unipotential progenitors with progressive, stochastic loss of potencies. The same authors regarded that the roles of the haemopoietic microenvironment and humoral regulators are still to be defined (McCulloch, 1983; Ogawa et al, 1983). When a satisfactory in vitro assay for pluripotent stem cell is established, it will be a powerful tool for the study of stem cell physiology and pathology. Fig 1.2.1 shows a scheme of the inter-relationships among the different stem cells and progenitor cells and is adapted from a review by Ogawa et al (1983).

Whilst CFU-s have considerable ability to self-replicate, this is not inexhaustible, at least in the in vivo serial transplantation model (Siminovitch et al, 1964). Exposure to alkylating agents decreases the capacity of murine CFU-s for self-renewal and limits the repopulating potential of stem cells (Botnick et al, 1978). The limitation induced by such agents on the proliferative capacity of haemopoietic stem cells may have clinical relevance in the determination of the late sequelae of chemotherapy and irradiation.

The haematological defects in the S1/S1d (McCulloch et al, 1965) and the W/Wv mice (Lewis et al, 1967), cyclic neutropenia in the gray collie dog (Dale et al, 1972) and bulsuphan induced hypoplastic anaemia (Morley et al, 1975) are all examples of haemopoietic stem cell disorders with human counterparts. The myeloproliferative disorders, aplastic anaemias and the hereditary Diamond-Blackfan syndrome (Chronic pure red cell aplasia) are all examples of human stem cell diseases.
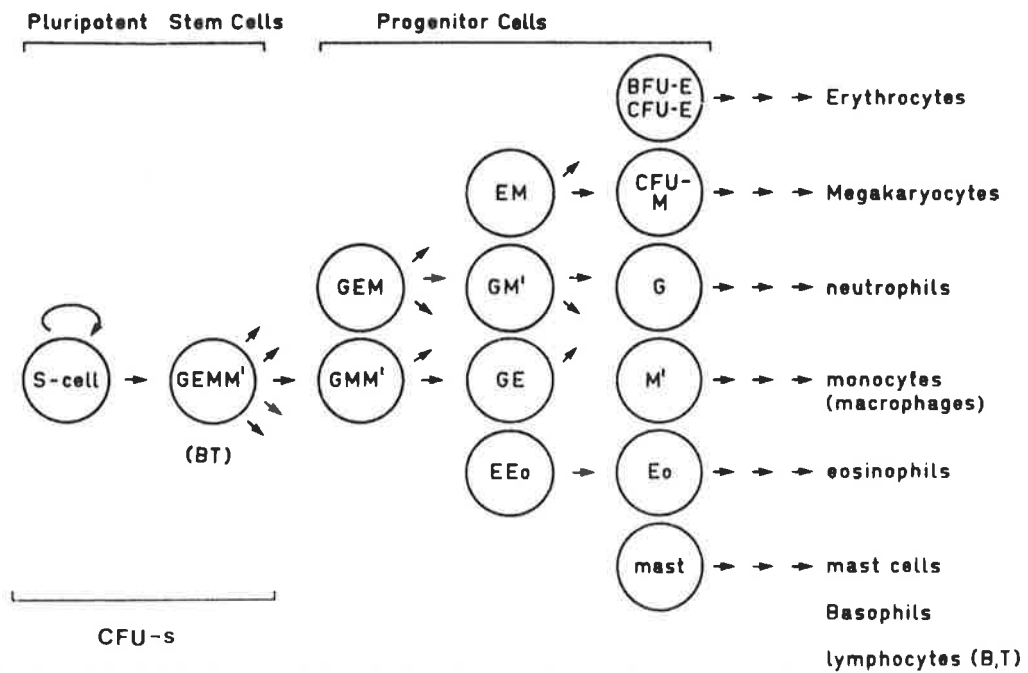
Fig. 1.2.1   A schematic presentation of a model of differentiation
             for haemopoietic stem cells assayable in culture
             adapted from Ogawa et al (1983).

Abbreviations:

CFU-s   Colony-forming unit - spleen.

S-cell   An early CFU-s which give rise to undifferentiated
             splenic colonies.

GEMM'   A late CFU-s which give rise to differentiated
             splenic colonies.

G   Granulocyte

E   Erythroid

M   Megakaryocyte

M'   Monocyte-macrophage

Eo   Eosinophil

Mast   Mast cell

B   B lymphocyte

T   T lymphocyte

The ability of the pluripotent haemopoietic stem cell to maintain its viability and repopulating potential when cryopreserved with dimethyl sulfoxide in liquid nitrogen (Gray and Robinson, 1972; Lewis and Trobaugh, 1964) has important therapeutic implications which will be discussed later in this chapter.

In summary, the existence of a pluripotent haemopoietic stem cell has been established but further study of this important cell in the haemopoietic system is hampered by the lack of satisfactory assay techniques. In contrast, unipotent progenitor cell assays are better standardised and established.

2. Unipotent Progenitor Cells

Among the various assays for unipotent progenitor cells, the myeloid progenitor cell (Colony Forming Unit in Culture, CFU-c, CFU-GM) assay is the most commonly used and is the method employed in this thesis so only this assay will be discussed below.

CFU-GM was first described in 1966 by Bradley and Metcalf in an in vitro semi-solid culture system. Murine bone marrow cells were cultured in 0.3% agar using mouse kidney cell feeders. After 7 days in incubation, groups of cells consisting of developing granulocytic and monocytic cells were found. The cells which gave rise to such 'colonies' in the culture system were named 'colony-forming unit in culture, CFU-c'. Pike and Robinson later adopted the system for human bone marrow cells (1970). Liquid

culture systems have also been developed (Summer et al, 1972) but are not in routine use.

The single-cell origin of granulocyte-monocyte colonies has been established by cell-transfer studies which showed that one cell is capable of giving rise to both granulocytes and monocytes (Moore et al, 1972).

CFU-GM, like the CFU-s, resembles transitional lymphocytes morphologically (Moore et al, 1972) but behaves in a kinetically different manner with a higher proliferation rate (30-50% c.f. <10% in CFU-s) (Rickard et al, 1970; Iscove et al, 1970; Morley et al, 1971). These cells can be partially separated from CFU-s by velocity, density and adherence techniques (Haskill et al, 1970). They have little capacity for self-renewal compared with CFU-s (Moore and Williams, 1974).

The growth of CFU-GM in vitro is dependent on the continued presence of Colony-Stimulating Activity (CSA). Human CSA represents a family of glycoproteins with varying molecular weight from 24,000 to 50,000 Daltons (Burgess and Metcalf, 1980; Newton et al, 1982). Macrophages and monocytes (Golde and Cline, 1972; Chervenick and LoBuglio, 1972), activated lymphocytes (Cline and Golde, 1974) and endothelial cells (Knudtzon and Mortensen, 1975) can produce CSA. Human placenta in culture is also a rich source of CSA (Burgess et al, 1977; Burgess and Metcalf, 1980) and the cellular origin of CSA has been traced to the trophoblasts (Ruscetti et al, 1982). Several cell lines, transformed cells or malignant tissues can also produce CSA in vitro, but the physiological relevance of this phenomenon is uncertain (Austin et al, 1971). In vivo, the role of CSA is

still undefined although most situations in which granulocytosis and monocytosis occur are associated with high serum and tissue levels of CSA.

While CSA provides the stimulatory effect on CFU-GM, there are several compounds which appear to provide a counteracting inhibitory influence in vitro, and may act as negative feedback regulators of granulopoiesis in vivo. Chalones are low molecular peptides derived from mature granulocytes (Rytomaa 1973) having an inhibitory effect on granulopoiesis. Another granulocyte product, lactoferrin, appears to exert its inhibitory effect on granulopoiesis by the inhibition of human monocyte CSA production (Broxmeyer et al, 1978a). The physiological significances of chalone and lactoferrin are not yet established (Moore, 1979) but various monocyte products have been described which may have more physiological significance.

In studies on the diffusible monocyte-derived CSA, maximum colony stimulation occurred at a monocyte concentration of 1×10^5 cells/ml (Kurland et al, 1978a). At higher concentrations of monocytes, inhibition started to occur and no further increase in colony formation was observed. Addition of the Prostaglandin Synthetase inhibitor, indomethacin, to the monocyte underlayers resulted in an increase in colony formation. It was further demonstrated that the inhibition of colony formation was due to Prostaglandins of the E series (PGE) synthesised by monocytes and the PGE production was enhanced in the presence of CSA. PGE may therefore exert a regulatory effect on granulopoiesis by negative feedback (Kurland et al, 1978b).

Acidic iso-ferritins are another family of monocyte products which may have important physiological influence on granulopoiesis. First discovered as the leukaemia-associated inhibitory activity (Broxmeyer et al, 1978b), they were subsequently isolated from monocytes and their production by monocytes in vitro requires the presence of a critical number of monocytes so cellular interactions are probably important for their production (Broxmeyer et al, 1982a). They suppress the formation of colonies from CFU-GM which are in S-phase (Broxmeyer et al, 1982b) and may therefore behave like a negative feedback inhibitor. Antibodies raised against acidic iso-ferritins abolished the inhibitory effect on CFU-GM and it was suggested that acidic iso-ferritins may confer a proliferative advantage to leukaemic stem cells over normal stem cells because the former appear to be resistant to its inhibitory effect (Broxmeyer 1982a). A recent review by Jacob (1983) cautioned that further work was required before acidic iso-ferritins could be accepted as physiological modulators of granulopoiesis.

While the physiological control of granulopoiesis remains to be elucidated, the knowledge gained from the in vitro CFU-GM assay system has increased our understanding of normal and abnormal haemopoiesis (Greenberg, 1980). Both animal studies and experience in human bone marrow transplantation have shown that the CFU-GM content of a specimen of bone marrow correlates well with its haemopoietic reconstitutive capacity, indicating that CFU-GM measurement may provide a reliable, albeit indirect estimation of the pluripotent haemopoietic stem cell

*ADDENDUM

The usual correlation between pluripotent stem cell and CFU-GM was not found in one animal study. In a murine model using bone marrow to study changes in repopulating (pluripotent) stem cells and progenitor cells in the first few days following a dose of 5-Fluorouracil, Hodgson et al (1982) found that progrenitor cells were reduced more than pluripotent stem cells. This dissociation between the two populations is, however, a result of acute drug-induced perturbation and does not necessarily reflect steady state conditions. No data are available on the pluripotent stem cell: CFU-GM ratio in the bone marrow of man during recovery from drug-induced marrow depression.

Hodgson, G.S., Bradley, T.R. and Radley, J.M. (1982): "The Organization of Hemopoietic Tissue as inferred from the Effects of 5-Fluorouracil". Exp Hematol, 10: 16-35.

content. In man, haemopoietic reconstitution can be reliably achieved with the infusion of 2×10^8 bone marrow nucleated cells/Kg or 4×10^4 CFU-GM/Kg after supralethal chemoradiotherapy (Spitzer et al, 1980).✱

Bone marrow CFU-GM measurements, however, are not useful as an indicator of the haemopoietic activity in a subject. There is no simple and reliable way to relate the CFU-GM level of a bone marrow specimen to the total haemopoietic activity of the body (Parmentier et al, 1978). This is because the volume of active bone marrow is difficult to determine (Knospe et al, 1976) and the cell counts in marrow aspirates are highly variable depending on the site examined (Parmentier et al, 1978; Gordon et al, 1976), the technique used and the volume aspirated (Holdrinet et al, 1980); furthermore, these problems are aggravated in disease states (Holdrinet et al, 1980; Knospe et al, 1976).

In contrast, the levels of the myeloid progenitor cell in peripheral blood (PB CFU-GM) can be measured without the problems of sampling error or major discomfort to the patient. There is also experimental evidence to suggest that the levels of PB CFU-GM are a reliable indicator of the pluripotent stem cell. Fliedner et al (1976) described an experiment in which beagle dogs were given a supralethal dose of total body irradiation to ablate haemopoiesis irreversibly. Autologous or allogeneic peripheral blood mononuclear cells were infused and the dogs monitored for subsequent haemopoietic recovery. The number of myeloid progenitor cells in the infused cells correlated well with their haemopoietic reconstitutive ability. As myeloid

progenitor cells have no self-renewal capacity and therefore cannot be responsible for the haemopoietic recovery, the correlation suggests that the circulating level of CFU-GM is a reliable indicator of the pluripotent stem cell. Abrams et al (1981) further demonstrated that the recovery following /cyclophosphamide treatment expands the circulating haemopoietic stem cell pool in dogs and this expansion can be measured by the CFU-GM.

Studies on the PB CFU-GM in man are few. While there is general agreement on the methodology and the normal ranges for bone marrow CFU-GM reported by different groups, considerable differences exist in the various reports on PB CFU-GM. The next part of this Chapter reviews the PB CFU-GM assay system and published reports.

CHAPTER 1   INTRODUCTION

PART 3 :   PB CFU-GM - THE IN VITRO ASSAY SYSTEM, LEVELS IN

NORMAL SUBJECTS AND IN PATIENTS WITH ACUTE

NON-LYMPHOBLASTIC LEUKAEMIA - A REVIEW

The presence of PB CFU-GM in man was first described
by several groups of workers independently in 1971
(Chervenick and Boggs; Kurnick and Robinson; McCredie et
al). Since then the majority of PB CFU-GM studies in man
have been performed either in normal subjects or in patients
with Acute Non-lymphoblastic Leukaemia (ANLL). There have
also been several studies performed in patients with normal
bone marrow during the recovery phase after cytotoxic
chemotherapy. These reports are reviewed later in this
Chapter while other isolated reports on patients with Down's
Syndrome (Standen et al, 1979) and hereditary spherocytosis
(Weetman et al, 1977) will not be further discussed.
Considerable differences in the methodology of the in vitro
assay system exist between the various reports and this will
be reviewed first.

COMPONENTS OF THE IN VITRO PB CFU-GM ASSAY SYSTEM

PB CFU-GM studies have been performed culturing either whole blood leucocytes or PB MNC in either agar or methylcellulose in the presence of one of the different forms of CSA with a supply of nutrients and serum. Groups of greater than 20 or 40 cells detected after 7 or 14 days' incubation have been variously considered to represent the progeny of the oligopotent myeloid progenitor cell. Each of these components will be discussed below.

Nutrient Media, Supporting media and Serum

The nutrient media supply simple sugars, amino acids, lipids and minerals in physiological concentrations to the culture system. The exact formulation varies slightly between different brands and various modifications are used in different centres (Eagle' medium, McCoy's medium, Dulbecco's mdium) but none is proven to be superior to the others. Most depend on the CO_2/bicarbonate system for buffering capacity. Antibiotics, most commonly penicillin and streptomycin, are added to discourage bacterial growth.

The two types of semi-solid supporting medium in common use are agar and methylcellulose. Methylcellulose is water-soluble so that colonies can be readily picked up and identified by staining. This is particularly important in studies involving identification of different colony types. In routine culture work, however, agar is more commonly used because it is easier to prepare and to use. Colonies in

agar cultures are also easier to score (Greenberg, 1980).
CFU-GM growth appears to be very dependent on the correct
concentration of agar. If the concentration is too low,
insufficient viscous support leads to the cells settling to
the bottom of the plate. Colony morphology is affected and
accurate scoring becomes difficult. If the concentration is
too high, CFU-GM growth is inhibited, leading to an
underestimation of the CFU-GM content (Metcalf, 1977).

Foetal calf serum is the most common form of growth
supplement used although human AB Rh-ve serum has also been
used. The growth promoting activity of foetal calf serum is
as yet unexplained and can only be tested by meticulous
batch-testing in the culture system (Metcalf, 1977).

Colony Stimulating Activity (CSA)

The in vitro proliferation of CFU-GM depends on the
continued presence of a source of CSA. Feeder layers using
cells immobilised in an underlayer of 0.5% agar were the
first type of CSA used (Bradley and Metcalf, 1966) but more
recently various conditioned media have been used because of
ease of preparation and storage.

Feeder Layers

Feeder layers of whole blood leucocytes are still
the standard against which other types of CSA are compared
but their routine use is limited by the variable levels of
CSA in different batches of feeders, the time and cost
involved in preparation and their short shelf-life
(Metcalf, 1977). The level of CSA in different batches of

feeder layers may vary according to the subjects donating the cells, and may also vary at different times in the same subject. It is therefore important that feeder layers from at least two subjects are used in parallel. Feeder layers must be used within seven or eight days otherwise the level of CSA decreases and the incidence of contamination increases. Feeder layers are, furthermore, a relatively expensive source of CSA. Besides doubling the quantity of media and petri-dishes used, preparing enough feeder layers for 4 sets of PB CFU-GM assays involves two hours of work. When large number of assays are performed, the frequent venesection of volunteers, the work and the expense involved in preparing feeder layers often becomes an enormous burden. Bearing in mind that ten or more assays might be performed each week, the preparation time will be one full working day in five. Feeder layers are therefore best used as a standard source against which other types of CSA are tested (see below). Feeder layers are also a better source of CSA than Human Placental Conditioned Media (HPCM) when cryopreserved cells are cultured (Schlunk et al, 1981). They suggested that monocytes are important in the processing of HPCM and cryopreservation may have impaired this ability. While the actual mechanism is still to be elucidated, feeder layers should be used in order not to underestimate CFU-GM in cryopreserved cells.

Human placental conditioned medium (HPCM)

Burgess et al (1977) first described CSA activity in conditioned medium prepared from human placentae.

Ruscetti et al (1982) showed that a trophoblastic cell line can produce CSA identical to that in HPCM, thus providing evidence for the cellular origin of CSA in HPCM. The optimal method of preparation of HPCM was described by Schlunk and Schleyer (1980). It is easy to prepare and stable on storage. Up to 1 litre of HPCM can be prepared from each placenta (sufficient for up to 10,000 plates of culture) and is probably the most common form of CSA in use. Burgess and Metcalf (1980) reviewed the various species of human and murine CSA identified but the difficulty in purifying sufficient quantities for study has prevented their molecular structures and biological actions to be fully understood. Concentration procedures have been described (Nicola et al, 1978; Stanley and Guilbert, 1981; Okabe et al, 1982) but they may result in considerable loss in CSA so the methods are best applied for the processing of large volume of HPCM (e.g., 20 to 30 litres). The level of CSA in HPCM is standardised against feeder layers and/or another batch of HPCM with known activity.

Leucocyte conditioned medium (LCM)

LCM has been prepared from whole blood leucocytes with or without phyto-haemagglutinin (Cline and Golde, 1972; Price et al, 1975), from monocytes (Shah et al, 1979) and from spleen cells (Paran et al, 1970). These media are easy to prepare and stable on storage and are often used where placentae are not readily available. The level of CSA needs to be standardised as for HPCM.

Comparison between HPCM and various types of LCM

Newton et al (1982) reported that the potency and specificity of CSA in HPCM and various LCM are similar so that studies performed with these conditioned media are comparable.

Cell Type and Plating Number

Most of the early studies on PB CFU-GM were performed using whole blood leucocytes after the red cells had been removed by dextran sedimentation. However, most recent studies are performed culturing mononuclear cells obtained by Ficoll-paque density gradient separation because the more efficient removal of red cells by the latter method makes scoring easier.

A survey of the published reports on PB CFU-GM in normal subjects shows that most studies employ one or two plating numbers, usually 5 or 10 x 10^5 PB MNC per plate (Table 1.3.1). These plating numbers were 5 to 10 times higher than that in the bone marrow CFU-GM assay. Because the number of CFU-GM in peripheral blood is relatively low, higher plating numbers are used in order to have a higher number of colonies per plate so that the results are more suitable for statistical calculations. The few studies to investigate the relationship between the number of MNC plated and the number of colonies detected (Tebbi et al, 1976; Barrett et al, 1979) seemed to indicate that a linear relationship existed. The validity of these results and the importance of plating number will be further discussed later in this Chapter.

Incubation Conditions

All nutrient media depend primarily on the CO_2/bicarbonate system for buffering capacity so the cultures need to be incubated in a CO_2 enriched atmosphere. The concentration of CO_2 required depends on the concentration of bicarbonate in the media and 5% and 7.5% are the two levels most commonly used. The remainder of the gaseous environment is made up of oxygen and nitrogen in a ratio either as that present in air or lower. Bradley et al (1978) showed that better colony growth occurred in the presence of 7.5% oxygen and suggested that the lower oxygen tension may provide a more physiological environment so this level of oxygen tension has been adopted for studies in this thesis. The required gaseous environment may be provided by an incubator fed with a constant flow of CO_2 at the required concentration or by putting the culture plates in a container which is sealed after being flushed with the particular gas mixture.

Cultures are usually incubated for 10 to 14 days at 37°C. Studies comparing 7 and the 14 day cultures (Johnson et al, 1977) showed that colonies were formed by different cells and therefore carry different significance. Based on cell kinetics, cells that form colonies after 14 days are probably more primitive. Since most studies used the longer incubation period, the results reported in this thesis are based on 14 day cultures. The 14 days incubation period is more convenient than that of 10 days because it fits better into the pattern of a working week.

Scoring of Colonies and Expression of Results

The in vitro recognition of PB CFU-GM depends entirely upon the formation of a 'colony' of cells when a single cell suspension of peripheral blood cells is cultured in conditions described above. The most commonly used criterion is a group of 40 or more cells. If the mitotic divisions are symmetrical, 6 or more divisions would produce a group of more than 40 cells ($2^6 = 64$) so such a criterion would define CFU-GM as a cell capable of undergoing six or more cell divisions in 14 days. Colonies containing one thousand cells or more are occasionally observed showing that CFU-GM are capable of 10 or more divisions in 14 days ($2^{10} = 1024$). Some studies considered groups of 20 or more cells sufficient criterion for CFU-GM (Richman et al, 1976). Their results therefore included cells with lower proliferative potentials and may not be comparable with others using 40 or more cells as the criterion.

CFU-GM levels are often expressed as colonies per 10^6 mononuclear cells plated (Richman et al, 1976; Standen et al, 1979; Verma et al, 1980; Goldberg et al, 1980). Such an expression, however, is not suitable in clinical studies of PB CFU-GM levels because both the CFU-GM:mononuclear cell ratio and the number of mononuclear cells per ml of blood may be different among subjects and in the same subject at different times. For such purposes, CFU-GM/ml blood appears to be a more meaningful expression of CFU-GM levels. As the PB CFU-GM is a non-adherent, mononuclear cell

morphologically similar to a lymphocyte, it is reasonable to calculate CFU-GM results based on CFU-GM/10^6 lymphocytes. The number of CFU-GM/ml blood can then be derived by multiplying this value by the number of lymphocytes (in 10^6) per ml of blood.

Another instance where the expression CFU-GM per 10^6 mononuclear cells is inadequate is in studies involving fractionation of mononuclear cells by adherence. When the mononuclear cell population is further fractionated, the ratio of CFU-GM to total number of cells is different in the various cell fractions so that the CFU-GM/10^6 cells plated becomes misleading and inaccurate. As the CFU-GM:lymphocyte ratio should be much less affected by the adherence procedure than the CFU-GM:total cells ratio, the CFU-GM/10^6 lymphocytes is again a better expression (To et al, 1983a).

PB CFU-GM STUDIES IN NORMAL SUBJECTS

The normal ranges of PB CFU-GM in published reports are shown in Table 1.3.1. They are classified into two groups according to the type of cells cultured. There are other studies of PB CFU-GM in normal subjects that are not included either because the number of subjects studied was too small (Cline et al, 1977b; Kreutzman et al, 1979) or because the methodology was not clearly stated (Standen et al, 1979).

Considerable differences exist between the means and normal ranges of PB CFU-GM in the various reports. The

TABLE 1.3.1

PUBLISHED REPORTS OF PB CFU-GM IN NORMAL SUBJECTS

Plating Number (x10^5/plate)	Number of Subjects	Supporting Media	CSA	Colony Size	CFU-GM (per ml)	Reference
1. Dextran separated Whole Blood Leucocytes						
5	30(M) 28(F)	AGAR	FL/LCM	>50 cells	98, 8-300 44, 0-260	Barrett (79)
10	37(M) 15(F)	AGAR	FL	>50 cells	215 ±13* 182 ±13*	Ponassi (79)
2. Ficoll-paque separated Mononuclear Cells						
2	14	MC	LCM	>20 cells	120, 25-600*	Richman (76)
5	6	AGAR	FL	>40 cells	45±15*	Goldberg (80)
5	?	AGAR	HPCM	>50 cells	11-61	Lohrmann (78)
5	30	AGAR	LCM	>50 cells	78, 15-410*	Beran (80)
5-10	15	AGAR	HPCM	>40 cells	13 ±13(AM)* 28± 28(PM)*	Verma (80)
5-10	9	AGAR	FL	>50 cells	29, 0-70	Jehn (83)
10	?	AGAR	FL	>50 cells	93± 45*	Peschel (83)

TABLE 1.3.1 (continued)

MC   =   Methyl-cellulose
CSA =   Colony Stimulating Activity
FL   =   Feeder Layers
LCM =   Leucocyte Conditioned Media
HPCM =   Human Placental Conditioned Media
* denotes calculated value: the levels in the original report were given as
  CFU-GM/10^6 cells plated. The result shown here are calculated assuming
  that the mean numbers of leucocytes and mononuclear cells are 6.5 x 10^3/µl
  and 2.5 x 10^3/µl respectively.

difference between the highest and lowest means is more than tenfold (215 and 13). The upper limit of the normal range varies from 61 to 600 and the lower limit is 0 or even less than 0 in some of the reports. A normal range including negative values does not make sense. While all these differences may be real it is more likely that the assay system is not yet fully optimised and may not be measuring the actual levels of PB CFU-GM (To et al, 1983a). Monocytes have been shown to exert both stimulatory and inhibitory influence on the in vitro proliferation of CFU-GM (Kurland et al, 1978a). The monocyte secretory products CSA, PGE and acidic iso-ferritins have opposing effects on CFU-GM so any change in the monocyte population may cause an apparent change in the level of CFU-GM. The number of monocytes in the PB CFU-GM assay when 5 or 10 x 10^5 MNC are cultured is high, up to 100 times that in the BM assay (Table 1.3.2) and is similar to the number causing inhibition when added to the bone marrow CFU-GM assay (Kurland et al, 1978a). Thus it is quite probable that the assay system is not measuring the actual levels of PB CFU-GM owing to the inhibitory effect of large numbers of monocytes. Indeed, the levels of PB CFU-GM detected when 5 or 10 x 10^5 mononuclear cells/plate were cultured were lower than that when 2 x 10^5 cells/plate were cultured (Richman et al, 1976). The standardisation of the PB CFU-GM assay taking into account the effect of monocytes is therefore the first major subject addressed in this thesis. Only when PB CFU-GM can be measured accurately can a reliable normal range be established.

Two large studies (Barrett et al, 1979; Ponassi et

TABLE 1.3.2

A COMPARISON OF THE CELLULAR COMPOSITION OF CELLS USED FOR
BONE MARROW AND PERIPHERAL BLOOD CFU-GM ASSAY

	BONE MARROW NUCLEATED CELLS $(x10^5)$	PERIPHERAL BLOOD MONONUCLEAR CELLS $(x10^5)$
TOTAL NO. OF CELL/PLATE	1	5 – 10
MYELOID CELLS	0.6 – 0.8	-
ERYTHROID CELLS	0.15 – 0.35	-
LYMPHOCYTES	0.03 – 0.15	3 – 8
MONOCYTES	0 – 0.04	1 – 4
CFU-GM	< 0.1%	< 0.1%

al, 1979) found that PB CFU-GM levels were higher in males than in females while others quoted a common mean and normal range for both sexes. It is important to take into account such differences if small samples of patients are studied or if only minor changes in the levels are expected (Ponassi et al, 1979). Ponassi et al (1979) also found a mean difference of 19% (range 3 to 39%) between the morning and afternoon PB CFU-GM levels but no definite trend of increase or decrease was present. Verma et al (1980) found a more than twofold increase in PB CFU-GM levels in the afternoon compared to those in the morning. It is obviously important to determine whether the time of specimen collection has a bearing on the results of the assay.

Two groups of workers (Barrett et al, 1979; Kreutzman et al, 1979) found evidence of long-term cycling in a small number of normal subjects studied over a period of time and suggested a cycling time of 3 to 4 weeks and 19 to 25 days respectively. Ponassi et al (1979) also found considerable long-term fluctuations in PB CFU-GM in the same subject, with more than threefold difference in 11% (2/11) of subjects studied. However, no serial studies covering more than 3 months have been reported except in one subject studied twice 7 months apart. Furthermore, two- to four-fold increases in PB CFU-GM have been described after vigorous physical exercise, injection of adrenocorticotrophic hormone (Barrett et al 1978) and endotoxin (Cline et al, 1977b), suggesting that there is a readily mobilisable pool of CFU-GM and rapid exchanges can occur between the mobilisable and the circulating pools of CFU-GM. Thus, it would be quite difficult to determine

whether variations in PB CFU-GM levels are the result of all these known and other yet unknown factors or are genuinely related to the proposed cycling phenomenon (see also Chapter 4 Discussion).

PB CFU-GM STUDIES IN ACUTE NON-LYMPHOBLASTIC LEUKAEMIA (ANLL)

ANLL is primarily a bone marrow disease so both the diagnosis and the monitoring of response depend on findings of bone marrow examinations. Most CFU-GM studies in ANLL patients have therefore been performed using bone marrow cells, and not peripheral blood cells. They were most often undertaken to study whether the in vitro growth pattern carried any prognostic significance. Greenberg (1980) reviewed the reports by Moore et al (1974), Curtis et al (1975), Spitzer (1976), Vincent et al (1977), Bro-Jorgensen and Knudtzon (1977), Goldberg et al (1978) and Beran et al (1980) and found there is general agreement (except Curtis et al) that patients with decreased or no colony growth at diagnosis have higher complete remission rates compared to those with increased or abnormal (clusters and single persisting cells) growth. However, overall clinical responses in the early studies were low by current standards and included various treatment regimens. There is little current interest in the use of the in vitro CFU-GM growth patterns as a prognostic indicator. Other studies showed that the cells from ANLL patients may have defects in production of CSA in vitro (Francis et al, 1981) but the

physiological explanation is unclear.

In studies of PB CFU-GM in ANLL patients, Moore et al (1974) and Beran et al (1980) found that the in vitro growth pattern of peripheral blood cells was similar to that of bone marrow cells. In contrast, Jehn et al (1983) found that the levels of peripheral blood and bone marrow CFU-GM tended to vary in opposite directions and suggested that this was a result of displacement of normal stem cells from bone marrow to blood. This explanation seems simplistic because the bone marrow CFU-GM pool is so much greater than that of the peripheral blood (seventy-fold or more) that a much greater increase in PB CFU-GM would be expected than that described by these workers. Peschel et al (1983) studied PB CFU-GM levels during long-term remission and found the levels were either normal or low. All these studies, however, were performed without allowing for the effect of monocytes and the results need to be regarded with caution.

Richman et al (1976) demonstrated increased levels of PB CFU-GM in patients with solid tumours during recovery from the myelosuppressive effect of cytotoxic chemotherapy. Lohrmann et al (1979) performed serial studies on patients receiving adjuvant chemotherapy for breast cancer and showed that PB CFU-GM and leucocytes exceeded their respective normal ranges during the recovery phase. In addition, the rise in PB CFU-GM regularly and predictably preceded the rise in granulocytes. ANLL patients usually exhibit a rapid rise in platelet and leucocyte counts when they first enter remission after induction chemotherapy. During this very early remission phase, the platelet count commonly doubles

daily and may reach levels of $1,000 \times 10^3/\mu l$ or higher. This observation suggested that a similar rise in PB CFU-GM might be seen in these patients with primary bone marrow disease. A preliminary study by Juttner et al (1982a) showed high PB CFU-GM levels in seven ANLL patients during very early remission but no systematic studies of the serial changes in this phase have previously been reported. The therapeutic potentials of harvesting circulating stem cells during very early remission for later autologous haemopoietic reconstitution will be discussed in the next section.

CHAPTER 1   INTRODUCTION

PART 4 : A REVIEW OF THE CURRENT TREATMENTS OF

ACUTE NON-LYMPHOBLASTIC LEUKAEMIA (ANLL)

Acute Non-lymphoblastic leukaemia (ANLL) is the most common form of acute leukaemia in the adult population with an annual incidence of 3.5 per 100,000 of population, a rate similar to that of malignant melanoma and brain tumours. Most of the patients are middle-aged or older. There is good evidence that ANLL is a clonal disorder of haemopoietic stem cells characterised by uncontrolled proliferation and defective maturation of the abnormal clone (Quesenberry and Levitt, 1979c). Clinical manifestations are due to leukaemic infiltration, metabolic and nutritional disturbances and haemopoietic failure. Prognosis is poor, with patients meeting their demise in weeks if untreated. Until the advent of improved supportive care and combination chemotherapy containing Daunorubicin and Cytosine Arabinoside, only 20-30% of patients achieved complete remission and the remissions tended to be short-lasting. At present, 70% or more of ANLL patients achieve complete remission (Gale and Cline, 1977). However, the majority of these patients relapse within one to two years and long-term disease-free survival occurs in less than 20% of patients (Lister and Rohatiner, 1982; Santos and Kaizer, 1982).

Leukaemic relapse appears to occur because cytotoxic drugs are relatively ineffective against non-cycling cells (Carter and Livingston, 1982) and residual leukaemic cells in the body lead to eventual relapse. Maintenance therapy has not proved effective in prolonging remission although intensive consolidation regimes have been claimed by some workers to produce longer remissions (Lister and Rohatiner, 1982; Weinstein et al, 1983). Ionising irradiation is effective against leukaemic cells so that another approach is to combine high-dose chemotherapy and total body irradiation (TBI) to eradicate leukaemic cells. To counteract the irreversible haemopoietic failure that results, haemopoietic reconstitution using a source of haemopoietic stem cells either from a histocompatible donor (allogeneic), an identical twin (syngeneic), or patient's own cells (autologous) is an essential part of such treatment.

A group of patients with drug resistant end-stage acute leukaemia has been treated with high dose chemotherapy, TBI and allogeneic or syngeneic bone marrow transplantation (BMT) and 10% - 15% of such patients survived more than five years and are probably cured (Thomas, 1982). These results suggest that supralethal chemoradiotherapy can eradicate leukaemia. Indeed, allogeneic BMT in first remission for young patients with a histocompatible donor has been shown to produce a 70% long-term survival rate with possible cure and is becoming an accepted form of treatment (Thomas, 1982). There has been concern, however, in subjecting patients who may have been cured by chemotherapy to allogeneic BMT and potential complications such as Graft-versus-Host Disease (GvHD) and

prolonged immunosuppression. GvHD, in particular, tends to
be more severe in older patients. The alternative is to
perform allogeneic BMT in second remission but the incidence
of leukaemic relapse is much higher then (Blume et al,
1981). Nonetheless, most ANLL patients do not have
histocompatible donors or are too old for allogeneic BMT to
have much chance of success. BMT performed with haplotype
mismatched donors are still being evaluated but many
problems exist (Powles et al, 1983).

These limitations have led to interest in using
autologous stem cells harvested and cryopreserved during
stable remission for rescue after supralethal
chemoradiotherapy (Dicke et al, 1979). The viability of
cryopreserved pluripotent stem cells were confirmed by
haemopoietic reconstitution on re-infusion and the number of
CFU-GM appeared to be a reliable indicator of the
pluripotent stem cell (Spitzer et al, 1980). Since
autologous cells are used, this procedure can be offered to
all patients who achieve remission without the risk of GvHD
and prolonged immunosuppression. The main drawback is
leukaemic recurrence. Recurrence may occur because of
failure to eradicate all leukaemic cells in the patient or
the leukaemic contamination in the stored cells, or both.
This has led to studies of autologous BMT performed during
first remission rather than at first relapse as the
supralethal chemoradiotherapy should be more effective
against a smaller number of leukaemic cells in the patient
(Dicke, 1983). There are, however, patients who relapse
before bone marrow cells can be stored. Furthermore, the
question of leukaemic contamination in the stored cells

remains.

There is no satisfactory method at present to detect low levels of leukaemic contamination in the stored cells. The leukaemic cell load during remission has been estimated to be 10^9 or less while the total number of nucleated cells in the marrow is around 10^{12}, so that leukaemic cells occur at a frequency of 1:1000 or less. Such a low frequency is well beyond the capacity of ordinary morphological examinations to detect leukaemic cells. Cytogenetic study of the stored cells is only useful when the leukaemic population has an abnormal karyotype and does not provide quantitative information. No specific markers for ANLL have been identified so the use of leukaemia-specific antibodies to eliminate contaminating leukaemic blasts, analogous to the approach used in Acute Lymphoblastic Leukaemia (Netzel et al, 1978; Ritz et al, 1982), is not yet feasible in ANLL. Physical method using density gradient separation (Dicke et al, 1978) and pharmacological method using 4-Hydroperoxycyclophosphamide (Sharkis et al, 1980) to eradicate contaminating leukaemic blasts are still being evaluated but seems to be less promising as they are not leukaemia-specific.

The long-term outlook for ANLL patients is therefore still unsatisfactory. Despite the >70% complete remission rate with current induction programmes, most of the patients will suffer leukaemic relapse and die in the following one to three years whether they receive maintenance therapy or not. Allogeneic BMT performed in first remission can be curative in the small number of patients who are young and have histocompatible donors, but a significant percentage of

these patients will suffer from GvHD. Autologous BMT avoids GvHD and is applicable to more patients but the problem with leukaemic relapse due to leukaemic contamination in the stored cells is far from being solved. New treatment strategies are needed that can be applied to the majority of ANLL patients who are ineligible for allogeneic BMT.

The preliminary report of high levels of PB CFU-GM found in several ANLL patients during very early remission (Juttner et al, 1982a) raises the possibility of harvesting peripheral blood cells for later autologous stem cell rescue in association with supralethal chemoradiotherapy. If such an approach proves to be feasible, all patients going into remission (which includes 70% or more of those receiving standard chemotherapy) can be treated. Several questions, however, need to be considered.

There have been doubts whether circulating haemopoietic stem cells are as effective as bone marrow derived stem cells for haemopoietic reconstitution. Micklem et al (1970) compared the use of blood and bone marrow cells for haemopoietic reconstitution in serial passage studies in lethally irradiated mice. Fewer and smaller splenic colonies were found in mice receiving blood cells, although the colonies were morphologically similar to those derived from bone marrow cells. It was therefore suggested that murine blood-derived CFU-s have a lower self-renewal capacity. Chertkov et al (1982) also found that the self-maintaining capacity of circulating CFU-s is less than that of bone marrow CFU-s. Gidali et al (1974) showed that 30% of circulating CFU-s were in proliferative cycle compared to 20% for bone marrow-derived CFU-s and the

radiosensitivity of circulating CFU-s was lower, suggesting

that the two populations were different. Studies using

rabbit anti-mouse brain serum showed that the percentage of

murine blood-derived CFU-s not reacting to the anti-serum

may be as high as 56% compared to 14% of bone marrow-derived

CFU-s. Assuming that differentiation in the CFU-s

compartment is associated with the loss of the antigen

recognised by the anti-serum and assuming such

differentiation is associated with a decrease in

self-renewal capacity, it was argued that blood-derived

CFU-s may be less satisfactory than bone marrow-derived

CFU-s for haemopoietic reconstitution (Monette and Stockel,

1980). However, Rencricca et al (1970) used a different

experimental system and found the same determinant on the

oligopotent progenitor cells as well. Until the actual

function and significance of the antigen recognised by the

anti-mouse brain antibody are better understood, such

extrapolations remain speculative.

Studies in other species are limited by the lack of

a CFU-s assay while the CFU-GEMM assay is still in the

developmental stage. Thus the CFU-GM is often employed as

an indirect measure of the pluripotent stem cell. Studies

using beagle dogs have shown that peripheral blood cells

have haemopoietic reconstitutive capacity similar to bone

marrow cells when given in adequate numbers to lethally

irradiated dogs as measured by the number of CFU-GM

(Nothdruft et al, 1977). Gerhartz and Fliedner (1980) also

showed that CFU-GM in the blood of dogs which were

leukaphereses and given dextran sulphate were larger than

those before, more closely resembling those of CFU-GM in

bone marrow. If this finding is extrapolated to the pluripotent stem cells, the haemopoietic reconstitutive capacity of circulating stem cells collected during periods of increased haemopoietic proliferation would be comparable to that of bone marrow stem cells. This is supported by findings reported by Abrams et al (1981) who found that peripheral blood cells collected from dogs during the recovery phase after cyclophosphamide treatment contained an increased number of stem cells and this increase could be measured by the increase in CFU-GM. In baboons, a primate species, it has also been shown by cross-perfusion experiments that circulating stem cells have haemopoietic reconstitutive capacity (Storb et al, 1976). In man, autologous haemopoietic reconstitution in patients with Chronic Myeloid Leukaemia using peripheral blood cells had been successful (Goldman and Lu, 1982) but this finding cannot be extrapolated to normal haemopoietic stem cells becasue the reconstitution was by an abnormal clone of cells. Two attempted haemopoietic reconstitutions using normal blood cells both failed, most probably because of the low number of stem cells infused as measured by the number of CFU-GM (Hersko et al, 1979; Abrams et al, 1980). So the question whether circulating and bone marrow haemopoietic stem cells are functionally equivalent with regard to haemopoietic reconstitution is still unanswered (McCarthy and Goldman, 1984). While studies in mice suggest strongly that circulating stem cells are inferior, the studies in dogs and baboons suggest that the two types of cells are similar and no valid data are available in man.

In man, repeated leukaphereses to increase the yield

of stem cells have been attempted but was limited by the development of thrombocytopenia (Korbling et al, 1980). The high levels observed during the recovery phase after chemotherapy may facilitate the collection of sufficient stem cells (Richman et al, 1976) but no such collections have been reported except for a preliminary report of leukaphereses during the very early remission phase in an ANLL patient (Juttner et al, 1982b). The feasibility and safety of harvesting circulating stem cells are still to be studied.

The second question is whether cells at very early remission has a lower leukaemic contamination and are therefore more suitable for autologous stem cell rescue than bone marrow cells collected later during stable remission. It is generally believed that the leukaemic process causes bone marrow failure not just by physical infiltration and replacement but also by direct inhibition of the normal haemopoietic cells through substances like the leukaemia-associated inhibitory activity (Broxmeyer et al, 1978b). Remission occurs when the number of leukaemic cells is reduced by induction chemotherapy, allowing normal cells to proliferate and replete the bone marrow. Since leukaemic cells are more sensitive to the cytotoxic injury, less efficient in repairing sublethal injuries and proliferate more slowly (Arlin et al, 1978), normal cells most probably predominate during this period of intense haemopoietic regeneration. If the number of contaminating leukaemic cells in the stem cell harvest is low, haemopoietic reconstitution using these autologous stem cells at relapse may lead to much longer second remissions than are seen with

conventional autologous bone marrow transplantation using stem cells harvested later, in stable remission.

Thus the use of peripheral blood cells collected during very early remission of ANLL may overcome some of the limitations of autologous and allogeneic BMT and improves the outlook of the majority of ANLL patients but this approach has yet to be investigated in a systematic study.

CHAPTER 1   INTRODUCTION

PART 5 :   THE CRYOPRESERVATION OF HAEMOPOIETIC STEM CELLS

Freezing is lethal to most though not all living systems in nature; yet it can also preserve cells and their constituents. Cells are damaged during freezing because of intracellular ice crystal formation and cellular dehydration when water is removed as ice. Ice formation causes disruption of membranes and organelles while dehydration leads to solute concentration, precipitation and the denaturing of protein. Freezing injury, however, can be minimised by controlling the rate of freezing as well as the use of cryoprotectants (Mazur, 1970). Successful application of this knowledge has enabled the cryopreservation of organelles, viruses, bacteria, formed blood components, haemopoietic stem cells, sperms, embryos and organs like the parathyroid gland.

The optimal rate of cooling differs in different biological systems and appears to be the result of the interaction between the two events of intracellular freezing and dehydration in the particular type of cells involved, and modified by the cryoprotectant used. The optimal cooling rate is one that is slow enough to prevent

production of intracellular ice and yet is rapid enough to minimize the length of time cells are exposed to the dehydration effect.

There are two classes of cryoprotectant and they seem to have different mechanisms of action. Low molecular weight hydrophilic solutes like glycerol (Polge et al, 1949) and dimethylsulfoxide (DMSO) (Lovelock and Bishop, 1959) protect on a molar basis and probably act by permeating into cells to reduce the electrolyte concentration in the residual unfrozen solution in and around a cell at any given temperature, thereby protecting against the dehydration effect. Glycerol is one of the earliest cryoprotectants used and is still the agent of choice for cryopreservation of red cells. DMSO, however, is the one most commonly used for cryopreservation of haemopoietic stem cells. Macromolecules like polyvinylpyrrolidone and dextran have also been used but their mechanisms of action are still unclear. They are generally less effective than the low molecular weight cryoprotectants and are little used.

Cell survival is also affected by the rate of thawing. Slow thawing permits more gradual equilibration and reconstitution but enhances 'grain growth' due to fusion of small crystals leading to disruption of membranes. As a result cells that are frozen quickly are more susceptible to slow thawing because they are less dehydrated with a higher intracellular water content. Thus it is important to determine the optimal cooling and thawing rate for each type of target cells (Mazur, 1970).

For human haemopoietic stem cells, viability can be measured by the in vitro recovery of the CFU-GM and the in

vivo haemopoietic reconstitutive capacity on re-infusion. Highest viability is achieved by suspending cells in 10% DMSO and 15-20% autologous plasma, a cooling rate of 1 to 3°C per minute, storage in liquid nitrogen at -196°C, and a rapid thawing rate of 100°C per minute (Ma et al, 1981). To achieve a constant freezing rate, it is important that the latent heat released at the eutectic point is quickly dissipated. A pre-programmed controlled rate freezer that will automatically deliver an increased amount of liquid nitrogen into the cooling chamber when the temperature is between -18° and -25°C to neutralise the latent heat released is recommended. DMSO is a strong organic acid and releases heat while dissolving in water so it has to be added to the cells slowly at 4°C to avoid damage to cells. When the cells are thawed, the DMSO is either removed by immediate washing or diluted by infusing into the patient. Washing of thawed cells to remove DMSO has been shown to cause an up to 20% loss of CFU-GM in cryopreserved bone marrow cells (Ma et al, 1981) and the infusion of small amount of DMSO does not cause harmful effects so it seems reasonable to infuse thawed cells directly into the patient without washing.

Several workers have shown that it is important to use feeder layers but not other conditioned media as CSA when cryopreserved bone marrow cells are cultured otherwise falsely low CFU-GM viability may result (Ellis et al, 1981; Schlunk et al, 1981; Gilmore, 1983) but no similar study on cryopreserved PB MNC has been reported.

Experience with autologous BMT shows that cryopreserved haemopoietic stem cells retain their

haemopoietic reconstitutive capacity for more than 3 years
after storage (Juttner, unpublished data). However, there
are unresolved problems such as the slow platelet recovery
often observed after autologous bone marrow transplantation
and the clumping of bone marrow cells on thawing which
renders further in vitro processing difficult (Juttner,
unpublished data). A 40% loss of peripheral blood derived
pluripotent stem cells after cryopreservation has been
reported (Lasky et al, 1982). Whether using peripheral
blood mononuclear cells as the source of stem cells may
provide solutions to some of these problems remains to be
seen.

CHAPTER 1   INTRODUCTION

PART 6 :   THE AIMS OF THIS STUDY

1. To study the effect of monocytes in the in vitro PB
   CFU-GM assay in order to establish an accurate assay for
   the PB CFU-GM.

2. To establish the normal range for PB CFU-GM.

3. To study the alterations in the level of PB CFU-GM under
   various physiological conditions.

4. To study the alterations in the level of PB CFU-GM in
   ANLL patients.

5. To study the harvest and cryopreservation of haemopoietic
   stem cells from peripheral blood.

   5.1. To study the optimal timing for harvesting
        circulating stem cells in ANLL patients entering
        remission.

   5.2 To study the use of continuous flow leukapheresis
        for harvesting circulating stem cells in normal
        subjects and ANLL patients entering remission.

   5.3. To study the cryopreservation of circulating stem
        cells.

6. To study the use of cryopreserved peripheral blood
   mononuclear cells for haemopoietic reconstitution after
   supralethal chemoradiotherapy in ANLL patients at
   relapse.

CHAPTER TWO   MATERIALS AND METHODS

Part 1 : Nutrient and Supporting Media

Part 2 : Colony Stimulating Activity

Part 3 : Preparation of Cells for Culture

Part 4 : CFU-GM Assay

Part 5 : ANLL Patients

Part 6 : Exercise Test

Part 7 : Continuous Flow Leukapheresis

Part 8 : Controlled Rate Freezing

Part 9 : The Harvest and Cryopreservation of Bone Marrow
          Cells

Part 10 : Statistical Methods

PART 1 : NUTRIENT AND SUPPORTING MEDIA

Nutrient Media

Alpha-modified Eagle's Media (aMEM)

A concentrated aMEM stock solution was first prepared without glutamine or sodium bicarbonate because they are unstable on long-term storage. Aliquots of the stock solution were used to prepare the working solution.

The aMEM stock solution was prepared by adding the content of a 10 litre pack of aMEM powder (Flow Laboratories, Rockville, Md, USA) to 1490 ml of deionised water (Commonwealth Serum Laboratories, Vic, Australia). 10 ml of 0.5% phenol red (Commonwealth Serum Laboratories, Vic, Australia) was added as an indicator. 100 ml of essential vitamins (Flow Laboratories, Rockville, Md, USA) was added as vitamins supplement. The suspension was stirred at room temperature until the aMEM powder was all dissolved. Additional deionised water was then added to bring the osmolality of the stock solution into the range of 1270-1290 mOsm/l. The stock solution was sterilised by filtering through a 0.22 m filter. Aliquots of 70 ml were dispensed into 100 ml bottles and stored at -18°C. The stock solution remained stable in storage for at least six months.

The aMEM working solution was a double-strength preparation with 30% foetal calf serum (Flow Laboratories, Rockville, Md, USA), using the CO_2-bicarbonate system as the buffer. Glutamine was added as a nutritional

supplement.   Penicillin and streptomycin were included to retard bacterial growth.

The recipe for the working solution :

aMEM stock solution	61 ml
penicillin (200,000 units/ml)	0.15 ml
streptomycin (500 mg/ml)	0.25 ml
glutamine (29.22 mg/ml)	5 ml
deionised water	77 ml
0.9% sodium chloride solution	32 ml
foetal calf serum	75 ml
sodium bicarbonate powder	1.34 gm

Total :	250 ml

The pH of the working solution was adjusted to between 7.15 and 7.2 if necessary by adding either 10M hydrochloric acid (if pH too alkaline) or 10M sodium hydroxide (if pH too acid).   It was then sterilised by filtering through a 0.22 µm filter.   The osmolality was measured using an osmometer (Model 3DII, Advanced Instruments Inc, Needham Heights, Mass, USA) and the level of bicarbonate was measured using a SMAC II multichannel analyser (Technicon Inst, Tarrytown, NY, USA).   The osmolality should be in the range of 570-600 mOsm/l.   The level of bicarbonate should be in the range of 60 to 66 mMol/l.   Those two criteria were the most reliable quality check because the osmolality reflected whether the correct amount of the stock solution was added and the correct bicarbonate level is crucial for the maintenance of a physiological pH in culture.   Because the pH of the working

solution as well as the concentration of bicarbonate tended
to change after a period of storage, only 250 ml of working
solution was prepared at a time.

Foetal Calf Serum (FCS)

Foetal Calf Serum (Flow Laboratories, Rockville, Md,
USA) is one of the most critical component in this and other
cell cloning systems. However, different batches of FCS may
have different growth supporting activity so batch-testing
is essential. Batch-testing involves setting up parallel
assays using several batches of FCS with each batch of FCS
being tested at different concentrations. Bone marrow cells
were used as the target cell and parallel cultures were set
up using 2%, 5%, 10% and 15% of each of batch of FCS. The
batch which supported the highest CFU-GM growth was selected
for use in the CFU-GM assay. FCS selected by this process
was also used for the preparation of Human Placental
Conditioned Medium.

Media for Washing of Cells

Dulbecco Phosphate Buffered Solution (DPBS,
Commonwealth Serum Laboratories, Vic, Australia) is an
inexpensive and simple solution for washing of cells. Its
pH is stable in the physiological range because of the
phosphate present. It does not contain glucose or any other
nutrient so it is suitable for short term cell culture work
only.

Hanks Balanced Salt Solution (HBSS, Commonwealth
Serum Laboratories, Vic, Australia) is another simple
solution for washing of cells. It contains glucose as a

source of nutrient but its pH is less stable because it depends on the CO_2/bicarbonate system for buffering. It is mainly used as a constituent of the freezing mixture added to cells to be cryopreserved.

RPMI 1640 (Commonwealth Serum Laboratories, Vic, Australia) is an inexpensive nutrient medium so is often used in procedures requiring short-term incubation such as adherence procedure (see below). It also depends on the CO_2/bicarbonate system for buffering.

Supporting media

0.3% agar for CFU-GM assay

Agar powder (Bacto-agar, Difco Laboratories, Detroit, Mich, USA) was added to deionised water, in the ratio of 0.66 gm agar to 100 ml of water. Usually 500 ml is prepared each time. The suspension was then heated to boil. Once the agar was completely dissolved, it was dispensed in 80 ml aliquots into 100 ml bottles. Lids were lightly applied and the agar was sterilised by autoclaving at 140°C for 5 minutes. Lids were tightened after autoclaving to maintain sterility. The 0.66% agar resumed a gel state at room temperature. When needed, the agar was melt* by standing the bottle in a 100°C water bath. When the agar had melt*, the temperature of the water bath was adjusted to between 50 to 60°C, so that the agar was kept in the liquid state. For each plate, 0.5 ml of 0.66% agar was mixed with 0.5 ml of cells suspended in aMEM, with 0.1 ml of HPCM added. The final concentration of agar therefore became 0.3% in a volume of 1.1 ml.

0.5% agar for feeder layers

The same method of preparation was followed but the agar:water ratio was 1:100 instead. When mixed with an equal volume of media (see below), the final concentration became 0.5%.

PART 2 : COLONY STIMULATING ACTIVITY

Human Placental Conditioned Medium (HPCM)

HPCM was prepared according to the method described by Schleyer and Schlunk (1980). Placentae obtained by Caesarian Section were preferred because the placentae were not contaminated. Only healthy placentae were used. When there were suggestions of placental insufficiency, e.g., history of moderate or severe toxaemia in pregnancy, small placentae or presence of multiple infarcts and calcification, the placentae were discarded.

Placentae were obtained by arrangement with the Obstetric Unit of the Modbury Hospital. A suitable placenta was placed in a sterile bucket containing approximately 400 ml of RPMI 1640 cell culture medium and sent directly to the IMVS. Preparation of HPCM was commenced on receiving the placenta, usually within five hours of delivery.

The placenta was first separated from its membrane. Cubes of placental tissue 1 cm^3 in size were cut out, avoiding large vessels and connective tissue septae. These cubes were washed in a large beaker containing DPBS and cut into smaller pieces. Blood was washed clear by rinsing with DPBS. Six to ten small pieces were added to a 50 ml tissue

culture flask containing 15 ml of RPMI with 5% FCS. The flasks were incubated at 37°C in an incubator which was continuously flushed through with 5% CO_2 in air, in a horizontal position with the caps only lightly applied to allow for gas equilibration between the inside and outside of the tissue culture flask.

After seven days, tissue debris were removed by straining through a mesh gauze and the supernatant was centrifuged at 14,000g for 30 minutes to remove the remaining fine sediments and sterilised by filtering through successive 0.65, 0.44 and 0.22 μm filters and stored at -18°C. Aliquots were thawed when required.

The level of CSA of HPCM prepared by this method was compared to that of feeder layers (see below) and another HPCM with known CSA. Dose titration study were done comparing the growth-stimulating effect of 0.05 ml, 0.1 ml, 0.2 ml of HPCM. 0.1 ml HPCM added to each 1 ml culture was usually found to be optimal. The failure of higher concentration of HPCM to stimulate growth was thought to be due to the presence of inhibitors to CFU-GM growth in HPCM.

Feeder Layers of Whole Blood Leucocytes

Feeder layers of whole blood leucocytes from normal subjects were used as a standard source of CSA both for the culture of fresh blood cells as well as cryopreserved cells. To prepare 80 feeder layers, 20 ml venous blood was mixed with an equal volume of DPBS and 4.4 ml of dextran 150 in 0.9% sodium chloride (Fisons, Loughborough, England). The cells were allowed to settle at unit gravity at 37°C for 30-45 minutes. When the agglutinated red cells had settled,

the supernatant containing the leucocytes was taken off. The cells were washed in DPBS twice and re-suspended in a volume of 3-4 ml. A cell count was obtained by manual counting using a haemocytometer. The appropriate volume of aMEM working solution and 1% agar were added to the cell suspension so that the cells were present at 1×10^6/ml in 0.5% agar. One ml aliquots were put into 35 mm plastic petri-dishes (Kayline, South Australia, Australia). The feeder layers were incubated at 37°C in a fully humidified atmosphere containing 7.5% O_2, 7.5% CO_2 and 85% N_2.

Feeder layers were used after 4 to 7 days. The plates were removed from the container and the lids of the petri-dishes were replaced with new, labelled lids. The target cells were layered onto the feeder layer and incubated as described in Part 4. It is important to change the lids to reduce the incidence of contamination of the cultures. Water tended to condense on the inside of the old lid when the plates were brought out from 37°C to room temperature; when it dripped back onto the culture, it might introduce fungal contamination. Changing the lids prevented this from happening. The level of CSA in different batches of feeder layers may vary according to the subjects donating the cells, and may also vary at different times in the same subject. It is therefore important that feeder layers from at least two subjects are used in parallel.

PART 3 : PREPARATION OF CELLS FOR CULTURE

Peripheral Blood Mononuclear Cells (PB MNC)

Blood collected by venepuncture was diluted with an

equal volume of DPBS and was then layered onto Ficoll-Paque (S.G. 1.077, Pharmacia, Uppsala, Sweden) and centrifuged at 400g for 30 minutes. The mononuclear cells at the interface were collected and washed three times with RPMI 1640. The cells were resuspended at a concentration of between 15 to 30×10^6/ml. An exact cell count was obtained by manual counting using a haemocytometer and the cells were ready for plating.

When CFU-GM assays were performed on cryopreserved PB MNC, direct plating without washing step was used because washing has been shown to cause an up to 20% loss of CFU-GM (Ma et al, 1981). Furthermore, non-viable cells were often removed by washing steps and falsely high post thaw viability may result from calculating the CFU-GM recovery based only on cells which are viable. Preliminary experiments showed that the viability of CFU-GM is not affected if direct plating is performed within 20 minutes of thawing.

However, when the cryopreserved cells were fractionated by adherence (see below), the cells were diluted 1:10 in RPMI 1640 with 15% FCS as soon as thawing was complete to reduce the concentration of DMSO to 1% which was non-toxic to CFU-GM (Ma et al, 1982). The cells were then washed once with RPMI 1640 before the adherence procedure.

Adherence Procedure

The mononuclear cells were fractionated by adherence to plastic (To et al, 1983a). Cells were added to RPMI 1640 supplemented with 15% FCS, in 90mm plastic petri-dishes

(Disposable Products, Australia) to give a cell concentration of 1.4-1.7 x 10^6 cells/ml in a volume of 10 ml and incubated at 37°C in 5% CO_2. After one hour the supernatant containing the non-adherent cells was transferred to another petri-dish and the adherence procedure repeated. The non-adherent cells were then recovered by centrifugation of the supernatant.

Fresh medium was added to the cells which adhered to the bottom of the plastic dishes and incubation was continued for one more hour. The supernatant was discarded and the adherent cells were recovered by scraping.

The percentages of granulocytes, lymphocytes and monocytes were determined on cyto-centrifuge preparations stained by (1) the Jenner-Giemsa method and (2) the combined specific and non-specific esterase method. At least 400 cells were counted in each of these preparations.

PART 4 : PB CFU-GM ASSAY

Assays performed on Fresh PB MNC

Volumes of cells were mixed with the appropriate volumes of aMEM with 15% FCS (v/v) in 0.3% agar so that cells were plated at 0.625, 1.25, 2.5, 5 and 10 x 10^5 per ml, 1 ml per plate (35mm petri-dishes, Kayline, South Australia). It is important to use such a range of plating numbers so that the maximal level of PB CFU-GM can be detected (To et al, 1982, 1983a). 0.1 ml of HPCM was added to each plate as the source of CSA. Quadruplicate plates were set up for each cell concentration. Two plates with no CSA added were also set up as control at each

concentration.

Assays performed on Cryopreserved PB MNC

Cryopreserved cells were assayed for CFU-GM by direct plating after thawing without washing steps (To et al, 1984). An ampoule of cryopreserved cells was removed from liquid nitrogen and thawed in a 37°C water-bath. The ampoule was then kept at 4°C and aliquots of the cells were taken for CFU-GM assay which was set up as soon as thawing was complete. Cells were plated at 1.25, 2.5, 5 and 10×10^5 cells per plate based on the cell counts prior to cryopreservation, with four plates at each point. The actual number of cells in the thawed sample was subsequently determined with a Coulter S Plus Counter and a correction applied to the plating number if necessary. Since the number of cells plated was calculated according to the pre-freeze cell count and the post-thaw count include both viable and non-viable cells, only minor corrections were usually required.

Incubation and Scoring

The cultures were incubated at 37°C in a closed humidified environment with an atmosphere of 7.5% CO_2, 7.5% O_2 and 85% N_2. Aggregates of more than 40 cells at day 14 were scored as colonies.

Expression of Results

A. CFU-GM Assays on Fresh Blood Cells

The culture results were expressed as the number of CFU-GM/10^6 lymphocytes (Corrected Colony Count, CC)

calculated as follows :

CC = (N X 10 / P) X (100 / LC)

CC = number of CFU-GM per 10^6 lymphocytes.
(Corrected Colony Count)

N = number of colonies per plate

P = number of cells per plate x 10^5

LC = percentage of lymphocytes in cell fraction

In fractionation and mixing experiments CC was used because the CFU-GM:lymphocyte ratio should be much less affected by the adherence procedure than the CFU-GM:total cells ratio (To et al, 1983a). To enable valid comparisons of actual levels of PB CFU-GM in normal subjects and in patients, as well as in the same subject at different times, the number of CFU-GM per ml of blood, derived from the CC, was used.

CM = CC X WC X LD/100

CM = number of CFU-GM per ml of blood

CC = number of CFU-GM per 10^6 lymphocytes

WC = white cell count in 10^6 per ml of blood

LD = percentage of lymphocytes as determined by a differential count

B. CFU-GM Assays on Thawed PB MNC

Since the thawed cells were cultured without any washing steps so that no cells were lost, the number of CFU-GM/10^6 total nucleated cells plated should be an indicator of CFU-GM viability after cryopreservation (To et al, 1984) :

% CFU-GM viability

$$= \frac{\text{number of CFU-GM}/10^6 \text{ thawed cells}}{\text{number of CFU-GM}/10^6 \text{ non-cryopreserved cells}} \times 100\%$$

PART 5 : ANLL PATIENTS

Diagnostic Criteria and Treatment Protocol

The diagnosis of ANLL was made according to the FAB classification (Bennett et al, 1976). Induction chemotherapy was a modification of the "TAD" regime reported by Gale and Cline (1977) and has been described (To et al, 1983b). It consisted of cytosine arabinoside 100 mg/sq m every twelve hours as a 30 minute infusion for seven days, thioguanine 100 mg/sq m every twelve hours orally for seven days and daunorubicin 60 mg/sq m intravenously daily on days 1, 2 and 3 of the seven day cycle. Patients were nursed in a protected environment with prophylactic non-absorbable antibiotics and received a low bacteria diet. Systemic antibiotics were given for documented or suspected infections and therapeutic granulocyte transfusions given for infections not responding to the above therapies. Platelets were given for bleeding episodes. Marrow examination was performed on days 14 and 21 from the start of chemotherapy to determine the response to therapy and a second cycle was given if necessary. Consolidation therapy consisted of three or four shorter and less intensive cycles with five days of cytosine arabinoside and thioguanine and one dose of daunorubicin on day one, given at the same dosage as in the induction cycle. No maintenance therapy was used.

PART 6 : EXERCISE TEST

Four volunteers were subjected to the standard

multistage treadmill exercise test using a Programmed
Exercise Control Treadmill (Quinton Instruments, Seattle,
Washington). The exercise programme consisted of 5
successive stages of treadmill running of increasing speed
and angle of incline. Each stage lasted 3 minutes. Each
volunteer ran on the treadmill either until exhausted or
after completing all five stages. The exercise test was
supervised by a cardiologist with continuous monitoring of
pulse rate, blood pressure and electrocardiographic
changes.

        PB CFU-GM and full blood counts were measured before
the exercise test as the baseline. They were again measured
immediately after exercise and at five and twenty four hours
afterwards to measure the changes in PB CFU-GM and blood
counts.

PART 7 : CONTINUOUS FLOW LEUKAPHERESIS

        A modification of the method reported by Korbling et
al (1980) was used (To et al, 1984). A continuous flow
blood cell separator (Celltrifuge, Model J4-6900D, American
Instrument Comp) was employed using a blood flow-rate of 50
ml/min and the centrifuge speed was set at 650 rpm. The
buffy coat was positioned just inside the white cell
collecting port to reduce the number of polymorphs collected
. The buffy coat was collected into a sterile plastic bag
(Tuta, Australia) at 2.5 to 3 ml/min. ACD-Formula B
solution (Intramel, W.A., Australia) was added to the blood
entering the Celltrifuge as an anticoagulant in the ratio of
one part of ACD(B) to 14 parts of blood. The actual

leukapheresis usually took 1 1/2 hours.

PART 8 : CONTROLLED RATE FREEZING

Cells collected by leukapheresis were diluted with an equal volume of DPBS and layered onto ficoll-paque in 50 ml centrifuge tubes (Nunc, Australia) and centrifuged at 400g for 30 minutes. The mononuclear cells at the interface were collected and washed twice with RPMI 1640. The freezing solution consisted of 20% DMSO, 20% autologous plasma and 60% Hank's Balanced Salt Solution. An equal volume of the freezing solution was added dropwise to the cell suspension which was kept at 4°C. The cell concentration in the final mixture ranged from 20 to 50 $\times$ 10^6 cells/ml. The final mixture was dispensed in 4 ml aliquots into screw-top ampoules (Filtrona, Australia). Cooling was then performed in a pre-programmed controlled rate freezer (Paton Industries, South Australia) at 1°C per minute to $-60°$ C and the ampoules were then transferred to the liquid phase of nitrogen for long-term storage. The programming is designed to allow for the release of the latent heat of fusion at $-20°C$ by increasing the in-flow of liquid nitrogen at that point.

PART 9 : THE COLLECTION AND CRYOPRESERVATION OF BONE MARROW
        CELLS

Marrow was obtained by multiple aspirations from the sternum and both posterior iliac spines when the patient was under general anaesthesia (To et al, 1984). One litre of

marrow was collected into a sterile plastic bag (Tuta, Australia) containing heparinised saline as an anti-coagulant. Dextran 150 was added to the bag so that the final concentration of dextran was 10% (v/v). The contents of the bag were allowed to settle at unit gravity for one hour. The red cell layer was removed after this time and cells remaining in the bag were centrifuged at 400g for 30 minutes. The buffy coat cells obtained by centrifugation were cryopreserved as described earlier. CFU-GM was assayed by culturing 1×10^5 nucleated cells in each plate. The results of the collection are expressed as the numbers of nucleated cells and CFU-GM per kilogram body weight.

PART 10 : STATISTICAL METHODS

The statistical formulae and tables used in this thesis were based on those recommended by Snedecor and Cochrans (1980) in "Statistical Method", 7th Edition, the Iowa State University Press, USA.

CHAPTER 3 : THE EFFECTS OF MONOCYTES IN THE PB CFU-GM ASSAY

INTRODUCTION

It has been suggested that monocytes are involved in the negative feedback control of granulopoiesis because they secrete obligatory growth factor(s) for the in vitro growth of CFU-GM (Colony Stimulating Activity, CSA) as well as inhibitory factors such as Prostaglandin E (PGE) (Kurland et al, 1978) and Acidic Iso-ferritins (AIF) (Broxmeyer et al, 1982a). The net colony stimulating activity is therefore a balance between these antagonistic influences. These original studies were performed using the bone marrow CFU-GM assay system but the number of monocytes required to inhibit CFU-GM growth, of the order of 10^5 monocytes per plate, is not normally present when 1 or 2 x 10^5 bone marrow cells are cultured. Thus, monocytes most probably do not exert a major modulatoy influence in the bone marrow assay system. By contrast, such numbers of monocytes are regularly present when 5 or 10 x 10^5 PB MNC are cultured per plate so that significant inhibition may result. It is therefore important to study the CFU-GM/monocyte interactions in the in vitro PB CFU-GM assay in order to measure the levels of PB CFU-GM accurately.

Since the freeze-thaw process has been found to

affect some monocyte functions (Weiner et al, 1981), the CFU-GM/monocyte interaction may be different when cryopreserved cells are cultured so it is important to study freshly separated monocytes and those which have been cryopreserved.

The aim of this Chapter is to investigate the effect of monocytes on the in vitro PB CFU-GM assay by studying CFU-GM growth in peripheral blood mononuclear cells cultured in the presence of different numbers of monocytes. Studies performed on freshly separated peripheral blood mononuclear cells are shown in Part 1 while studies performed on cells that have been cryopreserved are shown in Part 2. Cells from normal subjects and patients are studied.

PART 1 : STUDIES ON FRESHLY SEPARATED PERIPHERAL BLOOD
        MONONUCLEAR CELLS

MATERIALS AND METHOD

Blood was collected by venepuncture from seven healthy volunteers between 8:30am and 9:30 am. Three of the volunteers were studied twice. The mononuclear cells were obtained by density gradient separation using Ficoll-paque. The mononuclear cells were separated by adherence to plastic to a non-adherent fraction and an adherent fraction as described in Chapter 2.

Cells from each of the 3 fractions were plated at 0.625, 1.25, 2.5, 5 and 10×10^5 per plate in a volume of 1 ml. There were usually insufficient adherent cells so that only the lower concentrations could be cultured.

Mixing Experiments

A differential count was first performed on the mononuclear cells to determine the proportions of lymphocytes and the monocytes. Non-adherent cells and adherent cells were then mixed together in the following manner :

Set (1) were prepared as a control using a volume of non-adherent cells so that the number of lymphocytes in each of these plates would correspond to the number of lymphocytes present in 10×10^5 mononuclear cells.

Sets (2) - (5) : These plates contained the same number of non-adherent cells as in set (1) with increasing numbers of adherent cells so that the number of monocytes in each of the (2), (3), (4), (5) plates would correspond to 12.5%, 25%, 50% and 100%, respectively, of the number of monocytes in 10×10^5 mononuclear cells. Set (5) therefore contained the same number of lymphocytes and monocytes as when 10×10^5 mononuclear cells/plate were cultured.

CFU-GM levels were expressed in colonies/10^6 lymphocytes because the CFU-GM : lymphocyte ratio should be much less affected by the adherence procedure than the CFU-GM : total cells ratio.

Large Scale Study on Normal Subjects and Patients

A total of 104 assays were performed on 34 normal subjects. Peripheral blood mononuclear cells were cultured at 0.625, 1.25, 2.5, 5 and 10×10^5 cells/plate. The assay results were analysed according to the plating number used. Similar studies were performed on patients with Acute

Non-lymphoblastic Leukaemia (ANLL), Acute Lymphoblastic Leukaemia, Hodgkin's Disease without bone marrow involvement and drug induced granulocytosis.

RESULTS

CFU-GM Assay Results using Peripheral Blood Mononuclear Cells.

This mononuclear cell fraction consisted of 74 ± 9 % lymphocytes and 21 ± 8 % monocytes (mean ± 1 S D of 10 experiments). The effect of plating number on CFU-GM growth in this fraction is shown in Fig 3.1. The number of CFU-GM per 10^6 lymphocytes plated (Corrected CFU-GM count, CC) was much higher when smaller numbers of cells were plated than when the usual 5 or 10×10^5 cells were plated. A two-way analysis of variance showed significant differences between the CC when different numbers of cells per plate were cultured ($p < 0.001$). In all but four of the sets, the CC was maximal when either 1.25 or 2.5×10^5 mononuclear cells were plated. The CC at these two plating numbers was significantly higher than at 5 or 10×10^5 mononuclear cells/plate (CC at 2.5×10^5 mononuclear cells/plate compared with CC at 5×10^5 mononuclear cells/plate, $p < 0.005$, paired t test). In five of the experiments, there was also a decrease in CC when 0.625×10^5 PB mononuclear cells/plates were cultured. At this plating number, there were less than 0.2×10^5 monocytes per plate. For the three subjects studied twice, the same pattern was observed on both occasions. The control plates with no added HPCM showed only a small number of colonies or none at all.

NO. OF CFU-GM / 10^6 LYMPHOCYTES

MONONUCLEAR CELLS-
PLATING NUMBER EFFECT
(n = 10)

NO. OF MONONUCLEAR CELLS/PLATE

Fig. 3.1 CFU-GM assay results for peripheral blood
mononuclear cells. The results of 10 sets
of experiments are shown. Each line represents
the results of one experiment.

CFU-GM Assay Results using Non-adherent Cells

This non-adherent cell fraction consisted of 95 ± 3 % lymphocytes (mean $\pm$ 1 S D of nine experiments). The effect of plating number on CFU-GM growth in this fraction, as shown in Fig 3.2, was entirely different compared to that in the mononuclear cells fraction. A two-way analysis of variance showed significant differences between the CC when different numbers of non-adherent cells were plated ($p < 0.002$). In all experiments the CC was significantly higher at 5×10^5 or 10×10^5 cell/plate than at the lower cell numbers ('5×10^5' levels compared with '2.5×10^5' levels, $p < 0.005$, paired t test). The removal of monocytes from the original mononuclear cell preparation has resulted in higher levels of CFU-GM growth at high plating numbers. For the two subjects studied twice, the same pattern was observed on both occasions. The control plates showed only a small number of colonies or none at all.

CFU-GM Assay Results using Adherent Cells

This adherent cell fraction consisted of 79 ± 7 % monocytes (mean $\pm$ 1 S D of five experiments). No assays could be performed at the higher plating numbers because of lack of sufficient cells in this fraction. Culture at the lower plating numbers yielded usually very low numbers of colonies per plate. There were less than four colonies in 70% of the plates and no colonies could be detected in 30% of the plates. The culture results were therefore considered unsuitable for analysis with regard to the effect of plating number.
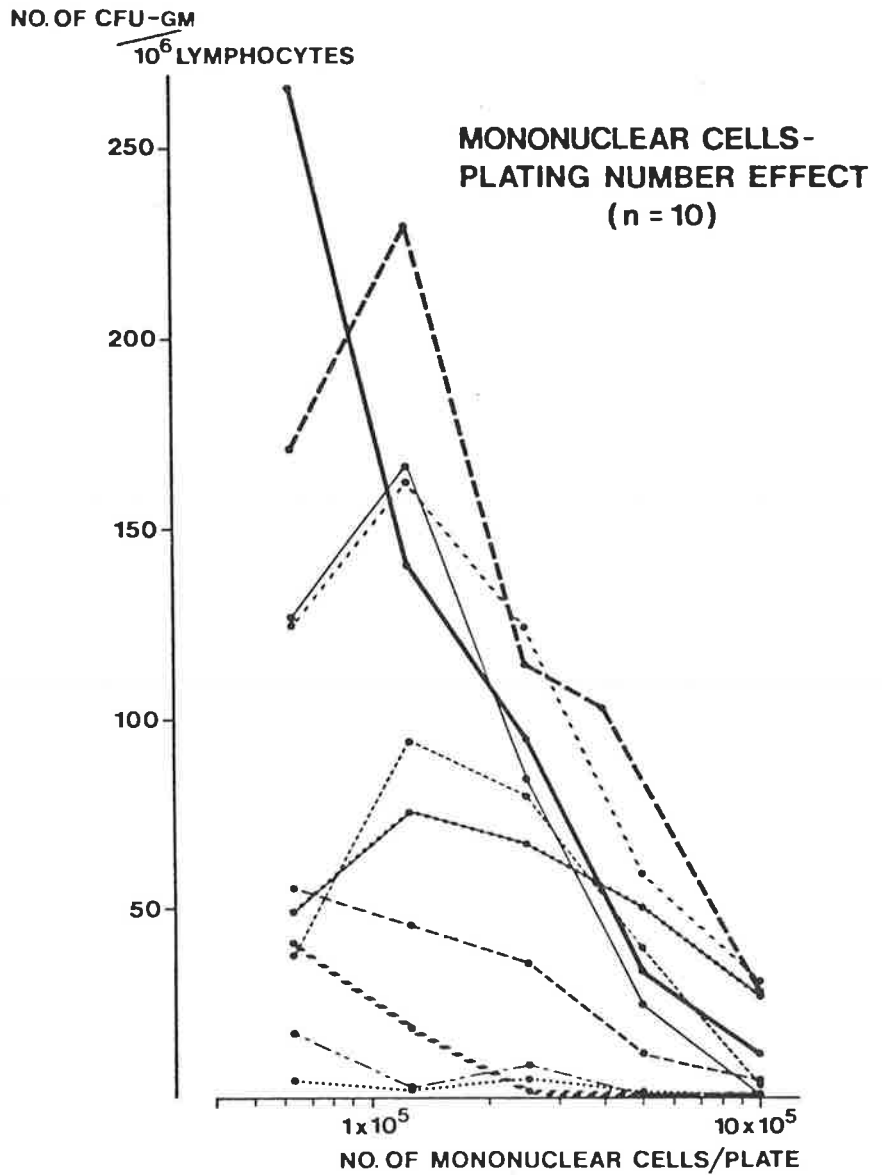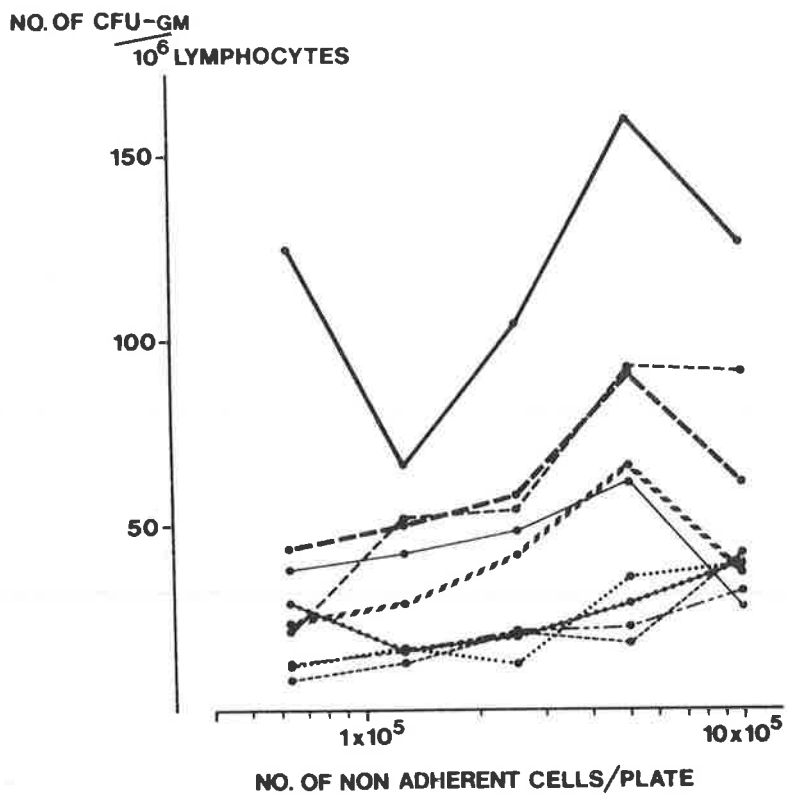
Fig. 3.2   CFU-GM assay results for nonadherent cells.
          The results of 9 sets of experiments are shown.
          Each line represents the results of one experiment.

CFU-GM Assay Results in the Mixing Experiments

The results of five mixing experiments are shown in Fig 3.3. Each line represents the effect of adding an increasing number of adherent cells to a constant number of non-adherent cells. The level of CFU-GM decreased significantly as more adherent cells were added; the CC at set (1) (no adherent cells added) compared with CC at set (5) (highest number of adherent cells added) showed a highly significant difference, $p < 0.0025$, using paired t test. This therefore virtually reproduced the pattern when mononuclear cells were cultured.

Summary of CFU-GM Assay Results in the Mononuclear Fraction, Non-adherent Fraction and Mixing Experiments

The mean CC at each of the five plating numbers of all the experiments was calculated. A mean value of the number of monocytes/plate was also calculated for each of the five plating numbers. The means and standard errors of CC at the five plating numbers were then plotted against the mean number of monocytes/plate at those points. This was done for CFU-GM assay results on the mononuclear cell fraction, non-adherent cell fraction and the mixing experiments. The three graphs are shown in Fig 3.4. From this figure, 0.15 – 0.5 $\times$ 10^5 monocytes per plate appear to be optimal for CFU-GM growth in this in vitro assay system.

Large Scale Study on Normal Subjects and Patients

The mean and 1 $\pm$ SE of 104 assays in 34 normal subjects at each of the five plating number are shown in Fig
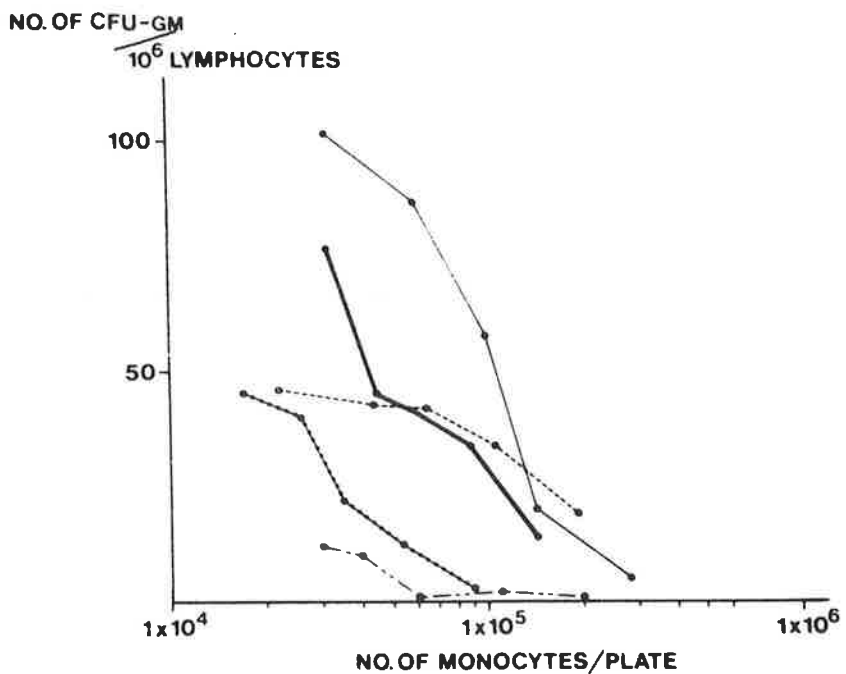
Fig. 3.3 CFU-GM assay results of mixing experiments.
The results of 5 sets of experiments are shown.
Each individual line represents the results of
one experiment. The leftmost point represents
the result when nonadherent cells only were
cultured. The rightmost point represents the
results when the highest number of adherent cells
were added. The results of the intermediate
cell mixing experiments are represented by the
points in between.
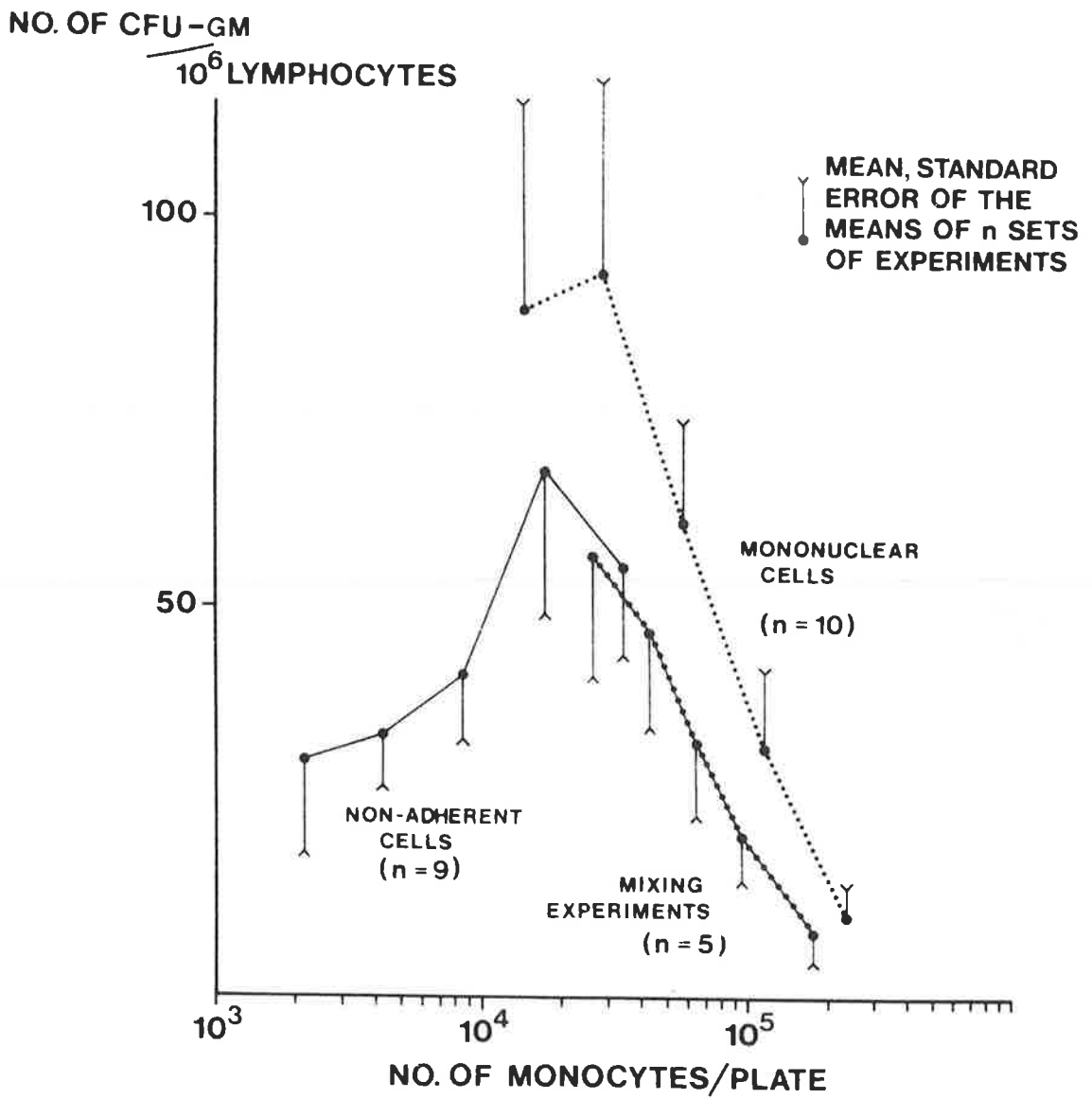
Fig. 3.4 The effect of monocytes in PB CFU-GM assay. A
composite graph incorporating the CFU-GM assay
results of PB MNC, nonadherent cells, and the
mixing experiments.
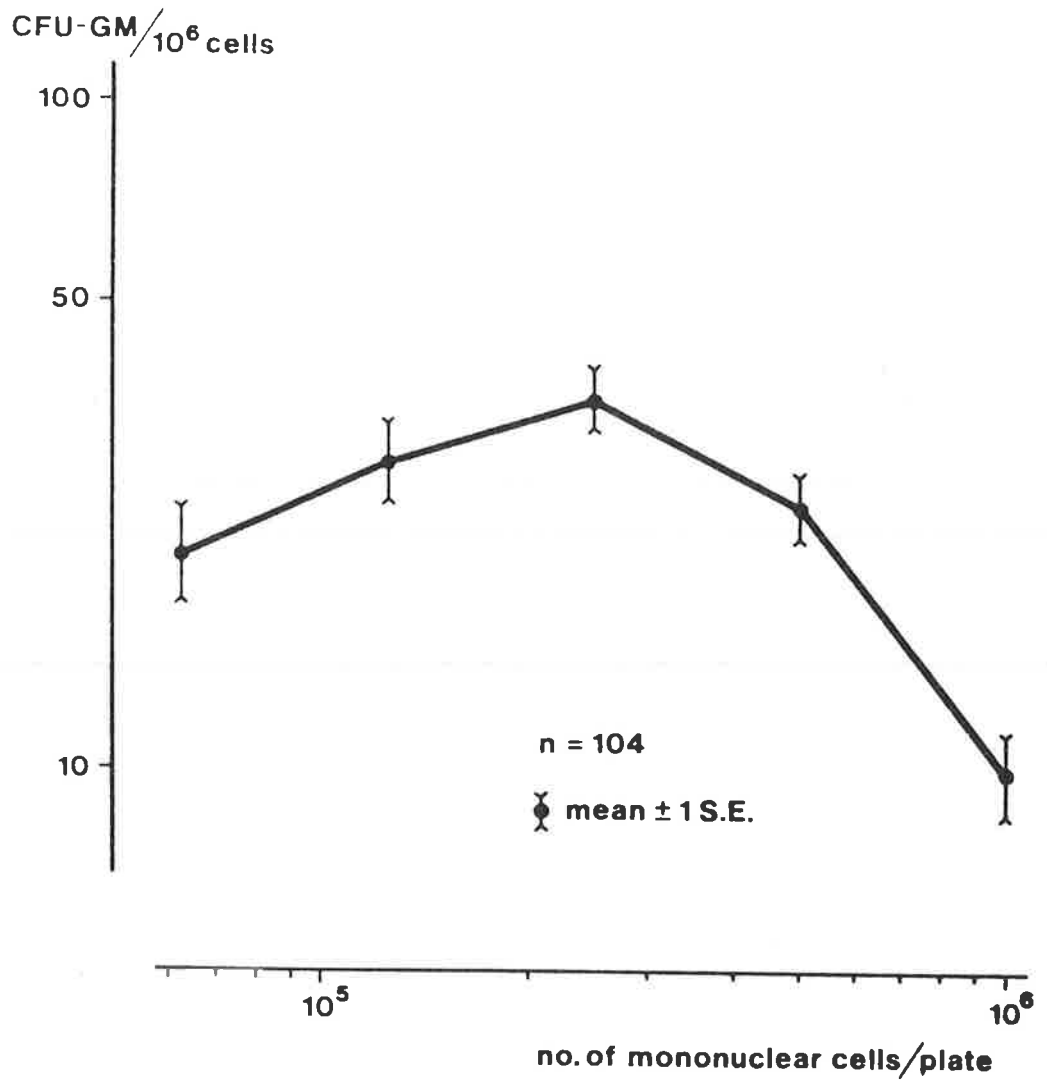
Fig. 3.5 The plating number effect in PB CFU-GM assays using
         PB MNC. The mean ± S.E. of 104 assays at each of the
         five plating numbers are shown.

3.5. The same plating number effect as described earlier with a small number of assays was again observed. While the mean result was highest at 2.5×10^5 mononuclear cells/plate, analysis of the individual results showed that the highest CFU-GM growth occurred at 10×10^5/plate in 6% of the assays, at 5×10^5/plate in 13%, at 2.5×10^5/plate in 39%, at 1.25×10^5/plate in 23% and at 0.625×10^5/plate in 20% of the assays. The number of monocytes present in the culture at which the highest CFU-GM growth occurred was $0.4 (0.18 - 1.0) \times 10^5$ monocytes/plate (Mean $\pm$ 1 SD). The optimal plating number varied between different subjects and even in the same subject studied at different times.

Forty-four assays in 8 ANLL patients during very early remission, consolidation and stable remission, 11 assays in 3 ALL patients, 7 assays in 3 HD patients and 2 assays on 1 patient with drug-induced agranulocytosis were performed. The same pattern of plating number dependent CFU-GM proliferation was found. In 75% of the assays the highest CFU-GM growth occurred when 0.625 to 2.5×10^5 cells/plate were cultured.

DISCUSSION

The significant decrease in CFU-GM growth when large numbers of peripheral blood mononuclear cells were plated has not been reported before. There is one report claiming that the number of colonies grown per plate increased as the number of PB mononuclear cells plated was increased (Tebbi et

al, 1976) which is contrary to our findings. Their assay system, however, was different because the presence of added CSA did not increase CFU-GM growth. There is another report showing an increase in the number of colonies grown per plate as the number of whole blood leucocytes plated was increased (Barrett et al, 1979) but the same report also showed an unusual finding in that the number of colonies grown per 10^6 whole blood leucocytes was similar to the number of colonies grown per 10^6 PB mononuclear cells. The discrepancy between these findings may at least in part be due to the effect of monocytes on the PB CFU-GM assay as discussed below.

The pattern of decreased CFU-GM growth at high plating numbers of PB mononuclear cells suggests the presence of an inhibitory influence related to the number of cells plated. The inhibition appears to be due to the large number of monocytes present because their removal resulted in a marked increase in CC. Furthermore, this pattern of monocyte-dependent inhibition was reproduced by adding the monocyte-rich fraction to the lymphocyte-rich fraction in the mixing experiments. Increasing inhibition of CFU-GM growth was evident when increasing numbers of adherent cells were mixed with a constant number of non-adherent cells. The same pattern of inhibition was observed when higher concentrations of HPCM was used so it is not due to excess utilization of CSA by monocytes. The same culture system has supported the growth of over 400 colonies per plate so the decreased CC at higher plating number is not caused by nutritional deprivation. All the findings appear to suggest that monocytes present in a concentration of 0.5×10^5/plate or

more are inhibitory to CFU-GM growth in the in vitro PB CFU-GM assay system.

There are also indications that too few monocytes/plate may be suboptimal for CFU-GM proliferation. A decrease in CC was observed when the number of monocytes/plate was less than 0.2×10^5 in five out of ten experiments (Fig 3.1). The CC in the non-adherent fraction also decreased when the number of monocytes/plate was less than 0.15×10^5 /plate (Fig 3.2). P4.

There is a distinct advantage in using CC rather than CFU-GM/total cells to express the assay results when results from different fractions of cells were compared. Assay results expressed in CFU-GM/total cells would be uninterpretable as they showed entirely different trends in the different fractions : the correlation between colonies per plate and plating number is negative in the mononuclear cell fraction, positive in the non-adherent cell fraction and zero in the mixing experiments.

The importance of plating number on CFU-GM growth in the PB CFU-GM assay was confirmed by the results of large scale study on normal subjects and patients with various haematological disorders with and without marrow involvement. The optimal number of monocytes based on 104 assays was in a similar range at 0.4×10^5 monocytes/plate, corresponding to 1.25 to 2.5×10^5 mononuclear cells/plate. However, the variations in the optimal plating number at which the highest CFU-GM growth occurred varied between different subjects and even in the same subject studied at different times suggest that the effects of monocytes may vary and performing assays at one or two plating numbers may lead to underestimate of

the actual level of CFU-GM. Experimental evidence is emerging that subsets of monocytes exist (Weiner et al, 1981; Akiyama et al, 1983). If the different subsets have different effects on the in vitro proliferation of CFU-GM then variations in the relative numbers or activations of the subsets may affect the pattern of CFU-GM/monocyte interactions in relation to plating numbers. The processing of cells in vitro prior to culture may also contribute to the variable state of monocyte stimulation/inhibition. Thus, it is important to culture PB MNC at several plating numbers in order to measure PB CFU-GM accurately.

The monocyte-macrophage system has been shown to have important regulatory influences on CFU-GM growth (Kurland et al, 1978a). Monocytes are an important source of CSA which is essential for the proliferation and maintenance of CFU-GM. It has also been claimed that monocytes may be important in the 'processing' of CSA (Schlunk et al, 1981). Until recently it has been thought that monocytes play a definite though small stimulatory role in the CFU-GM assay system (Messner et al, 1973). This conclusion is, however, based on the BM system where the number of monocytes is very small. When the effect of a larger number of monocytes on the marrow system was studied, it was found that monocytes secreted PGE which inhibited CFU-GM proliferation; this PGE secretion was greatly enhanced by the presence of CSA (Kurland et al, 1978a). While such high number of monocytes are not usually present in the BM CFU-GM assay, this regularly occurs in the PB CFU-GM assay. AIF derived from monocytes have also demonstrated to be inhibitory to CFU-GM proliferation. Moreover, AIF production is also dependent on the presence of

a critical number of monocytes (Broxmeyer et al, 1982a). With this dual stimulatory/inhibitory role of monocytes in mind, a model can be constructed to explain the particular growth pattern of CFU-GM (Fig 3.6).

When small numbers of PB mononuclear cells are plated (region I) the stimulatory effect of exogenous CSA predominates (line 1). Optimal CFU-GM growth, however, is not achieved. This could be due to an inadequate cell-cell interaction (Bentley, 1981) or an insufficient number of monocytes for the processing of CSA.

As the number of cells per plate is increased, the number of monocytes plated is also increased and optimal CFU-GM growth is achieved (region II). When the number of cells per plate is increased further resulting in the presence of more than the optimal number of monocytes, there is a sudden decrease in CFU-GM growth (region III); this effect may be due to humoral inhibitors secreted by monocytes like PGE (Kurland et al, 1978a) or cell-cell interaction leading to release of humoral inhibitors and/or contact inhibition at high cell number/plate (Broxmeyer et al, 1982a; Price and McCulloch, 1978). The net colony stimulatory activity in the culture system decreases and the apparent level of CFU-GM (or CC in practical terms) consequently decreases.

Most reported studies on PB CFU-GM are based on assays performed on PB mononuclear cells without allowance for the effect of monocytes judging from the plating numbers used (Table 1.3.1). The present study showed that the measured level of PB CFU-GM can be affected by the number of monocytes per plate so results reported by different workers
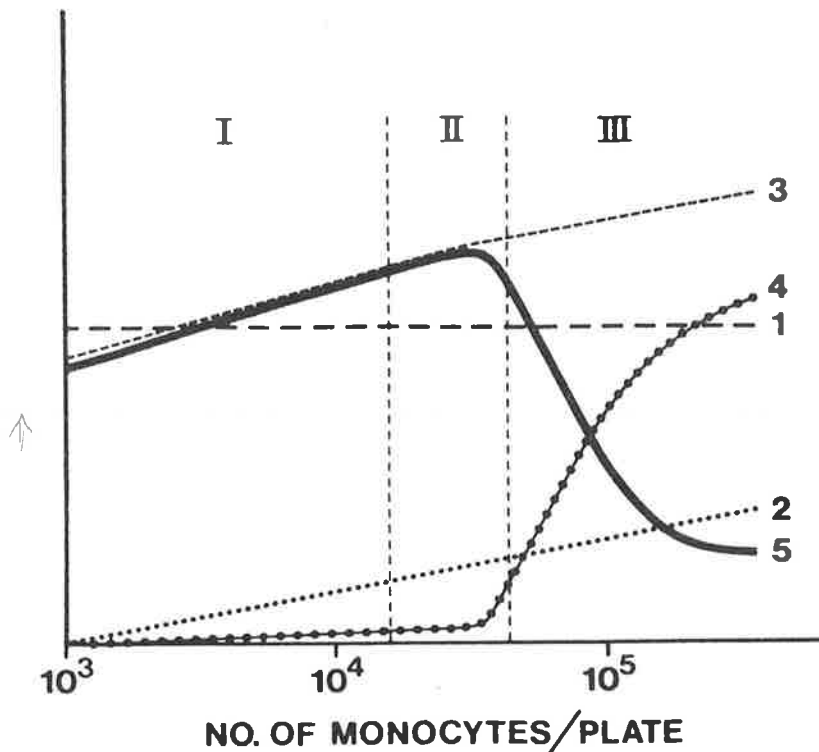
Fig. 3.6 A model depicting the proposed regulatory role of
monocytes in PB CFU-GM assay. Line 1 (— — — —)
represents the exogenous colony-stimulating
factor(s) added to the assay system in the form of
HPCM. Line 2 (·········) represents the monocyte-
derived colony-stimulating factor(s), the activity
of which is proportional to the number of monocytes
present. Line 3 (--------) represents the total
colony-stimulating activity in the assay system.
This is the sum of the exogenous and the endogenous
colony-stimulating factors. Line 4 (—•—•—•—)
represents the inhibitory activity in the assay
system, such as humoral inhibitors secreted by
monocytes or due to cell-cell interaction. Line 5
(———————) represents the net colony-stimulating
activity in the assay system as reflected by the
observed CFU-GM/10^6 lymphocyte level. In effect,
line 5 is the difference between the total colony-
stimulating activity (line 3) and the inhibitory
activity (line 4).

may not be comparable to one another. Furthermore, the high plating numbers used in the majority of these studies might lead to inaccurate results. These will be dealt with in the next Chapter.

This study represents a significant step towards the establishment of a quantitative and reproducible assay of the PB CFU-GM. It is important to culture PB MNC at several plating numbers to measure PB CFU-GM accurately. The present study also provides strong evidence that the monocyte-macrophage system may play an important regulatory role in granulopoiesis. Further advance in this direction may provide more insight into the control of haemopoiesis in vivo.

PART 2 : STUDIES ON CRYOPRESERVED PERIPHERAL BLOOD
              MONONUCLEAR CELLS

MATERIALS AND METHODS

Cryopreserved Cells from Normal Subjects

Peripheral blood cells were harvested from 5 normal subjects by continuous flow leukapheresis and cryopreserved (see Chapter 2 Parts 7, 8 and Chapter 6). The number of

CFU-GM in the cryopreserved cells after various periods of storage (5 to 9 months) was determined by direct plating after thawing without any washing steps (see Chapter 2 Part 4). Cells were plated at 1.25, 2.5, 5 and 10 x 10^5 cells per plate based on the cell counts prior to cryopreservation. Ten plates were set up at each plating number, four with feeder layers as CSA, four with HPCM as CSA and two with no added CSA as controls. Six studies were performed, one study on cells from each of four subjects and two on cells from the fifth subject. Results were expressed as number of CFU-GM per 10^6 cells plated.

Cryopreserved Cells from ANLL Patients

Peripheral blood cells were harvested by 3 or 4 leukaphereses from each of 5 ANLL patients during very early remission and cryopreserved (see Chapter 6). Cells from twelve of these leukapheresis runs were assayed for CFU-GM after various periods of storage (1 week to 5 months). The assay conditions were identical to those described for the studies on normal subjects.

Mixing Experiments

The design of the mixing experiments was similar to that in Part 1 of this Chapter but with three modifications. Firstly, monocytes obtained from cryopreserved PB MNC as well as monocytes from fresh blood were used in order to study whether the freeze-thaw process alters the CFU-GM/monocyte interaction. Secondly, unfractionated

cryopreserved PB MNC were used as the target cells instead of the non-adherent fraction in order to avoid the washing step which may lead to CFU-GM loss (Ma et al, 1981). Thirdly, feeder layers were used as the source of CSA.

In each mixing experiment, the adherent cells and the target cells from the same subject were used. One mixing experiment was performed adding adherent cells obtained from thawed PB MNC (see Chapter 2 Part 3) to unfractionated cryopreserved PB MNC. Two mixing experiments were performed using non-cryopreserved adherent cells obtained from fresh PB MNC as in Part 1 of this Chapter. Each mixing experiment consisted of five sets, the first set was the control with 5×10^5 unfractionated cells per plate while the second to the fifth sets contained the same number of unfractionated cells plus a volume of the adherent cells containing 12.5%, 25%, 50% and 100% respectively of the estimated number of monocytes in the unfractionated PB MNC. Six plates were prepared for each set, four with feeder layers and two with no added CSA as controls. The colony growths were expressed as a percentage of control value.

The mixing experiments were performed using cells from normal subjects. No mixing experiments were performed with cells from ANLL patients because the cells were reserved for possible later autologous stem cell rescue.

RESULTS

The Monocyte Effect in Cryopreserved PB MNC from Normal Subjects and from ANLL Patients

The number of CFU-GM observed when cryopreserved PB MNC were cultured at different plating numbers are shown in Fig 3.7. The results from normal subjects and those from ANLL patients are shown. Since the colony numbers in feeder layer-stimulated cultures and in HPCM-stimulated cultures were different, they are shown separately for both groups of subjects. Maximal growth usually occurred when 5 or 10 $\times$ 10^5 cells were plated, irrespective of the type of CSA used. When cryopreserved cells from normal subjects were assayed, the maximal growth occurred at 5 or 10 $\times$ 10^5 cells/plate in 92% (11/12) of assays. When cells from ANLL patients were assayed, the maximal growth occurred at the same two plating numbers in 79% (19/24) of assays. The difference in colony growth at various plating numbers was statistically significant ($p < 0.005$ for both types of cells and for both types of CSA, two-way analysis of variance) and the colony growth at the higher plating numbers was significantly higher than at the lower plating numbers (CFU-GM/10^6 cells at 5 $\times$ 10^5 cells/plate vs that at 2.5 $\times$ 10^5 cells/plate, $p < 0.01$ for both types of cells, paired t test).

In the control plates with no added CSA, about 30-60% of the maximal level of CFU-GM was regularly found and in a small number of cultures, levels as high as those in the feeder layer stimulated plates could be found. This
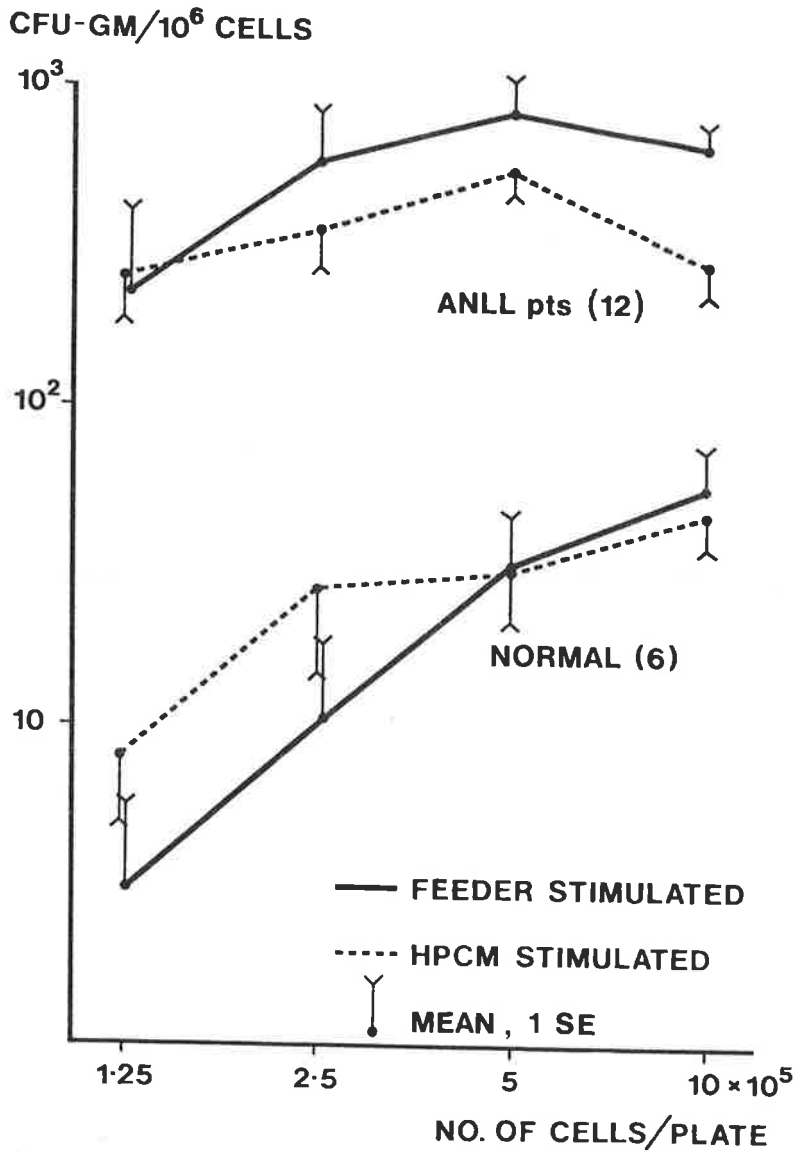
Fig. 3.7 The plating number effect in the CFU-GM
assay when cryopreserved PB MNC from ANLL
patients (top two lines) and normal subjects
(bottom two lines) were cultured. The
numbers in parenthesis denote the numbers of
assays performed. The mean ± S.E. are shown.
Two types of CSA, feeder layer and HPCM,
were used. Assay results from feeder layer
stimulated cultures are shown by the solid
lines (————). Assay results from HPCM-
stimulated cultures are shown by the dotted
lines (••••••).

was mostly found in plates with 5 or 10 x 10^5 cells.

Comparison between Feeder Layers and HPCM as the Source of CSA for Cryopreserved PB MNC

Feeder-layer stimulated cultures showed higher numbers of CFU-GM than HPCM stimulated cultures when cryopreserved PB MNC from ANLL patients were cultured. The mean of the maximal levels in 12 sets of feeder-layer stimulated cultures was significantly higher than that in the corresponding HPCM-stimulated cultures (1040 CFU-GM/10^6 cells vs 584 CFU-GM/10^6 cells, $p < 0.001$, paired t test). A similar trend was observed when cryopreserved PB MNC from normal subjects were cultured but the difference did not reach statistical significance (62/10^6 cells vs 50/10^6 cells).

Mixing Experiments

The addition of increasing number of adherent cells from cryopreserved PB MNC to cryopreserved cells from the same subject did not cause any significant decrease in the colony growth compared to the control as shown in line (a) in Fig 3.8.

The addition of increasing numbers of adherent cells obtained from freshly prepared PB MNC to cryopreserved PB MNC caused a progressive decrease in colony growth compared to the control value as shown in lines (b) and (c) in Fig 3.8. The percentage decrease in colony growth when increasing adherent cells were added was statistically

Fig. 3.8
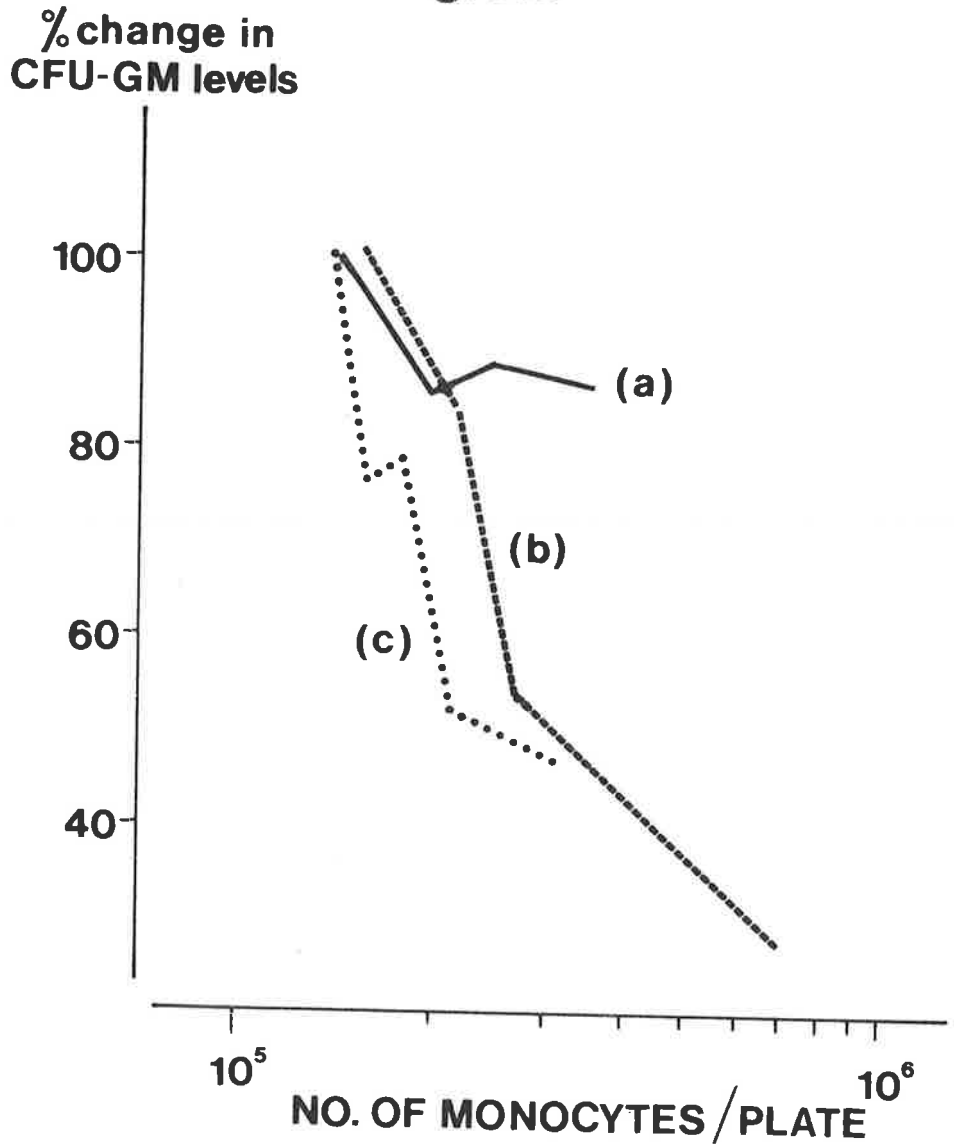
Fig 3.8   CFU-GM results of mixing experiments. The
results of 3 sets of experiments are shown.
Line (a) represents the results of adding adherent
cells obtained from cryopreserved PB MNC. Lines (b)
and (c) represent results of adding adherent cells
obtained from fresh PB MNC to unfractionated,
cryopreserved PB MNC. The leftmost point represents
the result when unfractionated cryopreserved PB MNC
only were cultured, serving as a control. The
rightmost point represents the result when the
highest number of adherent cells were added. The
results of the intermediate mixing experiments are
represented by the points in between.

significant (p < 0.025, two-way analysis of variance). The number of CFU-GM/10^6 cells was significantly higher in the control plates than those in the fifth set to which the highest number of non-cryopreserved monocytes were added (p < 0.05, t test). The control plates with no added CSA also showed a progressive decrease in colony growth when increasing numbers of adherent cells were added.

DISCUSSION

This study shows that when cryopreserved PB MNC were cultured, the maximal CFU-GM growth occurred most commonly at 5 or 10 × 10^5 cells/plate, whether the cells were from normal subjects or from ANLL patients. There was a remarkable similarity between the growth pattern of these cryopreserved cells and that of non-cryopreserved, non-adherent cells (Fig 3.2), suggesting that the inhibitory effect of monocytes was not operating in cultures of cryopreserved cells. Weiner et al (1980) found that several functions of monocytes were affected by the freeze-thaw process but the CFU-GM/monocyte interaction was not studied. Schlunk et al (1981) suggested that monocytes which have been cryopreserved might be defective in the 'processing' of HPCM. The mixing experiments showed that the addition of cryopreserved monocytes from normal subjects did not exert a significant inhibitory effect on the in vitro proliferation of CFU-GM but the addition of non-cryopreserved monocytes did. The lack of inhibition of CFU-GM when large numbers of cryopreserved PB MNC were cultured was therefore not due to alterations of the CFU-GM by the freeze-thaw process but is

a result of the susceptibility of monocytes to the freeze-thaw process so that they are no longer inhibitory to CFU-GM even when present in large numbers. Although no mixing experiments were performed with cryopreserved cells from ANLL patients, it is most probable that the above explanation is also applicable.

It is also interesting to note that 30 - 60% of the maximal CFU-GM growth occurred in the control plates with no added CSA when cryopreserved PB MNC were cultured and that this phenomenon was usually observed at 5 or 10×10^6 cells/plate. This suggests that the net level of endogenous CSA activity was quite high at those plating numbers. Thus, the net inhibitory effect of monocytes is reduced by the freeze-thaw process, giving rise to a shift to the right of region II depicted in Fig 3.6 so that the optimal number of monocytes now occurs at the higher plating numbers.

The change in the optimal numbers of monocytes when cryopreserved PB MNC were cultured may also be explained by postulating that most monocytes are destroyed by the freeze-thaw process so that the number of viable monocytes is reduced in the thawed cells relative to that in the non-cryopreserved cells. As a result, the optimal CFU-GM growth occurred at higher plating numbers. Monocytes, however, have been shown to remain viable after the freeze-thaw process (Weiner et al, 1980) so the change in the monocyte effect is most probably due to changes in the functional capacity of the thawed monocytes but not due to cell death.

Another important finding from the above studies is the importance of using feeder layers as the source of CSA

when cryopreserved cells from ANLL patients are cultured.
HPCM, while adequate as a source of CSA for freshly prepared
cells, provides a submaximal stimulus to the in vitro
proliferation of CFU-GM in cryopreserved PB MNC. This
difference has been shown when both cryopreserved bone
marrow cells from normal subjects and from patients with
malignancy were cultured (Ellis et al, 1981; Schlunk et al,
1981; To, unpublished data). No study to compare the
efficacy of HPCM and feeder layers when cryopreserved PB MNC
are cultured has been previously reported. Since the CFU-GM
assay is used as the in vitro measure of stem cell viability
after cryopreservation, using HPCM as the source of CSA may
lead to an underestimate of stem cell viability. The
reported 40% loss of PB CFU-GM after cryopreservation (Lasky
et al, 1982) may have resulted from the use of a HPCM
stimulated assay system as the source of CSA was not
specified. Although the study using cryopreserved cells
from normal subjects did not show a statistically
significant difference between feeder layers and HPCM as the
source of CSA, this is most probably due to the small number
of assays performed. In view of the significant differences
demonstrated above, it seems advisable to use feeder layers
as the source of CSA even when cryopreserved PB MNC from
normal subjects are cultured.

In summary, the present study shows that the
monocyte effect differs depending on whether the monocytes
are fresh or cryopreserved. This difference is probably
caused by non-lethal damage to the monocytes during the
freeze-thaw process. This study has also demonstrated the
importance of using feeder layers as the source of CSA when

cryopreserved PB MNC from ANLL patients are cultured in order not to underestimate the number of CFU-GM and it is suggested that feeder layers also be used when cryopreserved cells from normal subjects are cultured.

CHAPTER 4 : PB CFU-GM IN NORMAL SUBJECTS

INTRODUCTION

Considerable differences exist in the normal ranges of PB CFU-GM reported by different workers and large physiological fluctuations have been described even in the same individual (see Chapter 1.3). However, most of the studies were performed culturing 5 or 10 x 10^5 PB MNC per plate without taking into account the inhibitory effect that large numbers of monocytes may exert in such assay systems (To et al, 1983a). Results described in Chapter 3 Part 1 showed that it is important to perform the assay using several plating numbers from 0.625 to 10 x 10^5 PB MNC per plate in order to measure PB CFU-GM accurately.

The aims of this Chapter are firstly, to establish a more accurate normal range for PB CFU-GM taking into account the monocyte effect; secondly, to study the diurnal, day-to-day and the long-term variations of PB CFU-GM in the same individuals and thirdly, to study the changes of PB CFU-GM during and after vigorous physical exercise. The establishment of such a normal range and an increased understanding of the physiological fluctuations that may

occur would enable changes in disease states to be interpreted with greater precision.

MATERIALS AND METHODS

Healthy laboratory workers of both sexes who have normal blood counts were recruited for the study. After a rest period of 20 minutes, 30 ml of blood was collected by venesection between 8:30 am and 9:30 am. 5 ml of blood was used for the determination of blood counts using a Coulter S plus Counter and manual differential counting. The remainder of the blood was used to set up a PB CFU-GM assay as described in Chapter 2.

PB CFU-GM in Normal Subjects.

Thirty-four normal subjects were studied to provide data to establish a normal range. Some subjects were studied more than once as part of the studies to measure short-term and long-term variations.

The Short-term and Long-term Variations in PB CFU-GM in Normal subjects

In studies comparing the morning and afternoon levels of PB CFU-GM, an additional blood sample was collected between 1:30 pm and 2:30 pm on the same day. A total of thirteen studies was performed on six subjects. The morning and afternoon levels were compared by the paired t test.

In studies of the day-to-day variations of PB CFU-GM, assays were performed on three consecutive days in

each of four subjects and on two consecutive days on one subject. The percentage variations were calculated and the results were tested with a two-way analysis of variance.

In studies of long-term variations, serial studies were performed on seven subjects over a period of two years. The coefficient of variance (standard deviation/mean x 100%) was calculated for each subject to assess the amount of variation.

PB CFU-GM in Normal Subjects after Exercise.

Four healthy volunteers underwent the standard multistage treadmill exercise test (see Chapter 2, Part 6). They exercised either to exhaustion or until the completion of the fifth and final stage of the treadmill exercise. Venous blood was collected before, immediately after and at 4 hours and 24 hours after the exercise test to measure blood counts and PB CFU-GM levels.

RESULTS

The Normal Range for PB CFU-GM

A total of 104 CFU-GM assays was performed on 34 normal subjects. Equal numbers of males and females were studied and the mean age was 35 (range 20 to 57) in males and 30 (range 20 to 54) in females. For subjects who were studied more than once, the mean of all the results were used in the calculation. The mean levels for the 34 normal subjects are shown in Fig 4.1. There is a significant difference between the levels in male and female subjects (p < 0.001, t test). Separate means and ranges based on 95%
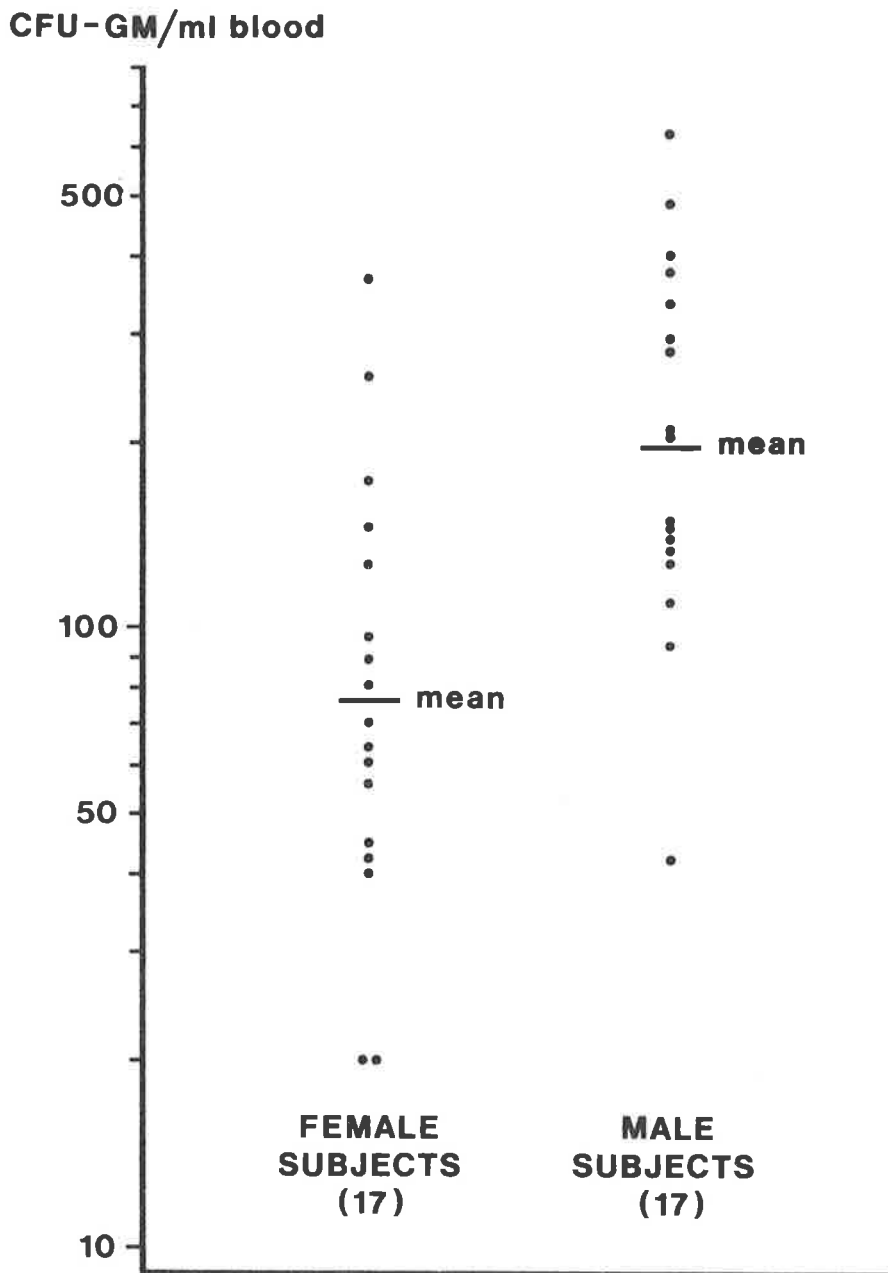
Fig. 4.1 The distrubution of the mean PB CFU-GM levels in 34 normal
subjects are shown. A total of 104 studies are done, 73
in males and 31 in females. For subjects who were studied
more than once, the mean of all results is shown. The
levels in male subjects are significantly higher than those
in females (p < 0.001, t test).

confidence limits were therefore calculated for male and female (Table 4.1). In both the female and the combined groups, the distribution of non-transformed data was positively skewed and did not conform to a normal distribution ($p < 0.01$, test of skew). Log-transformation allowed the data to fit into a normal distribution (p = not statistically significant, in Table 4.1). The data in the male group were therefore also log-transformed to maintain uniformity. Since the levels of PB CFU-GM followed a log-normal distribution, results were calculated using log-transformed data.

The 'apparent' levels (mean ± SE) of CFU-GM detected by culturing PB MNC at each of the five plating numbers in the 104 assays are shown in Fig 4.2. The highest CFU-GM levels were most commonly detected at 2.5×10^5/plate (39%), but might occur at any of the plating numbers : 1.25×10^5/plate (23%), 0.625×10^5/plate (20%), 5×10^5/plate (13%) and 10×10^5/plate (6%). The mean levels at the last three plating numbers were significantly lower than that at 2.5×10^5/plate while that at 1.25×10^5/plate was not. Moreover, the mean level at 2.5×10^5/plate was significantly lower than the mean of the 'actual' levels based on choosing the maximal levels out of the five plating numbers ($p < 0.01$, t test). The following data were therefore reported using the 'actual' levels rather than the levels at any one or two of the plating numbers in order not to underestimate the levels of PB CFU-GM.

TABLE 4.1.

PB CFU-GM LEVELS IN NORMAL SUBJECTS

	Non-transformed Data			Log-transformed Data		
Subjects	Mean†	S.D.	Skew	Mean †	Range (95% Confindence Limit)	Skew
Male (n=17)	244	159	0.86	198	46-855	-0.28
Female (n=17)	103	91	1.6*	76	14-411	0.14
Combined (n=34)	174	146	1.29*	122	21-734	-0.17

* $p < 0.01$, test of skew

† CFU-GM/ml
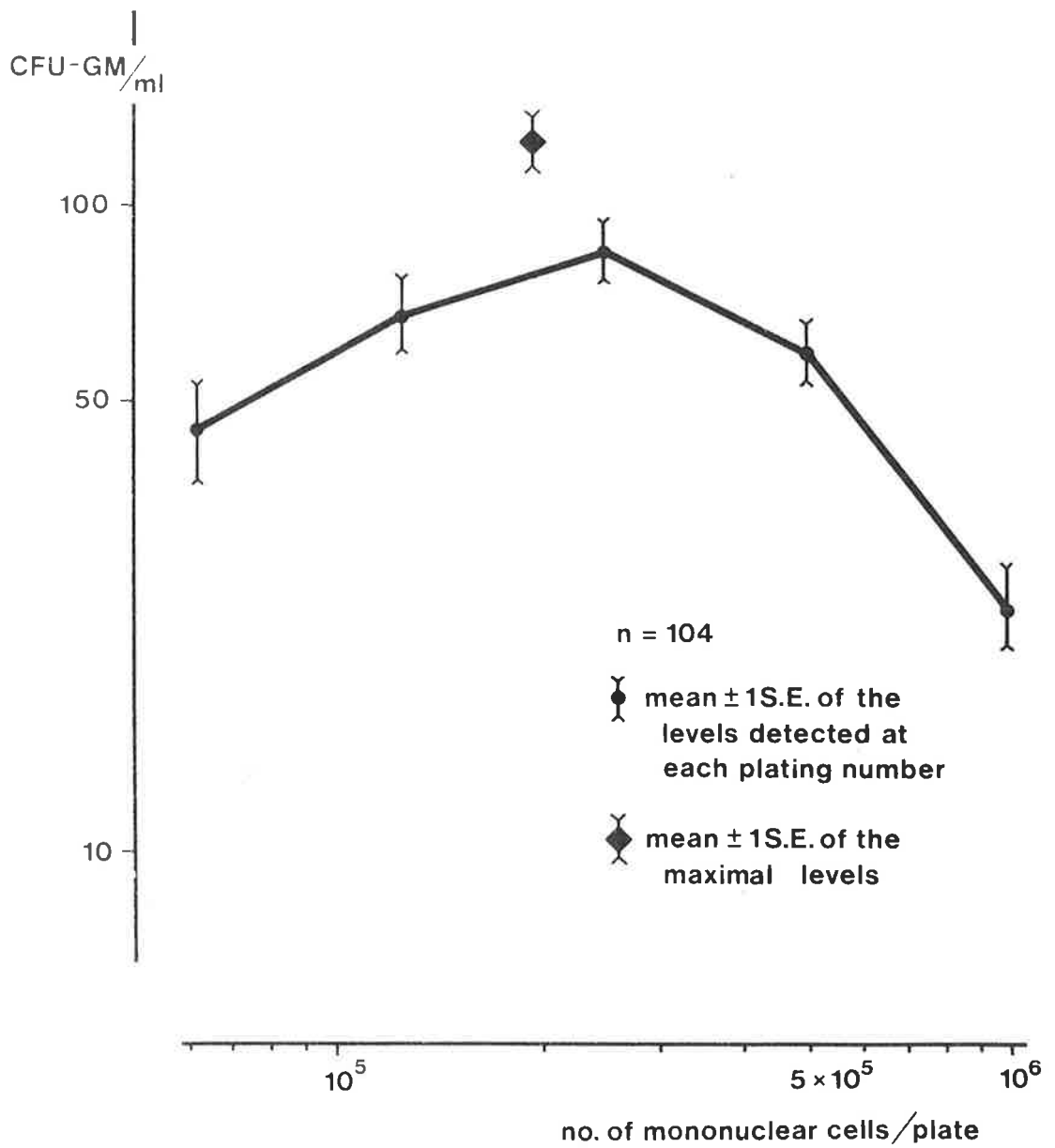
Fig. 4.2 The apparent levels of PB CFU-GM detected at the
different plating numbers. The results from
104 assays are shown. These levels were significantly
lower than the mean of the maximal level.

The Short-term and Long-term Variations in PB CFU-GM in Normal Subjects.

Morning and Afternoon PB CFU-GM Levels

The results of 13 pairs of assays performed on 6 subjects are shown in Table 4.2. No consistent pattern of variation was present. The mean ($\pm$ SE) percentage increase was 35% ($\pm$ 14%) but there was no significant difference between the levels of PB CFU-GM measured at 9 am and 2 pm (paired t test). The optimal plating number was the same in 6 out of the 13 studies and did not vary by more than four-fold in any of the studies.

Day-to-day Variations of PB CFU-GM

PB CFU-GM levels on three consecutive days in four subjects and on two consecutive days in one subject are shown in Table 4.3. While the mean ($\pm$ SE) day-to-day percentage variations of PB CFU-GM was 60% ($\pm$ 20%), the variation was not statistically significant (two-way analysis of variance, log-transformed data). In only one out of nine pairs did the optimal plating number vary by more than two-fold on consecutive days.

Long-term Variations

Seven subjects were studied over periods between 9 and 23 months (mean 19.5 months). Between 4 and 10 assays (mean 7 assays) were performed on each individual. The results are shown in Table 4.4. In five subjects, the maximal fluctuation as reflected by the difference between the highest and the lowest recorded levels was less than

TABLE 4.2

A COMPARISON OF MORNING & AFTERNOON PB CFU-GM LEVELS
IN NORMAL SUBJECTS

SUBJECTS	9 AM CFU-GM/ml (Plt no)	2 PM CFU-GM/ml (Plt no)	Variation
GC	518 (1.25)	441 (0.625)	-15%
	108 (1.25)	175 (5)	62%
	144 (2.5)	416 (0.625)	189%
DH	157 (10)	151 (10)	-4%
	193 (5)	173 (10)	-10%
	218 (2.5)	304 (2.5)	39%
JO	41 (2.5)	41 (2.5)	0%
RP	104 (0.625)	117 (2.5)	13%
	181 (0.625)	152 (0.625)	-16%
TR	197 (2.5)	187 (5)	-5%
	142 (2.5)	134 (2.5)	-6%
TO	59 (2.5)	25 (2.5)	-58%
	65 (5)	39 (1.25)	-40%

Plt no = Plating number ($x10^5$/plate)

TABLE 4.3

DAY-TO-DAY VARIATIONS IN PB CFU-GM LEVELS IN NORMAL SUBJECTS

Subjects	Day 1 CFU-GM/ml(Plt no)	Day 2 CFU-GM/ml(Plt no)	Day3 CFU-GM/ml(Plt no)	% Change D1/D2	D2/D3	D1/D3
TO	59 (2.5)	26 (2.5)	65 (5)	-56	+150	+10
TR	197 (2.5)	208 (5)	142 (2.5)	+6	-32	-28
DH	193 (5)	216 (1.25)	218 (2.5)	+12	+1	+13
GC	108 (1.25)	289 (1.25)	144 (2.5)	+168	-50	+33
RP	104 (0.625)	181 (0.625)	N.D.	+74	N.D.	N.D.

Plt no = Plating number ($\times 10^5$/plate)

N.D. = Not done

TABLE 4.4

LONG-TERM VARIATIONS IN PB CFU-GM LEVELS IN NORMAL SUBJECTS

Subjects	No of Assays	Duration (months)	CFU-GM/ml Mean (1.S.D.)	CFU-GM/ml Min	Max	Optimal plating number (10^5/ml) 0.625	1.25	2.5	5.0	10
AB	5	19	144 (49)	84	216	1	4	0	0	0
GC	8	23	250 (182)	96	518	2	4	2	0	0
DE	4	19	171 (34)	133	214	2	1	1	0	0
DH	10	19	205 (55)	138	332	0	5	2	1	2
RL	4	9	332 (180)	142	543	2	1	1	0	0
TR	7	22	209 (52)	142	293	1	1	2	3	0
TO	9	21	44 (17)	23	65	1	2	3	2	1

Min = Minimal

Max = Maximal

three-fold over the duration of study. The mean ($\pm$ SE) coefficient of variance in the seven subjects was 35% ($\pm$ 7)%. In only one subject was the maximal fluctuation up to five-fold. It is also evident from Table 4.4 that the optimal plating number varied in the same individual studied at different times. Nevertheless, in 61% (29/48) of the assays, the maximum CFU-GM level was detected at 1.25 or 2.5 $\times 10^5$ cells/plate. The variations in PB CFU-GM between the different subjects were highly significant (p < 0.001, one-way analysis of variance).

The Effect of Exercise on the Levels of PB CFU-GM

The serial changes in heart rates, blood counts and PB CFU-GM levels before, immediately after, 4 hours after and 24 hours after exercise in four subjects are shown in Table 4.5. There were significant rises in the level of PB CFU-GM in all four subjects, with a mean increase of 229% over the pre-exercise level (p < 0.05, paired t test, log-transformed data). In three of the subjects, the CFU-GM:lymphocyte ratio remained unchanged after exercises. In the fourth subject (TO), however, there was an actual increase in CFU-GM above that of the lymphocyte count, as reflected in a higher CFU-GM:lymphocyte ratio immediately after exercise compared to that in the pre-exercise, the 4 hours post exercise and the 24 hours post exercise assays. In all four subjects the rise in PB CFU-GM was only temporary as the levels were back to the pre-exercise level after 4 hours, in parallel to the changes of the leucocyte counts.

TABLE 4.5

PB CFU-GM AND BLOOD COUNTS CHANGES DURING AND AFTER EXERCISE

NAME	EXER*	HR# (/min)	HB (g/dl)	WCC ($10^3/\mu l$)	NEUT ($10^3/\mu l$)	LYM ($10^3/\mu l$)	MONO ($10^3/\mu l$)	PLAT ($10^3/\mu l$)	CFU/L6**	CFU-GM/ML	% CHANGE
TO	PRE	78	15.2	5.5	3.9	1.2	0.28	226	23	26	
	POST	199	15.5	8.3	5.0	2.6	0.42	278	69	178	+585
	4 HR	80	14.9	5.8	4.1	1.3	0.29	243	52	66	
	24 HR	72	15.0	6.5	4.0	1.2	0.39	239	47	65	
TR	PRE	65	14.1	6.8	3.3	2.9	0.27	391	73	208	
	POST	190	14.8	12.1	5.6	5.3	0.61	542	79	440	+117
	4 HR	66	12.8	7.6	4.3	2.7	0.30	430	78	208	
	24 HR	68	14.2	7.6	3.7	2.9	0.61	389	49	142	
DH	PRE	72	14.8	5.2	3.5	1.5	0.10	181	148	216	
	POST	180	16.5	9.6	5.0	4.1	0.38	259	110	454	+110
	4 HR	68	14.8	5.5	3.7	1.4	0.22	172	120	165	
	24 HR	70	14.3	5.5	3.5	1.7	0.17	199	128	218	
GC	PRE	60	15.5	5.8	3.9	1.3	0.35	205	226	289	
	POST	180	16.5	10.3	7.0	2.6	0.72	392	229	589	+104
	4 HR	62	14.6	6.4	4.4	1.5	0.38	219	243	374	
	24 HR	60	14.9	5.3	3.3	1.5	0.27	218	94	144	

Mean % CFU-GM increase   229%

*   Time of blood collected relative to the Exercise Test

HR# = Heart Rate

**   CFU-GM/10^6 LYMPHOCYTES

A Comparison of the Normal Ranges for PB CFU-GM from This Study and from Other Published Studies

The results from published reports (Table 1.3.1, group 2) are compared with those from the present study at the corresponding plating number in Table 4.6. It is evident that when similar plating numbers were used, the results from published reports are quite comparable to those from the present study but lower than the 'actual' levels.

DISCUSSION

This study describes the levels of PB CFU-GM in normal subjects using an improved assay method to allow for the effect of monocytes. Based on such an assay system, the distribution of levels of PB CFU-GM in normal subjects fits a log-normal distribution. The mean and the range (95% confidence limit) were therefore calculated using log-transformed data. Several published reports (Verma et al, 1980; Peschel et al, 1983) were based on non-transformed data but if their results were expressed as the 95% confidence limit range, negative levels would have resulted, indicating that their ranges were also skewed.

The optimal plating number varied in the same individual studied at different times as shown in Table 4.4. This variation may be due to the variable state of stimulation/inhibition of the monocyte populations as discussed in the last Chapter. Until these factors are understood and controlled, it is important to culture the mononuclear cells using several plating numbers in order to measure PB CFU-GM accurately.

TABLE 4.6

COMPARISONS BETWEEN RESULTS FROM THIS STUDY AND PUBLISHED REPORTS CLASSIFIED ACCORDING TO THE PLATING NUMBERS USED

Plating number ($\times 10^5$/plate)	Results from this study Mean*	95% Range	Results from published reports Mean*	Range	Reference
2.5	88	13 - 597	$120^\dagger$	$25 - 600^\dagger$	Richman (1976)
5	60	6 - 566	not stated	11 - 60	Lohrmann (1978)
			78	15 - 410	Beran (1979)
			29	0 - 70	Jehn (1983)
	95 ± 83#		$45 ± 15$#†		Goldberg (1979)
10	53 ± 64#		$13 ± 23(AM)$#†		
			$28 ± 28(PM)$#†		Verma (1980)
			$93 ± 45$#†		Peshel (1983)

Mean of maximal levels from this study

128	25 - 645	(Log-transformed data)
173 ± 136		(Non-transformed data)

* CFU-GM/ml

$\dagger$ Denotes calculated values (see Table 1.3.1)

\# The mean ± 1 SD is used here in the comparison instead of the mean and 95% range because this is the format used in the published reports.

The normal range for PB CFU-GM was found to be wide and the levels varied between subjects as well as in the same subjects studied at different times. However, the levels from each subject tended to cluster around his own mean (Table 4.4) such that some subjects always have PB CFU-GM levels in the higher section of the normal range while others have levels in the lower section of the range, as if each subject has his own setting of 'normal'. Very little is known about how the levels of PB CFU-GM are controlled but since they are higher in males than in females (Fig 4.1) hormonal differences and genetic make-up may be responsible. The log-normal distribution pattern in mathematical terms also suggests that multiple interacting factors are involved. The difference between the sexes confirms previous reports (Barrett et al, 1979; Ponassi et al, 1979).

This study shows that PB CFU-GM levels in particular cases may vary up to three-fold between morning and afternoon and between consecutive days and may vary up to five-fold over a period of one to two years although the diurnal and day-to-day variations were not statistically significant. These results confirm findings by Barrett et al (1979) and Ponassi et al (1979). The finding of higher PB CFU-GM levels in the afternoon by Verma et al (1980) is difficult to interpret since the assay system employed appears to be suboptimal, detecting only low levels of CFU-GM. The rapid rise and return to normal in the levels of PB CFU-GM during and after vigorous physical exercise shows that a reserve pool of CFU-GM exists and rapid shifts between this and the circulating pool occur. The extra

CFU-GM appear to have come mainly from the same pool of cells as the leucocytes, as evidenced by a similar rise in white cell count and a constant CFU-GM:lymphocyte ratio in three of the four subjects studied. Thus the cells may have come from the splanchnic and the peripheral circulations as suggested by Barrett et al (1978). In the fourth, there was an actual increase in the CFU-GM:lymphocyte ratio, so CFU-GM from other sites such as bone marrow may also contribute to the mobilisable pool (Barrett et al, 1978; Gerhartz and Fliedner, 1980). Such a mobilisable pool has been demonstrated in dogs after the administration of endotoxin and dextran sulphate (Ross et al, 1978; Fliedner et al, 1979), and in man after an injection of adrenocorticotrophic hormone (Barrett et al, 1978) and endotoxin (Cline et al, 1977). Thus it is quite probable that other factors such as emotional stress may also cause rapid shifts of cells between the circulating and the mobilisable pools leading to changes in the measured levels of PB CFU-GM (Verma et al, 1980).

There have been claims that the levels of PB CFU-GM undergo cyclical changes in normal subjects (Barrett et al 1979; Kreutzmann and Fliedner, 1979) and the cycle lengths are different in the various individuals studied, ranging from 19 days to 28 days. While the number of assays performed here did not permit actual testing of such claims, the presence of a readily mobilisable pool of CFU-GM allowing for rapid shifts between the circulating and the mobilisable pools makes the claims difficult to verify. Until the investigator is able to control all the known and the yet unknown factors that cause rapid, non-cycling-

related shifts between the two pools, it is not possible to study long-term cycling. The mere fact that the results can be fitted into a mathematical model of cycling with different cycle lengths does not necessarily mean that such cycling exists. Mathematical significance does not always imply biological or clinical significance.

The intrinsic variability of a biological assay system has also to be considered as contributing to the fluctuations observed. While it is impossible to exclude such an influence, a quality control program to standardise the system at regular intervals has been followed to reduce intrinsic variability to a minimum.

The studies described in this and the previous chapter show that the level of PB CFU-GM detected by culturing PB MNC depends on the plating number used. The comparison of the normal range from this study and other published studies classified according to the plating numbers used (Table 4.6) suggests strongly that the difference in the normal ranges reported is merely a reflection of the various plating numbers but not the actual levels in the subjects. Furthermore, results based on one or two plating number points would underestimate the actual level of PB CFU-GM. Other workers (Table 1.3.1, group 1) culturing whole blood leucocytes (WBL) reported results more similar to those in this study. While no studies have been carried out to investigate the effect of monocytes on in that assay system, the number of monocytes present when 10×10^5 WBL are cultured, of the order of 0.2 to 0.8×10^5, is similar to the optimal number of monocytes described in the previous chapter. Thus the plating number employed in

these reports is probably quite optimal.

In summary, the mean and range (95% confidence limit) of PB CFU-GM were 198 CFU-GM/ml (46 - 855) in males and 76 CFU-GM/ml (14 - 411) in females, based on 104 assays in 34 normal subjects. It is important to culture PB MNC at several plating numbers in order to measure PB CFU-GM levels accurately. The levels varied among subjects and in the same subject studied over a period of time but they tended to cluster around each individual's own mean. Vigorous physical exercise led to a mean three-fold increase in PB CFU-GM levels which were back to normal after 4 hours, suggesting that there is a mobilisable pool of PB CFU-GM. While the physiological basis of the long-term fluctuations remains unclear, it is probable that this may in part be due to the presence of a mobilisable pool of CFU-GM. The validity of published results using only one or two plating numbers are in question because they have not allowed for the monocyte effect.

Having established a reliable normal range, it is then possible to study the alterations in the levels of PB CFU-GM in ANLL patients.

CHAPTER 5 : THE CHANGES IN THE LEVELS OF PB CFU-GM IN

PATIENTS WITH ACUTE NON-LYMPHOBLASTIC LEUKAEMIA

(ANLL)

INTRODUCTION

Richman et al (1976) demonstrated increased levels of PB CFU-GM in patients with solid tumours during recovery from the myelosuppressive effect of cytotoxic chemotherapy. Lohrmann et al (1979) performed serial studies on patients receiving adjuvant chemotherapy for breast cancer and showed that PB CFU-GM and leucocytes exceeded their respective normal ranges during the recovery phase. In addition, the rise in PB CFU-GM regularly and predictably preceded the rise in granulocytes. Stiff et al (1983) reported similar changes but noticed that the high PB CFU-GM levels were accompanied by a monocytosis.

Patients with Acute Non-Lymphoblastic Leukaemia (ANLL) usually exhibit a rapid rise in platelet and leucocyte counts when they first enter remission after induction chemotherapy. During this very early remission phase, the platelet count commonly doubles daily and may

reach levels of $1,000 \times 10^3/\mu l$ or higher. This observation suggested that a similar rise in PB CFU-GM might be seen in these patients with primary bone marrow disease. A preliminary study by Juttner et al (1982a) showed high levels of PB CFU-GM in seven ANLL patients during very early remission but no systematic studies of the serial changes in this phase have been described.

The aims of this chapter are to study the changes in PB CFU-GM in ANLL patients during very early remission and to compare these changes to those during consolidation chemotherapy, stable remission and at relapse.

MATERIALS AND METHODS

PB CFU-GM levels were measured in 15 patients during the recovery phase after DAT induction chemotherapy (see Chapter 2 Materials and Methods). In the first two patients, only one PB CFU-GM assay was performed. Serial studies were performed in the subsequent 13 patients starting from as early as day 8 after the end of induction chemotherapy. Bone marrow CFU-GM were also measured in the last two patients during the recovery phase.

In 4 patients, serial studies were also performed during the recovery phase after consolidation chemotherapy. PB CFU-GM were measured at least twice a week in the three weeks between two courses of consolidation chemotherapy.

Six patients in stable remission from ANLL as well as 4 patients in relapse were also studied.

PB CFU-GM were assayed by plating peripheral blood mononuclear cells at 0.625, 1.25, 2.5, 5, and 10 $\times$

10^5/plate in order to be certain the maximal level of PB CFU-GM were detected. However, some of the studies were carried out before the plating number effect were recognised and assays were usually performed at 5 or 10 x 10^5/plate.

RESULTS

1. PB CFU-GM during Very Early Remission

Thirteen of the 15 patients went into remission after one course of induction chemotherapy. Table 5.1 shows the highest recorded PB CFU-GM levels in these patients during very early remission after induction chemotherapy. The mean of the recorded peak level was 2793 CFU-GM/ml, a 25-fold increase above the mean level in normal subjects. Studies on the four patients in Group 1 were performed using 5 or 10 x 10^5 PB MNC/plate only so the levels shown may be underestimates. Studies on the nine patients in Group 2, however, were performed using all five cell concentrations and the mean of the recorded peak was 4803 CFU-GM/ml. In two of the patients, the recorded peak levels were up to sixty and seventy times that of normal subjects. PB CFU-GM were very low or undetectable in all assays performed before the rise in platelet count. The levels of PB CFU-GM remained high for several days and then returned to normal. The morphology of these in vitro colonies were normal and all three types of neutrophil, macrophage and eosinophil colonies were present. The highest levels were recorded at the time when the platelet count was rising rapidly, between days 14 and 29 from the end of chemotherapy. Two typical examples of the serial changes are shown in Fig 5.1 and Fig

TABLE 5.1

PB CFU-GM LEVELS IN ANLL PATIENTS DURING VERY EARLY REMISSION

NAME	SEX/AGE	FAB *	DURATION OF STUDY[#]	HIGHEST CFU-GM/ml≈ (DAY POST TREATMENT)	Hb (g/dl)	WCC ($10^3/\mu l$)	NEUT (%)	LYM (%)	MONO (%)	Plt ($10^3/\mu l$)
A. COMPLETE REMISSION										
GP 1 : Assays performed with an incomplete range of plating numbers										
SN [†]	F/72	M2	20	1409 (20)	8.0	3.4	52	37	10	20
FWt [†]	M/61	M1	14	955 (14)	11.8	13.1	68	20	8	450
JM [†]	F/21	M3	8-21	370 (21)	9.2	3.8	75	15	2	159
SD [†]	F/30	M5	16-21	923 (21)	12.9	2.1	17	56	26	296
GP 2 : Assays performed with the full range of plating numbers										
GS	F/48	M2	17-22	4012 (22)	12.4	4.5	34	39	25	1090
MMc	M/20	M3	16,17	12000 (16)	12.3	2.7	18	65	13	677
TO [†]	M/63	M2	18-21	4116 (18)	10.2	2.4	39	28	24	309
PK	M/53	M2	25-31	2158 (31)	13.3	4.0	58	32	10	225
JP	M/55	M2	14-19	14520 (19)	9.3	8.7	60	22	18	988
CF	M/51	M2	16-21	3545 (18)	12.3	3.1	25	36	39	201
MC	M/50	M5	17-27	3456 (19)	13.3	6.0	69	10	18	692
KT	M/66	M2	15-18	5800 (18)	10.3	5.2	43	35	14	186
KJ	M/52	M2	15-18	3200 (18)	10.3	2.0	5	76	19	157
B. PARTIAL REMISSION										
FWm	M/62	M2	28-30	120 (28)	13.3	3.5	55	16	26	73
Aw	M/50	M1	13-16	12 (15)	11.9	1.4	73	20	1	396

Days post treatment

* FAB classification of ANLL

† Data kindly provided by Dr. C.A. Juttner and Mr. D.N. Haylock.

≈ Normal range for PB CFU-GM : Male: 46-855 CFU-GM/ml
Female: 14-411 CFU-GM/ml

5.2. Similar changes were present in all the patients studied who achieved complete remission.

In two patients, bone marrow CFU-GM were also measured during very early remission. The results were shown in Table 5.2. While the levels of PB CFU-GM were high at that time, the levels of CFU-GM/10^5 nucleated cells in the marrow aspirate were not increased above the normal range. The cell counts in the marrow aspirate were low and the estimated CFU-GM/ml in the marrow aspirate was not higher than the circulating levels in the blood at that time.

Patient (JM) did not show the marked increase seen in the other 12 patients. She had a difficult induction course with life-threatening septicaemia and adult respiratory distress syndrome. Recovery of peripheral blood counts was markedly delayed and the PB CFU-GM studies were terminated before she showed signs of remission, so the result shown may not reflect the highest level. Furthermore, studies on JM were not performed using the full range of plating numbers, and may have underestimated the actual levels of PB CFU-GM. She, incidentally, is the longest survivor in this group. She is now in her fourth year of complete remission and has given birth to two normal children.

The remission and survival status of these patients were shown in Table 5.3. There is no significant correlation between the recorded peaks and the durations of first remission or the durations of survival from the time of remission.
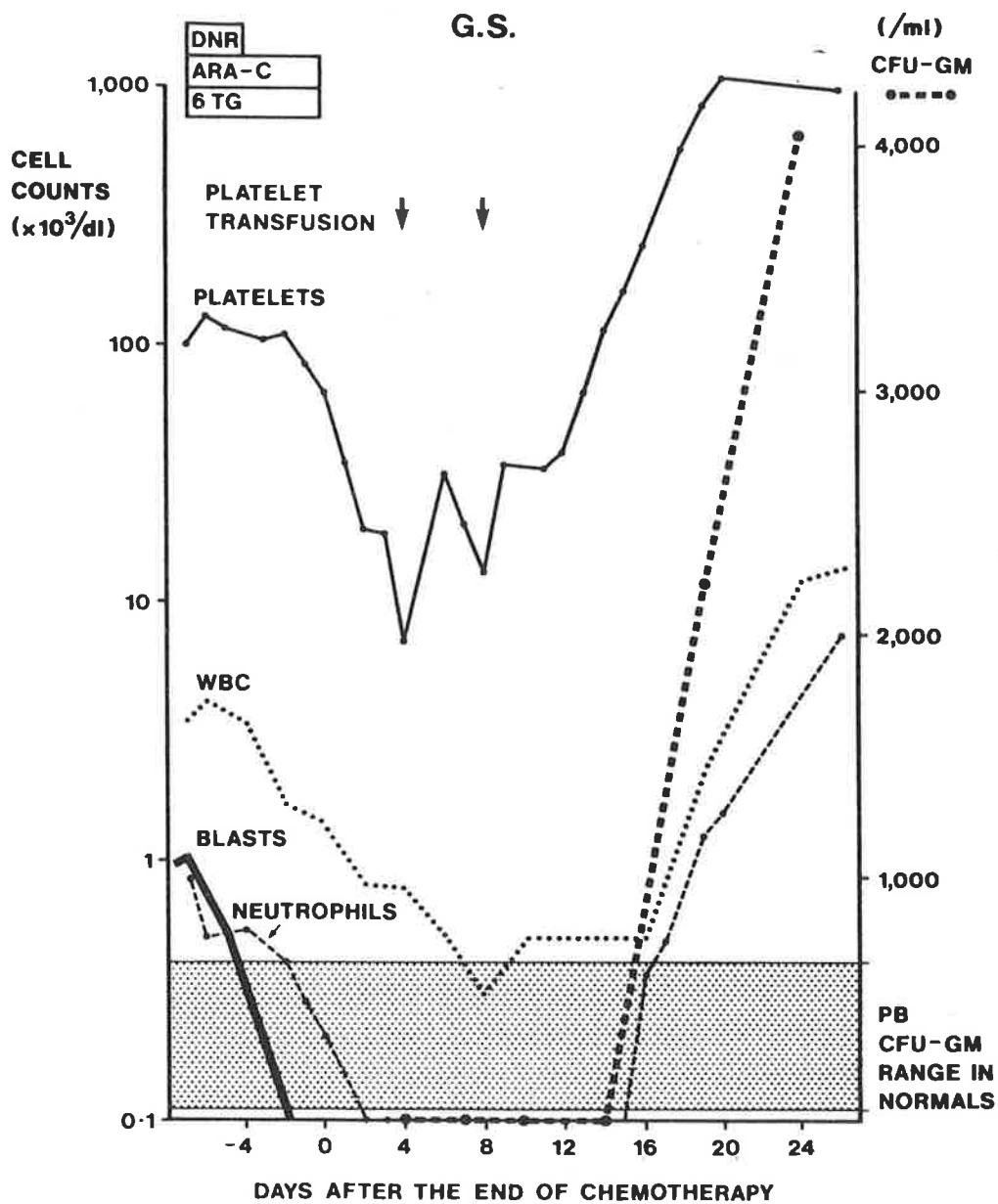
Fig. 5.1 Serial changes in blood counts and PB CFU-GM
levels during induction and very early remission
in patient G.S..
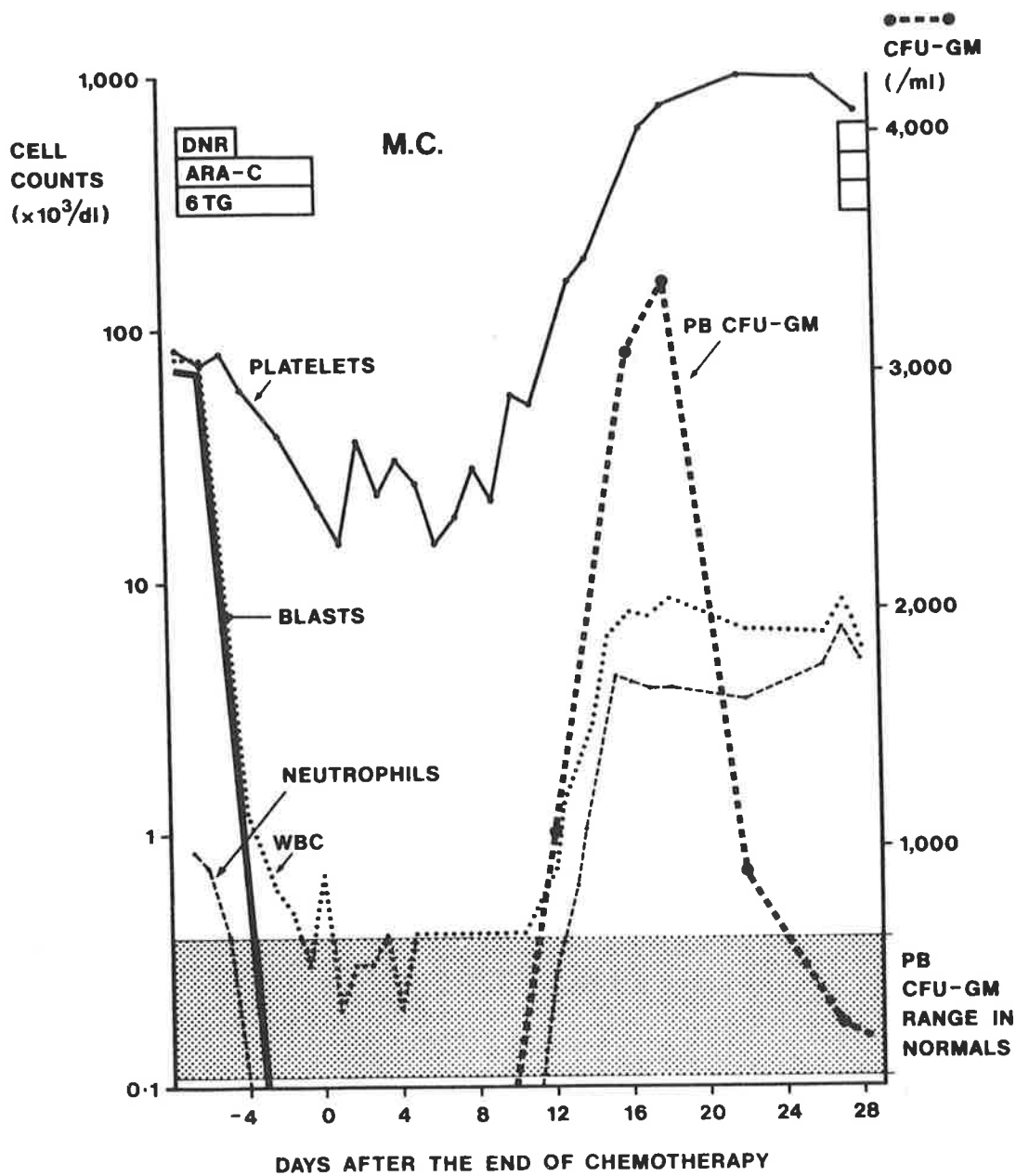
Fig. 5.2 Serial changes in blood counts and PB CFU-GM
          levels during induction and very early remission
          in patient M.C..

Fig 5.2   A COMPARISON OF THE CFU-GM LEVELS IN BONE MARROW
AND PERIPHERAL BLOOD IN 2 ANLL PATIENTS DURING VERY
EARLY REMISSION

Name	Days after End of Chemotherapy	PB CFU-GM (/ml)	Bone Marrow CFU-GM (/10^5 cells)	(/ml)
KT	18	5600	57	3210
	21	2692	16	1560
KJ	15	1074	30	1200
	18	2456	61	2500

TABLE 5.3.

THE CLINICAL STATUS OF THE 13 ANLL PATIENTS WHOSE PB CFU-GM LEVELS
WERE STUDIED DURING VERY EARLY REMISSION

Patient	PB CFU-GM Level	Duration of * First Remission (in months)	Duration of * Survival from Diagnosis (in months)
GROUP 1			
SN	1409	8	13
FWT	955	14	20
JM	370	42+	43+
SD	923	1	2
GROUP 2			
GS	4012	29+	30+
MMC	12000	7	16
TO	4116	8	21
PK†	2185	16+	17+
JP†	14520	13+	14+
CF†	3545	12+	13+
MC†	3456	5	10
KT†	5600	3	5
KJ†	3200	6+	7+

————————————————————————————————————— April, 1984. ————

* A '+' after the number of months denotes that the patient is
  still in remission and/or alive.

† Indicates that this patient has peripheral blood stem cell
  harvest performed during very early remission.

2. PB CFU-GM in Patients achieving Partial Remissions

Serial studies on the two patients who only achieved partial remission showed the highest levels of PB CFU-GM to be 120 and 10/ml, respectively. Colony morphology was normal but the colonies tended to be smaller in size compared to those who entered complete remissons.

3. PB CFU-GM during Consolidation Therapy

The levels of PB CFU-GM during the recovery phase after consolidation chemotherapy showed a much less marked increase compared to that after induction chemotherapy. Serial studies were performed after one course of consolidation chemotherapy in the first two patients, after two courses in the third patient and after three courses in the fourth patient. The highest recorded levels in the four patients were 920, 1089, 294 and 1730 CFU-GM/ml, respectively. The severe cytopenia and the overshoot in platelet count regularly observed following induction chemotherapy did not consistently occur following consolidation. On several occasions when only minimal cytopenia occurred, the PB CFU-GM level did not rise above the upper limit of the normal range.

4. PB CFU-GM during Stable Remission

The results of 10 studies on 6 patients during stable remission are shown in Table 5.4. The levels of PB CFU-GM were either below or within the normal range (mean 46 CFU-GM/ml, range 9 - 280). The colony morphology was normal.

TABLE 5.4

PB CFU-GM STUDIES IN ANLL PATIENTS IN STABLE REMISSION,
NOT ON MAINTENANCE CHEMOTHERAPY

Patient	No. of months in remission	PB CFU-GM/ml
OH*	3	59
JM†	11	21
	12	24
JB	22	24
	24	24
FH	24	23
GS	24	9
MB	30	24
	36	25
	37	220

* This patient has relapsed one month later
    and died.

† Data kindly provided by Dr. C.A. Juttner and
    Mr. D.N. Haylock.

5. PB CFU-GM during Relapse

Four studies were performed on four patients during relapse. No CFU-GM could be detected in two patients. In two other patients, abnormal colony growth occurred. In one patient (MC) studied when the blast count was 3% of 8,500 leucocytes/µl, PB CFU-GM was present at 151/ml, similar to the 158/ml detected one month earlier when there was no evidence of relapse. However, large numbers of clusters (>1000/ml) were also present. The growth of clusters appeared to be autonomous because they were present though in lower numbers even when no CSA was added. A similar pattern was seen when bone marrow cells were assayed for CFU-GM. In the other patient (KT) studied when the blast count was 64% of 14,700 leucocytes/µl, only one type of small, diffuse colonies each containing 30-50 cells were present at a level of >5000/ml. Similar diffuse colonies were present in the control plates. PB CFU-GM assayed two months earlier in remission was 142/ml with normal colony morphology.

DISCUSSION

This study shows that high levels of PB CFU-GM occur regularly in ANLL patients during very early remission after induction chemotherapy. While these findings are qualitatively similar to those reported by Richman et al (1976), Lohrmann et al (1979) and Stiff et al (1983) in patients with normal bone marrow, they have not previously been demonstrated in ANLL patients. Previous reports of PB CFU-GM in ANLL patients described the levels either at time

of diagnosis and relapse (Robinson et al, 1971; Moore et al, 1974; Beran et al, 1980; Jehn et al, 1983) or during long-term remission (Peschel et al, 1983) but not during this very early remission phase. This study also shows that such high levels did not regularly occur during consolidation therapy or stable remission, confirming the findings reported by Peschel et al (1983). Moreover, high levels were not found in patients who achieved only a partial remission. Thus, the increases in circulating stem cells during very early remission are most probably a result of the intense proliferative activity during recovery of the normal haemopoietic cells rather than a manifestation of the disease itself. The abnormal growth patterns noted in relapse are similar to the leukaemic growth pattern described (Moore et al, 1974; Beran et al, 1980; Jehn et al, 1983) and do not appear to have arisen from normal haemopoietic cells.

The timing of the occurrence of such high levels can be predicted by the rapid rise in platelet count. Richman and Lohrmann did not mention the changes in the platelet count while Stiff observed the high levels coincided with a monocytosis while the platelet count may be normal or high. It is thus quite possible that Stiff's findings are consistent with those described here. Nonetheless, a rising platelet count is easier to detect than a monocytosis when the total leucocyte count is low.

Lohrmann et al (1979) suggested that the high levels of PB CFU-GM observed during the recovery phase after myelosuppressive chemotherapy may be an overflow from the intensive regeneration occurring in the bone marrow. He,

however, could not find any consistent increase in the levels of CFU-GM in bone marrow studied at the same time. This study also showed that the levels of CFU-GM in bone marrow were not higher than the levels in peripheral blood, so the bone marrow may not be the origin of these CFU-GM but the high levels may reflect a migration of stem cells from extra-marrow sites to repopulate bone marrow. Migration of stem cells to repopulate the bone marrow has been described in mice which were given a lethal dose of total body irradiation with one of the limbs shielded. Stem cells from the shielded limb proliferated and migrated to repopulate the rest of the bone marrow (Hanks, 1964). In Man, migration of pluripotent stem cells occurs during foetal life from the yolk sac to the liver which acts as a haemopoietic organ from the third month to the sixth month of foetal life, and subsequently from the liver to the bone marrow. In post-natal life, both the liver and the spleen are capable of sustaining haemopoietic activity during severe haemopoietic stress as in thalassemia major and myelofibrosis, so it is possible that these organs possess a reserve of pluripotent stem cells which can be mobilised in times of severe demands such as during very early remission of ANLL. Further studies of the serial changes in the levels of CFU-GEMM and CFU-GM in the bone marrow and peripheral blood will be required to elucidate the biological basis of this recovery phenomenon.

The harvesting of circulating stem cells during recovery phase from chemotherapy has been suggested for the treatment of solid tumors (Barr and McBride, 1982; Zwaan 1982) but not for the treatment of ANLL. There have been

doubts whether circulating haemopoietic stem cells are as effective as bone marrow derived stem cells for haemopoietic reconstitution (McCarthy and Goldman, 1984). As reviewed in Chapter 1 Section 4, studies in mice suggested strongly that circulating stem cells have lower self-renewal capacity than bone marrow stem cells (Micklem et al, 1970; Monette and Stockel, 1980; Chertkov et al, 1982) but studies in baboons and dogs suggested that the two types of cells are similar (Storb et al, 1976; Nothdruft et al, 1977). Furthermore, Gerhartz and Fliedner (1980) suggested that circulating stem cells harvested during period of increased haemopoietic proliferation may have higher proliferative potential. Abrams et al (1981) demonstrated by transplantation experiments that peripheral blood cells collected from dogs during the recovery phase after cyclophosphamide treatment contained an increased number of stem cells and this increase could be measured by the increase in CFU-GM. Thus the increase in PB CFU-GM during the very early remission phase of ANLL described in this Chapter is most probably accompanied by an increase in circulating pluripotent stem cells and the peripheral blood cells harvested at this time probably have increased haemopoietic reconstitutive capacity.

Stem cell harvest from peripheral blood by leukapheresis has been carried out in experimental animals (Fliedner et al, 1976) as well as in volunteers (Korbling et al, 1980, Lasky et al, 1982). The procedure is simple and the subject is not exposed to the risk of general anaesthesia as in the case of harvest from the bone marrow. To harvest sufficient stem cells from normal individuals,

however, requires five or more three hours leukaphereses because of the low frequency of CFU-GM in the peripheral blood. Two attempts at haemopoietic reconstitution using peripheral blood cells from normal donors failed (Hersko et al, 1979; Abrams et al, 1980) most probably because an insufficient number of stem cells was collected and infused, as indicated by the CFU-GM content. Repeated leukaphereses are not only inconvenient to the subject but lead to significant thrombocytopenia (Korbling et al, 1980). On the other hand, with the high levels of circulating stem cells present in very early remission from ANLL, only a small number of leukaphereses are required to harvest sufficient cells as described in one report of leukapheresis in an ANLL patient (Juttner et al, 1982b). These cells can then be cryopreserved to be used for autologous stem cell rescue later at relapse. The use of autologous cells avoids graft-versus-host disease which remains a major cause of morbidity and mortality in allogeneic bone marrow transplantation.

There may be another important advantage in using cells harvested during very early remission. It is generally believed that the leukaemic process causes bone marrow failure not just by physical infiltration and replacement but also by direct inhibition of the normal haemopoietic cells through substances such as leukaemia-associated inhibitory activity (Broxmeyer et al, 1978b). Remission occurs when the number of leukaemic cells is reduced by induction chemotherapy, allowing normal cells to proliferate and replete the bone marrow. Since leukaemic cells are more sensitive to the cytotoxic injury, less

efficient in repairing sublethal injuries and proliferate more slowly (Arlin et al, 1978), normal cells most probably predominate during this period of intense haemopoietic regeneration. The low levels of PB CFU-GM in the two patients who achieved only partial remission lend support to this view. When the reduction in leukaemic cell number is less, the rebound proliferation is also less vigorous. If the number of contaminating leukaemic cells in the stem cell harvest is low, haemopoietic reconstitution using these autologous stem cells at relapse may lead to much longer second remissions than are seen with conventional autologous bone marrow transplantation using stem cells harvested later, in stable remission. If this therapeutic approach can be combined with effective in vitro processing methods to eliminate any contaminating leukaemic cells, longer remissions and possibly cure may result.

CHAPTER 6 : THE COLLECTION AND CRYOPRESERVATION OF

CIRCULATING HAEMOPOIETIC STEM CELLS

IN NORMAL SUBJECTS AND IN PATIENTS WITH

ACUTE NON-LYMPHOBLASTIC LEUKAEMIA

IN VERY EARLY REMISSION

INTRODUCTION

The previous Chapter showed that high levels of circulating CFU-GM regularly occurred in ANLL patients during very early remission phase after successful induction chemotherapy. If the increase in circulating CFU-GM is accompanied by a similar increase in pluripotent stem cells, sufficient pluripotent stem cells can be harvested from the peripheral blood with only a small number of leukaphereses for autologous haemopoietic reconstitution later.

The aims of this chapter are to investigate the use of continuous-flow leukapheresis to harvest peripheral blood mononuclear cells in normal subjects and in ANLL patients during very early remission and to study the cryopreservation of these circulating stem cells.

MATERIALS AND METHODS

1. Subjects

a. Normal subjects

Five healthy volunteers aged between 22 and 33 with normal peripheral blood counts were used.

b. ANLL patients

All patients under the age of 65 with newly diagnosed ANLL seen in the Haematology Unit, Royal Adelaide Hospital, Adelaide, S.A., Australia, between November 1982 and August 1983 and who entered remission were considered for inclusion in this study. Of the six eligible patients seen during that period only one patient was excluded because of gross obesity and poor venous access.

2. Continuous Flow Leukapheresis

Peripheral blood mononuclear cells were harvested using a continuous flow blood cell separator (see Chapter 2 Part 7). The actual leukapheresis usually took 1 1/2 hours. Complete blood counts and PB CFU-GM levels were measured immediately before and after leukapheresis. Counts on the collected cells were also performed.

One leukapheresis was performed on each of the five normal volunteers. For ANLL patients, three or four leukaphereses were performed over a period of three to five days at the time when the patient was entering remission. This corresponded to the time when the platelet count was increasing rapidly.

3. Cryopreservation

Cells collected were separated using a Ficoll-paque density gradient. The mononuclear cells obtained were cryopreserved in 10% DMSO, 15% autologous plasma and 25% HBSS. Cooling was then performed in a controlled rate freezer at 1°C per minute to -60°C and the ampoules were then stored at -196°C in liquid nitrogen (see Chapter 2 Part 8).

4. CFU-GM Assay

When fresh blood cells were assayed for CFU-GM, the full range of plating number were performed using HPCM as the source of CSA. Cryopreserved cells were assayed for CFU-GM by direct plating after thawing without washing steps using feeder layers as the source of CSA. Cells were plated at 1.25, 2.5, 5 and 10 x 10^5 cells per plate based on the cell counts prior to cryopreservation.

PB CFU-GM levels were expressed in colonies per ml of blood. In studies of CFU-GM recovery after cryopreservation, the number of colonies/10^6 total nucleated cells plated was used for the purpose of comparison.

5. The Collection and Cryopreservation of Bone Marrow Cells

Fourteen patients with ANLL in stable remission and 11 patients with malignant lymphomas without bone marrow involvement had bone marrow cells harvested and cryopreserved as part of an ongoing autologous BMT program (see Chapter 2 Part 9).

RESULTS

Continuous Flow Leukapheresis : Normal Subjects

There was no significant change in the levels of PB
CFU-GM measured before and after leukaphereses (127 and 144
CFU-GM/ml respectively, paired t test). The total number of
CFU-GM harvested was low at 0.33×10^4 CFU-GM/Kg body
weight. The decrease in haemoglobin concentration and
platelet count were statistically significant ($p < 0.005$ in
both instances, paired t test) but the changes in total
leucocytes, neutrophils, monocytes and lymphocytes were not
(Table 6.1). No bleeding or hypocalcaemic symptoms were
encountered.

Continuous Flow Leukapheresis : ANLL Patients

The clinical details of the five patient are shown
in Table 6.2. Three leukaphereses were performed on each of
four patients and four leukaphereses on one patient between
day 15 and day 29 after the completion of induction
chemotherapy. Very high levels of PB CFU-GM were found in
all patients during this period, ranging from 832 to 14520
CFU-GM/ml, mean 4150 CFU-GM/ml. There were again no
significant fall in the levels of PB CFU-GM after
leukapheresis (paired t test). The pre- and post-
leukapheresis PB CFU-GM levels in the first three patients
were shown in Fig 6.1.

The number of CFU-GM cryopreserved in each of the
five patients are shown in Table 6.3. Three or four
leukaphereses provide cells containing 15 to 60×10^4
CFU-GM/Kg body weight for cryopreservation.

TABLE 6.1

THE CHANGES IN PB CFU-GM LEVELS AND BLOOD COUNTS AFTER LEUKAPHERESIS IN FIVE NORMAL SUBJECTS

	Haemoglobin (gm/dl)	Leucocyte $(x10^3/\mu l)$	Neutrophil $(x10^3/\mu l)$	Lymphocyte $(x10^3/\mu l)$	Monocyte $(x10^3/\mu l)$	Platelet $(x10^3/\mu l)$	CFU-GM $(x10^3/\mu l)$
Pre-Leukapheresis	14.5 ± 0.5	4.9 ± 0.3	3.1 ± 0.2	1.4 ± 0.1	0.22 ± 0.02	188 ± 15	127 ± 31
Post-Leukapheresis	13.2 ± 0.5	4.8 ± 0.3	2.9 ± 0.1	1.5 ± 0.2	0.28 ± 0.05	169 ± 12	144 ± 46
P value (paired t test)*	< 0.005	n.s.	n.s.	n.s.	n.s.	< 0.005	n.s.

Results are expressed in Mean ± 1 SE of five leukaphereses

n.s. = not significant

*comparing the pre- and post- leukapheresis values.

TABLE 6.2

CLINICAL DETAILS OF THE FIVE ANLL PATIENTS WHO HAVE
UNDERGONE LEUKAPHERESIS

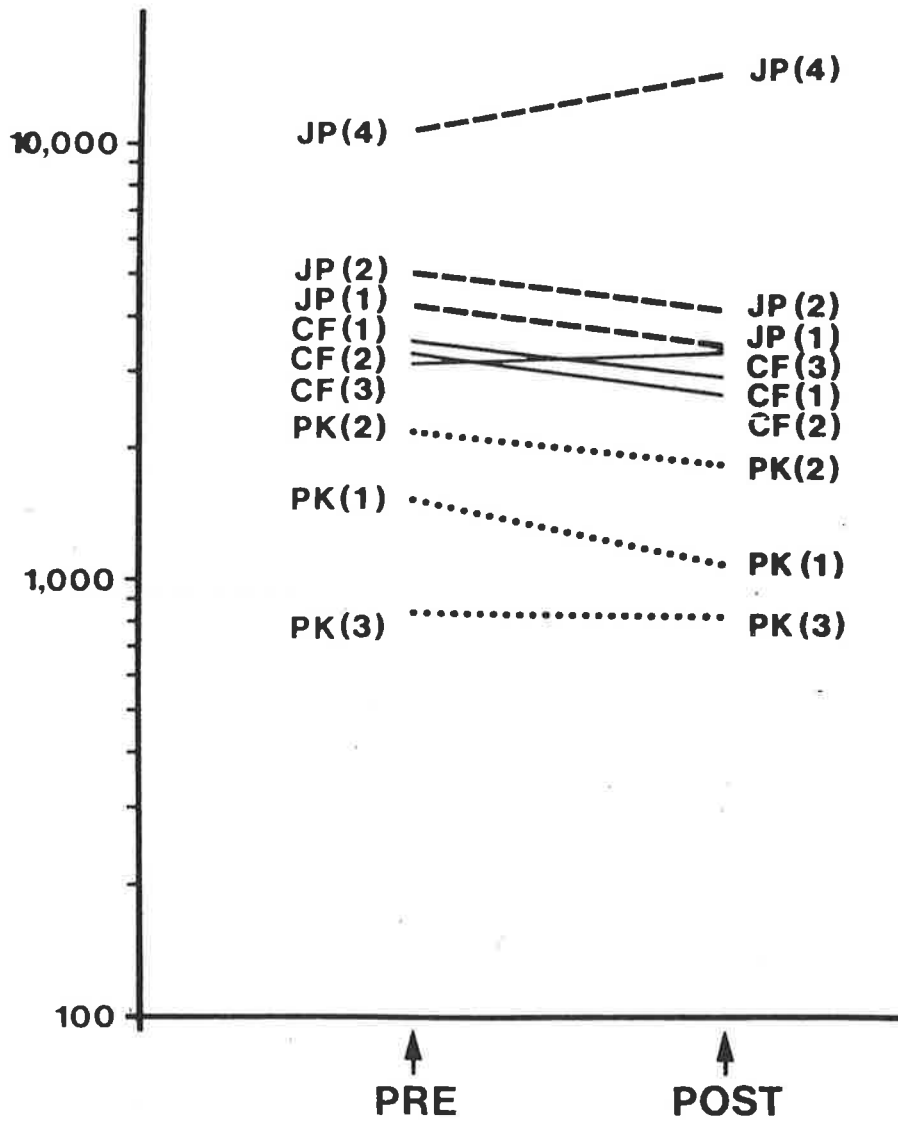| PATIENT	SEX/AGE	TYPE OF LEUKAEMIA*	MARROW FINDINGS AT DIAGNOSIS
PK	M/53	M2	Hypercellular, 75% blasts
JP	M/55	M2	Hypercellular, 67% blasts
CF	M/51	M2	Hypercellular, 38% blasts, with features of dysmyelopoiesis
MC	M/50	M5	Hypercellular, 95% blasts
KT	M/64	M2	Hypercellular, 47% blasts and marked fibrosis

*FAB Classification

Fig. 6.1 The changes in PB CFU-GM levels after a 1½ hour
continuous flow leukapheresis in 3 ANLL patients,
JP,CF and PK. Each line represents the change
in PB CFU-GM levels before and after one leukapheresis
run. The lines are identified by the patient's
initials followed by a number in parenthesis
indicating whether they were the first, second, third
or fourth leukapheresis run in that patient.

TABLE 6.3

COLLECTION OF HAEMOPOIETIC STEM CELLS BY LEUKAPHERESIS IN
ANLL PATIENTS DURING THE VERY EARLY PHASE OF REMISSION

Patient	Time of* Leukapheresis	Total Volume of cells (ml)	Number of Nucleated Cells Cryopreserved $(x10^8/Kg\ BW)$	Number of CFU-GM Cryopreserved $(x10^4/Kg\ BW)$
PK	Days 25,28,29	290	1.8	15
JP	Days 15-17,19	460	2.4	60
CF	Days 16-18	440	2.8	29
MC	Days 17-19	290	3.0	24
KT	Days 16,18,19	484	2.5	58

* Number of days after the completion of induction
  Chemotherapy.

Despite the large number of stem cells removed, levels of PB CFU-GM showed a steady increase over consecutive days in three patients and remained stable in the other two. There was a significant decrease in all mature blood cells except lymphocytes after each leukapheresis (paired t test). The pre- and post-leukapheresis blood counts in the first three patients were shown in Fig 6.2. The decrease in blood counts was most likely a result of the limited reserve of mature end cells at such time being unable to replace the large numbers of blood cells removed by leukapheresis (Table 6.4). However, the white cell and platelet counts increased over succeeding days, so the falls noted immediately after leukapheresis were temporary and leukaphereses did not interfere with the general pattern of recovery.

No undesirable side-effects were experienced and the last leukapheresis run in four of the five patients was performed after the patient was discharged. Two patients relapsed after 3 months (KT) and 5 months (MC) in remission while the other three patients were still in complete remission 13 months (CF), 14 months (JP) and 16 months (PK) following leukapheresis. Bone marrow cells have been harvested from these three patients during stable remission as a back-up source of stem cells.

Viability of PB CFU-GM on Long-term Storage

The viability of CFU-GM after cryopreservation in liquid nitrogen for various period of time is shown in Table 6.5. Cryopreserved CFU-GM from normal subjects remained viable up to ten months after storage. Studies on

Fig. 6.2 The changes in blood counts after a $1\frac{1}{2}$ hour leukapheresis in 3 ANLL patients, CF, JP, and PK. Each line represents the changes in one type of blood count before and after one leukapheresis run. The lines are identified by the patients' initials followed by a number indicating whether they are from the first, second, third or fourth leukapheresis run in that patient.
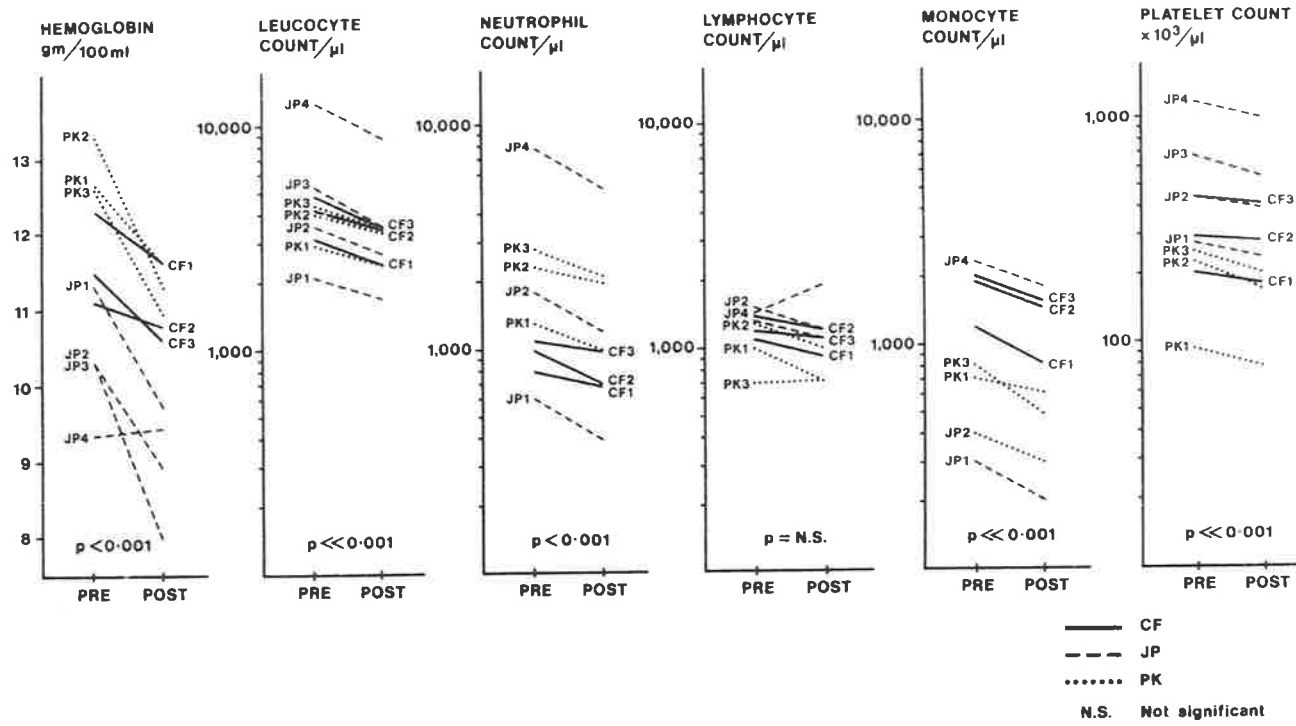
Fig. 6.2

TABLE 6.4

THE EXTRACTION EFFICIENCY OF LEUKAPHERESIS IN ANLL PATIENTS

	The Number of Cells Removed by Leukapheresis Expressed as the Equivalent Volume of Blood
Red Cells	158 ± 9 ml (6)
Neutrophils	1995 ± 234 ml (5)
Lymphocytes	2299 ± 187 ml (5)
Monocytes	3155 ± 275 ml (5)
Platelets	180 ± 24 ml (4)
CFU-GM	2266 ± 81 ml (10)

Results are expressed in mean ± 1 S.E.

The number in parenthesis represent the number of leukapheresis in which complete data are available.

TABLE 6.5

VIABILITY OF PB CFU-GM AFTER CRYOPRESERVATION

Duration of Storage	Normal Subjects	ANLL Patients
0 - 3 Months	84 ± 8% (4)	106 ± 17% (4)
4 - 7 Months	104 ± 19% (4)	94 ± 22% (4)
7 - 10 Months	101 ± 12% (4)	Not done

Results are expressed in mean ± 1 S.E. of the % CFU-GM recovery.

The numbers in parenthesis denote the number of tests performed.

cryopreserved cells from ANLL patients also showed that CFU-GM remained viable up to seven months after storage. While clumping of cells often occurred when cryopreserved bone marrow cells were thawed (unpublished observations), only minor clumpings occurred when these peripheral blood cells were thawed, most likely because of the much lower content of polymorphs.

Peripheral blood cells were harvested from another ANLL patient by discontinuous flow leukapheresis as a pilot study (Juttner et al, 1982b). Viability studies performed on the stored cells after 24 months in cryopreservation showed 96% CFU-GM recovery.

A Comparison of Stem Cell Collection by Leukapheresis and by Bone Marrow Aspiration

The number of CFU-GM collected by one leukapheresis run was compared to that collected by multiple bone marrow aspirations in lymphoma patients without bone marrow involvement and in ANLL patients in stable remission. This is shown in Table 6.6. Each leukapheresis yields almost twice the number of CFU-GM obtained by bone marrow aspiration. The CFU-GM:nucleated cell ratio is also much higher in cells collected by leukapheresis.

DISCUSSION

The high levels of PB CFU-GM in ANLL patients during the very early phase of remission provide a unique

TABLE 6.6

A COMPARISON OF THE COLLECTION OF HAEMOPOIETIC STEM CELLS
BY LEUKAPHERESIS AND BY BONE MARROW ASPIRATION

Subjects	Nucleated Cells $(x10^8$/Kg BW)*	CFU-GM $(x10^4$/Kg BW)*
Leukapheresis in Normal Subjects (n=5)	0.40 ± 0.05	0.33 ± 0.06
Leukapheresis in ANLL Patients in Early Remission (5 Patients, 16 Leukaphereses)	0.73 ± 0.1	11 ± 2
Bone Marrow Aspiration[†] in ANLL Patients in Stable Remission (n=14)	2.2 ± 0.2	6.5 ± 1.5
Bone Marrow Aspiration in Lymphoma Patients with Normal Marrow (n=11)	2.5 ± 0.3	6.7 ± 1.0

*   Results are expressed in mean ± 1 S.E.

[†]   Data kindly provided by Dr. C.A. Juttner
    and Mr. D.N. Haylock.

opportunity to collect and cryopreserve large numbers of haemopoietic stem cells from the peripheral blood. In contrast, leukapheresis in normal subjects results in the collection of low numbers of PB CFU-GM. The collection procedure was simple and caused no significant side-effects in the patients.

This study also shows that there was no significant loss of CFU-GM on long-term storage whether they were from normal subjects or from ANLL patients. Low CFU-GM viability after cryopreservation reported by Lasky et al (1982) may have resulted from the use of a HPCM stimulated assay system instead of a feeder layer stimulated system as the source of CSA was not specified. Other workers using feeder layer culture systems have not found a significant loss of CFU-GM after cryopreservation (Schlunk et al, 1981).

Because the proliferation of the pluripotent stem cell necessarily precedes the replenishment of the progenitor cell compartment, the maximum release of the pluripotent stem cell may not coincide with the maximum release of committed progenitor cells. Based on cell kinetics, it is probable that the pluripotent stem cell peak precede the CFU-GM peak by one or more days. However, high levels of PB CFU-GM persist for a number of days so there must be considerable overlap between pluripotent stem cell release and CFU-GM release. Stem cell collection was commenced as soon as haemopoietic recovery began and was performed over several days to minimise the possibility of missing the pluripotent stem cell peak. The levels of the pluripotent progenitor cell (CFU-GEMM) (Fauser and Messner, 1979; Ash et al, 1981) in peripheral blood may be a better

measure of the pluripotent stem cell.

The use of cells collected in very early remission for autologous haemopoietic reconstitution after supralethal chemoradiotherapy may give longer second remissions because of the low level of leukaemic contamination. In vitro processing of the cells collected to eliminate leukaemic blasts, while still in the developmental stage (Dicke et al, 1978; Sharkis et al, 1980; Herve et al, 1983; Ritz, 1983) may constitute an important element in this therapeutic approach.

Stem cells collected during the recovery phase after the last course of consolidation therapy may have even less leukaemic cell contamination. However, the rise in levels of circulating CFU-GM was not as marked during the recovery phase following consolidation therapy (see Chapter 5). This would render the collection of sufficient stem cells difficult.

Thus the collection of PB MNC by leukapheresis in the very early phase of remission appears to be a satisfactory method of harvesting stem cells. Adequate number of CFU-GM, and by inference, pluripotent stem cells can be harvested without discomfort or risk to the patient. Three or four leukaphereses yield four to fifteen times the amount usually required for haemopoietic reconstitution (Spitzer et al, 1980). Moreover, the cells collected by leukapheresis may have very low leukaemic cell contamination. There was no significant loss of CFU-GM after long-term cryopreservation.

The definitive proof of the efficacy of these stem cells will depend on clinical studies. Initially, the

peripheral blood stem cells will be used during leukaemic
relapse to demonstrate haemopoietic reconstitutive capacity
and to test whether long-lasting second remissions result.
If the initial studies are successful, the final objective
is to use these cells for autologous haemopoietic
reconstitution during first remission to produce
long-lasting remissions, and even cures, without most of the
risks of allogeneic BMT and with potential application to
the majority of ANLL patients who are ineligible for
allogeneic BMT because of age or lack of histocompatible
donors.

CHAPTER 7 : THE USE OF AUTOLOGOUS PERIPHERAL BLOOD CELLS
HARVESTED DURING VERY EARLY REMISSION FOR
HAEMOPOIETIC RECONSTITUTION IN PATIENTS WITH
ANLL

INTRODUCTION

Previous Chapters have demonstrated that high levels
of PB CFU-GM are found regularly in ANLL patients during
very early remission and large numbers of these cells can be
harvested by leukaphereses performed at that time. These
CFU-GM remain viable after two years of cryopreservation.
Two questions remain to be answered : firstly, are the high
levels of PB CFU-GM during very early remission accompanied
by high levels of pluripotent stem cells so that peripheral
blood cells harvested at that time could be used for stem
cell rescue after supralethal chemoradiotherapy to induce
remission when relapse occurs and secondly, are the
leukaemic contamination in these cells very low so that long
remissions may follow after haemopoietic reconstitution
using these cells. This chapter describes the use of these
cells for autologous stem cell rescue at relapse in two of
the five patients described in the last chapter. In the

first patient who relapsed after only three months remission, high dose melphalan was given because alkylating agents generally have a linear dose response curve so that increasing the dose should increase cell kill and high dose melphalan has no major toxicity other than prolonged myelosuppression (McElwain et al, 1978). In the second patient, supralethal chemoradiotherapy was used in an attempt to eradicate leukaemia in the patient.

CASE STUDY 1

KT, a 64 year old male, was hospitalised in July 1983 for the investigation of a pulmonary opacity found on chest radiography. During hospitalisation he was also found to be suffering from ANLL, M2 type.

He achieved remission after one course of DAT chemotherapy. Peripheral blood stem cell harvest was performed on days 16, 18 and 19 from the completion of chemotherapy. A total of 58×10^4 CFU-GM/Kg BW were cryopreserved. The start of consolidation therapy was delayed for two weeks until the area of pneumonic consolidation had resolved. After the first course of consolidation KT developed mycoplasma pneumonia and congestive cardiac failure. The second course of consolidation was therefore delayed for ten days and daunorubicin was omitted.

He suffered no complication after the second consolidation and the third consolidation was commenced on schedule. However, leukaemic blasts reappeared in the peripheral blood and the consolidation therapy was ceased

after 2 days. Bone marrow examination confirmed relapse with 81% blasts. Considering the rapid relapse while receiving consolidation chemotherapy and the poor cardiac reserve limiting the use of daunorubicin it was decided to attempt re-induction with high dose melphalan followed by autologous peripheral blood stem cell rescue.

High dose melphalan (200 mg/sq m) was given as an intravenous bolus while a forced alkaline diuresis was maintained. Eight hours after the completion of the melphalan infusion, half of his cryopreserved cells were thawed and infused intravenously, representing a dose of 29 x 10^4 CFU-GM/kg BW. The levels of PB CFU-GM was undetectable immediately before the infusion, then rose to 1544/ml at the finish of the infusion which took just under one hour. Five and twenty four hours afterwards, the levels were 507 CFU-GM/ml and 20 CFU-GM/ml respectively. Blasts disappeared from the peripheral blood after four days and severe cytopenia developed seven days after the high-dose melphalan infusion. Bone marrow examination showed marked marrow hypoplasia, although occasional normal myeloid and erythroid cells were present. On day 11, neutrophils started to appear in the peripheral blood, the platelet count started to rise and PB CFU-GM were present at 24 CFU-GM/ml.

By day 14, the blood counts were : Hb 8.6 gm/dl, WCC 1300/μl (neutrophils 47%, lymphocytes 38%, monocytes 10%) and platelets 24000/μl. CFU-GM assays showed that there were 23 CFU-GM/ml in the blood and 3 CFU-GM/10^5 nucleated cells in the marrow. Bone marrow biopsy showed numerous foci of haemopoietic cells against a background of

hypoplasia. Most of these foci were small, consisting of erythroid cells only, but a few contained more than one lineage of cells, e.g. erythroid-megakaryocyte, erythroid-myeloid. The larger foci were more pleomorphic in composition with all three lineages represented. On smears, both myelopoiesis and erythropoiesis appear active and normal. Myeloblasts constituted 3.5% of the cells present. Melphalan, however, was not very effective against leukaemic cells and they started to reappear less than three weeks after high dose melphalan and stem cell rescue. The serial changes in blood counts and levels of PB CFU-GM were shown in Fig 7.1. Further attempts to re-induce remission failed and the patient died of bilateral bronchopneumonia. No post mortem examination was performed.

CASE REPORT 2

MC, a 50 year old male, was diagnosed to have Acute Monoblastic Leukaemia (M5) when he developed symptoms of anaemia and easy bruising. He achieved remission with one course of DAT induction chemotherapy and cells containing 24 $\times$ 10^4 CFU-GM/kg BW were harvested by three leukaphereses. He received four courses of DAT consolidation chemotherapy, requiring red cell and platelet transfusions on several occasions because of cytopenia. At the time of the last consolidation chemotherapy, he began to notice progressive pain and paraesthesia radiating from his lower cervical region to the right shoulder. Later, similar radiating pain affected his left shoulder. The pain was not relieved by simple measures and the pain began to radiate further down
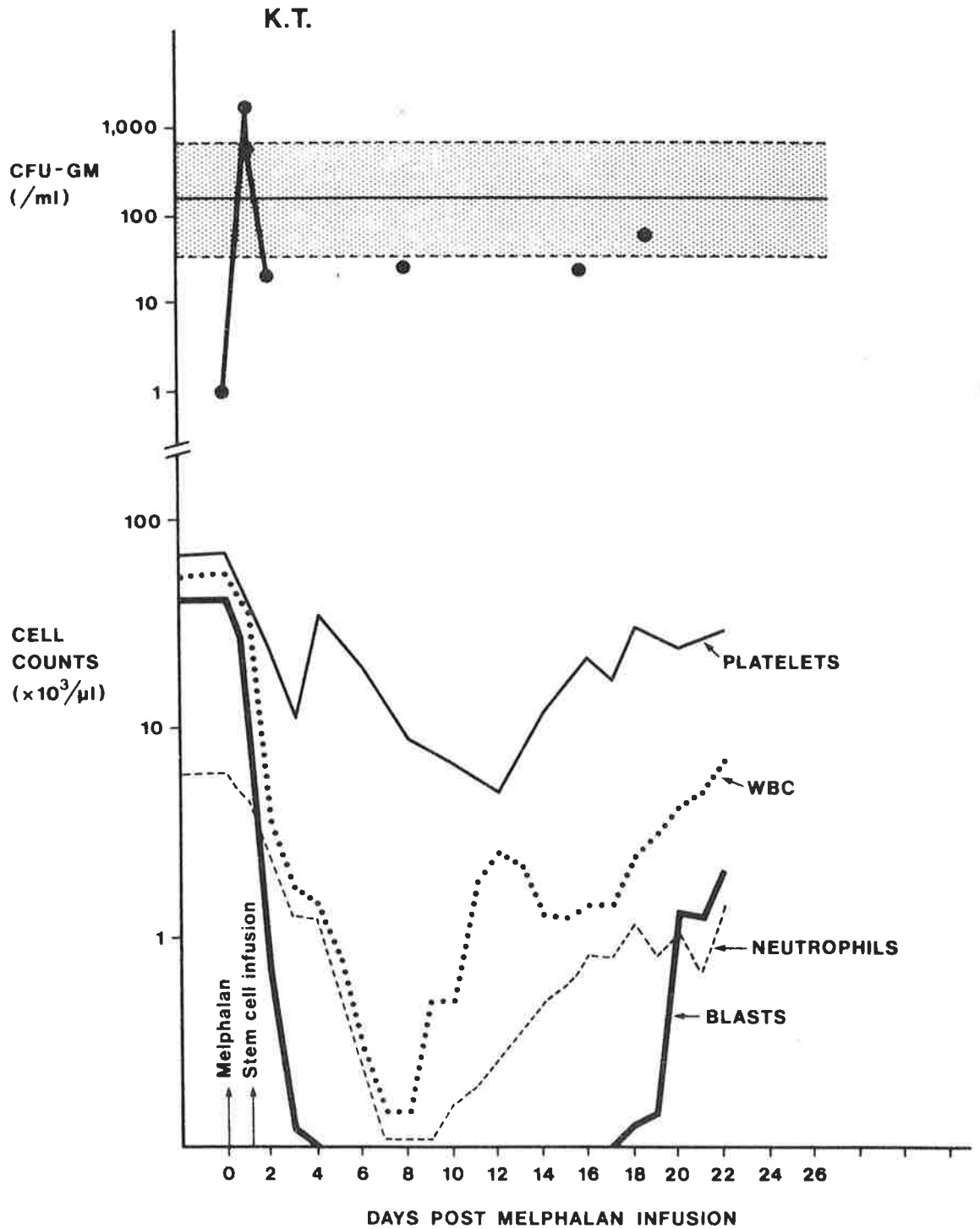
Fig. 7.1 The serial changes in PB CFU-GM levels and blood
counts in patient, K.T. who, at leukaemic relapse,
received high dose Melphalan followed by autologous
stem cell rescue using peripheral blood cells
harvested during very early remission. The shaded
area represents the mean and normal range for
PB CFU-GM.

both arms. Distal muscle weakness began to develop, worse on the right side. Four weeks after the fourth and the last course of consolidation chemotherapy, occasional blasts were noted in his peripheral blood although other blood counts were normal. PB CFU-GM were present at 151/ml, similar to the 158/ml detected one month earlier but large number of clusters were also present, even in cultures with no CSA added. Bone marrow examination showed normal cellularity but an increase in the percentage of monoblast to 20%. A similar leukaemic growth pattern was found when bone marrow cells were cultured. There were motor and sensory deficits in the C5, 6, 7 distribution bilaterally, but more severe on the right. Examination of the cerebro-spinal fluid (CSF) showed 220 blasts/μl, elevated protein concentration (1.4 gm/l, R 0.1-0.65) but normal glucose concentration (2.8 mmol/l, R 2.2-5.5). Thus, there was evidences for both systemic and central nervous system relapse .

His central nervous system leukaemia was treated by twice weekly intrathecal administration of Cytosine Arabinoside (Ara-C) 100 mg through an Omaya reservoir. Blast cells disappeared from the CSF after one dose and the pain and paraesthesia subsided after one week. After discussing with the patient the potential risk of supralethal chemoradiotherapy followed by autologous stem cell rescue, it was decided to proceed. The protocol is shown below :

Day -14   Ara-C 100 mg intrathecally

Day -11   Ara-C 100 mg intrathecally

Day -9   Ara-C 10 mg/kg intravenously over 24 hours

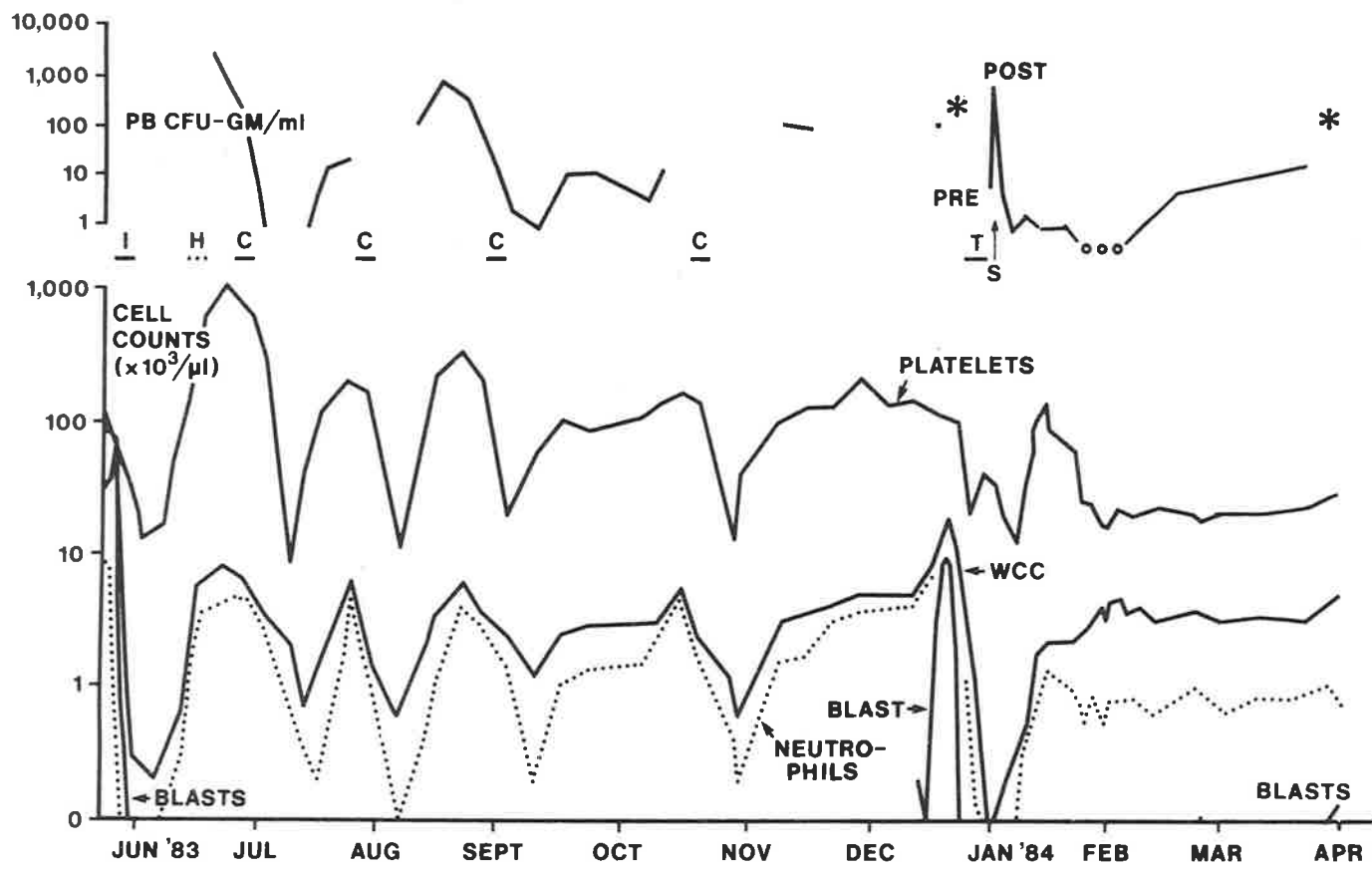Day -8   Ara-C 100 mg intrathecally

Ara-C 10 mg/kg intravenously over 24 hours

Daunorubicin 60 mg/m^2 intravenously

Day -7   Ara-C 10 mg/kg intravenously over 24 hours

Day -6   Cyclophosphamide 2.5 gm/m^2 intravenously

Day -5   Cyclophosphamide 2.5 gm/m^2 intravenously

Day -3 to Day -1

Total body irradiation 200 rads to the mid plane of the body every twelve hours delivered by a 10 MeV linear accelerator at a rate of 14 rads/min using right and left lateral fields. A total of 1200 rads was given in six fractions.

Day 0   Autologous stem cell rescue using cells collected during very early remission. An estimated 23 × 10^4 CFU-GM/kg BW were given intravenously over one hour.

The serial changes in PB CFU-GM levels and blood counts from the time of initial diagnosis are shown in Fig 7.2. At the time of stem cell infusion, no leukaemic blasts or CFU-GM could be detected in the patient's blood or bone marrow. PB CFU-GM levels rose to 768 CFU-GM/ml immediately post-infusion but fell to 4 CFU-GM/ml by day 3 and 1 CFU-GM/ml by day 6. On day 11, neutrophils started to appear in blood and the platelet count started to rise, as did the haemoglobin concentration. By day 16, Hb was 13.4 gm/dl, WCC 4,000/µl (neutrophil 1,400, lymphocyte 2,240, monocyte 360) and platelet count 154,000/µl. Bone marrow examination showed mild bone marrow hypoplasia but no evidence of leukaemia. The patient felt well and was

Fig. 7.2 The serial changes in PB CFU-GM levels and blood counts in patient M.C.
from diagnosis, remission induction, consolidation, first relapse,
re-induction followed by autologous stem cell rescue and second relapse.

10,000

1,000

100 — PB CFU-GM/ml

10

1

| I | H | C | | C | | C | | C |

POST

*****

PRE

T
S

CELL COUNTS (×10³/μl)

1,000

100 — **PLATELETS**

10 — ←WCC

1 — **BLAST→**

NEUTRO-PHILS

←BLASTS

0 — **BLASTS**

JUN '83 | JUL | AUG | SEPT | OCT | NOV | DEC | JAN '84 | FEB | MAR | APR

I — Induction chemotherapy

H — Harvest of circulating stem cells

C — Consolidation chemotherapy

T — Supralethal chemoradiotherapy

S — Stem cell rescue

***** — Leukaemic growth pattern

o — No. PB CFU-GM detectable

discharged home.

His blood counts, however, began to fall after day 16. PB CFU-GM levels were 1 CFU-GM/ml on day 17 and undetectable on day 20. By day 30, his Hb was 10 gm/dl, WCC 3,200/μl (neutrophils 540, lymphocytes 2,460, monocytes 120), platelets 18,000/μl and PB CFU-GM undetectable. Bone marrow examination showed marked bone marrow hypoplasia but still no evidence of leukaemia. His CSF remained clear of leukaemic blasts and the protein concentration had returned to normal although the distal weakness in both upper limbs had not improved. He did not have any bleeding tendency but required blood transfusions for symptoms of anaemia.

Eight weeks after stem cell rescue, his general condition remained stable, the neutrophil count had risen to 1,010/μl (24% of 4,200 white cells), the lymphocyte count was still normal and PB CFU-GM level had then risen to 13 CFU-GM/ml. However, the platelet count remained low at 19,000/μl and marked hypoplasia was still present on bone marrow examination. No leukaemic blast was found in blood or bone marrow.

Twelve weeks after stem cell rescue, he began to notice left retro-orbital discomfort and occasional blasts were present in his blood. However, his neutrophil count was still above 1,000/μl and the platelet count was 31,000/μl. Bone marrow examination showed a more cellular marrow with the re-appearance of normal myeloid, erythroid and megakaryocytic cells but the leukaemia had relapsed with 30% of the cells present being monoblasts. CFU-GM assays showed the same leukaemic pattern with numerous clusters in his blood and bone marrow. A partial left oculomotor nerve

palsy began to become evident but CSF examination did not detect any abnormalities and a computerised tomographic examination of the head did not reveal any mass lesion in or behind the left orbit. The patient was informed of the leukaemic relapse and the probable orbital or retro-orbital involvement but he declined any further treatment. The patient died at home three weeks later.

DISCUSSION

Experience with the use of high dose melphalan in patients with solid tumors has shown that haemopoietic recovery is unlikely to occur within three weeks without stem cell rescue (McElwain et al, 1979), so the temporal pattern of recovery observed in the first patient suggests engraftment of the infused stem cells. The morphological pattern of recovery with foci of haemopoietic cells in the marrow is also more similar to that seen after stem cell rescue than that seen after induction with chemotherapy. After successful DAT induction, the restoration of marrow cellularity occurred in a more diffuse, generalised manner and discrete foci of haemopoietic cells are seen uncommonly. In contrast, discrete foci of haemopoietic cells against a hypoplastic background have been described in the bone marrow of recipients of allogeneic BMT (Cline et al, 1977a). Animal models of supralethal irradiation followed by autologous stem cell rescue have also shown that the pattern of marrow recovery was related to the number of cells infused (Calvo et al, 1976). Marrow regeneration was focal when low numbers of cells were infused while general diffuse

regeneration occurred when higher numbers of cells were infused. DAT chemotherapy is not marrow-ablative and marrow recovery is mediated by the mobilization and proliferation of the residual stem cells the number of which, in all likelihood, far exceeds that infused in the stem cell rescue situation. Thus the pattern of marrow recovery observed in this case is more consistent with that seen with stem cell rescue after marrow ablative therapy rather than that due to residual endogenous stem cells. The early leukaemic regrowth, however, prevents any definite conclusion to be drawn.

The supralethal chemoradiotherapy used in the second patient (MC) was marrow ablative and any haemopoietic recovery could only be attributed to the infused cells. The first phase with early recovery starting on day 10, reaching a peak between day 14-16 before declining rapidly followed a time course remarkedly similar to that observed in the in vitro culture system. This phase is most probably a reflection of the proliferation and then exhaustion of oligopotent progenitor cells which possess little or no self-renewal capacity. Such a phase has not been described in patients receiving autologous and allogeneic BMT where haemopoietic recovery usually does not become evident until 17-21 days after bone marrow cell infusion. This difference may be due to the lower number of progenitor cells infused in BMT where cells containing an average of $4-6 \times 10^4$ CFU-GM/kg BW are usually given compared to the 23×10^4 CFU-GM/kg BW given in this patient. Thus, the number of progenitor cells given in BMT may not be sufficient to cause a noticeable peak. The second phase with low but persistent

haemopoietic activity indicates that stem cells with self-renewal capacity exist but either their number is too low or their proliferative capacity is limited, or both. Clinical experience with BMT using cryopreserved bone marrow cells has shown that pluripotent stem cells remain viable after cryopreservation, so the incomplete recovery in this patient suggests that there are considerably fewer pluripotent stem cells per CFU-GM in the peripheral blood cells harvested during very early remission compared to that in bone marrow cells collected in stable remission. This is in contrast to the finding in animals as reported by Abrams et al (1981) but suggests that the progenitor compartments are more expanded than the pluripotent stem cell compartment in the peripheral blood of ANLL patients during recovery from bone marrow depression. To achieve a more satisfactory haemopoietic recovery, stem cell rescue should be attempted with more stem cells. Two of the remaining patients have cells containing 29 and 60 × 10^4 CFU-GM/kg cryopreserved so the haemopoietic recovery with a higher cell dose may be studied. Furthermore, as leukaphereses were well tolerated, more cells can be collected in future by more and longer leukaphereses and by starting as early as possible because the rise in pluripotent stem cells may precede the rise in oligopotent progenitor cells. The levels of CFU-GEMM may identify the period of maximal availability of pluripotent stem cells. The CFU-GEMM assay in man, however, has not been fully established so no reliable quantitative information of the serial changes of CFU-GEMM in very early remission is yet available.

There were evidences that the stem cell graft

function was starting to improve after 8 weeks with the rises in PB CFU-GM levels and neutrophil count. By the twelveth week, the platelet count had also risen and normal haemopoietic cells were identifiable in the bone marrow. Such a delayed recovery has been observed in patients receiving autologous BMT (Ritz et al, 1983; Juttner, unpublished data) but the biological explanation is still not known. The relapse of leukaemia, however, made it impossible to know whether the recovery would continue.

Thus peripheral blood cells collected during very early remission do possess haemopoietic reconstitutive activity but the pluripotent stem cell:CFU-GM ratio appears to be much lower than that in bone marrow cells during stable remission. The minimum CFU-GM cell dose required has to await further clinical studies.

The relapse of leukaemia 3 months after supralethal chemoradiotherapy and stem cell rescue could have arisen either because of the failure of the chemoradiotherapy to eradicate all leukaemic cells in the patient or because of the leukaemic cells in the infused cells, or both. The short first remission duration with simultaneous central nervous system and systemic relapse is consistent with the more aggressive course in Acute Monoblastic Leukaemia as noted by some workers (Weinstein et al, 1983) and supralethal chemoradiotherapy are less effective when given at relapse (Blume et al, 1981) so it is quite probable that there were residual leukaemic cells in the patient. Whether leukaemic cells in the infused cells contributed to the relapse is more difficult to determine but the pattern of early extra-marrow involvement at second relapse tends to

support that the relapse is due to residual disease.

The second question posed at the beginning of this Chapter, whether these cells harvested in very early remission contain fewer leukaemic cells than bone marrow cells harvested during stable remission, is yet to be answered. Since no maintenance treatment is used, the duration of chemotherapy-induced remissions probably reflect the residual leukaemic cell load at the finish of induction-consolidation therapy. Similarly, the duration of remission after supralethal chemoradiotherapy and autologous stem cell rescue probably depends on the efficacy of the chemoradiotherapy in eradicating leukaemic cells in the patient and the degree of leukaemic contamination in the infused cells. Experience with allogeneic BMT in first relapse or second remission shows that 30-40% of patients become long-term survivors (Blume et al, 1981). In other words, 60-70% of patients given autologous circulating stem cell rescue would relapse due to residual leukaemic cells in the body, irrespective of the leukaemic contamination in the infused cells. It is in the other 30-40% of patients that the durations of second remission would serve as an indicator of the leukaemic contamination in the infused cells. Thus it is necessary to study the durations of remission after this form of stem cell rescue in a large number of patients before a reliable conclusion can be drawn about the question of leukaemic contamination. After conventional chemotherapy second remissions are almost always shorter than the first. If the remission duration after this form of stem cell rescue is consistently shorter than the first remission, the postulation of low leukaemic

contamination can be considered disproved. On the other hand, if this remission duration is longer than the first, the use of cells collected in very early remission may represent a real therapeutic advance for the majority of ANLL patients who are not eligible for allogeneic BMT.

In the future, leukaemic contamination may be further reduced by in vitro processing of the harvested cells to identify and eradicate contaminating leukaemic cells. One of the approaches depends on the immunological recognition of leukaemia-specific antigens. Recently, both heterologous and hybridoma antibodies (Netzel et al, 1978; Ritz et al, 1980) have been raised against the Common Acute Lymphocytic Leukaemia Antigen (CALLA). The use of such antibodies to eliminate contaminating leukaemic blasts in autologous bone marrow cells has led to some long-lasting remissions although there is no significant difference compared to the control group who received untreated cells (Ritz et al, 1982). To date, no specific markers for ANLL have yet been found. Antibodies active against ANLL blasts tend to cross-react with normal myeloid cells (Strauss et al, 1983) and hence are unsuitable for use in in vitro purging.

Other workers have employed physical methods using density gradient separation (Dicke et al, 1978) and pharmacological methods using 4-Hydroperoxycyclophosphamide (Sharkis et al, 1980) to eradicate contaminating leukaemic blasts but such approaches are not leukaemia-specific and do not seem promising.

Another potentially useful approach exploits the fact that the different biological behaviour of normal and

leukaemic cells is a result of their different patterns of metabolism. Metabolism is governed by the protein composition of the cell which is in turn a reflection of the mRNAs which encode for proteins. Thus the mRNA pattern in leukaemic cells should be different from that of normal cells. With recombinant DNA technology, such differences have been explored and confirmed. Birnie et al (1983) reported a specific RNA sequence which is 10 to 50 times more abundant in chronic granulocytic leukaemia than in chronic lymphocytic leukaemia and normal cells. If specific mRNA patterns can be identified in ANLL blasts, it would be possible to detect even small numbers of contaminating leukaemic cells. Since total cellular mRNA is used as the substrate, this approach is likely to be more sensitive than merely looking for surface membrane characteristics as in heterologous or monoclonal antibody techniques. The amino-acid composition can further be deduced from such sequences and the corresponding peptide chain synthesised. The synthetic peptide may then be used for the production of anti-leukaemic antibodies of both heterologous and hybridoma types. Similar approaches have led to synthetic vaccines produced against the virus of foot-and-month disease (Bittle et al, 1982) and it should be feasible to apply this approach to leukaemias.

It is also important to ensure that the processing does not damage the pluripotent stem cells. While CFU-GM viability is the most commonly used measure of stem cell viability, the CFU-GEMM assay when fully established may be a better measure of the pluripotent stem cell than the CFU-GM assay.

The case studies in this Chapter show that cells harvested from the peripheral blood of ANLL patients during very early remission possess haemopoietic reconstitutive capacity but that they probably contain fewer pluripotent stem cell per CFU-GM than bone marrow cells collected at stable remission. The minimum number of cells which can achieve complete haemopoietic reconstitution and the question of leukaemic contamination may be answered by continuing clinical studies.

CHAPTER 8 : DISCUSSION

This thesis describes the development of an accurate in vitro assay for PB CFU-GM, studies of PB CFU-GM in normal subjects and ANLL patients and the result of the use of circulating haemopoietic stem cells for haemopoietic reconstitution.

Kurland et al (1978a), using a bone marrow CFU-GM assay system, found that monocytes exert both stimulatory and inhibitory effect on the in vitro proliferation of CFU-GM depending on the number of monocytes present. However, the number of monocytes that inhibits CFU-GM growth, of the order of 10^5 monocytes per plate, is not present when bone marrow cells are assayed. Thus monocytes do not exert a significant modulatory influence in the bone marrow assay system. By contrast, such numbers of monocytes are regularly present in the PB CFU-GM assay system when 5 or 10×10^5 PB MNC are cultured per plate, so significant inhibition may result and the levels of PB CFU-GM detected may not reflect the number present (To et al, 1983a). However, most published studies on PB CFU-GM were performed using such high plating numbers (Lohrmann et al, 1978; Standen et al, 1979; Beran et al, 1980; Goldberg et al,

1980; Verma et al, 1980; Jehn et al, 1983; Peschel et al, 1983) and therefore may be inaccurate. The studies in Chapter 3 Part 1 demonstrate for the first time that there is a critical number of monocytes in the PB CFU-GM assay system above and below which CFU-GM growth falls. The highest CFU-GM growth occurred at 1.25 or 2.5×10^5 cells per plate in most subjects, but in some it occurred at the other plating numbers. Hence it is necessary to assay PB MNC at several concentrations from 0.625 to 10×10^5 PB MNC/plate in order to measure PB CFU-GM accurately. This variability in the optimal plating number may be caused by fluctuations in the monocyte population in vivo, or the effect of in vitro manipulations during culture, or both. This plating number effect was found in assays performed in normal subjects as well as in patients with a variety of haematological diseases with or without bone marrow involvement including both Acute Lymphoblastic and Non-lymphoblastic leukaemias in remission, Myelofibrosis, Hodgkin's Disease and drug induced agranulocytosis during the recovery phase.

The range of PB CFU-GM was wide in the 34 normal subjects studied (Chapter 4). The levels were significantly higher in males as found by Barrett et al (1979), so different means and ranges (95% confidence limit) were necessary : 198 CFU-GM/ml (46 - 855) for males and 76 (14 - 411) for females. The levels followed a log-normal distribution, so calculations were performed using log-transformed data. The differences between the normal ranges in most published reports can be explained by the plating numbers employed in the studies (Richman et al,

1976; Lohrmann et al, 1978; Standen et al, 1979; Beran et al, 1980; Goldberg et al, 1980; Verma et al, 1980; Jehn et al, 1983; Peschel et al, 1983). These reports underestimate the levels of PB CFU-GM because the assays were performed using only one or two plating numbers. The results in this study are similar to those in the past that found moderate fluctuations in the same subject studied at different times (Barrett et al, 1979; Kreutzmann and Fliedner, 1979; Ponassi et al, 1979) and the presence of a mobilisable extravascular pool (Cline et al, 1977; Barrett et al, 1978). While the physiological basis of the fluctuation in the same subject studied at different times remains unclear, this may in part be due to the rapid shifts that can occur between the circulating and the mobilisable CFU-GM pools. The occurrence of such rapid shifts also makes it difficult to determine whether PB CFU-GM levels undergo any cyclical changes as suggested by Barrett et al (1979) and Kreutzmann and Fliedner (1979).

Weiner et al (1981) showed that several monocyte functions were affected by the freeze-thaw process and Schlunk et al (1981) suggested that cryopreserved monocytes may be less effective in processing HPCM but whether the monocyte/CFU-GM interaction is altered after cryopreservation has not been answered. Studies in Chapter 3 Part 2 showed that when cryopreserved PB MNC were cultured, the highest CFU-GM growth usually occurred at 5 or 10×10^5 cells per plate. Furthermore, the addition of cryopreserved monocytes to cryopreserved PB MNC did not cause any decrease in colony growth but the addition of monocytes from fresh blood did. Thus the difference in the monocyte effect

appears to be a result of injury to monocytes occurring during the freeze-thaw process. Feeder layers have been shown to be better than HPCM as a source of CSA when cryopreserved bone marrow cells were cultured (Schlunk et al, 1981). The present study showed the same difference when cryopreserved PB MNC were cultured.

The PB CFU-GM assay developed in this thesis may thus be used to study perturbations in different disease states but the necessary two weeks' delay for a result limits the routine, diagnostic application of this assay system where information is required quickly, e.g., assessing severity of marrow suppression or where the clinical situation is changing quickly, e.g., monitoring the recovery from drug induced agranulocytosis. Thus the PB CFU-GM assay is most suitable for research or specific project applications.

High levels of PB CFU-GM have been found in patients with solid tumours during the recovery phase after cytotoxic-induced marrow depression (Richman et al, 1976; Lohrmann et al, 1979; Stiff et al, 1983) and preliminary studies in ANLL patients suggested that high levels were also present during very early remission (Juttner et al, 1982a). The harvesting of circulating haemopoietic stem cells during such time for later autologous stem cell rescue has been suggested for the treatment of solid tumours (Barr and McBride, 1982; Zwaan 1982)) but not for the treatment of ANLL. In ANLL, current chemotherapy cures only 10-20% of patients. Allogeneic BMT in first remission may cure up to 70% of selected patients but most patients are not eligible either because no histocompatible donors are available or

because they are too old and would be at a high risk of developing Graft versus Host Disease. Autologous BMT using bone marrow cells collected during stable remission avoids the risks associated with the use of allogeneic cells but a proportion of patients relapse before bone marrow cells can be harvested. Even when bone marrow cells could be harvested during stable remission, the cells are most probably contaminated with leukaemic cells that could lead to relapse when reinfused. On the other hand, if circulating stem cells harvested during early remission possess adequate haemopoietic reconstitutive capacity, all patients going into remission (which includes 70% or more of those receiving standard chemotherapy) can have their cells stored and thus be eligible for autologous stem cell rescue after supralethal chemoradiotherapy at the time of relapse. Since normal cells probably predominate during the recovery phase, the leukaemic contamination of these cells is probably low and longer lasting remissions may follow the use of such cells. No systematic studies of PB CFU-GM levels or their harvesting during very early remission in ANLL have previously been described.

In 13 patients with ANLL entering remission, high levels of PB CFU-GM were found during very early remission, i.e., 15 to 29 days after the completion of induction chemotherapy. No such increase was found in another two patients who did not enter complete remission (Chapter 5). Such high levels have not previously been reported because they are present for only a few days, so PB CFU-GM assay performed outside those few days would have missed the peak. Furthermore, assays performed with only one or two plating

numbers may also underestimate the levels. The high levels most probably reflect the intense proliferative activity of the recovering normal haemopoietic cells after significant reduction of leukaemic cell-load, and may have arisen from the mobilisation of stem cells from extra-marrow sites. The normal or low levels found during stable remission and the abnormal growth patterns found in relapse confirmed previous reports (Moore et al, 1974, Vincent et al, 1977; Beran et al, 1979; Jehn et al, 1983; Peschel et al, 1983).

It was found that cells containing large numbers of circulating CFU-GM could be harvested by three or four continuous flow leukaphereses during very early remission of ANLL at the time when the platelet count was rising rapidly (Chapter 6). In the five patients studied, a mean of 37 x 10^4 CFU-GM/kg body weight were harvested by three or four leukaphereses. The yield of CFU-GM was five times that obtained by bone marrow aspiration under general anaesthesia. There were no major side-effects and no delays in haemopoietic recovery. These harvested cells were cryopreserved with DMSO in liquid nitrogen. There was no loss of stem cells on storage as measured by the viability of the CFU-GM from an ANLL patient for more than two years and from normal subjects for more than eleven months since storage. The reported 40% loss of CFU-GM on cryopreserved PB MNC collected from normal subjects (Lasky et al, 1982) is probably related to the use of a suboptimal assay depending on HPCM instead of feeder layers as the source of CSA, leading to an underestimate of CFU-GM in the cryopreserved cells.

Clinical studies are necessary to resolve the two

questions of haemopoietic reconstitutive capacity and leukaemic contamination. Haemopoietic reconstitution using circulating stem cells have been achieved in mice (Micklem et al, 1975; Cherktov et al, 1982), dogs (Nothdurft et al, 1977) and baboons (Storb et al, 1977) but was unsuccessful in man (Hersko et al, 1979; Abrams et al, 1980). In the present study, two of the five ANLL patients who have had circulating stem cells harvested and stored have relapsed (Chapter 7). The first patient (KT) relapsed after three months while still receiving consolidation chemotherapy. He was given high dose melphalan chemotherapy followed by autologous stem cell rescue using the stored cells. Haemopoietic recovery started 11 days after stem cell infusion, much earlier than the three to four weeks seen in other patients treated with such high doses of melphalan but not given stem cell rescue. However, leukaemic cells regrew quickly so no definite conclusion about haemopoietic reconstitution can be drawn. The second patient (MC) relapsed after five months before his bone marrow cells could be stored. He was given autologous peripheral blood cells containing 23×10^4 CFU-GM/kg BW following supralethal chemoradiotherapy. This number of CFU-GM represents six times the number of CFU-GM usually given in allogeneic BMT (Spitzer et al, 1980). Early haemopoietic recovery was again observed, starting on the 10th day following stem cell infusion. The haemopoietic activity, however, was not sustained and blood counts started to fall from day 17 onwards. By 4 weeks, the neutrophil count had fallen to 800/μl and the platelet count had fallen to below 20,000/μl. By 8 weeks, the neutrophil count and PB CFU-GM

levels started to improve. By 13 weeks, there was further
improvement in the stem cell graft function with a rise in
platelet count and an increase in normal haemopoietic cells
in the bone marrow but the leukaemia had also relapsed.

The biphasic course of haemopoietic recovery in
patient MC is of considerable biological interest. The
first phase of early recovery followed by rapid decline is
most likely a reflection of the proliferation and then
exhaustion of the large number of progenitor cells which
have little or no self-renewal capacity. The second phase
with low but persistent haemopoietic activity indicates that
stem cells with self-renewal capacity were present in the
peripheral blood at very early remission but there were
probably considerably fewer pluripotent stem cells per
CFU-GM compared to that in bone marrow cells collected in
stable remission. This is in contrast to the finding in
animals as reported by Abrams et al (1981) and suggests that
the progenitor cell compartments are more expanded than the
pluripotent stem cell compartment in the peripheral blood of
ANLL patients during recovery from bone marrow depression.
The use of more stem cells may achieve a more satisfactory
haemopoietic recovery. As leukaphereses were well
tolerated, more cells can be collected by starting earlier
and perform more and longer leukaphereses. In future, the
levels of CFU-GEMM may identify the period of maximal
availability of pluripotent stem cells.

The second question of whether these cells harvested
at very early remission contain fewer leukaemic cells is yet
to be answered. This can only be answered by comparing the
durations of remission using this form of stem cell rescue

in a large number of patients with the second remission durations after conventional chemotherapy. After conventional chemotherapy second remissions are almost always shorter than the first. If the remission duration after this form of stem cell rescue is longer than the first, the use of cells collected in very early remission may represent a real therapeutic advance for the majority of ANLL patients who are not eligible for allogeneic BMT.

In the future in vitro processing of the harvested cells may reduce the leukaemic contamination even further. Different approaches based on immunological recognition, recombinant DNA technology, physical separation and pharmacological purging are being developed.

The studies in this thesis demonstrate that to measure PB CFU-GM accurately, it is necessary to culture PB MNC using a range of plating numbers in order to allow for the monocyte effect. PB CFU-GM studies in normal subjects showed that levels are higher in males than in females, both short-term and long-term variations occur and a mobilisable pool exists. It was also shown that very high levels of PB CFU-GM occur regularly in ANLL patients during very early remission and peripheral blood cells containing large numbers of CFU-GM can be collected by a small number of leukaphereses during this phase. Autologous stem cell rescue using these stored cells after supralethal chemoradiotherapy at relapse showed that these cells do possess haemopoietic reconstitutive capacity. The minimum number of cells required to achieve complete haemopoietic reconstitution and the question of leukaemic contamination may be answered by continuing clinical studies.

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