INVESTIGATION OF THE EFFECTS OF *IN VITRO* CYTOKINE EXPOSURE ON SHORT AND LONG TERM RECONSTITUTING HAEMOPOIETIC STEM AND PROGENITOR CELLS IN A MURINE MODEL

by

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DEDICATION

This thesis is dedicated to my parents, Mary and Trevor, and to Andy.

DECLARATION

Unless otherwise stated, I declare that the work presented in this thesis was my own.

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List of Abbreviations

ABMT	autologous bone marrow transplantation
ARA-C	cytosine arabinoside
BFU-E	burst forming unit-erythroid
BMT	bone marrow transplantation
CAFC	cobblestone area forming cell
CCE	counterflow centrifugal elutriation
CFC	colony forming cell
CFU	colony forming unit
CFU-A	Colony forming unit-type A
CFU-GEMM	colony forming unit-granulocyte-erythroid-macrophage-megakaryocyte
CFU-GM	colony forming unit-granulocyte-macrophage
CFU-mix	colony forming unit-mix
CFU-S	colony forming unit-spleen
CHO	Chinese hamster ovary
CLL	chronic lymphocytic leukaemia
CM	conditioned medium
CRA	competitive repopulation assay
CSF	colony stimulating factor
DHS	Donor horse serum
Epo	erythropoietin
FACS	fluorescence activated cell sorting
FBC	full blood count
FCS	fetal calf serum
FISH	fluorescence in situ hybridisation
5-FU	5-fluorouracil
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
HPP-CFC	high proliferative potential-colony forming cell
HSC	haemopoietic stem cell
IL	interleukin
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
LTBMC	long term bone marrow culture
LTCIC	long term culture initiating cell
LTRC	long term repopulating cell
MCP-1	monocyte chemotactic factor-1
M-CSF	macrophage-colony stimulating factor
MIP-1α	macrophage inflammatory protein-1α
MRA	marrow repopulating ability
OSM	oncostatin M
PBPC	peripheral blood progenitor cell
PDGF	platelet derived growth factor
PF4	platelet factor 4
PHA	phytohaemagglutinin
RBC	red blood cell
SCA-1	stem cell antigen-1
SCF	stem cell factor
SEM	standard error of the mean
TBI	total body irradiation
TGF-β	transforming growth factor-β
TNF	tumour necrosis factor
WBC	white blood count

ABSTRACT

There is increasing interest in the possibility of expanding haemopoietic stem and progenitor cells *ex vivo*. Successful expansion of pluripotent haemopoietic stem cells would extend the use of high dose chemotherapy with autologous rescue to include those patients for whom we are currently unable to harvest sufficient stem cells for transplantation. It is likely that the efficiency of gene transduction would be significantly enhanced under conditions in which stem cells were proliferating. For cancer treatments, using high dose but non-myeloablative chemotherapy, progenitor cell expansion should reduce harvesting requirements and, thereby, tumour cell contamination. Finally, the primary aim of these studies was to expand, *ex vivo*, those progenitors cells thought to mediate the early phase of engraftment in order to reduce the duration of both neutropenia and thrombocytopenia following bone marrow transplantation (BMT).

We have attempted to determine optimum *ex vivo* culture conditions which allow maximum amplification of murine transient engrafting stem cells. The short term (6 days) incubation of unfractionated bone marrow in liquid culture with Stem Cell Factor (SCF), Interleukin-11 (IL-11), with or without Macrophage Inflammatory Protein-1 alpha (MIP-1α) produced a 50 fold amplification of those cells which were shown to rescue lethally irradiated recipients in a BMT model. Following *ex vivo* expansion, ten to twenty fold fewer cells were required to rescue lethally irradiated mice. When transplanted in cell doses above threshold for engraftment, bone marrow cells expanded *ex vivo* resulted in significantly more rapid haemopoietic recovery. Thus, we were able to demonstrate expansion of progenitor cells *ex vivo* and to assess the engraftment potential of the expanded cells. The results suggested two potential benefits for patients with cancer if similar data could be obtained with human haemopoietic cells.

Although we had demonstrated expansion of progenitor cells, it was equally important to assess the effect that *ex vivo* culture had on those stem cells responsible for long term reconstitution following BMT. In a serial transplantation model, unmanipulated bone marrow was only able to consistently sustain secondary BMT recipients but bone marrow expanded *ex vivo* sustained quaternary BMT recipients which remained alive and well more than 120 days after BMT. These findings have important implications for transplantation and gene transfer studies since expansion of clonogenic cells accompanied by maintenance of long term reconstituting stem cells will result, not only in improved early engraftment, but also in sustained long-term reconstitution following transplantation and may result in enhanced transduction efficiencies with genes of interest.

Following tertiary and quaternary BMT many of the recipients of expanded cells developed B-cell chronic lymphocytic leukaemia (B-CLL). The group transplanted with SCF / IL-11 / MIP-1α expanded marrow developed leukaemia earlier and with a greater frequency that those transplanted with SCF / IL-11 expanded cells. The leukaemia was shown to have arisen in host haemopoietic cells rather than in the donor bone marrow which had been expanded *ex vivo*. The mechanism underlying the development of these leukaemias is not clear and will be the subject of future study within our group.

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INTRODUCTION

Chapter 1

1.1 Haemopoiesis

In mammalian development, HSC sequentially occupy the embryonic yolk sac, fetal liver, spleen and finally the adult bone marrow (Zon, 1995). In the bone marrow, haemopoiesis appears to be a complex cellular and biological process which is carefully controlled and rarely gives rise to disease. The regulation of haemopoiesis is dependent upon interactions between the bone marrow microenvironment, HSC, progenitor cells and an array of both stimulatory and inhibitory factors.

The system can be arbitrarily divided into several types of cell population: pluripotent stem cells, multipotent progenitors, committed progenitors and maturing cells. The hierarchy of these cell subsets is established on the basis of decreasing ability to generate new stem cells (self-renewal), decreasing proliferative potential and increasing lineage commitment.

The production and maturation of blood cells of the eight major lineages is a continuous process, largely controlled by specific glycoprotein regulators. These cytokines, or growth factors, promote the survival and proliferation of stem and progenitor cells and in their absence the cells undergo apoptosis and die (Ogawa, 1993; Sachs & Lotem, 1994). Under steady state conditions total blood cell production to maintain homeostasis in humans is approximately 350-400 x 10^9 cells per day (Moore, 1995) and this can be amplified up to ten fold under conditions of stress. Although cytokines appear to play a crucial role in cell survival, proliferation and differentiation, regulation of haemopoiesis is dependent also on the interaction of the

haemopoietic stem and progenitor cells with the cells making up the bone marrow microenvironment.

1.2 The bone marrow microenvironment, long term bone marrow culture (LTBMC) and the role of stroma

HSC are found in close contact with stromal cells in the haemopoietic microenvironment, both *in vitro* and *in vivo*. Most knowledge regarding stromal cell function has been derived from *in vitro* systems, in particular the LTBMC (Dexter *et al.*, 1977). The basis of the LTBMC assay is the development of a complex marrow stromal cell layer containing fibroblasts, adipocytes, endothelial cells and macrophages among which HSC and progenitor cells reside.

Stromal cell lines cloned from murine LTBMC have proven invaluable in revealing the role of locally produced cytokines and adhesion molecules in the regulation of haemopoiesis. In LTBMC the more mature precursors and terminally differentiated cells are found in the supernatant fluid and are termed "non-adherent". Haemopoietic stem cells and immature precursor cells adhere tightly to the stroma and are termed "adherent" (Coulombel *et al.*, 1983). The adhesion molecule VLA-4 plays a major role in the interaction between HSC and stroma by binding to VCAM-1 (Simmons *et al.*, 1992; Williams *et al.*, 1991). Monoclonal antibodies which prevent this binding have been shown to release HSC from the stroma both *in vitro* and *in vivo* (Simmons *et al.*, 1992; Williams *et al.*, 1991). VLA-4 is not the sole mechanism by which HSC adhere to stroma and other interactions involve cytokine receptors on HSC which bind to membrane or extracellular matrix associated factors, such as SCF (Williams & Majumdar, 1994).

It is well established that a proportion of murine stem cells can persist for several weeks without differentiation in LTBMC (Fraser *et al.*, 1992) and a variety of cloned stromal cell lines support HSC to a varying degree. The

S17 line appeared more efficient, even than primary murine stromal layers, in supporting HSC and HSC cultured on S17 competed efficiently with freshly isolated marrow in the competitive repopulation assay (CRA) (Wineman *et al.*, 1993) (see later for explanation of assay systems). However, this study also showed that the majority of stromal cell lines failed to support HSC which retained the capacity to compete with fresh marrow in the CRA, therefore, stromal cells capable of supporting primitive stem cells may be infrequent in bone marrow or may lose this capacity on culturing *in vitro*.

Whether support is mediated by the stromal cells themselves or by secreted molecules, remains unclear. Those stromal layers which support murine haemopoiesis, in general, also support human HSC and, therefore, the mechanism responsible for HSC support may not be species specific. Since many early acting cytokines are structurally conserved between species, but later acting cytokines are often species specific, this suggests a possible role for some cytokines in the support of HSC in stromal cultures. However, Wineman et al have failed to find a relationship between the cytokines produced by stromal layers and HSC support and similarly the ability to maintain HSC does not appear to correlate with the extent of expansion of mature cells induced by cytokines (Muller-Sieburg & Deryugina, 1995).

Finally, a recent study suggested that very primitive HSC were in fact refractory to the effects of combinations of cytokines in suspension culture (Berardi *et al.*, 1995). This suggested that the mechanisms which support HSC and those which promote maturation and expansion of their progeny may be separable. Elegant work by Verfaillie and co-workers suggested that, for human HSC, direct contact between the HSC and the stroma was not required for long-term haemopoiesis and that a high molecular weight molecule, derived from medium conditioned by stroma, in combination with the stimulatory cytokine IL-3 and the inhibitory chemokine MIP-1α may

substitute for primary stroma (Verfaillie, 1992; Verfaillie, 1993; Verfaillie *et al.*, 1994).

It appears, therefore, that stromal cell derived factors rather than stromal cells themselves, are necessary for HSC support and that it may be possible to develop stroma free *in vitro* culture systems which not only support maturation, expansion and proliferation of progenitor cells, but also support HSC maintenance and / or self-renewal.

1.3 Stem Cells and CFU-S

In the 1960s, Till & McCulloch described the colony forming unit spleen (CFU-S) assay (Till & McCulloch, 1961), based on the observation that macroscopic colonies derived from primitive cells can be observed in spleens of lethally irradiated mice 8-14 days after the inoculation of bone marrow. These early experiments raised the possibility that RBC, WBC and megakaryocytes were all derived from the same precursor but could not prove this in the absence of a marker such as a cytogenetic abnormality (Till & McCulloch, 1961). Two years later Becker et al reported cytogenetic evidence which further supported this conclusion (Becker *et al.*, 1963), however it was not until 1967 that Wu et al succeeded in demonstrating that each spleen colony was a clone and that cells for these three lineages were derived from a single precursor (Wu *et al.*, 1967). Further studies, using chromosomal markers, confirmed that a progenitor cell capable of myeloid differentiation was also capable of forming T and B lymphocytes (Abramson *et al.*, 1977). In their study, bone marrow cells with chromosomal abnormalities induced by radiation were used to transplant stem cell deficient mice (W/W^v). Several months later bone marrow, spleen and thymus were examined for the presence of cells having the same chromosomal marker. In at least five mice a unique marker was observed in CFU-S, in T cells stimulated by phytohaemagglutinin

and in B cells stimulated by lipopolysaccharide. This provided firm evidence that myeloid and lymphoid cells derived from a pluripotent stem cell.

Self-renewal of CFU-S was demonstrated using secondary transfer of spleen colonies, showing that CFU-S was capable of forming other cells like itself (Siminovitch *et al.*, 1963). From this work, Till & McCulloch formed the following definition for the HSC: "HSC are, at the single cell level, capable of differentiation into all blood cell elements as well as extensive proliferation and self-renewal".

In murine marrow HSC are thought to be present at a frequency of 1 in 10^4 to 1 in 10^5. Since true stem cells are so rare, their presence is, in general, deduced by the appearance of differentiated cells in the assay systems available (see below). *In vivo* long-term repopulation assays are considered to be the most stringent test for very primitive stem cells. The majority of HSC are considered to be quiescent under steady state haemopoiesis but to be capable of extensive proliferation and self-renewal in situations when marrow regeneration is required, for example following chemotherapy or irradiation.

1.4 Stem cells are a heterogeneous population

If one considers the definition of HSC as pluripotent and able to give rise to all haemopoietic lineages, then the stem cell population is functionally heterogeneous. During the late 1960s and 1970s it became apparent that CFU-S was not a homogeneous stem cell population but was heterogeneous, made up of cells at varying levels of commitment (Worton *et al.*, 1969). For example, it was shown that CFU-S day 8 were less primitive than CFU-S day 12 (Magli *et al.*, 1982). Furthermore, Hodgson & Bradley demonstrated the existence of pre-CFU-S as a class of stem cells which, following primary bone marrow transplantation (BMT), homed to the bone marrow and gave rise to day 12 CFU-S upon secondary transfer (Hodgson & Bradley, 1979). This property of stem cells became known as marrow repopulating ability (MRA).

More recently, Harrison et al, using the CRA, demonstrated that early after BMT, a number of pluripotent cells contributed to repopulation, whereas many months later only a few were active (Harrison *et al.*, 1993). Similar data have been obtained using stem cells marked using retroviruses (Dick *et al.*, 1985; Lemischka *et al.*, 1986). This work was corroborated by demonstrating heterogeneity, even within highly enriched stem cell populations (Sca-1^+ Thy-1^{lo} Lin^-), by limiting dilution analysis *in vivo*. Most stem cells in this population ceased to contribute to repopulation after only a few weeks (Smith *et al.*, 1991; Uchida & Weissman, 1992). It appears, therefore, that only a minority of "stem cells" are capable of long term persistence.

1.5 CFU-S are separable from HSC

For 30 years following the original description by Till & McCulloch (Till & McCulloch, 1961), the CFU-S assay was used as an indicator of stem cell frequency in marrow and spleen suspensions. However, the ability to separate bone marrow cells, on the basis of a variety of physical and phenotypic properties, demonstrated that the vast majority of CFU-S did not have long term repopulating ability. In addition, bone marrow cells depleted of CFU-S were shown to be responsible for long-term reconstitution (Jones *et al.*, 1990; Ploemacher & Brons, 1989). Thus, CFU-S appeared to contribute to early engraftment following BMT, whereas "pre-CFU-S" were responsible for long-term reconstitution.

In vitro Assays

In the 1960s, groups in Israel and Australia developed *in vitro* clonogenic assays for the growth of normal murine bone marrow cells (Pluznik & Sachs, 1965; Bradley & Metcalf, 1966; Ichikawa *et al.*, 1966). These culture systems were then extended to the cloning of human myeloid cells and to erythrocytes, B cells and T cells (Sachs, 1992). Such assays facilitated the discovery of a

family of cytokines which have been found to regulate cell viability, proliferation and differentiation. There is now a wide range of *in vitro* assay systems available to investigators. The most appropriate choice of assay will depend on the experimental question to be answered, but also tends to depend on the preference of the investigator for a particular assay(s). *In vitro* assays have now been developed which measure progenitors at different stages of commitment. For example, committed assays measure myeloid precursor cells committed to the granulocyte, eosinophil, macrophage, megakaryocyte or erythroid lineage, whereas HPP-CFC and CFU-A measure progenitor cells which are still multipotential. The most primitive progenitor cells detected *in vitro* are measured indirectly by using long term culture techniques. A number of assays which are in frequent use are described in some detail below.

1.6 Committed progenitor assays

Committed progenitor assays including CFU-GM, BFU-E and CFU-Mix became well established in routine haemopoietic cell culture as a result of work performed in the 1960s (Pluznik & Sachs, 1965; Bradley & Metcalf, 1966) and developed thereafter. The colonies which grow in these cultures arise from progenitor rather than stem cells.

A number of cytokines have direct stimulatory effects on haemopoietic cells in these assays (GM-CSF, G-CSF, M-CSF, IL-3 and erythropoietin (Epo)), whilst others have no or modest stimulatory effects when used alone, but may exert powerful synergistic effects when used in combination with the CSFs mentioned above (IL-1, IL-4, IL-5, IL-6, IL-11, IL-12 and SCF) (Heyworth & Spooncer, 1993).

Since CSFs and interleukins may themselves be produced by cells present in bone marrow it is important to look at effects on highly purified cell populations, depleted of accessory cells, before concluding that an effect is mediated directly. Additionally, unknown components present in serum, in

combination with growth factors, may exert effects on haemopoietic cells and therefore cell culture performed under serum free or serum deprived conditions may be of additional value under certain circumstances.

Although culture of purified cells, in strictly defined media, describes the ideal, in general, routine experiments are performed using unfractionated bone marrow in the presence of serum, and often conditioned media replace purified growth factors. To answer specific questions, however, defined conditions should be employed.

The essential components for these assays include a semi-solid medium, either agar or methylcellulose, a source of pre-tested serum (or specifically devised serum free medium), a source of growth factor(s) and a cell population for testing. CFU-Mix detect multipotent myeloid cells, BFU-E primitive and more mature erythroid progenitors, and CFU-GM, committed granulocyte and macrophage progenitor cells. Variations of these assays may be developed to detect megakaryocyte or eosinophil progenitors (Heyworth & Spooncer, 1993).

Although the majority of cells comprising these colonies are maturing and post-mitotic, a low incidence of clonogenic progenitors may be demonstrated by replating the colonies into identical conditions and observing for the development of further colonies.

1.7 High Proliferative Potential Colony-Forming Cells (HPP-CFC)

Following the description of the *in vivo* CFU-S assay in 1961 (Till & McCulloch, 1961) the *in vitro* detection of committed progenitor cells was described by two groups (Pluznik & Sachs, 1965; Bradley & Metcalf, 1966). Attempts to study the factors responsible for the proliferation and differentiation of more primitive cells was, however, hampered by the lack of a suitable *in vitro* assay system. In 1979, Bradley & Hodgson described the HPP-CFC assay which detected primitive progenitors with properties similar

to stem cells (Bradley & Hodgson, 1979). Following the initial description in 1979, the HPP-CFC assay was further developed and shown to detect at least three subpopulations of progenitors stimulated by differing combinations of cytokines (McNiece *et al.*, 1986). These were termed HPP-CFC-1, 2 and 3 and appeared to be developmentally distinct. The various types were shown to be responsive to a variety of cytokines including SCF, GM-CSF, M-CSF, G-CSF, IL-1, IL-3, IL-4 and IL-6. HPP-CFC-1, the most primitive, were resistant to 5-FU and were capable of generating the more mature HPP-CFC-2 & 3 (Bradley & Hodgson, 1979; Bradley *et al.*, 1986; McNiece *et al.*, 1986). HPP-CFC-1 correlated closely with pre-CFU-S (Hodgson & Bradley, 1984), were able to generate cells which formed CFU-S and cells which possessed marrow repopulating ability (McNiece *et al.*, 1987). The incidence of HPP-CFC-1 was estimated at only 1 in 2380 normal murine bone marrow cells (Bradley & Hodgson, 1979). Combining different methods of stem cell enrichment (see below) Bertoncello et al were able to resolve HPP-CFC-1 and 2 (Bertoncello *et al.*, 1985; Bertoncello *et al.*, 1989). For example, HPP-CFC-1 were rhodamine^{dull} but HPP-CFC-2 were rhodamine^{bright} (Bertoncello *et al.*, 1991).

1.8 Colony forming unit-Type A (CFU-A) assay

Although single cytokines are unable to induce proliferation of more primitive progenitor cells, it is clear that synergistic cytokines may interact to induce cycling, proliferation and differentiation of such cells *in vitro*. In 1988, Pragnell et al first described an *in vitro* clonogenic assay which detected a multipotential progenitor cell with characteristics very similar to CFU-S day 12 (Pragnell *et al.*, 1988). In this assay normal murine bone marrow cells were grown in agar with serum supplemented medium and a source of synergising growth factors. In the original description the growth factors were from conditioned media shown to contain high concentrations of both M-CSF and

GM-CSF (L929 (Stanley & Heard, 1977) and AF1-19T-CM(Franz *et al.*, 1985)) although subsequently the assay was reproduced using recombinant factors (Pragnell *et al.*, 1988). Under these conditions macroscopic (2-5mm) colonies, containing on average 4.5 x 10^4 cells per colony, developed after 11 days in culture. The incidence of colony formation ranged from 145-200/10^5 bone marrow cells depending on the mouse strain. These were shown to be derived from multipotential progenitor cells and contained precursors for granulocytes and erythroid cells in addition to macrophages (Pragnell *et al.*, 1988). On further examination, colonies <2mm were found to be derived from cells in cycle, whereas colonies >2mm were derived from minimally cycling cells. For this reason a 2mm cut off was used for CFU-A determination.

CFU-A were compared with CFU-S day 12 in a number of ways (Pragnell *et al.*, 1988; Lorimore *et al.*, 1990). Their recovery profile following a single dose of 5-FU showed a main peak at days 12-15, an identical pattern to CFU-S day 12. CFU-A were found to be replatable and to produce a low number of CFU-A colonies >1mm in diameter. Furthermore, CFU-A plucked at day 5 of culture were capable of producing CFU-S day 12, suggesting a similar capacity for self-renewal as that described for CFU-S. CFU-A and CFU-S day 12, derived from normal bone marrow were found to be out of cycle (<10% in S phase), whereas those derived from regenerating bone marrow were actively cycling (30% in S phase). When these two respective populations (i.e. quiescent and proliferating) were exposed to a proliferation stimulator (Lord *et al.*, 1977) or inhibitor (Lord *et al.*, 1976) respectively, both CFU-A and CFU-S day 12 responded in an identical manner. The quiescent progenitors entered cell cycle and the proliferating progenitors became quiescent (Pragnell *et al.*, 1988). Further studies compared the buoyant cell density and response to ionising radiation of CFU-A and CFU-S and both parameters were found to be similar for the two progenitor populations, but different to the more committed CFU-GM progenitor (Lorimore *et al.*, 1990).

Murine CFU-A therefore derive from that part of the stem cell compartment measured by the *in vivo* CFU-S day 12 assay. The incidence for CFU-A is nearly 10 fold greater than for CFU-S but this may be explained by the fact that only a proportion of the bone marrow innoculum actually seeds to the spleen in the CFU-S assay. CFU-A appear to overlap with that part of the stem cell compartment detected by the more mature HPP-CFC. Since the original description, the murine CFU-A assay has proven indispensable for studies of stem cell regulation and led to the identification and purification of a stem cell inhibitor now known to be MIP-1α (Graham *et al.*, 1990). More recently an equivalent *in vitro* assay for the detection of human CFU-A was reported (Holyoake *et al.*, 1993).

In transplantation studies, CFU-S day 12 have been shown to be crucial for transient engraftment following BMT (Jones *et al.*, 1989; Jones *et al.*, 1990), therefore an *in vitro* correlate of CFU-S day 12 should be capable of measuring that part of the stem cell compartment responsible for the early phase of bone marrow engraftment.

1.9 CFC-blast assay

The original description of murine blast cell colonies was of small colonies consisting of only blast cells on day 16 of cultures supported by pokeweed mitogen stimulated spleen cell conditioned medium (Nakahata & Ogawa, 1982a; Nakahata & Ogawa, 1982b). These colonies were derived from cell cycle dormant progenitors and, unlike the majority of colonies which develop in semisolid cultures, could contain up to 100% replatable CFU-C (Suda *et al.*, 1983). CFU-blast were slow to proliferate in culture and were more primitive than CFC that readily form colonies in semi-solid cultures (Suda *et al.*, 1983). This assay, therefore, provides an excellent means of studying the commitment and differentiation of primitive cells.

1.10 Cobblestone Area Forming Cell (CAFC) and Long Term Culture Initiating Cell (LTCIC) assays

Cobblestone areas are clones of haemopoietic cells situated beneath the stromal layer in LTBMC (Dexter *et al.*, 1977). They can be differentiated from the stromal cells and from more mature cells sitting on top of the stroma by phase-contrast microscopy. The production of mature CFC in LTBMC has been shown to be associated with the presence of these cobblestone areas (Ploemacher *et al.*, 1989). These observations prompted Ploemacher et al to develop the CAFC assay as a means of scoring stem cell subsets (Ploemacher *et al.*, 1989). The CAFC assay is a miniaturised murine LTBMC performed in limiting dilution. It allows detection of subsets of the stem cell compartment depending upon the time of appearance of the cobblestone areas. Detection and quantification of these *in vitro* cobblestone areas was shown to correlate closely with stem cell subsets detected *in vivo* (Ploemacher *et al.*, 1989; Ploemacher *et al.*, 1991). CAFC detected on day 10 and 28 of culture correlated with CFU-S day 12 and long term repopulating cells respectively (Ploemacher *et al.*, 1989; Ploemacher *et al.*, 1991). The"test" cells are overlaid on pre-established stromal layers in 96-well microtitre plates following a limiting dilution protocol. At weekly intervals, or more often, the wells are scored for the presence/absence of cobblestone areas. Poisson statistics are employed to determine the stem cell frequencies. In normal murine bone marrow CAFC numbers per 10^5 cells range between 100-300 CAFC-10 (i.e. very similar to CFU-A), 1-5 CAFC-28 and 0.3-1.0 CAFC-35 (Ploemacher, 1994).

The LTCIC assay is an indirect means of quantifying primitive HSC by assessment of the number of clonogenic progenitors they produce after at least 4 weeks in LTBMC (Van der Sluijs *et al.*, 1990; Sutherland *et al.*, 1990). In the presence of a stromal layer, the clonogenic cell output was shown to be linearly related to the input cell number over a wide range of cell

concentrations (Sutherland *et al.*, 1990). The frequency of LTCIC may be determined by limiting dilutions techniques similar to those employed for the CAFC except that instead of scoring cobblestone areas, the cultures are fed weekly and then both the supernatant cells and the adherent layer are sacrificed and replated into clonogenic assays to determine the clonogenic progenitor output (Sutherland & Eaves, 1994). CAFC have been correlated with LTCIC by first scoring cobblestone areas and then plating the contents of the well into CFU-GM assay (Ploemacher *et al.*, 1989). The proliferative capacity of individual LTCIC was shown to be highly variable with a range of 1-30 CFU-GM produced per LTCIC (Sutherland *et al.*, 1990). Therefore, both CAFC and LTCIC assays detect a range of stem cell subsets. The CAFC appearing late in culture and the LTCIC with high proliferative capacity representing the more primitive subsets.

1.11 Self-renewal versus differentiation

Two major models are proposed to explain the concept of stem cell self-renewal versus differentiation. Stochastic models, supported, for example, by Ogawa (Ogawa, 1993), suggest that the decision of HSC, either to self-renew or to differentiate may be a random process with survival and proliferation of progenitor cells controlled by cytokines.

On the other hand, deterministic models, supported, for example, by Uchida et al (Uchida *et al.*, 1993) suggest that the HSC pool is heterogeneous and the outcome for an individual HSC is dependent on some unknown property of that cell such as the number of divisions already undergone. Commitment to differentiation may then be influenced by the surrounding microenvironment and the cytokines / inhibitors present. Currently available evidence tends to support the latter model.

There is ample evidence that the HSC pool is a heterogeneous population comprised of largely quiescent HSC, self-renewing stem cells and

multipotential cells which do not self-renew, but expand to give rise to committed progenitor cells (Ploemacher & Brons, 1989; Spangrude & Johnson, 1990; Uchida *et al.*, 1994). Heterogeneity has been demonstrated *in vivo* by transplantation of highly purified cells whose progeny can be detected as donor derived at varying time points after BMT (Uchida *et al.*, 1993). In this model, the proliferative life history of a single HSC limits its potential for further self-renewal, i.e. an individual HSC "ages" after a finite number of divisions and leaves the HSC compartment to differentiate.

Evidence suggesting that HSC may be developmentally regulated may be found in B and T cell differentiation and in erythroid development.

Firstly, it appears that fetal HSCs are able to give rise both to conventional B cells and to CD5 positive (Ly-1) B cells, whereas adult bone marrow HSCs may give rise only to conventional B cells (Hayakawa *et al.*, 1985).

Secondly, Ikuta et al have shown that fetal liver derived HSCs have a predetermined developmental potential for T cell differentiation which differs from that of adult bone marrow derived HSCs (Ikuta & Weissman, 1991).

Thirdly, fetal erythrocytes differ from adult erythrocytes in terms of size, the presence of a nucleus and the expression of fetal-type rather than adult-type globin (Fantoni *et al.*, 1967). These findings, taken together, suggest that fetal HSCs have the developmental potential to give rise to both fetal-type and adult-type cell lineages, but that adult HSC may only differentiate into adult-type lineages.

Finally, it has been proposed that loss of telomeric DNA from the ends of human chromosomes at the time of cell division, sequentially leads to ageing / senescence of that cell. Since telomeres are critical for chromosome stability, loss of telomeric length may be responsible for cells exiting from cell cycle after a "pre-determined" number of divisions. Recently, Vaziri et al demonstrated that highly purified human candidate stem cells at varying

stages of development (i.e. cells from fetal liver, umbilical cord blood and adult bone marrow) had differing telomeric lengths, suggesting that those with shorter telomeres (adult bone marrow) had reduced proliferative potential as a result of ageing (Vaziri *et al.*, 1994).

These four examples strengthen the argument that stem cells are regulated, in part, by pre-determined factors which are integral to the individual stem cell subset, be it fetal derived or adult derived. Cytokines may then act to modify stem cell responses depending on the requirement for differentiated progeny at any point in time.

1.12 Haemopoietic growth factors (cytokines)

The first CSFs to be discovered included M-CSF, G-CSF, GM-CSF and IL-3 (Sachs, 1992). However, over recent years an array of CSFs, interleukins and other cytokines have been cloned. These soluble factors are capable of affecting haemopoietic cell growth and differentiation and, in general, combinations of synergising growth factors are required for optimum proliferation. The function of cytokines is not restricted to the haemopoietic system and these factors have important roles in defence against infection, wound healing, angiogenesis and inflammation.

Haemopoiesis may be a flexible enough system to respond rapidly when an increase in cell production is necessary, but equally to "quench" the system when the requirement for increased cell production is over. This suggests the need for both inducers and inhibitors of proliferation. Each cytokine is the product of multiple cell types and many appear to exhibit functions which overlap with other cytokines (i.e. redundancy). Furthermore, many cytokines are polyfunctional, with multiple effects both within and outwith the haemopoietic system. This produces a network effect which includes positive stimulation by CSFs and interleukins, and inhibition by negative regulators such as TGF-β, MIP-1α and TNF. Normal myeloid

precursor cells depend on exogenous cytokines for viability as well as proliferation and differentiation. In the absence of cytokines, these cells die by programmed cell death or apoptosis (Williams *et al.*, 1990b).

There are a numbers of observations which, together, suggest redundancy amongst the proliferative regulators of haemopoiesis.

Firstly, several cytokines show common proliferative actions. For example, G-CSF, GM-CSF, M-CSF, IL-3, SCF and IL-6 may all promote the growth of small granulocyte colonies (Metcalf, 1993).

Secondly, cytokines tend to share a range of biological effects and may not only effect cells from different lineages in the haemopoietic system but have a range of target cells outwith haemopoiesis. For example, LIF, IL-6, IL-11 and OSM share biological effects and are able to promote thrombopoiesis, stimulate the production by hepatocytes of acute phase reactants, alter neuronal signalling and suppress lipid transport (Metcalf, 1993). Furthermore, IL-6, IL-11 and LIF produce similar effects on thrombopoiesis *in vivo*.

Thirdly, many cytokines share receptor subunits. The receptors for M-CSF, and SCF are protein tyrosine kinase containing receptors which show structural similarity to the platelet derived growth factor (PDGF) receptor (Qiu *et al.*, 1988). The receptors for many growth factors are members of a cytokine receptor superfamily. This family is characterised by conserved elements in the extracellular domains suggesting the possibility that these receptors are derived from a common ancestral receptor (Metcalf, 1993; Ihle *et al.*, 1994). These receptors have a specific α-chain but, whereas GM-CSF, IL-3 and IL-5 share the same β-chain, IL-6, LIF, OSM and IL-11 all share another common β-chain, the gp130 molecule (Metcalf, 1993; Ihle *et al.*, 1994). Since the β-chain appears dominant in intracellular signalling, this may explain the similarities of action of GM-CSF, IL-3 and IL-5 and of LIF, IL-6, OSM and IL-11. Why then should individual cells require multiple receptors which share and signal via the same β-chain?

The question of cytokine redundancy has, in part, been answered by the study of animals in which the gene in question has been deleted or functionally inactivated (knockouts). To date, cytokine knockouts of G-CSF, GM-CSF, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, LIF, TGF-β and others, yet to be published formally, have been engineered (Brandon *et al.*, 1995). In addition, mice with a genetic defect causing osteopetrosis (op/op) have been shown to have inactivation of the M-CSF gene (Yoshida *et al.*, 1990), and both Steel (Sl) and white spotting (W) anaemic mice have now been shown to be genetically deficient in SCF and c-kit (its receptor) respectively (Russell, 1979).

The availability of gene knockout animals and mutations occurring spontaneously *in vivo*, have allowed investigators to learn more regarding the role of individual cytokines. For example, G-CSF knockouts suffer severe neutropenia but some normal mature neutrophils are still present suggesting that other cytokines are able to partially compensate for the lack of G-CSF (Lieschke *et al.*, 1994). GM-CSF knockouts show pulmonary pathology, but, surprisingly, have apparently normal haemopoiesis (Stanley *et al.*, 1994). C-Mpl is a proto-oncogene and a member of the cytokine receptor superfamily (Gurney *et al.*, 1994). The ligand to c-Mpl, named thrombopoietin, was recently cloned and found to have potent effects on megakaryocytopoiesis (de Sauvage *et al.*, 1994). C-Mpl knockouts have reduced platelet numbers and increased serum thrombopoietin but are still able to produce some functional platelets in the absence of signalling via c-Mpl (Gurney *et al.*, 1994). Likewise, op/op M-CSF deficient mice exhibit a major deficiency in macrophage-derived osteoclasts and partial deficiency of other macrophage populations which are relieved by injection of M-CSF. However, since these mice are not completely devoid of macrophages, other cytokines must be partly able to compensate for the deficiency (Yoshida *et al.*, 1990). Sl and W

mice show similar phenotypes including severe anaemia and deficiency of very primitive HSC.

The conclusions which may be drawn, from the knowledge currently available, are that in certain instances cytokines are essential for normal development and blood cell production. Although cytokines have a number of such unique effects which cannot be compensated for by other cytokines they also possess redundant actions for which alternative cytokines may substitute.

1.13 Stem Cell Factor

The cloning of SCF provided a major advance to the understanding the cytokine interactions which controlled the proliferation of early haemopoietic stem cells. SCF, first identified in 1990 (Anderson *et al.*, 1990; Copeland *et al.*, 1990; Huang *et al.*, 1990; Martin *et al.*, 1990; Williams *et al.* 1990a; Zsebo *et al.*, 1990a; Zsebo *et al.*, 1990b), is the ligand for the receptor encoded by the c-kit proto-oncogene (Qiu *et al.*, 1988). In mice, mutations may either effect the locus of c-kit (White Spotting W locus) or its ligand (Steel Sl locus), resulting in variable defects in pigmentation, fertility and haemopoiesis (reviewed by Williams *et al.*, 1992).

SCF exists in both soluble and membrane bound forms. Sl mutant mice synthesise soluble SCF, which is active *in vitro*, but fail to make the membrane bound form. Since these mice exhibit an obvious phenotype, this suggests that membrane bound SCF has a critical role in stem cell-stromal interactions in the intact organism that is not duplicated by the soluble molecule. The fact that defects in the production or action of SCF lead to bone marrow failure in mice, suggests that this cytokine is likely to play a major physiological role in stem cell development and in haemopoiesis. Ogawa et al used an anti-c-kit antibody to demonstrate that nearly all *in vitro* clonogenic cells, including CFU-S, derived from bone marrow or spleen

expressed c-kit (Ogawa *et al.*, 1991). Furthermore, bone marrow stromal cells actively produce both soluble and membrane bound SCF.

As predicted, SCF has been shown to be a potent stimulator and regulator of early events in haemopoiesis (de Vries *et al.*, 1991; Ogawa *et al.*, 1991). SCF may shorten the dormant phase of stem cells (Tsuji *et al.*, 1991) and enhanced survival of highly enriched murine progenitor cells under serum free conditions (Heyworth *et al.*, 1992). To date, no convincing evidence has yet been published indicating that SCF promotes self-renewal of HSC.

Acting alone, SCF has little proliferative activity, however it can synergise with a number of cytokines to stimulate growth of haemopoietic progenitor cells *in vitro*. For example, the addition of SCF, which did not act alone, to low levels of combinations of M-CSF, G-CSF, GM-CSF, IL-1α and IL-3, which without SCF showed little or no clonogenic capacity, consistently increased HPP-CFC colony number and size to the degree produced when all 5 growth factors were used at plateau concentration (Lowry *et al.*, 1991; Lowry *et al.*, 1992). In addition to potent effects on granulocyte and macrophage precursors, SCF has been shown to exert effects on erythropoiesis (Anderson *et al.*, 1991; Dai *et al.*, 1991), mast cell generation (Huang *et al.*, 1990; Williams *et al.*, 1990a) , T and B cell lymphopoiesis (Williams *et al.*, 1992), and megakaryopoiesis (Briddell *et al.*, 1991). Although all these lineages are affected by SCF *in vitro*, and often *in vivo*, the impact of mutations in Sl mice is preferentially seen in erythroid and mast cell differentiation, with minimal effects on the remaining pathways. These findings suggest that SCF independent pathways exist for megakaryopoiesis and for both granulopoiesis and macrophage production (Williams *et al.*, 1992).

1.14 Interleukin-11

IL-11 was first cloned from a primate bone marrow derived stromal cell line, PU-34, as a factor which stimulated proliferation of an IL-6 dependent murine plasmacytoma cell line (Paul *et al.*, 1990). A second group, working separately, cloned a novel adipogenesis inhibitory factor from the human bone marrow derived cell line KM-102, which they later found to be identical to IL-11 (reviewed in Kawashima & Takiguchi, 1992).

Both the murine and human IL-11 receptors have recently been cloned and show 82% sequence homology (Hilton *et al.*, 1994; Cherel *et al.*, 1995). IL-11 belongs to the family of cytokines that use the gp 130 transducing subunit in their high affinity receptors.

IL-11 is a multifunctional cytokine with a range of biological activities which resemble those of IL-6. These effects include stimulation of T cell dependent development of immunoglobulin producing B cells (Anderson *et al.*, 1992), synergism with other cytokines to support murine and human megakaryopoiesis (Paul *et al.*, 1990) and erythropoiesis (Quesniaux *et al.*, 1992), the ability to shorten the dormant period of stem cells (Musashi *et al.*, 1991), induction of secretion of acute phase proteins in the liver and the inhibition of adipose conversion in the bone marrow (Kawashima & Takiguchi, 1992).

Like SCF, IL-11 has little activity as a single factor but was shown to act synergistically with other cytokines, such as IL-3 and SCF, to increase murine blast and CFU-GEMM colony formation (Tsuji *et al.*, 1992). When added to LTBMC, IL-11 stimulated expansion of CFU-GM and CFU-S day 12, but at the expense of LTRC, suggesting that, in this setting, it may have increased commitment of HSC into the multipotential progenitor compartment (Du *et al.*, 1995).

In vivo, in normal rodents, IL-11 stimulated a marked increase in the numbers of bone marrow megakaryocytes and circulating platelets (Du &

Williams, 1994). Given to mice following BMT, neutrophil and platelet recovery was enhanced, and this was accompanied by increased numbers of progenitors in the bone marrow and spleen (Du *et al.*, 1993b). Haemopoietic recovery, following administration of cytotoxic agents, was also accelerated by IL-11 administration (Du & Williams, 1994). More recently IL-11 was shown to protect clonogenic stem cells in murine gastrointestinal mucosa from the effects of radiation and chemotherapy (Keith *et al.*, 1994; Potten, 1995).

IL-11 is, therefore, a stromal derived cytokine with a distinct biological profile from IL-6. It shows pleiotropic effects on haemopoiesis, presumably depending on the surrounding cellular and cytokine environment. Some of its effects are consistent however, and include stimulation of megakaryopoiesis and early HSC.

1.15 SCF / IL-11 interactions *in vitro* and *in vivo*

SCF and IL-11 are both stromal derived cytokines which, though relatively inactive as single factors, synergise with a range of cytokines to promote proliferation and differentiation of HSC. SCF, used alone, promoted HSC survival but did not induce their proliferation (Katayama *et al.*, 1993). In combination with other cytokines, both SCF and IL-11 were able to shorten the dormant period of HSC and so trigger their entry into cell cycle (Musashi *et al.*, 1991; Tsuji *et al.*, 1991). Both cytokines have effects on early and late stages of myelopoiesis, including erythropoiesis and megakaryopoiesis.

It would be expected, therefore, that combination of these two cytokines should stimulate potent synergistic effects in terms of progenitor expansion *in vitro*. Several groups have demonstrated that this is, indeed, the case. Tsuji et al showed that SCF and IL-11, in the presence of serum, interacted to support formation of multilineage colonies (Tsuji *et al.*, 1992). Quesniaux et al, showed, that in the presence of either IL-3 or SCF, IL-11 had profound stimulatory effects on primitive multilineage progenitor cells and on

various stages of erythroid differentiation. In addition, SCF with IL-11 stimulated the growth of very large, highly proliferative, erythroid colonies which, upon morphological examination, were pure erythroblast colonies (Quesniaux *et al.*, 1992). Neben et al examined the effects of SCF, IL-3, IL-11 and IL-6 as single factors and in different combinations for their ability to induce expansion of CFU-S, CAFC and LTRC. Although several combinations promoted expansion of more committed progenitors, the recovery of LTRC was found to be enhanced four fold when SCF and IL-11 were combined, compared with the use of SCF alone (Neben *et al.*, 1994).

In vivo studies have also shown that SCF and IL-11 synergise, when administered in combination, to promote accelerated haemopoietic recovery, following BMT and to promote mobilisation of HSC (Du *et al.*, 1993a; Mauch *et al.*, 1995).

1.16 Macrophage Inflammatory Protein-1α

MIP-1α is one member of the chemokine (chemotactic cytokine), or platelet factor 4 (PF4) superfamily. The members of this group of cytokines are related by structural similarities and by the conservation of a four cysteine motif. The family is further subdivided on the basis of this cysteine motif, with the "C-X-C" branch characterised by separation of the first two cysteines by an intervening amino acid, and the "C-C" branch having the first two cysteines directly adjacent. MIP-1α belongs to the "C-C" branch of the family (Schall, 1991).

In 1988, Wolpe and co-workers identified MIP-1, (now known to be composed of MIP-1α and MIP-1β), in mouse macrophages (Wolpe *et al.*, 1988). At that time, MIP-1 was described as an acidic, heparin-binding protein, that resolved as a doublet of 8, 000 daltons on SDS-PAGE. This protein caused inflammatory reactions in the footpads of mice. In 1990, Graham et al, identified and characterised a potent, reversible, inhibitor of that

part of the stem cell compartment measured by CFU-S day 12/CFU-A, which was shown to be identical to the major component of the doublet, MIP-1α, and to the previously reported CFU-S inhibitory activity obtained from primary cultures of normal bone marrow (Lord *et al.*, 1976; Graham *et al.*, 1990;).

Members of the chemokine family exhibit a wide range of diverse functions including monocyte, T-cell and neutrophil activation (Schall, 1991). Although MIP-1α 's primary property appears to be as an inflammatory mediator, it may also regulate haemopoietic stem cell proliferation under certain circumstances (Graham *et al.*, 1990; Graham *et al.*, 1992;). In the original report (Graham *et al.*, 1990), conditioned medium from normal bone marrow had been shown to inhibit the development of macroscopic colonies in the murine CFU-A assay. The macrophage cell line J774.2 was used as a source of conditioned medium, and the CFU-A assay, as a read-out for stem cell inhibition, to follow purification of MIP-1α to homogeneity. The active fractions inhibited CFU-A colony formation and reduced the proportion of CFU-A or CFU-S day 12 that were in cycle. The components of the doublet were resolved as MIP-1α and MIP-1β.

Investigations performed by our group (Graham *et al.*, 1993) revealed that members of the C-C family including MIP-1α, MIP-1β, LD78 and ACT-2 were all active as stem cell inhibitors in the murine CFU-A assay. These more recent data, using purified MIP-1β, were likely to be more accurate than earlier reports (in which MIP-1β levels were not accurately measured) which suggested that the chemokine was inactive as a stem cell inhibitor (Graham *et al.*, 1990). Although definitely active, both MIP-1β and its human homologue ACT-2 were 10-20 times less active than MIP-1α or its human homologue LD78. The remaining C-C chemokines including RANTES, MCAF and TCA-3 and the C-X-C chemokines IL-8 and MIP-2 were all inactive when used in the CFU-A assay (Graham *et al.*, 1993).

The studies described above were performed using murine cytokines (or their human homologues) on a restricted target population (i.e. murine CFU-A). Broxmeyer et al have reported data which appear to conflict those of our own group (Broxmeyer *et al.*, 1990; Broxmeyer *et al.*, 1991). In an early report by Broxmeyer et al, murine MIP-1β was found to be inactive on either murine or human CFU-GEMM, BFU-E and CFU-GM, whereas under identical circumstances MIP-1α was shown to be inhibitory. Since Broxmeyer et al have also reported stimulation of more mature CFU-GM by MIP-1α whilst we have seen no such effect, these differences are likely to reflect variation in the assay systems used (Broxmeyer *et al.*, 1990). For example, since CFU-A may be inhibited nearly 100% by MIP-1α but require 20 fold more MIP-1β for the same effect it is possible that more mature progenitors described by Broxmeyer (which are only inhibited by approximately 50%) may not respond to the weaker inhibitor MIP-1β at the concentrations used. Broxmeyer also observed no effect with MIP-1β when used *in vivo* (Maze *et al.*, 1992). The more recent study by Broxmeyer et al was even more difficult to compare with our own work since only human cytokines were tested for their effects on human haemopoietic cells (Broxmeyer *et al.*, 1993). In that study, chemokines from both the C-C and C-X-C families were assessed for their ability to inhibit colony formation (CFU-GEMM, BFU-E and CFU-GM). The chemokines were each tested at concentrations up to 1000ng/ml. MIP-1α (C-C), MIP-2α (C-X-C), PF4 (C-X-C), IL-8 (C-X-C) and MCAF (C-C) all inhibited colony formation by approximately 50% of control whereas MIP-1β (C-C), MIP-2β (C-X-C), GRO-α (C-X-C), NAP-2 (C-X-C) and RANTES (C-C) were inactive. Using the murine CFU-A assay, our group has never demonstrated inhibition by members of the C-X-C family but consistently observed inhibition by MIP-1β within the concentration range tested by Broxmeyer et al (Broxmeyer *et al.*, 1993). Using the more mature progenitor assays (compared with CFU-A), Broxmeyer was unable to demonstrate 100%

inhibition even if five active chemokines were combined, each at 100ng/ml (Broxmeyer *et al.*, 1993). This suggests that the target population detected by these assays contains progenitors which are both responsive and unresponsive to the effects of chemokines. In the same report, Broxmeyer showed that those chemokines with inhibitory properties were able to synergise when combined with each other to produce the same level of inhibition (i.e. 50%) even at concentrations reduced by 2, 500 fold (Broxmeyer *et al.*, 1993). The inhibitory effects of IL-8 and PF4 on human myeloid cells have been confirmed in a further very recent report by the same group (Daly *et al.*, 1995). In addition a novel member of the C-C family called MIP-related protein-2 has also been reported to inhibit the growth of both human and murine myeloid colonies (Youn *et al.*, 1995).

It is unfortunate that the assay systems used by Broxmeyer's group and by our own group were so different. In addition the majority of the work performed by Broxmeyer was on human rather than on mouse cells. Nevertheless it seems surprising that the differences observed appeared so great. It is, however, possible that chemokines may be suppressive, stimulatory or have no effect depending both on the target cell involved and the surrounding environment including both stromal elements and other cytokines present.

A range of investigations have revealed a further area of conflict between our results and those of Broxmeyer. When present in solution at relatively high concentration, MIP-1α is known to aggregate. The MIP-1α monomer is only 8kDa, however the more usual polymer is often >100kDa in size. Graham et al have produced non-aggregating mutants of MIP-1α which showed an activity identical to the wild type protein (Graham *et al.*, 1994). The mutant was no more active than the wild type protein, suggesting that the aggregation seen at high concentrations resolved upon addition to a solution or to the CFU-A assay when a large dilution factor was operating. Both mutant

and wild type MIP-1α were active *in vivo* at equivalent doses. On the other hand, Broxmeyer's group suggested that polymerised MIP-1α was completely inactive and that the inhibition observed was produced by tiny (<0.3% of total) quantities of MIP-1α present in monomeric from (Mantel *et al.*, 1993). Broxmeyer used MIP-1α diluted in acetonitrile to maintain the monomeric form. In one report he demonstrated that monomeric MIP-1α was fully active at concentrations as low as 0.5ng/ml *in vitro* and 4-8ng per mouse when used *in vivo* (Cooper *et al.*, 1994). Even using the preparation diluted in acetonitrile, our group has been completely unable to reproduce these data.

The issues discussed above are clearly difficult to resolve. There are no other investigators who have performed such in depth studies of chemokines in general, and of MIP-1α in particular, to obtain further information from. The inhibitory effects of the different family members may only be resolved by performing the same progenitor assay, with the same human or murine cells and the same cytokine preparation. To my knowledge, this has never been done. If possible, it would be better to work with a formulation of MIP-1α that did not aggregate. We have no way of determining whether the non-aggregating mutant produced in our laboratory would show the same potency as the wild type monomer diluted in acetonitrile and investigated in the laboratory of Broxmeyer et al. It remains questionable whether such tiny quantities of MIP-1α could be truly active. No other groups working in the area have confirmed this aspect of Broxmeyers work.

The primary structure of the family of receptors for chemokines revealed that they are members of the superfamily of G protein-coupled receptors containing seven transmembrane domains. The first receptors to be cloned belonged to the C-X-C family (discussed in Graham *et al.*, 1993), however, more recently, Neote et al cloned a C-C receptor which bound human and murine MIP-1α, human MIP-1β, human MCP-1 and RANTES (Neote *et al.*, 1993). This receptor, C-C CKR-1, was expressed by a variety of

haemopoietic cell lines and was widely expressed in different haemopoietic cells. Since the chemokines, MIP-1α in particular, showed a range of biological activities, it was possible that not all these activities were mediated via the cloned C-C receptor. However, since the stem cell inhibitory activities of MIP-1α were not species specific and C-C CKR-1 bound both human and murine MIP-1α with equal affinity, this suggested that the C-C receptor was probably responsible for affects on stem cell proliferation (Neote *et al.*, 1993). This question requires further investigation.

Although the effects of MIP-1α on haemopoietic cells were initially thought to be restricted to inhibition of stem cell proliferation (i.e. CFU-S/CFU-A), Broxmeyer et al demonstrated that MIP-1α could be either inhibitory or stimulatory, depending on the combination of cytokines selected to promote colony growth. For example, when single cytokines, such as M-CSF or GM-CSF, were selected to promote the development of mature progenitor cells, the addition of MIP-1α resulted in enhanced colony formation. However, when GM-CSF was combined with IL-3 to promote the proliferation of immature progenitors, the effect of MIP-1α was consistently inhibitory (Broxmeyer *et al.*, 1990). These apparently opposing effects are likely to reflect differences between more mature or more primitive progenitor cells. For example, primitive cells may express a higher number of receptors for MIP-1α, or the same number of receptors may be upregulated on primitive cells. Equally it is possible that different receptors exist for inhibition versus stimulation. Finally, the response of an individual cell is dependent on a number of factors acting in concert. Its overall response (i.e. inhibition versus stimulation) may therefore reflect its ability to interact with the haemopoietic microenvironment and with other synergising cytokines.

The Vancouver group demonstrated control of primitive progenitor cell proliferation by negative regulators in LTBMC. Both TGF-β and MIP-1α were shown to be produced in the adherent layer of LTBMC, in which the

HSCs resided (Eaves *et al.*, 1991; Otsuka *et al.*, 1991). In undisturbed cultures, the primitive stem cells in the adherent layer were maintained in a quiescent state, presumably by regulators such as MIP-1α and TGF-β. However, upon fresh medium change, or addition of stimulatory cytokines, these cells were shown to transiently enter cell cycle (Cashman *et al.*, 1985; Cashman *et al.*, 1990). It appeared, therefore, that the cycle status of primitive progenitors was dependent on the balance of inhibitors and stimulators, either produced endogenously in, or added exogenously to, the culture. This was confirmed in a series of experiments where either TGF-β or MIP-1α, added exogeneously at the time of fresh medium change, was able to prevent entry of primitive progenitors into cell cycle. These inhibitors were also able to prevent the entry into cell cycle induced by addition of IL-1 (Cashman *et al.*, 1990; Eaves *et al.*, 1993). These effects were restricted to primitive progenitors (CFU-GM and BFU-E of larger size and appearing later in culture) with no effect on more committed cells.

The inhibitory effects of TGF-β, in LTBMC, were reversible by the addition of anti-TGF-β antibody which could prolong or reactivate primitive progenitor proliferation when added to either previously stimulated or quiescent cultures. The latter effect, on quiescent cultures, was not consistently reproducible, perhaps because MIP-1α, or another unknown inhibitor, was present in sufficient amounts to maintain stem cell quiescence, even in the absence of TGF-β, in some of the cultures (Eaves *et al.*, 1991). Similar experiments, using an antibody to MIP-1α, were never performed. Instead, MIP-1β was used to reverse the effects of MIP-1α, as described by Broxmeyer et al (Broxmeyer *et al.*, 1991). MIP-1β, which binds to the same receptor and therefore competes with MIP-1α for receptor binding, was able to maintain progenitors in the activated state several days after fresh medium change, suggesting that endogenously elaborated MIP-1α was at least partly responsible for the return to quiescence which had been observed (Eaves *et*

al., 1993). These findings strongly suggested that both TGF-β and MIP-1α, produced by bone marrow stroma, were required to maintain primitive progenitors in a quiescent state. Exactly how these two inhibitory proteins interact is unknown, but it seems possible that both play a crucial role in stem cell regulation.

In 1995, Cook et al described experiments performed with mice in which the gene encoding MIP-1α had been disrupted. In view of the inhibitory effects of MIP-1α on haemopoietic stem cells, these researchers looked carefully for evidence of effects on haemopoiesis in the "knock-out" animals. The -/- mice were shown to lack any overt haemopoietic abnormalities, however they were resistant to Coxsackie virus induced myocarditis and had reduced pneumonitis during influenza infection. These experiments confirmed the requirement for MIP-1α for an inflammatory response and confirmed that no other chemokines fulfilled that function in the absence of MIP-1α (Cook *et al.*, 1995). The lack of a haemopoietic phenotype in the null animals was not surprising. As discussed earlier, cytokines are often pleiotropic with overlapping functions and apparent redundancy. It is most likely that MIP-1α plays a role in stem cell inhibition *in vivo,* but that this role may be performed by an alternative negative regulator in the absence of MIP-1α.

It is possible that the interaction of negative regulators and stimulatory cytokines could be manipulated *in vitro* to encourage expansion of stem and progenitor cells, without exhausting pluripotent stem cells. Whether TGF-β and MIP-1α require interaction with stroma to exert their effects and whether the effects observed are due solely to the negative regulators, or to interactions with these and other molecules / factors secreted by marrow stroma remains unclear. These questions have been addressed, in part, by Verfaillie and colleagues.

Verfaillie et al have carried out a series of experiments using highly purified human stem and progenitor cells. Careful comparisons have been

made between cytokine supplemented, but stroma free, cultures, standard LTBMC with the haemopoietic cells in direct contact with bone marrow stroma, "stroma non-contact" cultures, in which the stromal layer was separated from the stem and progenitor cells by a 0.4μm porous membrane called a Transwell insert and stroma free cultures supplemented with conditioned medium derived from bone marrow stroma (SCM). The major endpoints of these experiments were to determine stroma and cytokine requirements which would allow expansion of colony forming cells *ex vivo,* but with maintenance or expansion of LTCIC. Cultures were maintained for 5-8 weeks.

The most important findings from these studies were as follows: conditions in which human CD34 positive HLA-DR negative (CD34+ DR-, 0.1-0.4% of bone marrow) stem and progenitor cells were cultured, in direct contact with stroma for 5 weeks, allowed only 20% of input LTCIC to be maintained, however maintenance was improved to 50% for stroma non-contact cultures (Verfaillie, 1992). This finding suggested that a proportion of HSC maintained in direct contact with stroma were unable to proliferate sufficiently to allow maintenance of LTCIC. No efforts have been made, in the studies reported to date, to prove whether this was due to close proximity of HSC to locally high concentrations of negative regulators such as TGF-β and MIP-1α. When CD34+ DR- cells were cultured in the presence of cytokines, but in the absence of stroma or SCM, maintenance of LTCIC was poor (Verfaillie, 1993). These findings suggested that a soluble factor(s), which was able to pass through a Transwell insert, was required to induce maturation of primitive progenitor cells (to maintain CFU-GM output) and to prevent terminal differentiation of the primitive LTCIC pool (LTCIC maintenance) (Verfaillie, 1993). This possibility was strengthened by further experiments (Verfaillie, 1993). CD34+ DR- cells were cultured in stroma free, cytokine free cultures which were then supplemented daily with SCM. In

these experiments, LTCIC were maintained at 50% of input, equivalent to a stroma non-contact culture, confirming that soluble factors were sufficient, in the absence of either stroma or additional cytokines to maintain LTCIC at 50% of output.

The following experiments were aimed to further increase maintenance of LTCIC. A range of stimulatory cytokines were used to supplement stroma non-contact cultures, either singly or in combination. IL-3 had no effect on LTCIC maintenance at 5 weeks, but caused LTCIC exhaustion by 8 weeks compared with cultures without cytokines. Multiple cytokines consistently produced exhaustion of the cultures with loss of LTCIC (Verfaillie, 1993). These results suggested that the "anti differentiation" capacity of stromal derived factors may be overwhelmed by persistent stimulation by high concentrations of growth promoting cytokines.

Verfaillie et al then reasoned that, since basal haemopoiesis appears to be regulated, not only by stimulatory cytokines, but also by inhibitory factors, it might be necessary to combine positive and negative regulators of haemopoiesis to optimise both progenitor output and LTCIC maintenance during *ex vivo* culture. When TGF-β was combined with IL-3 and used to supplement stroma non-contact cultures, both CFC output and LTCIC maintenance were significantly decreased compared with unsupplemented cultures (Verfaillie *et al.*, 1994). Although MIP-1α added alone had no effect, when combined with IL-3, 100% of input LTCIC were maintained for at least 8 weeks in stroma non-contact cultures. This result was not reproducible in the absence of bone marrow stroma unless the culture was supplemented with SCM (Verfaillie *et al.*, 1994).

From all these experiments, it appeared that MIP-1α, in combination with another stromal derived factor(s), was able to prevent the terminal differentiation of LTCIC which had been induced to proliferate by IL-3. Until the unknown factor(s) is identified it will be impossible to determine whether

the effects observed were directly due to MIP-1α and IL-3. Once identified and purified it should be possible to investigate the combination of IL-3, the unknown factor and MIP-1α for effects on LTCIC maintenance in stroma free cultures of highly purified cells.

The major emphasis of this groups' current work is aimed at examination of the role of extracellular matrix components (e.g. fibronectin, thrombospondin and proteoglycans) in these cultures. It is likely that such components may prove important for normal haemopoiesis. Murine LTBMC have been shown to synthesise several types of glycosaminoglycans including heparan sulphate, hyaluronic acid and chondroitin sulphate. In an early study, heparin and heparan sulphate enhanced the biological activity of acidic and basic fibroblast growth factors and prevented their inactivation (Gospodarowicz & Cheng, 1986). One year later, Gordon et al showed that bone marrow derived glycosaminoglycans were able to bind haemopoietic growth factor activity, in particular GM-CSF (Gordon *et al.*, 1987). A later study demonstrated that the binding of haemopoietic cells to stromal cells involved heparan sulphate (Siczkowski *et al.*, 1992). Such experiments raise the exciting possibility that, by careful manipulation of culture conditions, it may be possible to expand HSC *ex vivo*. They also suggest that negative regulators, and in particular MIP-1α, may play a crucial role in preventing stem cell differentiation and raise the possibility that extracellular matrix derived factors may be equally important.

Whether MIP-1α is essential for stem cell control may be revealed by the study of -/- animals (Cook *et al.*, 1995). A recent report, in abstract form, showed that LTBMC initiated with bone marrow from MIP-1α "knock-out" animals continued to proliferate with production of supernatant cells for longer than cultures initiated with wild type bone marrow (Boggs *et al.*, 1995). At first, this result could be interpreted to suggest that the presence of MIP-1α had an adverse effect on stem cell maintenance (the opposite of the effects

shown by Verfaillie et al) however, the known inflammatory properties of MIP-1α must be taken into account. It is quite possible that, in the absence of MIP-1α, there was much less macrophage proliferation in the LTBMC which would therefore be expected to behave quite differently from wild type cultures.

Several studies have reported the use of MIP-1α *in vivo* in animal models. When given to normal animals, MIP-1α was shown to decrease the proportion of primitive progenitor cells in active cell cycle (Dunlop *et al.*, 1992; Maze *et al.*, 1992). These effects appeared to be both dose dependent and reversible. These studies raised the possibility that MIP-1α could be used to prevent cycling of normal progenitor cells during administration of cycle specific cytotoxic agents and thereby prevent the myelosuppression which is the primary adverse effect of these drugs when used clinically. MIP-1α has been shown to be useful in this setting in two animals models. In the first study, in mice given two doses of cytosine-arabinoside, MIP-1α was able to protect haemopoietic stem and progenitors cells and to allow faster neutrophil recovery in treated animals (Dunlop *et al.*, 1992). In the second study, MIP-1α was able to protect the CFU-S compartment from the toxic effects of hydroxyurea (Lord *et al.*, 1992).

To summarise, although MIP-1α is predominantly an inflammatory mediator, it has been shown to have inhibitory effects on the proliferation of immature haemopoietic progenitor cells. These effects appear to be directed towards a relatively restricted part of the stem cell compartment, represented by CFU-S day 12 and CFU-A. The fact that MIP-1α knockout animals appear healthy and have normal blood counts suggests that the inhibitory properties of the protein may overlap with those of other negative regulators suggesting another example of apparent redundancy. The protective effects of MIP-1α on cytotoxic induced myelosuppression and the "antidifferentiation" effects

demonstrated by Verfaillie and colleagues suggest potentially exciting roles for this protein in therapeutic use.

1.17 Stem cell purification strategies

Whilst the development of both *in vitro* and *in vivo* assay systems enhanced the study of regulation of the stem cell compartment, the endogenous production and induction of cytokines by accessory cells present in unfractionated bone marrow cell cultures prevented definitive conclusions regarding the direct actions of individual cytokines added exogeneously. Cytokines produced by accessory cells may have direct actions on stem and progenitor cells and may also act in synergy with added cytokines. Furthermore, in unfractionated bone marrow, HSC are present at very low frequencies and it is therefore necessary to enrich or purify stem cell subsets to allow accurate study of the mechanism of action of certain cytokines and cytokine combinations and to carry out biochemical studies. Cell separation techniques, which are now available, are based on both physiochemical and immunological approaches. These methods rely on very diverse cell characteristics which include density, affinity for plant lectins, differential sensitivity to cytotoxic agents, cell surface antigen expression and uptake of a fluorescent indicator of metabolic activity (i.e. rhodamine).

Counterflow centrifugal elutriation (CCE) can separate large numbers of cells, primarily by size and density (Sanderson & Bird, 1977). The cells are placed in a revolving chamber and as they reach equilibrium, the smaller cells migrate to the centre of the chamber while the larger ones sediment towards the outer end. With changes in the flow rate of elutriation liquid to overcome the centrifugal force, cells in different size categories can be collected.

The basis of fluorescence activated cell sorting (FACS) is to label cells with one, or a variety, of fluorescent markers to select cell populations, firstly on the basis of density, then to sort the cells of interest using a flow cytometer

on the basis of forward angle light scatter (a measure of cell size) and right angle light scatter (a measure of cellular heterogeneity and fluorescence intensity).

In the mouse, lineage markers have been identified for B cells (B220, IgM), T cells (CD4, CD5 and CD8), myelomonocytic cells (Mac-1 and Gr-1) and many other cell phenotypes. More primitive cells express low levels of Thy-1.1 and are positive for Sca-1. Other markers used to define primitive cells include AA4.1 and c-kit. Therefore, by FACS, a subset of cells may be separated (Sca-1+ / Thy-1.1^{lo} / Lin-) which is highly enriched in activity in stem cell assays. As discussed above, despite being enriched, these subsets still show marked heterogeneity with respect to *in vivo* lifespan (Smith *et al.*, 1991; Uchida & Weissman, 1992). Sca-1+ / Thy-1.1^{lo} / Lin- cells may be further divided on the basis of rhodamine staining (Spangrude & Johnson, 1990). Rhodamine is a supravital, cationic fluorescent dye that has relatively high affinity for mitochondrial membranes and is retained much better in cycling cells than in quiescent cells (Bertoncello *et al.*, 1985; Bertoncello *et al.*, 1991). The mechanism underlying these observations is thought to be due to more efficient efflux of the dye from primitive cells by the gp 170 efflux pump. Rhodamine staining may, therefore, be used to discriminate primitive from more mature cells. For example, Bertoncello et showed that HSC with long term reconstituting capacity *in vivo* were rhodamine^{dull} whilst a large proportion of HPP-CFC and CFU-S were rhodamine^{bright} (Bertoncello *et al.*, 1985). In addition to the cell markers and methods mentioned above, there are a number of other techniques which are used by different research workers to enable purification of specific cell subsets.

The Ly-5 antigen (leucocyte common antigen / CD45) is expressed on all myeloid precursors and mature nucleated cells. Allelic differences at Ly-5 may be detected by monoclonal antibodies specific for Ly-5.1 and Ly-5.2, allowing donor cells (e.g. Ly-5.2) to be detected in Ly-5.1, congenic hosts.

This system allows transplantation without crossing the major histocompatibility barrier and has been used in the CRA (Smith *et al.*, 1991; Uchida & Weissman, 1992).

1.18 Phases of haemopoietic engraftment following BMT

Following lethal doses of radiation and infusion of bone marrow cells, engraftment is thought to occur in overlapping phases. During the early phase of "transient engraftment", a relatively large number of stem cells contribute to haemopoiesis, however after the first few weeks only a few stem cells continue to contribute (Dick *et al.*, 1985; Lemischka *et al.*, 1986; Smith *et al.*, 1991; Uchida & Weissman, 1992; Harrison *et al.*, 1993). Jones et al separated an enriched CFU-S day 12 population from more primitive stem cells by CCE and showed that this cell population, upon transplantation following lethal dose irradiation, was sufficient to enable rapid return of normal blood counts. However, this cell population was not sufficient for long term haemopoiesis. This study demonstrated that CFU-S were responsible for the early phase of engraftment following BMT but pre-CFU-S were essential for long term haemopoiesis (Jones *et al.*, 1990).

For transplantation with enriched cell populations, even small numbers of highly purified cells may give rise to sustained long term haemopoiesis (Uchida *et al.*, 1994). The work by Uchida et al argued that small numbers of Thy-1.1^{lo} Sca-1+ Lin- cells were sufficient, when transplanted alone, to produce both transient and long term reconstitution. However, the enrichment techniques used were known to enrich not only for HSC, but also for progenitor cells, especially CFU-S day 12, thereby producing a heterogeneous population which may well have contained both progenitor cells able to rapidly reconstitute early haemopoiesis and a further quiescent subset which gave rise to an increasing fraction of donor derived cells in each lineage with time (Uchida *et al.*, 1994).

This work was not incompatible with the earlier results described by Jones et al (Jones *et al.*, 1990). The cell population obtained by CCE in their study, which was virtually depleted in CFU-GM and CFU-S, was not able, alone, to rescue lethally irradiated animals. These results, taken together, suggest that there are indeed two phases of engraftment following BMT and that different progenitor populations may give rise to early haemopoiesis depending on the number and purity of the stem cell population transplanted.

1.19 Bone marrow transplantation and radiation protocols

In order to demonstrate "rescue" from the effects of radiation, a protocol which ensures close to 100% mortality in the absence of BMT but 100% survival with adequate BMT is required. Radiation damage is concentrated in rapidly proliferating tissues including the bone marrow, the lung and the gastrointestinal tract. The degree of damage is related to the total dose, dose rate and use of dose fractionation. It has previously been reported that dose fractionation may permit a high total dose of radiation with fewer fatalities due to gastrointestinal and pulmonary complications (Geraci *et al.*, 1977; Ferrara *et al.*, 1987; Spangrude & Scollay, 1990).

In vivo Assays

In vivo stem cell assays have been developed to allow study of the functional properties of stem cell populations. Mouse models are available with genetic markers that allow accurate measurement of the long-term function of transplanted HSC. A number of assays have been described including those for colony formation (CFU-S (Till & McCulloch, 1961)), radioprotective ability, MRA (Hodgson & Bradley, 1979), serial BMT (Jones *et al.*, 1989), and CRA (Harrison *et al.*, 1993). These assays vary considerably with respect to assay time and the chimeric mouse model employed and therefore comparison of data generated using different assay systems is not always

possible. Although most of these assays produce only comparative or qualitative data, the CRA is fully quantitative and may be accurately assayed over the entire lifespan of the recipient.

1.20 Competitive repopulation assay

The CRA, described in detail by Harrison et al (Harrison *et al.*, 1993), directly compares the long-term proliferative abilities of two populations of donor cells. The first population, the competitor, is a dose of fresh bone marrow which serves as a standard and is of sufficient repopulating ability to allow successful reconstitution if used alone. The second population, the donor, is of unknown HSC content and is measured relative to the competitor. Competitor and donor are derived from mice that differ at genes specifying quantitative markers and, therefore, the relative percentages of their progeny can be determined over time. This allows a very accurate assessment of the potential of different cell populations to rescue mice both early and late following radiation.

1.21 Serial BMT

Serial BMT may be used to study proliferation and self-renewal of HSC *in vivo* (Ogden & Micklem, 1976; Harrison *et al.*, 1978; Brecher *et al.*, 1988; Jones *et al.*, 1989; Harrison *et al.*, 1990). One of several models in use employs syngeneic BMT with male donors and female recipients. The recipients are lethally irradiated prior to transplantation and at varying time intervals following primary BMT, the reconstituted recipient mice are then used as donors in a second BMT procedure. With repeated serial transfers, the ability of the donor marrow to reconstitute is eventually lost. The underlying reason for this limited repopulating ability of serially transplanted bone marrow is unknown (see discussion).

The normal proliferative capacity of bone marrow stem cells is severely stressed when required to fully regenerate haemopoiesis following BMT. In previous studies, serial BMT performed with short intervals (8-10 weeks) between transfers produced a progressive impairment of the ability of donor cells to repopulate irradiated hosts (Siminovitch *et al.*, 1964; Cudkowicz *et al.*, 1964). By lengthening the interval between transfers the repopulating ability was improved, but even with very long (6-12 months) intervals, serial transfers could not be continued for more than five generations (Siminovitch *et al.*, 1964; Cudkowicz *et al.*, 1964; Ogden & Micklem, 1976; Harrison *et al.*, 1978).

Serial BMT is therefore a useful functional test of the ability of cells to reconstitute under circumstances requiring enormous stem cell reserve, but it cannot measure the number of reconstituting cells present. Although the assumption is generally made that serial transfer eventually fails because stem cells have a finite capacity for self-renewal, there are a number of other possible explanations for this observation (see discussion). The fact that leaving intervals of one year between transfers is insufficient to allowed more than five transfers suggests that the changes to the stem cell compartment induced by BMT are in part irreversible.

Ex vivo Expansion of Stem / progenitor Cells

1.22 Potential clinical applications

Over the last decade autologous bone marrow transplantation has been largely replaced by the use of mobilised PBPC (Kessinger & Armitage, 1991). Although HSC are present in peripheral blood under steady state conditions, their numbers are greatly increased during the recovery phase of treatment with myelosuppressive cytotoxic agents or following the administration of growth factors such as G-CSF and GM-CSF. More recently the use of PBPC has also been investigated for allogeneic transplantation. The key advantage of

using PBPC for transplantation compared with bone marrow is that PBPC significantly accelerate the rate of haematological recovery and hence the morbidity and mortality associated with the procedure (Kessinger & Armitage, 1991). Furthermore, PBPC provide the only source of HSC for patients whose bone marrow is fibrotic or involved with tumour and therefore unsuitable for harvesting.

The rapid engraftment experienced following PBPC transplantation is thought to reflect both the increased number of stem and progenitor cells which may be harvested from peripheral blood compared with bone marrow and alterations both in phenotype / cycle status and adhesiveness / homing of these cells (reviewed in (Henon, 1995)). Since PBPC collected during steady state haemopoiesis, as opposed to following chemotherapy +/- growth factor mobilisation, do not accelerate haemopoietic recovery compared with bone marrow, it is likely that the effects of growth factors, either administered directly, or produced endogenously during marrow regeneration, are in part responsible for the acceleration in engraftment which is observed. This raises the question whether stem cells could be manipulated *ex vivo*, in the presence of growth factors, to further increase their numbers and / or alter their characteristics to produce even more rapid engraftment upon transplantation. In certain clinical situations it would be of value to expand the number of progenitor cells available, whilst in others it would be more valuable to facilitate very rapid haemopoietic recovery.

To ensure rapid engraftment, a critical number of stem and progenitor cells must be collected. This usually requires multiple leucapheresis procedures, performed on consecutive days. Recent strategies to reduce the number of tumour cells contaminating PBPC collections for autologous transplantation have involved positive selection of stem and progenitor cells. CD34, first described by Civin et al, is an antigen present on the surface of all HSCs and progenitor cells (Civin *et al.*, 1984). The availability of monoclonal

antibodies directed to this antigen have facilitated the development of a range of commercially available CD34 selection devices which are based on separation of the CD34 positive cells from the remaining CD34 negative cells present in PBPC collections. These techniques are not fully efficient and although tumour cells may be depleted by 2-3 logs, approximately half of the stem and progenitor cells available are lost during the separation procedure.

Thus, considerable attention is currently directed at methods to expand stem and progenitor cells *ex vivo*. The obvious advantages of this approach would be to reduce the number and duration of leucapheresis procedures, particularly in situations when multiple transplants are planned, to expand cord blood derived HSC in order to provide sufficient cells for transplantation, to trigger cycling of primitive stem cells to facilitate gene transduction and then, perhaps, to selectively expand the transduced population prior to transplantation (reviewed in (Moore, 1995)). Intense investigation has also been directed towards developing *ex vivo* culture conditions which promote proliferation of progenitors cells which produce prompt engraftment upon transplantation. The mechanisms which underlie any observed acceleration in haemopoietic recovery are likely to reflect both quantitative and qualitative progenitor changes.

1.23 Homing of HSC: Potential modulation by growth factor exposure *ex vivo*

The mechanisms which regulate the circulation and homing of HSC, both during fetal and adult life, are largely unknown. HSC are known to be present in peripheral blood under steady state (i.e. non-mobilised) conditions. Such cells are able to provide complete and sustained haemopoietic reconstitution in transplant recipients (Kessinger & Armitage, 1991).

In murine studies, bone marrow cells administered intravenously home to the bone marrow and produce sustained engraftment, even in the absence of

myeloablation (Brecher *et al.*, 1982; Stewart *et al.*, 1993). These findings suggest that HSC constantly circulate from the bone marrow into the peripheral blood and vice versa. Homing to the bone marrow may be greatly increased by myeloablation of the transplant recipient. However, the homing which occurs following radiation / chemotherapy may differ from that occurring in steady state. Similarly, the HSC found in the circulation in steady state may differ from those remaining in the bone marrow and those recruited into the circulation by cytokines and / or the effects of chemotherapy may again be subtly different.

It is clearly of importance to try and establish which factors mediate HSC circulation and homing in order to try and manipulate the situation either experimentally or therapeutically. Experiments performed by Tavassoli and co-workers suggested that homing of HSC to the bone marrow was mediated by an unknown lectin on their surface which had an affinity for carbohydrate determinants present on stromal cells (reviewed in Tavassoli *et al.*, 1995). This group designated the lectin, the "haemopoietic homing receptor". They proceeded to develop synthetic glycoproteins which could be radiolabelled and which competed as ligands for the "homing receptor".

In vivo murine studies revealed that the survival of lethally irradiated, splenectomised mice was reduced if bone marrow cells were transplanted in conjunction with the synthetic glycoproteins. The effect on survival was much less impressive in the presence of a normal spleen, suggesting that homing of intravenously transplanted HSC to bone marrow, but not spleen, was mediated by an interaction between the lectin (homing receptor) and a glycoprotein ligand. In addition to effects on survival, bone marrow cellularity, CFU-S and CFU-GM were all reduced by competition with the synthetic glycoproteins (Aizawa & Tavassoli, 1988).

Further *in vitro* studies showed that the synthetic ligands competed reversibly with the "homing receptor" to reduce progenitor output in LTBMC

(Aizawa & Tavassoli, 1987). The "homing receptor" was shown to be present on the surface of stem cells, CFU-S and CFU-GM, but not mature granulocytes. Interaction of the "homing receptor" with its specific carbohydrate ligand present on stromal cells was necessary for binding of stem cells to stroma (Tavassoli *et al.*, 1995). These findings suggested that the level of expression of the "homing receptor" declined with stem cell maturation and differentiation and may explain why, in LTBMC, stem cells are found in the adherent layer, whilst more committed progenitor cells and mature cells are found in the supernatant. This also raises the possibility that if stem cells are manipulated *ex vivo* to produce more mature, committed progenitor cells, these may not express the "homing receptor" in sufficient quantity to permit homing to the bone marrow. If this were the case, these cells would be likely to be destroyed eventually, in the peripheral circulation.

In addition to this "homing receptor", further adhesive interactions are thought to play a role in retaining stem cells within the marrow. One major component of the extracellular matrix to which stem cells may bind is fibronectin (Williams *et al.*, 1991). Experiments performed using IL-3 dependent progenitor cell lines have demonstrated that these cells possess a β1 integrin in their membrane which is presumed to mediate adherence to fibronectin. This binding was shown to be cytokine dependent since adherence to fibronectin was dramatically, but reversibly, reduced by IL-3 removal (Tavassoli *et al.*, 1995). These findings suggest that cytokine manipulation of stem and progenitor cells *ex vivo* might alter their ability to adhere to fibronectin and, hence, to home to the bone marrow stroma upon reinfusion.

Proteoglycans are synthesised by stromal cells and are transiently present in the cell membrane before being released into the extracellular space. In addition, proteoglycans may be present on the HSC surface. Once outside the cell, they are able to adhere to the extracellular matrix. In this way proteoglycans may be able to present growth factors to stem and progenitor

cells (Tavassoli *et al.*, 1995). This question was the subject of the study performed by Gordon et al (Gordon *et al.*, 1987) in which bone marrow derived glycosaminoglycans were used to coat tissue culture plates. These were then used to extract GM-stimulating activity from 5637CM or to bind purified GM-CSF. The results strongly suggested a role for glycosaminoglycans in the binding of cytokines and subsequent presentation to stem cells. It remains unclear whether the *in vivo* situation within the bone marrow microenvironment may be reproduced *ex vivo*, simply by adding cytokines to stem cell populations. It seems likely that, in the absence of either stroma or proteoglycans, stem cell expansion and proliferation *ex vivo* may be sub-optimal.

The availability of monoclonal antibodies has allowed investigators to detect a range of known cytoadhesive molecules on HSC. Antigens expressed on the surface of progenitor cells include integrins, members of the immunoglobulin superfamily including intracellular adhesion molecule (ICAM-1) and lymphocyte function-related antigen (LFA-3), CD44 and L-selectin (reviewed in Tavassoli *et al.*, 1995). The inhibition of growth in LTBMC by antibodies to CD44 and $\alpha 4\beta 1$ integrin suggests an important role for these molecules in the localisation of stem cells to the stromal microenvironment (Tavassoli *et al.*, 1995). Since cytokines are known to be produced within the bone marrow microenvironment and are thought to play a major role in regulating haemopoiesis, it is likely that cytokines may act locally to effect the level of expression of certain adhesion molecules and thereby influence the localisation of stem and progenitor cells within the bone marrow. An example of this was demonstrated for SCF by Kovach et al (Kovach *et al.*, 1995). In their study treatment of MO7E cells with SCF produced a transient increase in adherence to human umbilical vein endothelium or to VCAM-1-transfected CHO cells with a peak at 30 minutes, however, more prolonged exposure to SCF induced a marked decrease in

integrin-mediated adherence with maximal inhibition at 24 hours. These findings may in part explain why cytokines such as SCF mobilise primitive haemopoietic cells from the bone marrow into the peripheral blood.

To investigate whether cytokines regulate stem cell homing and engraftment, a number of studies have been performed in which bone marrow cells were pre-treated with growth factors prior to transplantation into either lethally irradiated adult or "pre-immune" fetal recipients (Fabian *et al.*, 1987; Tavassoli *et al.*, 1991; Zanjani *et al.*, 1992). In these studies, short term exposure (a number of hours) of bone marrow or fetal liver progenitor cells to IL-3 or GM-CSF enhanced their capacity to reconstitute multilineage haemopoiesis in recipient animals. Since no expansion of stem and progenitor cell numbers could have occurred over such a short period of growth factor exposure, these results suggested that part of the capacity of cytokines to improve haematological reconstitution after transplantation with cells manipulated *ex vivo* related to effects on homing.

Very recently, however, Van der Loo & Ploemacher reported a similar study with contradictory results (Van der Loo & Ploemacher, 1995). In this study, a brief preincubation of bone marrow cells with either IL-3 alone or IL-3, IL-12 and SCF had a negative effect on the seeding of all haemopoietic subsets to bone marrow and spleen. Van der Loo argued that the medium only control used in the previous study by Tavassoli et al (Tavassoli *et al.*, 1991) may not have maintained stem cell viability since it was incubated at 37°C in the absence of cytokines. If this were indeed the case, then the cells incubated in the presence of cytokines would have appeared to produce enhanced homing compared with cells with poor viability. The control cells used in the latter study were maintained in the absence of cytokines, but on ice rather than at 37°C (Van der Loo & Ploemacher, 1995). These contradictory results are difficult to reconcile. In the absence of studies which use identical controls this issue remains open to debate, however, in view of the number of

investigators who have reported improvements in engraftment and / or homing of stem and progenitor cells following growth factor exposure *in vitro*, the balance of evidence is in favour of enhanced homing by cytokines. In additional experiments, Tavassoli and co-workers demonstrated that certain cytokines had direct effects on the level of expression of the "homing receptor" on FDCP-1 and FDCP-Mix murine haemopoietic progenitor cells (Tavassoli *et al.*, 1991; Shiota *et al.* , 1992). In these experiments, the number of homing receptors increased 2-3 fold during incubation with IL-3 or GM-CSF. Effects on other adhesive proteins in bone marrow have also been demonstrated by other groups. For example, the expression of ICAM-1 and VCAM-1 on stromal cells was shown to be increased by exposure to IL-1, IL-4 or TNF-α (Simmons *et al.*, 1992; Teixido *et al.*, 1992). These cytokine effects do suggest that stem cells could be manipulated *ex vivo* with the specific aim of modulating the interaction between molecules present on the stem cell surface and others present on stromal cells. This approach could then be exploited clinically to obtain enhanced engraftment following haemopoietic transplantation.

1.24 *Ex vivo* expansion: experimental results

In order to achieve, long term *ex vivo* expansion of HSC for any purpose, culture conditions which mimic the *in vivo* situation, as closely as possible, must be developed. It remains unclear whether combinations of stimulatory cytokines used alone may recruit primitive cells which have self-renewal capacity (e.g. LTRC) into cycle, or whether bone marrow stroma and / or stem cell inhibitors are required in addition.

In vivo, and in LTBMC, HSC are found in close association with the stroma and appear to be tightly regulated by both positive and negative signals. There are, therefore, concerns, that positive cytokines, used in the absence of stroma and negative regulators, may result only in terminal

differentiation of HSC with little or no maintenance of LTRC. Alternatively, HSC may be relatively resistant to the action of cytokines which may only provide a survival stimulus, such that HSC numbers are maintained, but not expanded. Over recent years, a wide range of both stimulatory and inhibitory cytokines have been discovered and a number of synergistic cytokine combinations have been identified.

In the absence of growth factors, HSC and progenitor cells have been shown to die by the process of apoptosis (reviewed by Koury, 1992). When undergoing apoptosis, cells decrease in size, their nuclei condense and eventually the cells become fragmented. Among the known growth factors, Epo, IL-3, GM-CSF and G-CSF have all been shown to suppress apoptosis in cells that are dependent upon them for survival (Koury, 1992; Williams *et al.*, 1990b). Katayama et al and Bodine et al have identified cytokines including IL-3, G-CSF and SCF, which appeared to support the survival of quiescent murine primitive progenitor cells when cultured *ex vivo* (Bodine *et al.* , 1991; Bodine *et al.*, 1992; Katayama *et al.*, 1993). These cytokines, used alone, supported survival but not proliferation of HSC. Further cytokines were required to recruit cells into active cell cycle. Ogawa's group followed the development of primitive "blast" cell colonies in response to both single and two factor combinations. These serial mapping studies revealed that certain cytokines were able to trigger proliferation of primitive progenitor cells and appeared to shorten the dormant (Go) period of HSC. These cytokines included SCF, IL-11, IL-6 and G-CSF (Musashi *et al.*, 1991; Tsuji *et al.*, 1991).

Based on these, and other observations, a number of combinations of synergising cytokines have been investigated which promote extensive amplification of both committed and multipotential progenitors. Prior to the cloning of SCF, the most potent combination was IL-3 and IL-6 (Bodine *et al.*, 1989) however, the inclusion of SCF promoted even greater stem and

progenitor cell amplification (Bodine *et al.*, 1992) and is currently included in most growth factor combinations.

Expansion of murine CFU-S *in vitro* has been achieved by several investigators using SCF containing combinations such as SCF / IL-1α (de Vries *et al.*, 1991), SCF / IL-6 (Luskey *et al.*, 1992), SCF / IL-3 / IL-6 (Bodine *et al.*, 1992), SCF / IL-1β (Muench *et al.*, 1992) and SCF / IL-11 (Neben *et al.*, 1994). Likewise, expansion of primitive multipotential progenitor cells detected by *in vitro* assays including HPP-CFC and CAFC has also been achieved with similar combinations (Muench *et al.*, 1992; Ploemacher *et al.*, 1993b).

Although these studies showed extensive progenitor expansion *in vitro*, *in vivo* experiments were required to assess the engraftment potential of such expanded populations. Exposure of progenitor cells to growth factors may enhance engraftment by two mechanisms. Firstly, such manipulation may provide an increased number of progenitors enriched in transient engrafting potential and, secondly, may effect the "homing" of stem and progenitor cells to the bone marrow microenvironment leading to more rapid engraftment and differentiation (Fabian *et al.*, 1987; Tavassoli & Hardy, 1990; Tavassoli *et al.*, 1991; Zanjani *et al.*, 1992).

In vivo experiments have been used to assess the effect of cytokine stimulation on both short term engraftment and LTRC. Significant acceleration of short term haemopoietic recovery following transplantation with cells expanded *ex vivo* has now been demonstrated convincingly, and confirmed by our own work (Muench & Moore, 1992; Muench *et al.*, 1993; Serrano *et al.*, 1994; Holyoake *et al* , 1996). Several studies have reported greater recovery of either the ability to rescue lethally irradiated animals, or of repopulating ability following *ex vivo* expansion of unfractionated bone marrow compared with purified stem cell populations (Okano *et al.*, 1989; Knobel *et al.*, 1994; Neben *et al.*, 1994). In the study by Neben et al, under

identical culture conditions, a significantly higher percentage of primitive stem cells was maintained in cultures of unfractionated 2 day post 5-FU bone marrow, than in cultures initiated with Sca+ Lin- 2 day post 5-FU bone marrow. Unfractionated bone marrow contains stromal elements which may elaborate, not only other cytokines, but also negative regulators of haemopoiesis. For example, MIP-1α is an inducible product of bone marrow macrophages and therefore is likely to be present in greater levels in cultures of unfractionated bone marrow. At least for human HSC, MIP-1α appears to play a role in the maintenance of primitive progenitors when used to supplement LTBMC initiated with highly purified progenitor cells in conjunction with IL-3 (Verfaillie *et al.*, 1994). The stroma present in unfractionated bone marrow cultures may also facilitate adherence of HSC to stromal elements and thereby interaction between the HSC and regulators produced by the stromal cells. These results emphasise that growth factors may have different effects depending on the cellular context of their action.

One of the difficulties encountered when attempting to interpret the extensive literature relating to effects of growth factor exposure on short and LTRC is the degree of variation in methodology employed in the different studies. For example, differences are encountered in the starting cell population, which may be normal unfractionated bone marrow, post 5-FU marrow or cells enriched to varying degrees; the number of growth factors and combinations employed; the culture conditions including the use of serum; the duration of incubation; the dose of radiation and method of administration; the donor and recipient murine strains used in the studies; the route of injection of the transplant and, finally, the control cells against which the expanded cells are compared.

Some investigators have compared the repopulating ability of cultured cells against fresh control cells (Okano *et al.*, 1989; Muench & Moore, 1992; Muench *et al.*, 1993; Knobel *et al.*, 1994; Rebel *et al.*, 1994; Serrano *et al.*,

1994) whilst others compared cells expanded with multiple cytokines against cells cultured with IL-3, IL-6 or SCF alone (Bodine *et al.*, 1989; Bodine *et al.*, 1991; Bodine *et al.*, 1992; Luskey *et al.*, 1992; Neben *et al.*, 1994). A more appropriate control may be cells which have been cultured under otherwise identical conditions, but in the absence of cytokines. This control has rarely been included because progenitor cells including CFU-S survive poorly in the absence of cytokines (Luskey *et al.*, 1992). Furthermore, Bodine et al have demonstrated that bone marrow cells cultured in the absence of cytokines are unable to compete against cells cultured with IL-3 alone in the CRA, suggesting that at least a single cytokine is required to maintain stem cell viability (Bodine *et al.*, 1992). The cytokines which appear to satisfy this requirement include IL-3, G-CSF and SCF (Bodine *et al.*, 1991; Bodine *et al.*, 1992; Katayama *et al.*, 1993).

Although certain cytokine combinations increased committed and multipotential progenitor content at the expense of stem cell function (Bodine *et al.*, 1991), combinations which allowed at least maintenance of LTRC have been determined (Bodine *et al.*, 1989; Bodine *et al.*, 1991; Bodine *et al.*, 1992; Muench *et al.*, 1993; Neben *et al.*, 1994; Rebel *et al.*, 1994; Serrano *et al.*, 1994). Many such studies compare cells expanded with multiple cytokines against control cells cultured with a single factor rather than with fresh uncultured marrow. Several of these and other studies have demonstrated the ability to transduce murine stem cells with genes of interest in the presence of such synergising growth factors with efficient infection of reconstituting haemopoieitc stem cells in the long term (Dick *et al.*, 1985; Lemischka *et al.*, 1986; Bodine *et al.*, 1989; Bodine *et al.*, 1991; Luskey *et al.*, 1992; Bernad *et al.*, 1994).

A number of conclusions may be drawn from the literature currently available regarding *ex vivo* expansion of murine haemopoietic stem and progenitor cells. Although single cytokines are necessary for cell survival,

synergising cytokine combinations are required to stimulate proliferation and expansion of progenitor cells. Cells which have been expanded *ex vivo*, using certain cytokine combinations, produce significantly more rapid haematological reconstitution compared with either fresh bone marrow, or bone marrow cultured with a single cytokine. Whether this improvement in early engraftment relates only to increases in progenitor cells responsible for transient engraftment, or to alterations in progenitor homing, or both, remains unclear. LTRC do appear to be maintained under certain *ex vivo* culture conditions, but whether this is due to slow turn-over with self-renewal of LTRC or to total quiescence of the original LTRC which are resistant to the effects of cytokines is not clear. Cytokine manipulation does appear useful for gene transduction and long term studies confirm that genes are still expressed at relatively high levels many months following transplantation, suggesting infection of LTRC, which, despite proliferating, have remained pluripotent. Finally, the reported differences, by some groups, comparing unfractionated bone marrow with highly purified populations, suggests that bone marrow stroma, and / or negative regulators, may play an important role in the maintenance of LTRC during *ex vivo* culture.

AIMS

Over recent years a wide range of stimulatory and inhibitory cytokines have been cloned and investigated for their biological effects on haemopoietic stem and progenitor cells. Many attempts have been made to develop *in vitro* systems which closely mimic *in vivo* haemopoiesis in order to establish the role that these cytokines have in the regulation of stem cell proliferation.

The first aim of the work presented in this thesis was to optimise, in the absence of stroma, *ex vivo* culture conditions which would promote proliferation and expansion of murine multipotential CFU-A progenitor cells. There was sufficient evidence that CFU-A / CFU-S day 12 were of crucial importance for the early, but transient phase of engraftment seen after BMT. It was hoped, therefore, that by transplanting a greatly expanded population of CFU-A, neutrophil and platelet recovery following BMT might be accelerated. In addition, if CFU-A numbers could be increased by *ex vivo* culture, then the number of bone marrow cells required for rescue from myeloablation could be reduced. If such endpoints could be achieved in a murine model, then there would be value in pursuing a similar approach clinically since, firstly, the major morbidity following autologous transplantation is related to prolonged myelosuppression and, secondly, in a significant proportion of patients, for whom autologous transplantation is indicated, insufficient stem cells can be harvested to mediate rescue from myeloablative therapy.

The second aim of these studies was to develop an appropriate *in vivo* model in which to test the engrafting potential of the progenitor cells which had been expanded *ex vivo*. Although the initial work was directed towards expansion of CFU-A and short term engraftment, it was also of importance to assess the effect that cytokine induced amplification had on stem cells which were responsible for long term reconstitution after BMT. Both for

transplantation studies and for gene therapy applications, maintenance, or preferably expansion, of pluripotent stem cells would be required to ensure sustained long term engraftment and gene expression.

The final aim, dependent on the results of preliminary work, was to develop suitable culture conditions to allow *ex vivo* expansion of human progenitor cells on a sufficiently large scale to generate enough cells for rescue following myeloablative therapy. Although the results of this clinical work are not discussed in this thesis, they are mentioned briefly in the discussion.

MATERIALS AND METHODS

MATERIALS

Tissue Culture

SUPPLIER	ADDRESS	REAGENTS
Beatson Institute Central Services	Beatson Institute	Penicillin (7.5mg/ml) (10, 000 units/ml) Streptomycin (10mg/ml) Sterile PBS-A (oxoid) Sterile Distilled H_2O (DW) Sterile Glassware and Pipettes
Bibby Sterilin Ltd.	Stone, Staffs, UK	3cm Dishes 6cm Dishes 9cm Dishes
Gibco Life Technologies	Paisley, Scotland, UK	Human Recombinant TGF-β1 Special Liquid Medium Fischer's Medium (10X) RPMI Medium (10X) 2.5% Trypsin 200mM Glutamine Alpha Minimal Essential Medium (α-MEM) MEM x 100 Vitamins Gentamicin Sulphate 7.5% Sodium Bicarbonate Foetal Calf Serum (FCS) Colcemid (10μg/ml) L-broth
Roche Products Amgen Roche	Welwyn Garden City UK	Human Recombinant G-CSF
R&D Systems	Oxon, UK	Murine Recombinant IL-3 Murine Recombinant IL-6 Human Recombinant MCP-1 Human Recombinant PDGF Human Recombinant IL-8 Murine Recombinant LIF Human Recombinant Rantes Murine Recombinant IL-7 Murine Recombinant IL-10
Genetics Institute	Cambridge, MA, USA	Murine Kit-Ligand (media conditioned by CHO cells expressing soluble Kit-Ligand) Human Recombinant M-CSF (CHO cells) Murine Recombinant GM-CSF (COS-1 cells)

		Human G-CSF (crude conditioned medium, CHO cell line) Human Recombinant IL-11 (E. Coli) Purified Human Recombinant MIP-1α (CHO cells)
Boehringer Mannheim Pharmaceuticals	Lewes, E. Sussex, UK	Human Recombinant Erythropoietin
Genzyme Diagnostics	Cambridge, MA, USA	Human Recombinant IL-1β
Costar	Cambridge, MA, USA	24 Well Culture Plates Cell Scrapers
A/S Nunc	Roskilde, Denmark	Tissue Culture Flasks Nunc Cryotubes 35mm Plates
Difco Laboratories	Detroit, Michigan, USA	Agar Noble
Sigma Chemical Co. Ltd.	Poole, Dorset, UK	Donor Horse Serum (DHS) Cytosine β-D-arabino-furanoside (ARA-C) 5-fluorouracil Rhodamine 123 Purified Rat anti-mouse PE-conjugated CD5
The Binding Site	Birmingham University	Sheep anti-mouse FITC-conjugated IgM
Harlan Olac Ltd.	Bicester, Oxon, UK	Male and Female B6D2F1 Mice
Fluka Chemika-Biochemika	Gillingham, UK	Methocel MC
Fisons Scientific Equipment	Loughborough, UK	Dimethyl Sulfoxide
Becton Dickinson (Falcon)	Cowley, Oxford, UK	Tissue Culture Flasks 50ml & 15ml Centrifuge Tubes 2ml, 5ml, 10ml, 20ml Plastipak Syringes 5ml, 10ml, 25ml Plastic Pipettes Cell Strainers
Coulter Electronics Ltd.	Luton, UK	Coulter Counter Zap-oglobin
Nycomed	Sheldon, UK	Nycodenz 1.150g/ml
DYNAL	Bromborough, UK	Dynabeads (M-450 sheep anti-rat IgG)
Pharmingen	San Diego, CA, USA	Purified Rat Anti-Mouse Monoclonal Antibodies Gr-1 B220 Mac 1

		CD5 CD8a FITC conjugated-Sca-1 FITC conjugated-D7 FITC conjugated-c-kit
Gelman Sciences	Michigan, USA	Acrodisc Syringe End Filters, 0.2 and 0.45μm Cell Lysing solution

Bacterial Hosts and Media

Gibco Life Technologies	Paisley, Scotland, UK	E. Coli Host Strain DH5α Ampicillin L-broth
Beatson Institute Central Services	Beatson Institute, UK	Sterile Glassware
Difco Laboratories	Detroit, Michigan, USA	Agar Bacto Bactotryptone
Beta Laboratories	East Molesey, UK	Yeast Extract

Plasmids & Inserts

Dr N. Hole	Edinburgh University, UK	pY353/B plasmid
Mr K. Ryan	Beatson Institute, UK	GAPDH insert

Kits, Columns

Pharmacia Biotech Ltd.	St. Albans, Herts, UK	Random-Primed DNA Labelling Kit NICK Columns Sephadex G-50 DNA grade
Qiagen Inc.	Chatsworth, CA, USA	Qiagen Plasmid Preparation Kits
Schleicher & Schuell	Dassel, Germany	Elutip-D Columns

Membranes, Paper and X-ray Film

Amersham International plc.	Amersham, Bucks, UK	Hybond N nylon Membrane
Vernon-Carus Ltd.	Preston, Lancashire, UK	Gauze Swabs
Schleicher & Schuell	Dassel, Germany	3MM Filter Paper
Eastman Kodak Co.	Rochester, NY, USA	X-OMAT AR X-ray Film

Nucleotides, Polynucleotides, DNA

Amersham International, plc	Amersham Bucks, UK	[α-^{32}P]-dCTP: 3000Ci/mmol
Sigma Chemical Co., Ltd.	Poole, Dorset, UK	Salmon Sperm DNA
Gibco Life Technologies	Paisley, Scotland, UK	1Kb DNA Ladder DNA Mass Ladder

Gels

Flowgen Instruments Ltd.	Sittingbourne, Kent, UK	Agarose and Low Melting Point Agarose (LMP)

Enzymes, Enzyme Buffers, Enzyme Inhibitors

Gibco Life Technologies	Paisley, Scotland, UK	Restriction Endonucleases, with Buffer Concentrates
NBL Gene Sciences Ltd.	Cramlington, UK	Ribonuclease A Proteinase K

Chemicals

Fisons Scientific Equipment	Loughborough, UK	Chloroform Methanol Acetic Acid Formaldehyde (40%)
Sigma Chemical Co. Ltd.	Poole, Dorset, UK	Ethidium Bromide Bromophenol Blue
James Burrough Ltd.	Witham, Essex, UK	Ethanol
Rathburn Chemicals Ltd.	Walkerburn, Scotland	Water-Saturated Phenol
Fluka Chemika-Biochemika	Gillingham, UK	Formamide
Northumbria Biologicals Ltd.	Cramlington, UK	Bovine Serum Albumin

Solutions

Alpha stock	5litre pack of α-MEM 1500ml DW 100mg Gentamicin sulphate 50ml MEM x 100 Vitamins Filter sterilise
Alpha Medium (x2)	DHS 25ml Alpha stock 21ml Sodium Bicarbonate 3ml Glutamine 1ml
PE	PBS 0.001M EDTA

Fischer's Medium	Distilled H_2O 87.5ml 10x Fischer's Medium 10ml L-Glutamine 1ml Sodium Bicarbonate 1.5ml
Special Liquid Medium	Supplemented Modified Eagles Medium 10% FCS 2mM L-Glutamine
Gel Loading Buffer	0.25% Bromophenol Blue 40% w/v Sucrose in Distilled H_2O
20 X SSC	3M Sodium Chloride 0.3M Tri-Sodium Citrate
DNA extraction buffer	0.1M Sodium Chloride 0.01M Tris (pH 7.4) 0.001M EDTA Ribonuclease A 20µg/ml 0.5% SDS
10 X TAE	0.4M Tris Acetate 0.05M Sodium Acetate (trihydrate) 0.01M EDTA
10 X TE	0.1M Tris/HCL (pH 8.0) 0.01M EDTA
High salt buffer	1.0M Sodium Chloride 0.02M Tris/HCL (pH 7.4) 0.001M EDTA
Low salt buffer	0.2M Sodium Chloride 0.02M Tris/HCL (pH 7.4) 0.001M EDTA
Depurination buffer	0.25M HCL
Denaturation buffer	1.5M Sodium Chloride 0.5N Sodium Hydroxide
Neutralisation buffer	1M Tris (pH 7.4) 1.5M Sodium Chloride
Blot washing buffer (1)	2 x SSC 0.1% SDS
Blot washing buffer (2)	0.1 x SSC 0.1% SDS
for DNA precipitation	3M Sodium Acetate
50 X Denhart's	1% (w/v) Ficoll-400 1% (w/v) Polyvinylpyrrolidone 1% (w/v) Bovine Serum Albumin (fraction V)
pre-hybridisation buffer	6 X SSC 5 X Denhart's 0.5% SDS 50% Formamide 10µg/ml Sonicated Salmon Sperm

SOC	2% w/v Bactotryptone 0.5% w/v Yeast Extract 0.01M Sodium Chloride 0.0025M Potassium Chloride 0.01M Magnesium Chloride 0.01M Magnesium Sulphate 0.02M Glucose
L-Amp broth	0.5% Yeast Extract 1% Bactotryptone 1% Sodium Chloride Ampicillin (50μg/ml)
Fixation medium for pathology	10% neutral buffered formaldehyde 40% Formaldehyde 100ml DW 900ml Disodium hydrogen phosphate dihydrate 8.19g Sodium dihydrogen phosphate dihydrate 4.52g

METHODS

Cell Culture

Mice

For all the work presented, the strain B6D2F1, an F1 hybrid of C57Bl/6 (females) and DBA2 (males) strains, was used. All mice were housed within the animal facility of the Beatson Institute. Female or male mice were used at age 6-12 weeks for all experimental procedures.

Murine Blood Sampling

Animals were sacrificed by carbon dioxide (CO_2) asphyxiation. All blood analyses were performed on blood obtained by cardiac puncture. This was performed by Mr. Stephen Bell (Beatson Institute). Full blood count analyses were performed using a Coulter counter. Blood films were stained using Leishmann stain.

Preparation of Murine Bone Marrow or Spleen Cells

Mice were sacrificed either by cervical dislocation or by CO_2 asphyxiation. Bone marrow cells were obtained from the femora of mice (Pragnell *et al.*, 1994). The femora were first cleaned of muscle tissue using a swab soaked in 70% ethanol, then crushed in Fischer's medium supplemented with 10% DHS (F/10), using a mortar and pestle. After crushing, the cell suspension was passed through a cell strainer into a 50ml centrifuge tube. After one wash in F/10, the bone marrow cells were re-suspended to a single cell suspension and counted in the Coulter counter after lysing the red cells with Zap-oglobin.

Spleens were carefully removed under sterile conditions using scissors and forceps. The spleens were disaggregated by crushing with the plunger end of a 2ml syringe through a cell strainer into a 50ml centrifuge tube. F/10 was

used to recover cells from the cell strainer. The cells were counted as for bone marrow.

Peritoneal Lavage

Animals were first sacrificed by CO_2 asphyxiation. Peritoneal lavage fluid was obtained by first injecting 4ml of PBS into the peritoneal cavity of mice and then carefully aspirating the fluid which was retained for further analysis.

Pathology of Spleens and Tibias

Samples of spleen or bone marrow were first placed in a solution of 1% neutral formaldehyde. Samples were then processed for histological section by standard methods. This was performed by Mrs Margaret O'Prey (Beatson Institute).

Antibody Labelling of Blood, Bone marrow and Spleen Cells

The test cells were first washed in sterile PBS. The required number (10^6 per test), was then re-suspended in 200µl of PBS for labelling. Antibody was added at the concentration recommended by the individual manufacturers. After 30 minutes incubation on ice, red cells were lysed if necessary (blood only) using 2ml cell lysing solution and, the cells were washed twice in PBS then re-suspended in 1% paraformaldehyde. For Rhodamine (Rh123) staining, Rh123 was dissolved in distilled water, stored frozen as a sterile 1mg/ml stock solution and serially diluted in PBS containing 5% FCS as required. The test cells were incubated in a final Rh123 concentration of 0.1µg/ml for 20 minutes at 37°C. Stained cells were washed twice, re-suspended and incubated at 37°C for 15 minutes to remove unbound fluorochrome. The cells were then washed and re-suspended in PBS ready for flow cytometry (Bertoncello *et al.*, 1991). Flow cytometric analyses were performed either on a FACScan (Becton Dickinson) or FACStar (Becton Dickinson) by Mrs Mary

Freshney and Mrs Elaine McKenzie (Beatson Institute) or Dr Kay Samuel (University of Edinburgh).

Maintenance of Cell Lines and Production of Conditioned Media (CM).

All cell lines were obtained from the frozen stocks of the Beatson Institute and were maintained in special liquid medium supplemented with 10% foetal calf serum (SLM/FCS). Cells were maintained in T75 tissue culture flasks. On reaching sub-confluence, cells were replated in fresh medium at one twentieth of the number at sub-confluence. Cells were detached from the surface of the flask using a 2 minute incubation with trypsin diluted 1:10 in PE. For the production of conditioned media (CM), the L929 (Stanley & Heard, 1977), and AF1-19T (Franz *et al.*, 1985) cell lines were grown in roller bottles in SLM/FCS to half-confluence. Spent medium was then removed, replaced with fresh medium, and the cultures allowed to grow for another three days. The CM was then removed, passed through 0.45 and 0.22μm filters and stored at -20°C. In the CFU-A assay (see below), CM from the murine L929 cell line was used as a source of M-CSF and AF1-19T-CM was used as a source of GM-CSF.

Clonogenic Assays

CFU-GM assay

For the CFU-GM assay, 5 x 10^4 bone marrow cells were added to supplemented α-MEM with 0.9% methylcellulose, 25% donor horse serum and recombinant murine GM-CSF (0.2ng/ml). 1ml cultures were plated into 3cm dishes and incubated at 37°C in 5% CO_2 in fully humidified conditions. After 6 days incubation, granulocyte, macrophage and granulocyte / macrophage colonies were scored if they contained ≥ 40 cells.

CFU-A assay

The protocol for CFU-A has been described in detail previously (Pragnell *et al.*, 1994; Pragnell *et al.*, 1988). Briefly, 10^4 bone marrow cells were suspended in α-MEM, supplemented with 25% DHS and 0.3% agar to a total volume of 4ml. This suspension was seeded onto a 4ml feeder layer consisting of 0.6% agar in the same medium with 10% L929- CM and 5% AF1-19T-CM as a source of synergising growth factors. Dishes were incubated in a humidified atmosphere containing 10% CO_2, 5% O_2 and 85% N_2 for 11 days. The "cut-off" for colonies to be included as CFU-A was ≥2mm in diameter and this was not varied. This assay detects multipotential progenitors with proliferative properties in common with CFU-S, and as such offers a means of measuring read out of transient engrafting populations (Jones *et al.*, 1990; Pragnell *et al.*, 1988). The only modification to the CFU-A assay, in some instances, was that for read out of lineage negative progenitors, L929-CM and AF1-19T-CM were replaced by rhM-CSF (6ng/ml), rmGM-CSF (0.2ng/ml) and rmSCF (12ng/ml).

Cycling / Suicide assay

To obtain estimates of the proportion of CFU-GM or CFU-A in S phase, bone marrow cells were incubated in vitro as follows. The test bone marrow cells were divided into two aliquots of 1-5 x 10^6 cells each and each was incubated in 1ml F/10 for 1 hour at 37°C; one of the paired tubes also containing 10^{-3}M ARA-C. The control aliquots were incubated in F/10 only. After incubation, cells were washed three times with F/10, re-suspended to appropriate cell concentrations, and used in the respective clonogenic assays mentioned above. CFU-GM or CFU-A numbers in these assays were calculated by averaging at least 10 plates. The CFU-GM and CFU-A proliferative status in each case was derived by comparison of the plus and minus ARA-C data to calculate the

proportion of progenitors sensitive to ARA-C and, hence, the proportion in S phase at the time of ARA-C treatment.

Selection of Lineage Negative Cells and Fluorescence Activated Cell Sorting (FACS) for Stem Cell Antigen (Sca) Positive Fractions

For experiments in which lineage negative populations were to be further purified to select Sca positive cells, bone marrow cells were harvested from tibia and femora of B6D2F1 mice 2 days after a tail vein injection of 5-Fluorouracil at 150mg/kg body weight. In other experiments normal bone marrow cells were used as the starting population. Bone marrow cells were re-suspended in Ca^{2+}, Mg^{2+} free PBS containing 2% foetal calf serum (PBS/2) and enriched by density gradient cell separation by layering 3ml of the cell suspension (2.5 x 10^7/ml) on top of 3ml Nycodenz mixture (density 1.077g/ml) and spinning at 1000g for 30 minutes at room temperature. The interface cells were harvested, washed once and re-suspended in PBS/2. These cells were further enriched for lineage negative progenitor content by using indirect immunomagnetic selection to remove mature cells according to previously described methods (Hirayama *et al.* , 1994; Shih & Ogawa, 1993). The antibodies used included Gr-1 to remove mature granulocytes, B220 to remove B cells, Mac 1 to remove monocytes and macrophages, CD5 and CD8a to remove T cells. Antibody labelling was performed for 45 minutes on ice. The labelled cells were washed three times and re -uspended in PBS/2, mixed with Dynabeads (M-450 sheep anti-rat IgG) at a 1:30 cell:bead ratio and incubated at 4°C for 45 minutes with end-over-end rotation. The tube containing the cells was then placed in a Dynal MPC-1 magnetic particle concentrator (DYNAL, Great Neck, NY, USA) for 10 minutes. The non-rosetted cells were harvested using a Pasteur pipette, washed once, re-suspended in PBS/2 and kept on ice for further enrichment by cell sorting. These cells were designated lineage negative. Prior to FACS, lineage negative

cells were incubated with Sca-FITC antibody for 45 minutes on ice. The cells were then washed three times and re-suspended in PBS/2 for sorting. Flow cytometric analyses and cell sorting were performed on a FACStar (Becton Dickinson) with an argon-ion laser tuned to 488nm at power 200mW. FITC fluorescence was measured through a filter arrangement with peak transmittance at 530nm. Cells with medium to high forward scatter intensity, low orthogonal scatter intensity and high fluorescent intensity were sorted into a 12 x 75 mm round bottom plastic tube containing 2ml of α-MEM. These cells were designated Sca+ lin-. Lineage negative populations labelled with Sca-FITC, contained approximately 11% (range 8-12.5) Sca positive cells.

Liquid Suspension Cultures for *ex vivo* Expansion

Bone marrow cells were suspended in α-MEM, supplemented with 25% DHS and the appropriate growth factors, in 24 well plates or in tissue culture flasks at a density of 0.5 x 10^6 per ml for unfractionated cells, 0.1 x 10^6 per ml for lineage negative populations and 0.05 x 10^6 per ml for Sca+ lineage negative cells. Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. For cultures maintained beyond 7 days, medium and growth factors were replenished at least every 7 days. Following expansion, the cells were harvested by vigorous pipetting, washed once in α–MEM and counted using a Coulter counter. The *ex vivo* amplifcation factor was derived from the increase in total cell number over time multiplied by the increase in the number of colonies per 10^5 cells plated.

Bone Marrow Transplantation (BMT)

B6D2F1 mice received fractionated radiation, titrated to produce 100% mortality from bone marrow aplasia by day 30 in control mice (no BMT). A total of 11.75-12.5Gy in two fractions three hours apart was given at a dose rate of 1.13Gy per minute using a Cobalt-60 source. Fractionated radiation

exposure is thought to allow some repair of rapidly proliferating tissues such as the gastrointestinal tract and lungs and hence to prevent early deaths due to radiation toxicity (Spangrude & Scollay, 1990). Using this protocol less than 5% of test animals (receiving a cell dose above threshold for engraftment) died before day 7 from radiation toxicity. The bone marrow infusions were given as a single injection (0.1ml) via the tail vein one hour after irradiation. All animals which received no bone marrow cells died from bone marrow aplasia between days 6-18 post-irradiation.

Transplantation of Spleen Cells

To investigate whether certain test cells were capable of producing a transplantable leukaemia, groups of mice were given a 2Gy dose of radiation. Spleen cells from a variable number of test animals were pooled and used to transplant sublethally irradiated animals (groups of 5 animals) by intraperitoneal injection of 10^6 cells per mouse. The transplanted animals were then observed for survival and upon showing signs of serious malaise were sacrificed by CO_2 asphyxiation. Analyses were performed for spleen and bone marrow pathology, full blood count (FBC), flow cytometric analyses and fluorescence in situ hybridisation.

Serial Transplantation and Assessment of Long Term Bone Marrow Reconstitution

To assess the ability of expanded populations to sustain haemopoiesis in the long term, serial syngeneic BMT, using different test bone marrow samples, was performed as follows. Male B6D2F1 mice were used as donors, females as recipients. Primary (1^o) BMT recipients received a cell dose of 5×10^5 (based on the number of cells at the start of *ex vivo* expansion culture). For secondary (2^o) and subsequent BMT the cell dose was 5×10^6. At various time points following BMT, analyses were performed for FBC, femur

cellularity and CFU-A content, percentage donor haemopoiesis by Southern blotting and ability to sustain serial transplantation.

Molecular Techniques

Propagation of Plasmid DNA in Bacterial Cells

DH5α competent cells, were transformed with the plasmid construct of interest. DH5α cells were first removed from -70°C and thawed on ice for 20 minutes. 20μl aliquots were dispensed into pre-chilled sterile 1.5ml eppendorf tubes, then three 1μl aliquots (neat, 1/10, 1/50 dilutions) of plasmid construct were added and incubated on ice for 30 minutes. The cells were then "heat shocked" at 42°C for 40 seconds exactly, returned to ice for 2 minutes and 80μl of SOC medium was added. This mixture was shaken at 225 rpm for 1 hour at 37°C to allow expression of the ampicillin resistance gene. The transformed cell mixture was spread evenly, using a glass spreader sterilised by flaming in ethanol, onto L-amp plates (5ml of 1% bactotryptone, 0.5% w/v yeast extract, 1% sodium chloride, 1.5% bactoagar) supplemented with 50μg/ml ampicillin. Plates were then inverted and incubated at 37°C overnight.

Growth of Plasmids

A single bacterial colony was plucked from an L-amp plate using a sterile loop and added to 10ml of L-amp broth in a universal container. This was incubated for 16 hours at 37°C shaking at 225 rpm. After incubation, 1ml of the mixture was added to 100ml of L-amp broth in a one litre conical flask and this was incubated under identical conditions for a further 16 hours. The suspension was then transferred to 50ml centrifuge tubes and spun at 3000 rpm for 10 minutes in a Beckmann J-6B centrifuge containing a GS-3 rotor. The supernatant was decanted and the pellet was then ready for plasmid preparation.

Plasmid Preparation

Plasmid preparation was performed using the commercially available Qiagen plasmid kit. The bacterial pellets were re-suspended in 5ml of re-suspension buffer (100mg/ml RNase A, 0.05M Tris/HCL, 0.01M EDTA, pH 8.0) in 50ml centrifuge tubes (Sorvall Instruments). 4ml of lysis buffer (0.2M NaOH, 1% SDS) was then added, the solution was mixed gently by inversion and incubated at room temperature for 5 minutes. 4ml of chilled neutralisation buffer (3M Potassium Acetate, pH 5.5) was added, mixed and incubated on ice for 15 minutes. Tubes were then spun at 4°C for 30 minutes at 10, 000 rpm in a Sorvall RC-5B superspeed centrifuge containing SS-34 rotors. The supernatant was then poured through a double layer of gauze swab to remove particulate material. To purify the DNA, a Qiagen-tip 100 column was equilibrated with 4ml of equilibration buffer (0.75M NaCl, 0.05M MOPS, 15% ethanol, 0.15% Triton X-100, pH 7.0) and the filtered supernatant was applied to the column. The column was then washed twice with 10ml of wash buffer (1M NaCl, 0.05M MOPS, 15% ethanol, pH 7.0). The DNA was eluted from the column using 5ml of elution buffer (1.25M NaCl, 0.05M Tris/HCL, 15% ethanol, pH 8.5). DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 10, 000 rpm at 4°C for 30 minutes as before. The DNA pellet was washed with 5ml of cold 70% ethanol, air dried for 5 minutes, and re-suspended in sterile distilled H_2O. The DNA concentration was then measured by spectrophotometry.

Purification of an Insert from a Plasmid

Plasmid DNA was digested in a solution containing 20µg DNA, 40µl of appropriate 10 x React™ buffer, 10µl of appropriate enzyme and H_2O to 400µl. Digestion was carried out for 2 hours at 37°C, after which the DNA was precipitated by adding 0.1 volumes of 3M sodium acetate and 2.5

volumes of ethanol. The tube was then placed on ice for 15 minutes and was spun at 13, 000 rpm for 10 minutes in a benchtop micro-Centaur centrifuge. The supernatant was carefully removed and the tube was respun for 1 minute only. Residual supernatant was removed and the pellet was washed once in 70% ethanol. Following this wash, the supernatant was again removed following a 10 minute and a 1 minute spin. The tube was then placed, open, in a 65°C heating block to allow any remaining ethanol to evaporate. The pellet was then re-suspended in 20µl of 1 x TE.

DNA restriction fragments were separated by 0.8% low melting point (LMP) agarose gel electrophoresis and visualised by ethidium bromide staining with ultraviolet illumination. The plasmid insert was excised from the gel and placed in a 1.5ml eppendorf tube. The tube was placed at 65°C in a heating block to melt the agarose. Low salt buffer (LSB)was equilibrated to 42°C in a water bath. After completely melting the agarose, this was added to 10ml of low salt buffer and returned to the 42°C water bath. Two Elutip-D-columns were equilibrated, first with 5ml of high salt buffer (HSB, room temperature), then with 5ml of LSB at 42°C, in each case by passing the buffer through the columns at high pressure using a 5ml syringe. The agarose / DNA solution was then applied to the columns (5ml per column) in a similar manner, after which the columns were washed with 5ml LSB at 42°C, before eluting the DNA from the columns with 0.6ml HSB. The resulting 1.2ml of DNA in HSB was precipitated with 3 volumes of ethanol, pelleted by microcentrifugation, washed with 70% ethanol, and re-spun to ensure removal of all residual ethanol. The sample was then re-suspended in 50µl of 1 x TE. The concentration of the re-suspended DNA insert was estimated by 1.5% agarose minigel electrophoresis and ethidium bromide staining, in comparison with a DNA mass ladder, run in parallel with the sample. A volume equivalent to 50ng was then used to make radioactive DNA probes.

Preparation of Radioactive DNA Probes

DNA inserts were first denatured by boiling for 2 minutes (DNA in solution) or 7 minutes (DNA in agarose) and placing on ice for 2 minutes. All DNA probes used for hybridisation to Southern blots were labelled for 15 minutes (DNA insert in solution) or 1 hour (DNA insert in agarose) at 37°C with [α-^{32}P]-dCTP, using a random priming kit according to the manufacturer's instructions. After labelling, the radioactive probe was separated from unincorporated ^{32}P-labelled nucleotides by passing through a Nick column, equilibrated with 1 x TE, according to the manufacturer's instructions. The probes were then denatured by boiling for 2 minutes, and placing on ice for 2 minutes.

Phenol Preparation

Phenol and chloroform were both used to remove protein contamination from DNA samples. Before use, phenol was equilibrated to a pH >7.8. For equilibration, an equal volume of 10 x TE was added and mixed thoroughly. After the two phases had separated, the upper phase was decanted and the procedure was repeated once or until pH > 7.8, with 1 x TE.

Phenol / Chloroform Extraction

An equal volume of phenol, phenol / chloroform or chloroform alone was added to the lysed cell suspension. The mixture was inverted gently and repeatedly for 10 minutes before spinning at 3, 000 rpm for 10 minutes. The top layer was aspirated and was used for the next step in the same way.

Preparation of Mammalian Genomic DNA

Cells were re-suspended in 1 x TE to a final concentration of 5 x 10^7 cells/ml, and 10ml of extraction buffer added per ml of cells. After 60 minutes incubation at 37°C, mixing gently on a Stuart TR-2 tube rotator, proteinase K

was added to a final concentration of 0.1mg/ml. This was gently mixed and placed in a 55°C water bath for at least 3 hours, swirling periodically. After cooling to room temperature, the protein fragments were removed by two phenol only, one phenol/chloroform and one chloroform only extractions. DNA was ethanol precipitated (2.5 volumes ethanol and 0.1 volume of 3M sodium acetate pH 5.2) and collected by spooling it onto a clean Pasteur pipette moulded into a hook shape over a Bunsen. The DNA was allowed to air dry for 15 minutes then slowly re-suspended at room temperature for at least 24 hours, in a volume of 1 x TE such that 1ml was included for every 2 x 10^7 cells in the original sample. The final solution was stored at -20°C: the DNA concentration was determined spectrophotometrically just prior to use.

Measurement of DNA Concentration by Spectrophotometry

The absorbence (A) at 260nm and 280nm of DNA in solution was measured in a 500µl quartz cuvette. Distilled water was used as a blank. An absorbence of 1 unit (i.e. one optical density (OD)) is equivalent to 50µg/ml of plasmid or genomic DNA. The $OD_{260/280nm}$ ratio gives an estimate of the purity of the DNA. Pure preparations of DNA have an $OD_{260/280nm}$ ratio of 1.8. If the OD at 260nm was greater than 1.0, the sample was further diluted in 1 x TE and the OD was repeated. If the 260/280nm ratio was significantly < 1.8, further phenol, phenol/chloroform, chloroform extractions were performed and the OD was repeated.

Digestion of Genomic DNA

Digests of genomic DNA were performed in eppendorf tubes at 37°C. The required amount (usually 10µg) of DNA was re-suspended in 1 x TE. 10 x buffer was added to 1/10 of the final volume. H_2O was added to make up to the final volume. The chosen restriction enzyme was added at 1/10 of the total digest volume in two aliquots, the first at the start of digestion and the second

after 2-4 hours. Digestion was usually carried out overnight. Following digestion a single phenol/chloroform extraction was performed. The DNA was precipitated by adding 2.5 volumes of ethanol and 0.1 volume of sodium acetate and placing on ice for 15 minutes. The sample was then centrifuged for 10 minutes and the supernatant removed. Following salt/precipitation, the DNA was washed once in 70% ethanol, then as much ethanol as possible was removed before resuspending in 1 x TE overnight.

Agarose Gel Electrophoresis

0.8% agarose gels were made by adding 2g agarose to 250ml 1 x TAE. 12.5µl of ethidium bromide was added to the gel (stock 10µg/ml) . Loading buffer (6X) was added to the DNA digests to 1/6 of the final voume and the DNA samples were loaded into the gel. A 1Kb molecular weight ladder was always included in the gel. The gels were run at 100 volts until good separation of the higher molecular weight fragments was obtained. The gels were then viewed and photographed under ultraviolet illumination. These photographs were retained to allow assessment of the quantity of DNA loaded into each well.

Detection of Specific Genomic DNA Sequences by Southern Blotting

After overnight digestion of the test DNA samples, gel loading buffer was added, and the DNA was electrophoresed for 4-6 hours at 100 volts on a 0.8% agarose gel. The gel was then visualised under ultraviolet light, photographed and trimmed to remove excess agarose. The gel was then depurinated by soaking in 0.25M HCL for 15 minutes with constant gentle agitation. After rinsing with deionised H_2O, the gel was denatured by soaking in denaturation buffer for 45 minutes, again with constant agitation. This was followed by soaking twice for 30 minutes in neutralisation buffer. Following neutralisation, the gel was inverted and placed on 3MM paper on a solid support over a reservoir of 20 x SSC. The edges of the 3MM paper were under

the surface of the 20 x SSC solution. 4 pieces of 3MM paper and in addition a piece of Hybond membrane, were cut to the size of the gel, soaked in 2 x SSC then placed on top of the gel with the Hybond membrane adjacent to the gel. Air bubbles were expressed by rolling the side of a Pasteur pipette across the membrane before applying the 3MM paper. Unwanted, used X-ray film was placed around the gel in such a way as to ensure that 20 x SSC from the reservoir would only diffuse through the gel. Paper towels were then stacked on top of the 3MM paper, a glass plate was placed on top and this was weighed down with a filled 500ml bottle. This was left overnight to allow capillary transfer of the DNA out of the gel and on to the membrane. The membrane was then carefully removed, the well positions were marked with a biro pen and the membrane was washed once in 2 x SSC to remove traces of agarose. The DNA was fixed to the membrane by exposure to ultraviolet light (UV Stratalinker). The membrane was incubated overnight in 25ml pre-hybridisation buffer, then hybridised overnight in 10ml of the same buffer, to which the radioactive probe of interest had been added. Following hybridisation, the membrane was sequentially washed in: 200ml washing solution 1 (2 x SSC, 0.1% SDS) for 15 minutes at room temperature; then in the same solution for 30 minutes at 68°C; then with washing solution 2 (0.1 x SSC, 0.1% SDS) for 30 minutes at 68°C for one or two washes depending on the level of background radioactivity remaining on the membrane. After washing, the membrane was sealed in a thin polythene bag and exposed to X-ray film at -70°C.

Assessment for Donor Derived Haemopoiesis in Transplantation Experiments

The percentage of male donor haemopoiesis was assessed by Southern blotting using a Y chromosome specific probe. pY353/B, which consists of a 1.5-kilobase (kb) fragment, was used to probe *Eco*R1 digested genomic blots

from the transplanted animals. In addition to the cognate 1.5-kb band, the probe detects four homologous bands of 5, 7, 9.5 and 15kb (Bishop *et al.*, 1985). Equal amounts (10μg) of DNA from individual mice were digested with *Eco*R1 and then separated on 0.8% agarose gels and transferred to Hybond N membrane by Southern blotting. No hybridisation was detected with female DNA. A standard titration of male DNA diluted into female DNA was included with all test samples to allow quantification of the proportion of male DNA (donor) to female DNA (recipient). The comparability of loaded DNA was determined as necessary by reprobing membranes with a probe for GAPDH. The radiographs were scanned as necessary by densitometry. Calculations of percentage male DNA were adjusted based on amounts of DNA loaded in each well.

Preparation of Chromosome Spreads for Fluorescence in situ Hybridisation (FISH)

Single cell suspensions were first prepared from either spleen or bone marrow in RPMI supplemented with 20% FCS, 1% glutamine and colcemid (final concentration 0.02μg/ml) to a cell concentration of approximately 5 x 10^7/ml. These cells were cultured at 37°C, 5% $C0_2$ and air for one hour. After incubation, the cells were spun at 1200 rpm for 10 minutes, the supernantant was decanted, and the pellet was re-suspended by tapping the tube. Freshly prepared hypotonic potassium chloride warmed to 37°C (0.5g KCl in 100ml DW) was then added dropwise, whilst flicking constantly to a final volume of 10ml. The cells were then incubated at 30°C for 20 minutes before spinning at 1000 rpm for 10 minutes. The hypotonic solution was then decanted and the pellet was re-suspended by flicking the tube. Freshly prepared fixative (methanol: acetic acid, 3: 1) was then added dropwise whilst flicking to a final volume of 5ml. Any obvious clumps were removed at this stage. The cells were left at room temperature for 10 minutes, then spun at 1200 rpm for 10

minutes, the supernatant fixative was decanted and the last step was repeated once, this time incubating the cells for 30 minutes instead of 10 minutes. The cells were then ready for storage at -20°C in 5ml of fixative.

FISH chromosome painting protocol

Chromosome painting was performed by Katherine Harper (MRC, Oxon) according to the method described in a recent report by Fennelly et al (Fennelly *et al.*, 1996).

Direct bone marrow chromosome preparations

Chromosome preparations were performed by Gwyneth Watson (MRC, Oxon) using the methods described by Watson et al (Watson *et al.*, 1996) and Piper & Breckon (Piper & Breckon, 1989).

RESULTS I

Chapter 3 Cytokine Interactions *In vitro*

The primary aim of this work was to determine whether haemopoietic stem and progenitor cells may be expanded *in vitro*, in the presence of growth factors, but in the absence of a fully formed bone marrow stromal layer. An important endpoint was to develop culture conditions which would encourage amplification of "transient engrafting stem cells" and to compare the engraftment potential of the cultured cells against unmanipulated bone marrow cells in an *in vivo* BMT model. Finally, if transplantation with cultured cells led to more rapid haematological recovery following BMT, compared with unmanipulated cells, then the effect of *in vitro* culture on the long term repopulating stem cells should be investigated.

The stimulatory cytokines may be divided into late-acting lineage specific, intermediate-acting lineage non-specific, factors which effect the kinetics of quiescent primitive progenitors and those which support survival without proliferation of primitive progenitors (Ogawa, 1993). Although useful in planning growth factor combinations for investigation, this division is somewhat arbitrary since, even within the haemopoietic system, cytokines have been shown to exhibit multiple functions affecting cells at different stages and there appears to be functional redundancy among cytokines, particularly those which are considered early-acting. The growth factor combination selected for study should include factors which support survival and / or proliferation of the most primitive progenitors and promote proliferation with limited differentiation of stem cells responsible for transient engraftment (CFU-GM, CFU-A, CFU-S). The growth factors considered to support survival of dormant primitive progenitors include SCF and IL-3 (Katayama *et al.*, 1993).

In the first series of experiments late-acting (EPO, G-CSF, M-CSF), intermediate-acting (GM-CSF, IL-3) and early-acting (SCF, IL-11, IL-6, G-CSF) cytokines were tested, either alone, or in combination, for their ability to amplify transient engrafting stem cells *in vitro*. Although IL-1 may be included with the early acting cytokines, there is evidence that its effects are indirect, perhaps mediated by either IL-6 or G-CSF (Leary *et al.*, 1988).

Unfractionated murine bone marrow cells were established in culture in α-MEM supplemented with 25% DHS and growth factors in 24 well plates. The duration of culture was kept constant at 6 days to allow comparison between different growth factor combinations. "Read-out" before and after expansion culture was for cell number, CFU-A and CFU-GM progenitors. The amplification factor was derived from the increase in both cell number and progenitor number. As shown in figure 3.1a, CFU-A progenitors were maintained at input numbers, even in the absence of growth factor addition. This suggests that DHS may contain sufficient amounts of certain growth factors to allow survival of CFU-A progenitors. The only growth factor which promoted expansion of CFU-A numbers when used alone was SCF. The SCF used in this series of experiments was in medium conditioned by CHO cells expressing soluble Kit Ligand, via truncation of the cDNA 5' to the transmembrane domain (Anderson *et al.*, 1990). Since this material was not purified, this result could be due to synergism between the SCF, known to be present in the conditioned medium, and small amounts of other unknown factors. In a small number of experiments purified murine recombinant SCF was tested, either alone or in combination with IL-11, for its ability to promote amplification of CFU-A progenitors. In these experiments, CFU-A amplification was of a similar degree to that achieved using the conditioned medium (data not shown).

Figure 3.1 a Cytokine combinations for the amplification of murine CFU-A progenitors in culture

Unfractionated murine bone marrow cells were cultured for 6 days in 24 well plates as described in materials and methods. Cytokines, either singly or in combination, were added at the start of culture only. IL-3 (50ng/ml), IL-6 (50ng/ml), IL-11 (100ng/ml), G-CSF (1:10,000), GM-CSF (25ng/ml), M-CSF (200ng/ml), Epo (2units /ml), SCF (18ng/ml), IL-1β (10ng/ml). Mean amplification factors (AF) (± SEM) for CFU-A progenitors. Each cytokine combination presented was tested in at least three separate experiments.

Figure 3.1 b Cytokine combinations for the amplification of murine CFU-GM progenitors in culture

Figure 3.1a

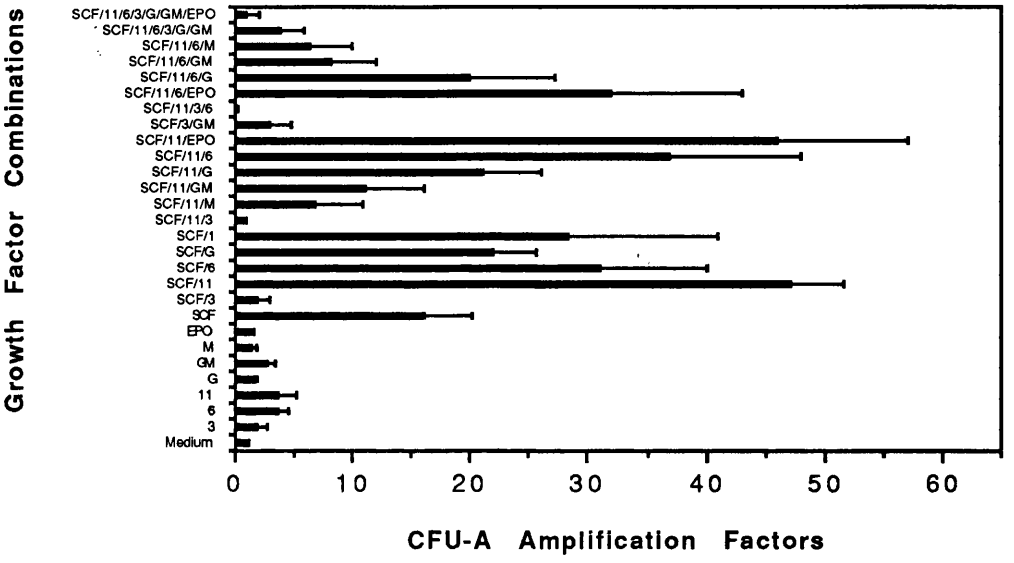
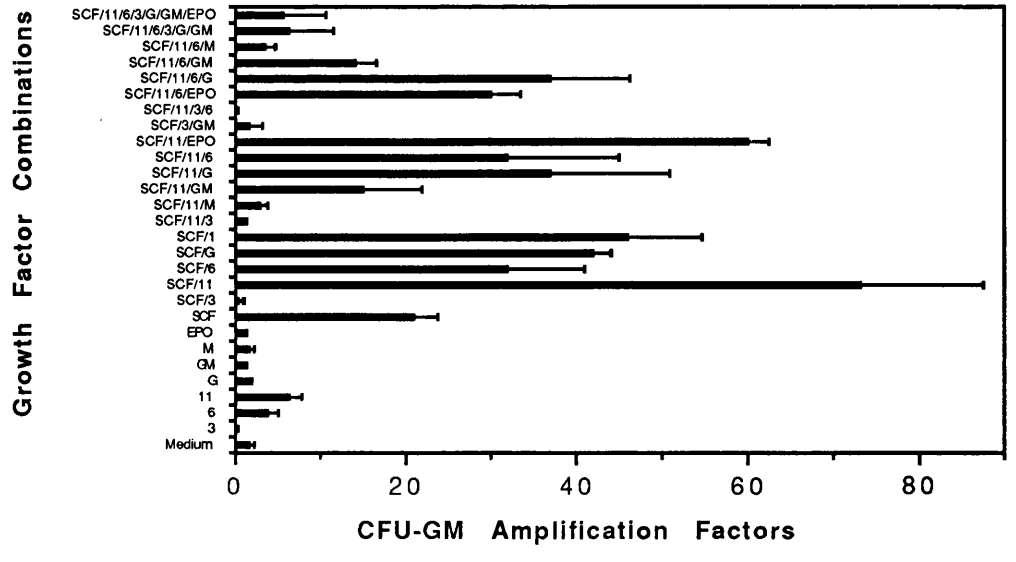


Figure 3.1b



As SCF was active in inducing expansion of CFU-A progenitors, growth factor combinations involving SCF plus a second early, or intermediate-acting, growth factor were investigated. As shown in Figure 3.1a, SCF synergised with IL-11, IL-6, G-CSF and IL-1β to promote CFU-A amplification. Although the combination of SCF and IL-11 consistently produced the greatest amplification, this was not significantly greater than for SCF with IL-6, G-CSF or IL-1β. SCF in combination with IL-3 produced no measurable CFU-A amplification and the baseline amplification seen with SCF alone was abolished. Similar results were seen when IL-3 was included in any combination containing two, three or more growth factors. When cell morphology was examined following expansion culture, conditions which promoted CFU-A amplification produced a population of myeloid precursors, the majority of which were blast cells and promyelocytes with smaller numbers of more mature cells. In cultures supplemented with IL-3, few, if any, blast cells remained, with neutrophils and macrophages predominating. Further investigation of SCF and IL-11 plus additional early, intermediate or late acting cytokines failed to detect a combination which produced greater CFU-A amplification than for SCF and IL-11 alone. Similar results were obtained for more committed CFU-GM progenitors (Figure 3.1b).

Having investigated a range of stimulatory cytokines, both early and late acting, experiments were designed to examine whether inhibitory cytokines could play a role in stem cell expansion. The negative regulators TGF-β and MIP-1α have been shown to have bi-directional effects (i.e. either stimulatory or inhibitory) depending on the maturational stage of the target population, whereas the interferons and TNF-α appear to be lineage non-specific inhibitory factors affecting progenitor cells at all developmental stages

(Ogawa, 1993; Keller *et al.*, 1994). For this reason, only TGF-β and MIP-1α were studied.

Unfractionated murine bone marrow cells were cultured for 6 days in 24 well plates as before. The optimum combination of SCF and IL-11 was used as a baseline for its known ability to promote amplification of both CFU-A and CFU-GM progenitors. Both negative regulators were added to cultures every 48 hours to ensure adequate concentrations throughout the culture period. As shown in Figure 3.2a-c, neither MIP-1α nor TGF-β, when used alone, stimulated proliferation of CFU-A or CFU-GM progenitors. When added to the combination of SCF and IL-11, MIP-1α produced a small but consistent increase in the degree of progenitor expansion achieved. This difference over SCF and IL-11 alone did not reach statistical significance. The addition of MIP-1α was never observed to produce an inhibitory effect under the culture conditions described. When TGF-β was added to SCF and IL-11 supplemented cultures, the opposite effect was seen. TGF-β completely abolished progenitor expansion, even in the presence of stimulatory cytokines. It is well recognised that these negative regulators may exert distinct effects depending on the differentiation status of the target cell and the other cytokines interacting with the cell.

A time course was then performed, using identical culture conditions and the same growth factor combinations, to determine whether the apparent stimulatory effect of MIP-1α was more obvious at an earlier stage of expansion culture. This time course was repeated twice (Figures 3.3 and 3.4) with similar results. Very little progenitor expansion was observed prior to day 6 of culture and the possible enhancing effect of MIP-1α was no more apparent earlier in the culture period. The inhibitory effect of TGF-β was seen

Figure 3.2 a-c Amplification factors for a. cell count, b. CFU-A progenitors and c. CFU-GM progenitors during *ex vivo* culture

Unfractionated murine bone marrow cells were cultured for 6 days in 24 well plates as described in materials and methods. SCF and IL-11 were added at the start of culture only. MIP-1α (100ng/ml) and TGF-β (10ng/ml) were added every 48 hours. Mean amplification factors ($\pm$ SEM) are shown for four replicate experiments.

Figure 3.2a
Amplifcation Factors for Cell Count

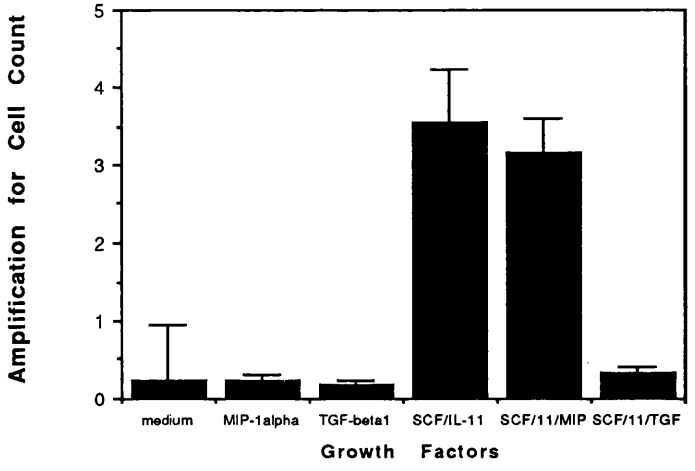


Figure 3.2b
Amplification Factors for CFU-A

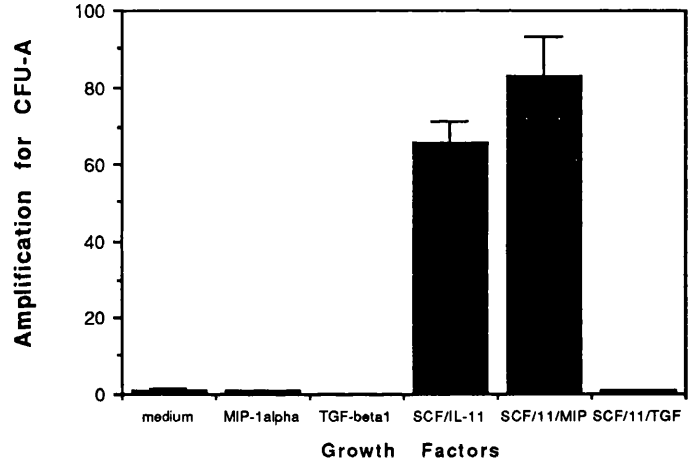
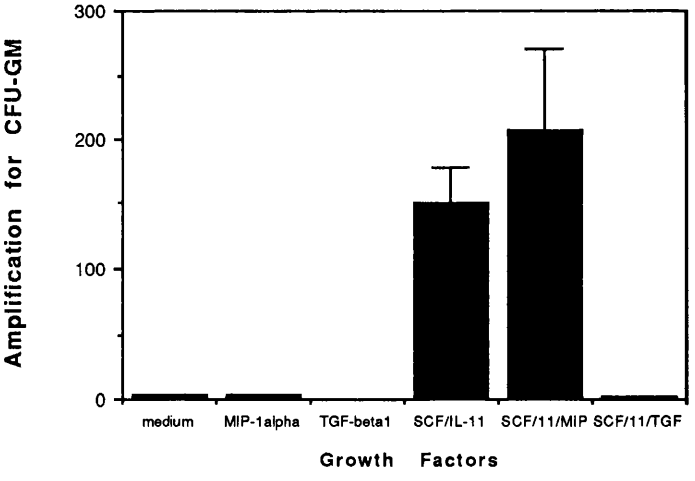


Figure 3.2c
Amplfication Factors for CFU-GM



Figures 3.3 a-c &3.4 a-c A six day time course for the amplification of a. cell count, b. CFU-A progenitors and c. CFU-GM progenitors

Unfractionated murine bone marrow cells were cultured in 24 well plates as described in materials and methods. SCF and IL-11 were added at the start of culture only. MIP-1α and TGF-β were added every 48 hours. Replicate wells were harvested on days 2, 4 and 6 to assess the optimum duration of *ex vivo* culture. The results shown are for the mean amplification factors ($\pm$SD) for a single experiment which was repeated on two occasions.

Figure 3.3a
Time course for amplification of cell count

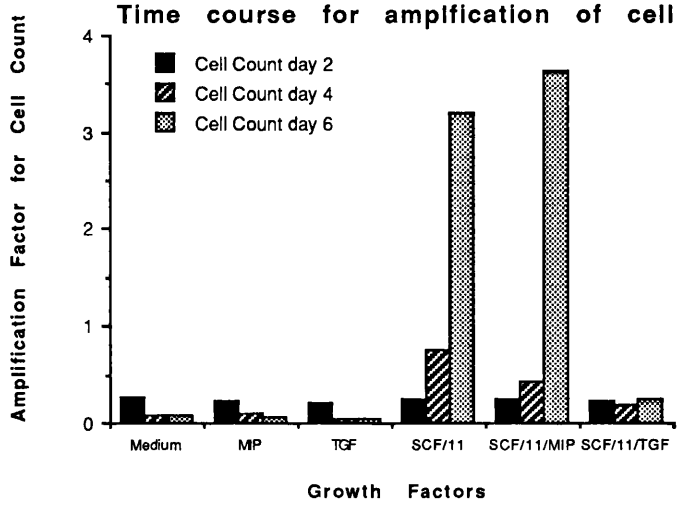


Figure 3.3b
Time course for amplification of CFU-A

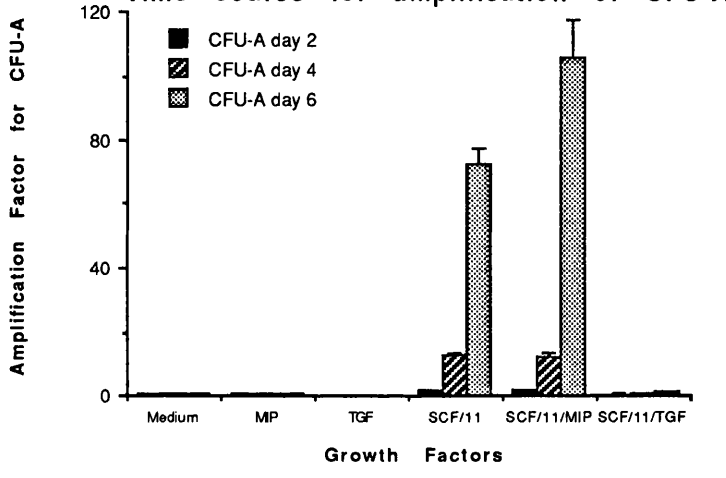


Figure 3.3c
Time course for amplification of CFU-GM

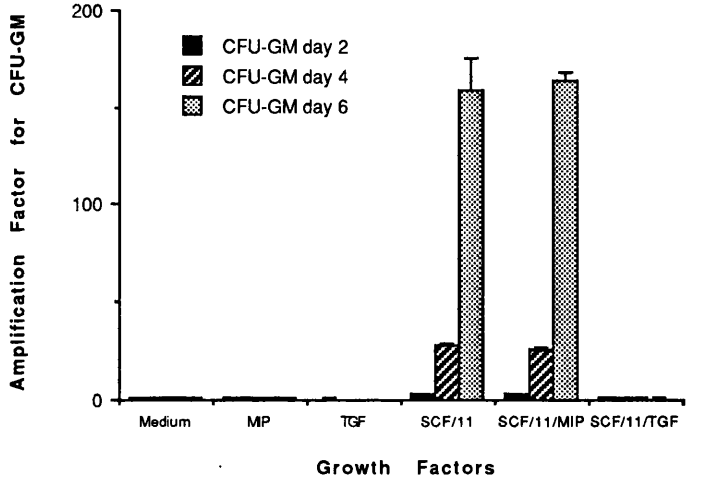


Figure 3.4a
Time course for amplification of cell count

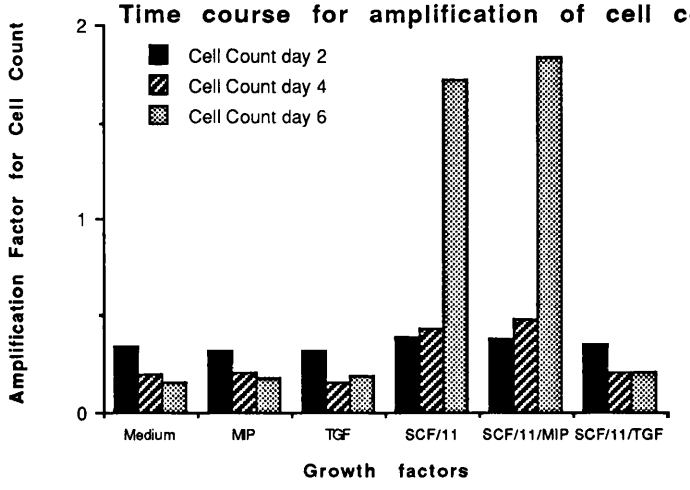


Figure 3.4b
Time course for amplification of CFU-A

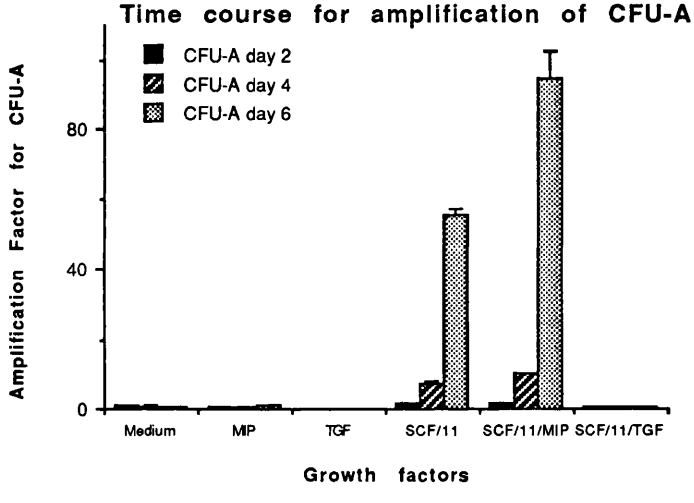
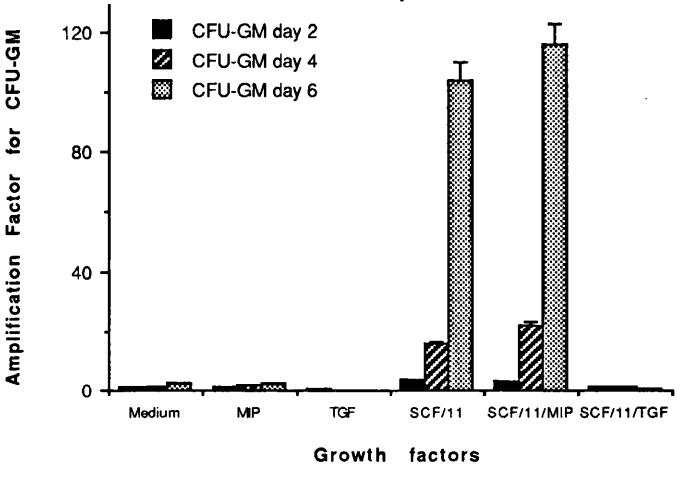


Figure 3.4c
Time course for amplification of CFU-GM



from the start of culture and was never overcome by the stimulatory cytokines present.

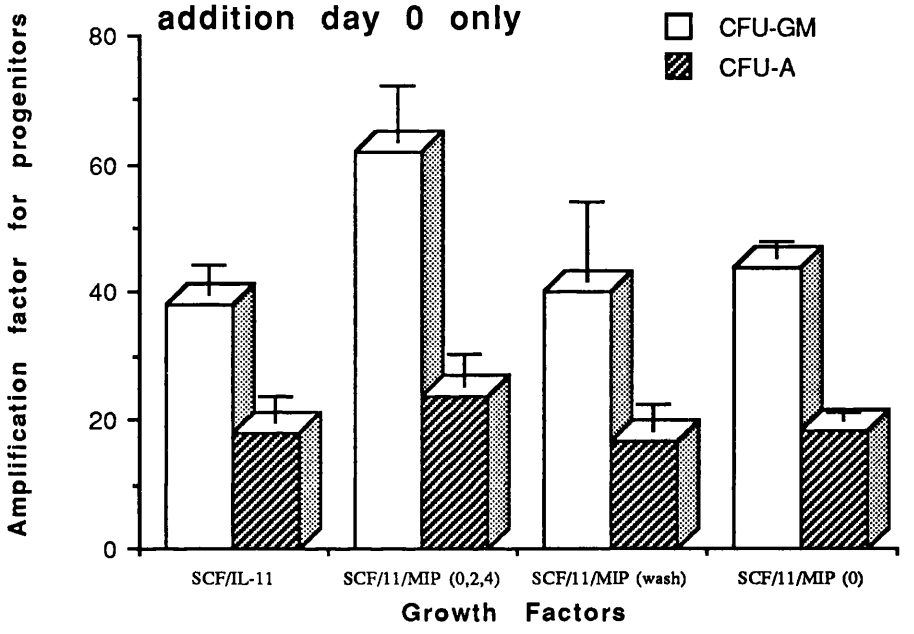
In the next experiment (Figure 3.5) the addition of MIP-1α to expansion cultures supplemented with SCF and IL-11 was examined in more detail. The first culture was supplemented with SCF and IL-11 at the start of incubation and was used to standardise the experiment. In the second culture, MIP-1α was added on days 0, 2 and 4, as before. In the third culture MIP-1α was added at the start of culture, and was then washed out on day 2 to determine whether its effect occurred very early during expansion culture and whether MIP-1α was unnecessary thereafter. In the fourth culture MIP-1α was added only on day 0 but was not washed out, to determine whether alternate day addition of MIP-1α was necessary to maintain an enhancing effect on progenitor expansion. The results of this experiment are difficult to interpret, because the degree of enhancement of progenitor expansion seen in the presence of MIP-1α is small and does not reach statistical significance, therefore any changes induced by altering the culture conditions, as done in this experiment, are even more difficult to demonstrate. From the results shown in Figure 3.5 it appears that MIP-1α should be added at least every 48 hours and that any enhancing effect by this cytokine is abolished if either the cytokine is washed out after the first 48 hours of culture, or if it is added only at the start of culture.

Having demonstrated that SCF and IL-11 in combination promoted amplification of both CFU-A and CFU-GM progenitors during a short (6 day) culture period, it was important to try to determine whether this result was due only to differentiation of a committed progenitor subset and therefore would disappear with prolonged culture or whether a more primitive stem cell subset had been triggered into cell cycle. Experiments were therefore designed to

Figure 3.5 A comparison of repeated addition of MIP-1α to *ex vivo* expansion cultures versus addition only at the start of culture

Unfractionated murine bone marrow cells were cultured for 6 days in 24 well plates as described in materials and methods. All experimental cultures were initially supplemented with SCF and IL-11. Except for the SCF/IL-11 only culture (columns 1 & 2), MIP-1α (100ng/ml) was also added on day 0. On day 2, culture three (columns 5 & 6) was harvested, washed once and was re-established in fresh medium, supplemented with SCF (18ng/ml) and IL-11 (100ng/ml). No further MIP-1α was added to this culture. Culture two (columns 3 & 4) was supplemented again with MIP-1α (100ng/ml) on days 2 and 4. Culture four (columns 7 & 8) was supplemented with MIP-1α on day 0 only. After 6 days culture all four experimental cultures were harvested in the usual way and were plated into CFU-A and CFU-GM. The mean amplification factors (± SEM) are shown for both CFU-GM and CFU-A progenitors. Results represent the mean of three replicate experiments.

Figure 3.5
Repeated addition of MIP-1alpha versus addition day 0 only



assess whether CFU-A and CFU-GM expansion could be maintained beyond 6 days and if so, what effect the addition of MIP-1α to SCF and IL-11 would have. Figure 3.6 shows the amplification factors for cell count, CFU-GM and CFU-A for unfractionated bone marrow cells cultured for five weeks with SCF and IL-11. At each time point the expanded populations were washed, counted, assayed for CFU-GM and CFU-A and an aliquot re-established in culture with fresh medium and growth factors at a cell density of 0.5 x 10^6/ml. The total number of cells in culture increased exponentially over time to a maximum 4, 500 (± 1152) fold by day 39. Although total CFU-GM numbers were still increasing (maximum 20, 000 (± 9839) fold) at day 39 of culture, total CFU-A amplification peaked at 1000 (± 347) fold at day 32 and the amplification potential was declining by day 39. The plating efficiency for CFU-A increased from 0.15% on day 0, to peak at 5.8% by day 6. Thereafter, plating efficiency was maintained above baseline to day 14 and was still close to baseline (0.12%) by day 32. Therefore, these cultures do expand over the first two weeks and do not appear to exhaust before day 32. These results show the profound ability of the SCF and IL-11 cytokine combination to expand the transient engrafting stem cell compartment *ex vivo* and suggest that this particular cytokine combination induced proliferation of a primitive cell. In the absence of a fully formed stromal layer, despite the regular addition of positively acting growth factors, these cultures eventually showed depletion of amplification ability. It is possible that some stromal elements formed in these cultures although this would have been disrupted with every medium change. This was not specifically looked at in these experiements.

The results for parallel cultures supplemented with MIP-1α in addition to SCF and IL-11 are shown in Figure 3.7. The cell count reached a maximum of 1014 (± 734) fold over baseline by day 32 and was falling by day 39. CFU-

Figure 3.6 The amplification of murine bone marrow progenitors in culture with SCF and IL-11

Unfractionated murine bone marrow cells were cultured for 39 days in tissue culture flasks as described in materials and methods. SCF and IL-11 were added at the start of culture and again with fresh medium at each time point. The amplification factors (logarithmic scale) for cell number, CFU-GM and CFU-A are shown against days in culture to day 39. Results represent the mean ($\pm$ SEM) for three replicate experiments.

Figure 3.6

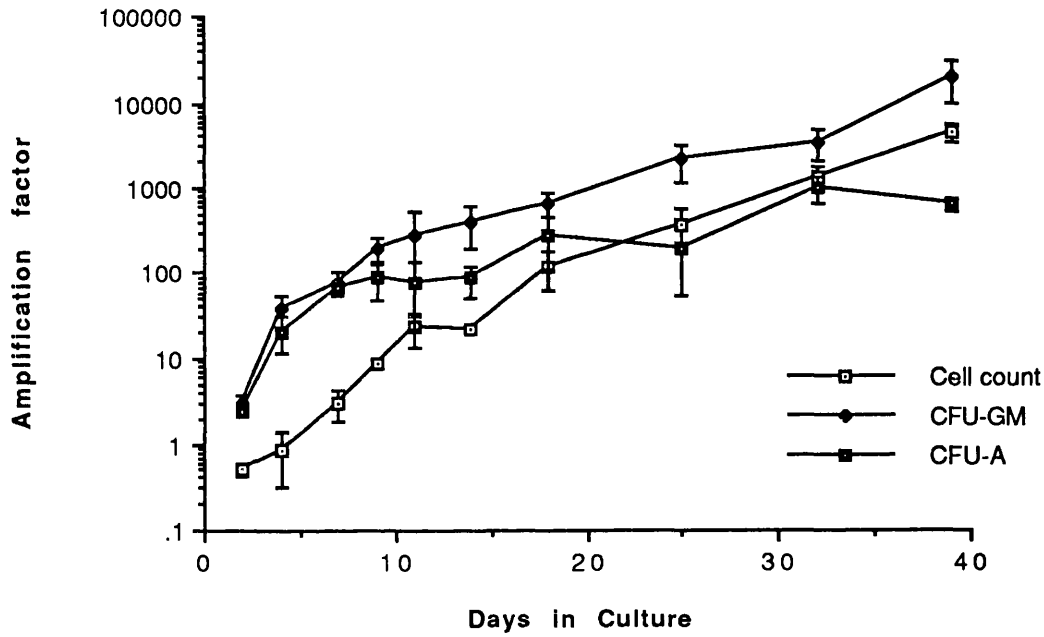
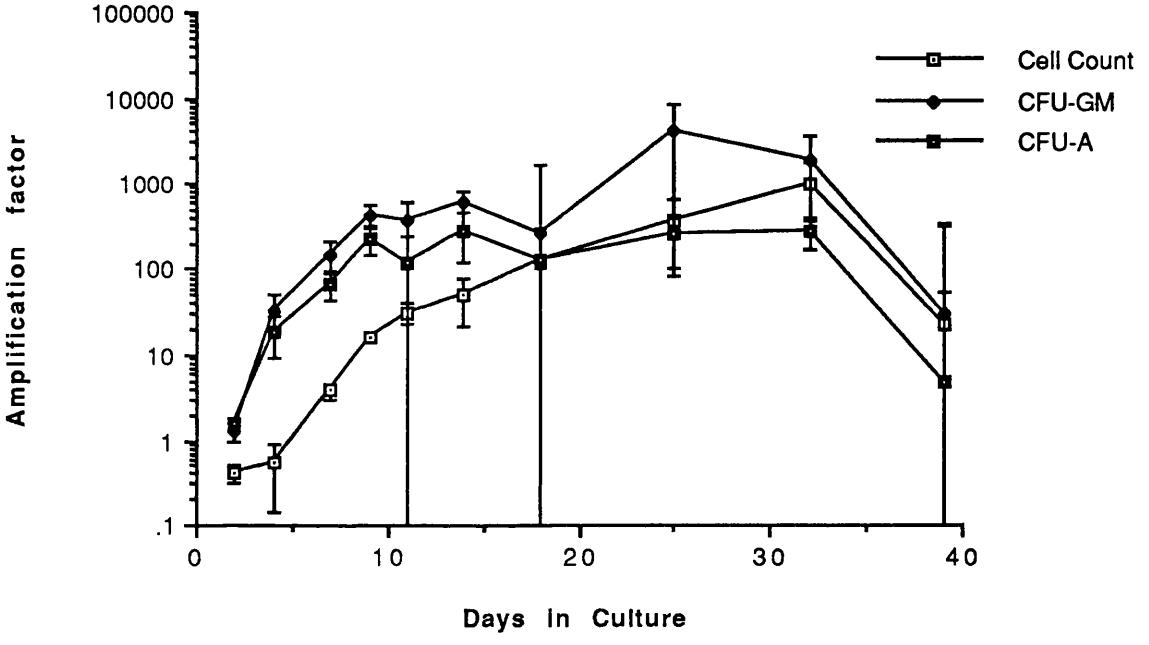

100000
10000
1000
100
10
1
.1
Amplification factor
0
10
20
30
40
Days in Culture
Cell count
CFU-GM
CFU-A

Figure 3.7 The amplification of murine bone marrow progenitors in culture with SCF, IL-11 and MIP-1α

Unfractionated murine bone marrow cells were cultured for 39 days in tissue culture flasks as described in materials and methods. SCF and IL-11 were added at the start of culture and again with fresh medium at each time point. MIP-1α was added every 48 hours. The amplification factors (logarithmic scale) for cell number, CFU-GM and CFU-A are shown against days in culture to day 39. Results represent the mean (± SEM) for three replicate experiments.

Figure 3.7


100000
10000
1000
100
10
1
.1
Amplification factor
0
10
20
30
40
Days In Culture
Cell Count
CFU-GM
CFU-A

GM peaked at 4261 (± 3867) by day 25, and then began to decline and CFU-A reached a maximum of only 270 (± 102) fold over baseline.

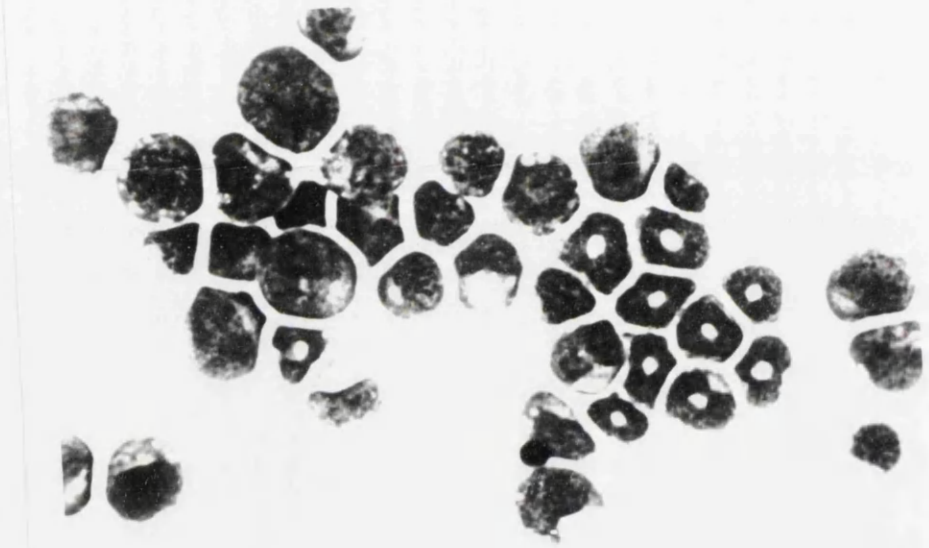
The reduced expansion potential in the presence of MIP-1α, compared with SCF and IL-11 alone, may be explained by the fact that one of the three replicate experiments showed extremely poor expansion from day 12 of culture. The inclusion of results from this experiment resulted in a lower mean amplification for the three replicate experiments and a much wider SEM for the later time points. In the early part of these parallel expansion cultures no difference could be detected in terms of progenitor expansion between cultures supplemented with only SCF and IL-11 and those with SCF, IL-11 and MIP-1α. Thereafter, there was no evidence that MIP-1α was stimulatory for progenitor output. Whether the MIP-1α cultures genuinely showed a decline in progenitor output earlier than the cultures without MIP-1α is unlikely since two of three MIP-1α supplemented cultures continued to show progenitor expansion at least to day 32.

Accessory cells in unfractionated bone marrow may either secrete growth factors independently, or may be induced to produce growth factors or inhibitory factrs by cytokines which are known to be present (i.e. an indirect effect). Both IL-1β and TNF-α appear to work indirectly by inducing cytokine production by accessory cells. MIP-1α has a role in the inflammatory process and as such could be anticipated to induce either growth factor or inhibitor secretion by accessory cells. One way to reduce the possible effects of accessory cells present in bone marrow is to purify stem and progenitor cells. An additional advantage of stem cell purification would be that if the activity of MIP-1α was directed at a primitive target cell then the effect on progenitor expansion may become more obvious in experiments with a relatively pure population of primitive cells. Day 2 post 5-FU bone marrow from a pool of

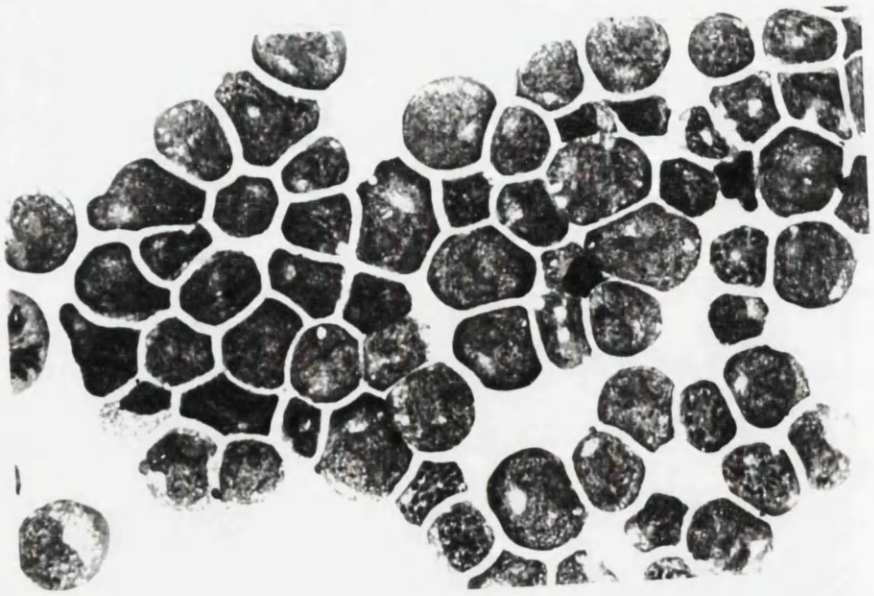
Expansion conditions	Experiment 1 (% blasts)	Experiment 2 (% blasts)	Mean (% blasts ± SD)
SCF/IL-11	22.7	37.1	30.0 ± 10
SCF/IL-11/MIP-1α	54.7	60.4	58.0 ± 4

Results based on 500 cell differential counts

SCF/IL-11



SCF/IL-11 + MIP-1α



twenty mice was used to prepare a lineage negative fraction as described in materials and methods. This fraction is depleted of B cells, T cells, mature myeloid cells, monocytes and macrophages. The lineage negative cells were then established in expansion culture with SCF and IL-11 +/- MIP-1α. The cells were incubated for 6 days as before. As shown in Figure 3.8, whereas, for unfractionated bone marrow, cell number increase was in the range of only 2-3 fold, lineage negative cells expanded dramatically in culture to around 200 fold by day 6. This increase in cell number reflects both the enrichment by using post 5-FU bone marrow and the depletion of mature cells. It is also likely that inhibitory proteins are produced in unfractionated cultures which would be absent or present at only low levels in more purified populations (e.g. TGF-β). CFU-A progenitors amplified to 400 fold over baseline by day 6 but no effect on CFU-A expansion was observed when MIP-1α was added to the combination of SCF and IL-11. The morphology of the starting lineage negative fraction was predominantly blast cells (>95%). At the end of 6 days expansion, lineage negative cells amplified in the presence of SCF and IL-11 showed differentiation down the myeloid lineage with some blast cells remaining, but also promyelocytes and more mature cells including neutrophils appearing. The addition of MIP-1α produced a striking change in morphology compared with SCF and IL-11 alone. The majority of the cells were still blast cells with some promyelocytes, but with far fewer mature cells such as neutrophils.

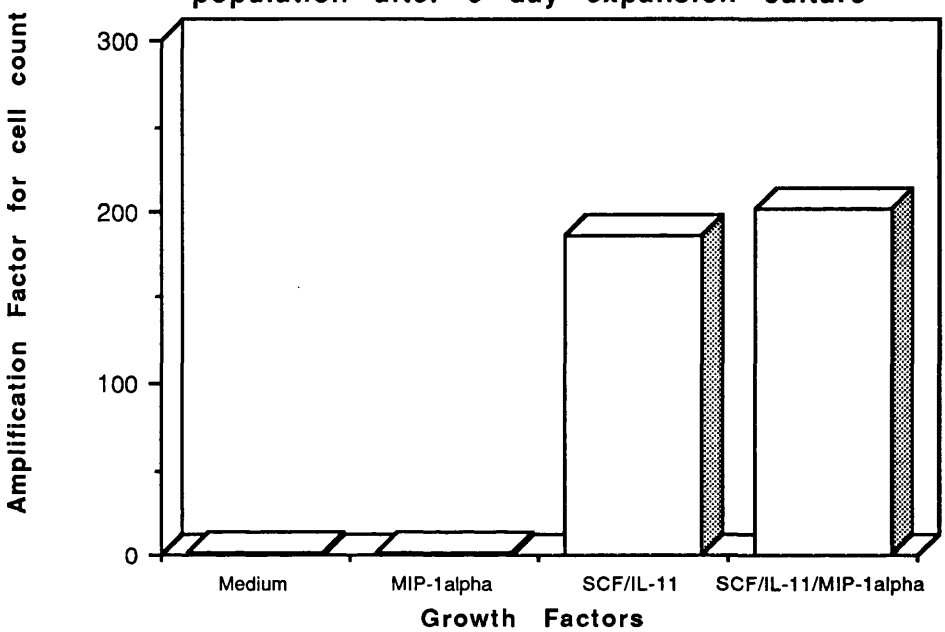
(see page facing)

To further investigate this finding, it was decided to further purify the lineage negative fraction by FACS for Sca+ cells. As shown in Table 3.1, lineage negative cells prepared from post 5-FU bone marrow were enriched for the Sca+ lineage negative fraction. This separation was performed on 4 separate occasions. Although the cloning efficiency of the final cell product was greatly enhanced over baseline (204-4456 fold), recovery of Sca+ cells was

Figure 3.8 Amplification of murine lineage negative bone marrow cells

Lineage negative, post-5 fluorouracil, murine bone marrow cells were prepared as described in materials and methods. These cells were cultured for 6 days in 24 well plates at a cell concentration of 0.1 x 10^6/ml as described in materials and methods. SCF and IL-11 were added at the start of culture only. MIP-1α was added every 48 hours from the start of culture. Amplification factors ($\pm$ SD) are show for cell count and CFU-A progenitors. Results represent a single experiment.

Figure 3.8
Amplification of lineage negative population after 6 day expansion culture



Amplification of CFU-A for lineage negative population after 6 day expansion culture

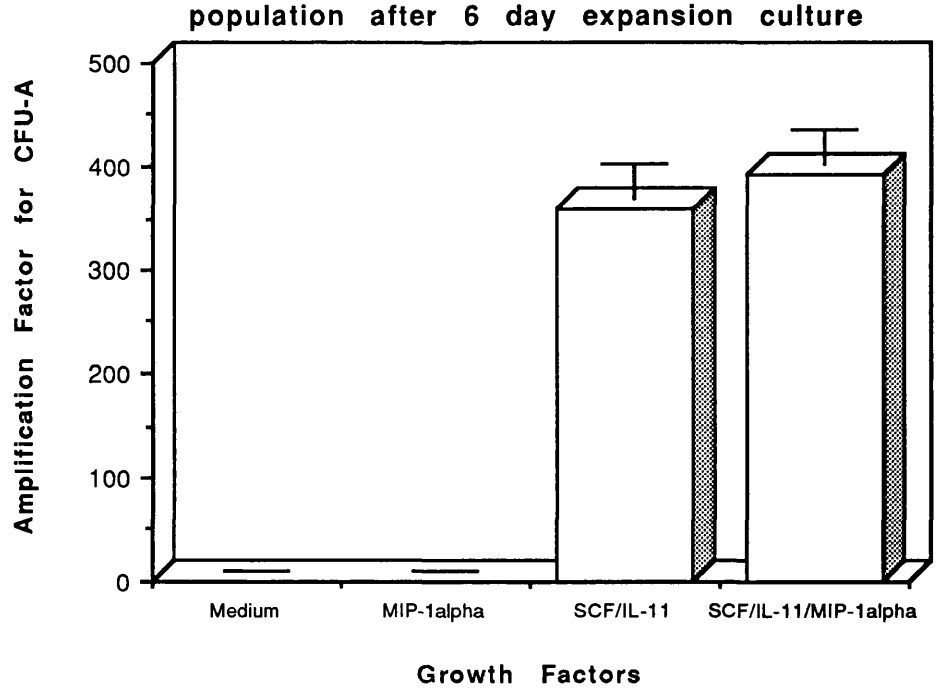


Table 3.1 Purification of a Sca+ lineage negative population from 2 day post 5-Fluorouracil murine bone marrow

2 days prior to investigation mice received 150mg/kg 5-FU intravenously. Bone marrow cells were enriched firstly for lineage negative cells by immunomagnetic depletion as described in materials and methods. The lineage negative population was then further purified by FACS for Sca+ progenitors. The results compare starting post 5-FU bone marrow with Sca+ lineage negative progenitors for CFU-A cloning efficiency, and show the recovery of CFU-A progenitors in the enriched fraction. Results represent four consecutive experiments.

Table 3.1

Experiment code	Cell type	CFU-A per 10^5	Total cell number (10^5)	Recovery (%)	Cloning efficiency (%)	Enrichment (fold over baseline)
1.	2dpost-5FU	57	2830	100	0.057	1
	Sca+ lin-	11600	0.7	5	11.6	204
2.	2d post-5FU	3.7	4440	100	0.0037	1
	Sca+ lin-	16500	0.45	45	16.5	4456
3.	2d post-5FU	5	4600	100	0.005	1
	Sca+ lin-	5500	0.82	19.6	5.5	1100
4.	2d post-5FU	15.3	4586	100	0.0153	1
	Sca+ lin-	15500	0.06	1.3	15.5	1013

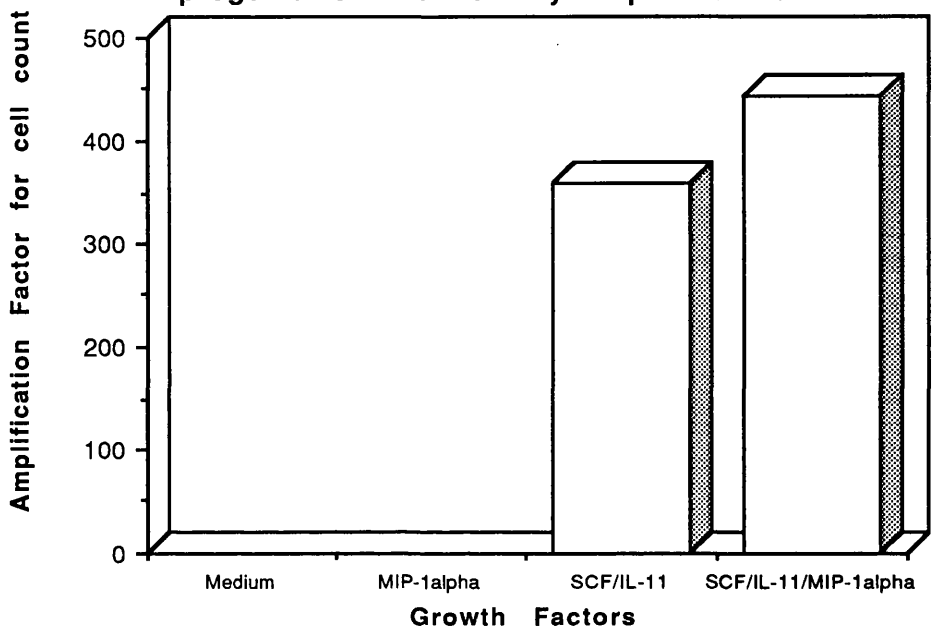
very poor and the number of cells available for investigation was limited. The Sca+ lineage negative cells purified in these four experiments were established in expansion culture with SCF and IL-11 +/- MIP-1α. One set of cultures showed bacterial contamination from the start of culture which probably occurred during the purification process which includes a great deal of cell manipulation. One set of cultures showed no expansion although there was no clear evidence of contamination. A third set amplified slightly but less than for lineage negative cells not further enriched for Sca+. The results shown in Figure 3.9 are for the final set of expansion cultures which showed excellent *in vitro* growth. Cell numbers increased 360 fold with SCF and IL-11 and 443 fold with SCF, IL-11 and MIP-1α. CFU-A amplified 520 (± 130) fold without MIP-1α and 750 (± 77) fold with MIP-1α. Once again morphology showed that there was less terminal myeloid differentiation in the presence of MIP-1α. In view of the low cell recoveries and the fact that, for technical reasons, only one of four experiments was successful, no further Sca+ lineage negative cells were purified.

The standard stem cell purification process described above was simplified to try to make it more applicable for regular use. A lineage negative fraction was prepared from normal mice (no 5-FU) and FACS for Sca was not performed. The consistent differences in morphology between MIP-1α supplemented and standard SCF / IL-11 cultures were of great interest. It appeared that MIP-α, by an, as yet, unknown mechanism, blocked differentiation of primitive cells. At this stage in my studies, the only formal "read-out" for the expanded populations had been cell number, CFU-A and CFU-GM. It could be reasoned that the target cell for MIP-1α in these culture conditions could represent a small proportion of the total progenitor population previously measured. Therefore, lineage negative cells were established in expansion culture as described in the protocol in Figure 3.10. Initial "read out" was for cell number,

Figure 3.9 Amplification of murine Sca+ lineage negative bone marrow cells

Sca+ lineage negative, post-5 fluorouracil, murine bone marrow cells were prepared as described in materials and methods. These cells were cultured for 6 days in 24 well plates at a cell concentration of 0.05 x 10^6/ml as described in materials and methods. SCF and IL-11 were added at the start of culture only. MIP-1α was added every 48 hours from the start of culture. Amplification factors ($\pm$ SD) are shown for cell count and CFU-A progenitors. Results represent a single experiment.

Figure 3.9
Amplification over baseline for Sca+ progenitors after 6 day expansion culture



Amplification over baseline for Sca+ progenitors after 6 day expansion culture

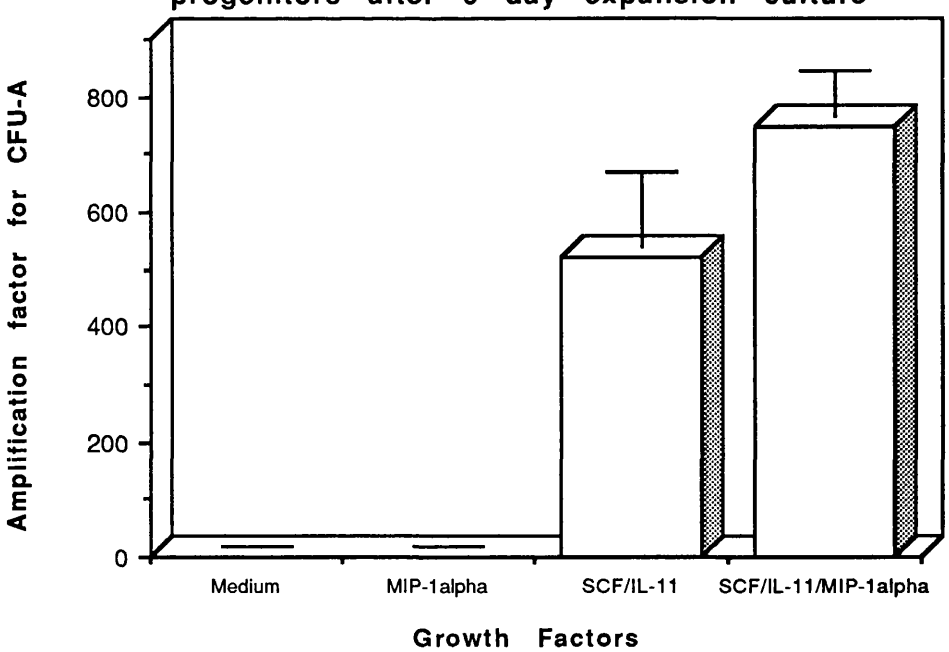
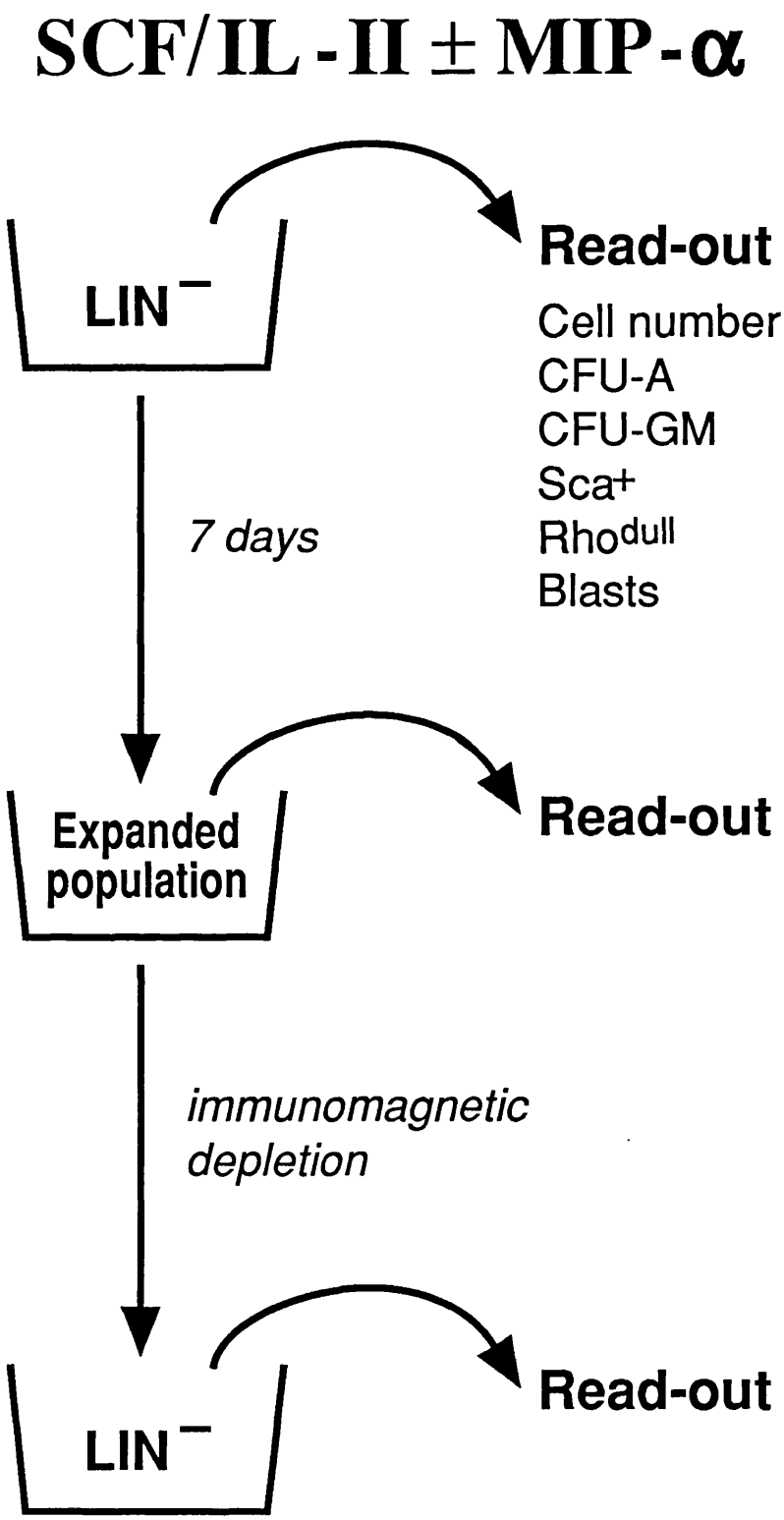


Figure 3.10 Protocol for amplification of murine lineage negative bone marrow cells

Lineage negative cells were first enriched from normal bone marrow by immunomagnetic depletion as described in materials and methods. Read-out was performed for cell number, CFU-A, CFU-GM, percentage Sca+ and Rhodamine^{dull} (flow cytometry) and percentage blasts cells (by morphology). The cells were then incubated for 7 days in tissue culture flasks in medium supplemented with SCF and IL-11 or SCF, IL-11 and MIP-1α. SCF and IL-11 were added at the start of culture only. MIP-1α was added at the start of culture and every 48 hours. Following 7 days culture, the cells were harvested, washed and again enriched for lineage negative cells. Read-out was performed on the starting lineage negative fraction, on the day 7 total cell population and on the day 7 lineage negative reselected fraction.

Figure 3.10

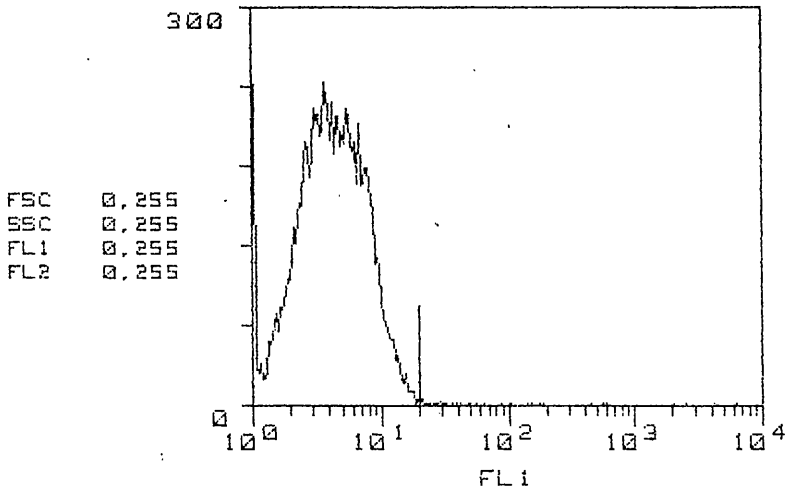

SCF/IL-II ± MIP-α
LIN⁻
Read-out
Cell number
CFU-A
CFU-GM
Sca+
Rhodull
Blasts
7 days
Expanded population
Read-out
immunomagnetic depletion
LIN⁻
Read-out

CFU-GM and CFU-A as before, but in addition for percentage Sca+ and Rhodamine^{dull} cells and percentage of cells of blast morphology. An example of baseline lineage negative cells labelled with the negative control antibody and with Sca is shown in Figure 3.11. Approximately 10% of the starting lineage negative fraction was Sca positive. The cells were then cultured for 7 days in either SCF and IL-11, or SCF, IL-11 and MIP-1α. At the end of incubation "read out" was performed for cell number, CFU-GM, CFU-A, percentage of Sca+, Rhodamine^{dull} and blast cells. To ascertain whether fewer cells had differentiated and become lineage positive in the presence of MIP-1α, the two expanded populations were once again depleted of lineage positive cells and "read out" was repeated. Two examples of the reselected lineage negative populations labelled with Sca are shown in Figure 3.12. It is clear that the proportion of cells which were Sca+ following expansion in the presence of MIP-1α was greater than without MIP-1α. In addition the total number of lineage negative cells was consistently greater in the presence of MIP-1α. Figure 3.13 shows an example of a reselected population stained with Rhodamine. On this occasion the percentage of cells which failed to retain Rhodamine (Rhodamine^{dull}) was roughly equivalent in the presence or absence of MIP-1α, however, since the number of cells recovered in the lineage negative fraction was around two fold higher in the presence of MIP-1α, the absolute number of lineage negative Sca+ cells was still greater. To allow for unavoidable cell losses during the lineage positive depletion step, "read out" for all parameters was performed on both the total expanded population and the reselected lineage negative fraction. As shown in Figures 3.14 a-g, this experiment was repeated in full on three occasions. The degree of amplification achieved in each experiment was highly variable, resulting in large SEM between the three experiments, however in each experiment the difference between adding MIP-1α and no MIP-1α was similar. The cell number increase was consistently slightly greater in the presence of MIP-1α

Figure 3.11 Flow cytometric analysis: example for baseline lineage negative cells

The percentage of cells expressing Sca was determined by flow cytometry as described in materials and methods. The top panel is the negative control using an irrelevant IgG antibody. The lower panel demonstrates that approximately 10% of the starting lineage negative cells expressed Sca.

FL1, on the X axis, represents fluorescence intensity for FITC-conjugated antibodies, in this case either the isotype control or anti-Sca. The Y axis represents relative frequency of events.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FL2 0,255
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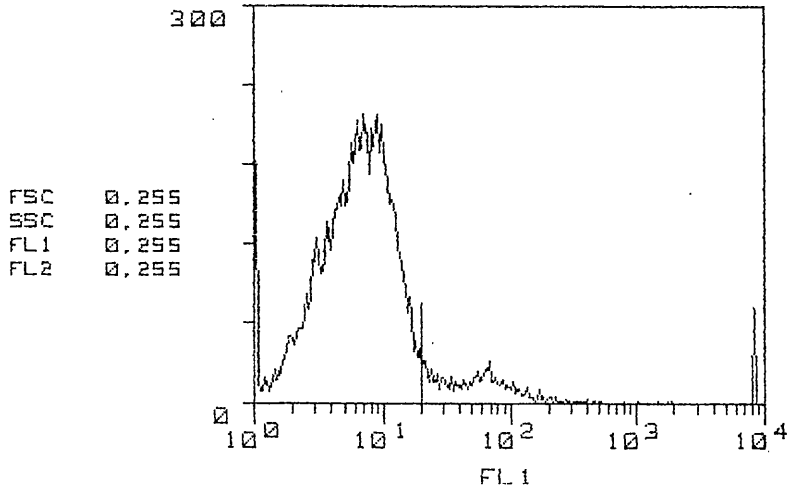
10^2
10^3
10^4
FL1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SSC 0,255
FL1 0,255
FL2 0,255
0
10^0
10^1
10^2
10^3
10^4
FL1

Figure 3.12 Flow cytometric analysis: 2 examples of Sca labelling for the reselected lineage negative population at day 7

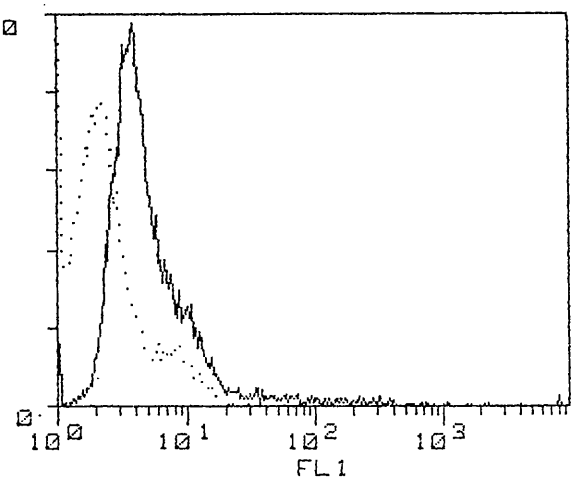
Following 7 days *ex vivo* expansion culture, the cells were harvested, reselected for lineage negative cells and labelled for flow cytometry. The top panel is the negative control. The middle panel shows expression of Sca in cells expanded with SCF and IL-11 alone. The lower panel shows expression of Sca in cells expanded with SCF, IL-11 and MIP-1α. The percentage of positive cells in this example was 10.3% for SCF and IL-11 and 31.2% for SCF, IL-11 and MIP-1α.

In the second example, from a separate experiment, 19.2% of cells were positive for Sca after expansion with SCF and IL-11 and reselection for lineage negative cells. 28.2% were sca+ after expansion with SCF, IL-11 and MIP-1α.

FL1, on the X axis, represents fluorescence intensity for FITC-conjugated antibodies, in this case either the isotype control or anti-Sca. The Y axis represents relative frequency of events.

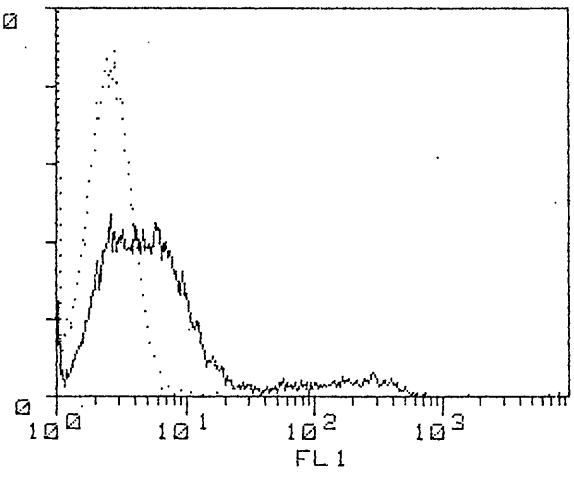
1.) MFØØ1

2.) MFØØ4 350



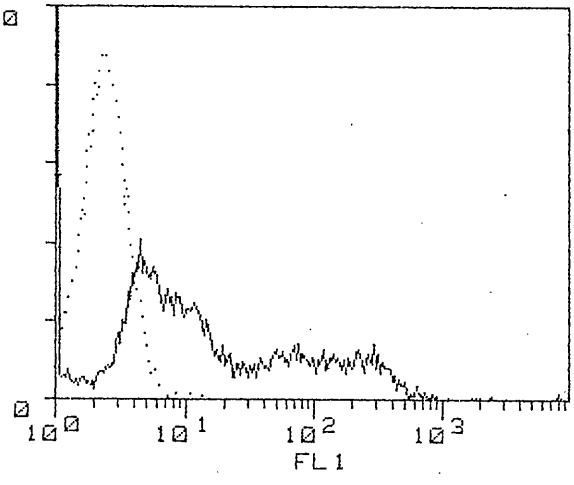
1.) MFØØ2

2.) MFØØ5 400



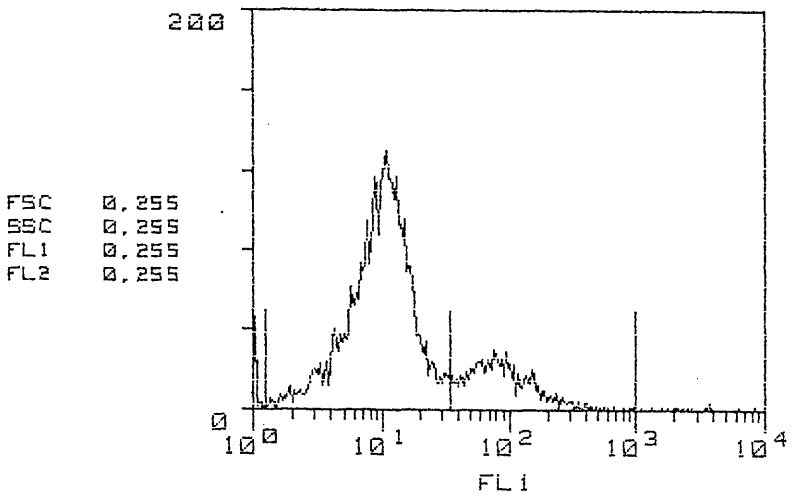
1.) MFØØ3

2.) MFØØ6 400



In the second example, from a separate experiment, 19.2% of cells were positive for Sca after expansion with SCF and IL-11 and reselection for lineage negative cells. 28.2% were sca+ after expansion with SCF, IL-11 and MIP-1α.

FL1, on the X axis, represents fluorescence intensity for FITC-conjugated antibodies, in this case either the isotype control or anti-Sca. The Y axis represents relative frequency of events.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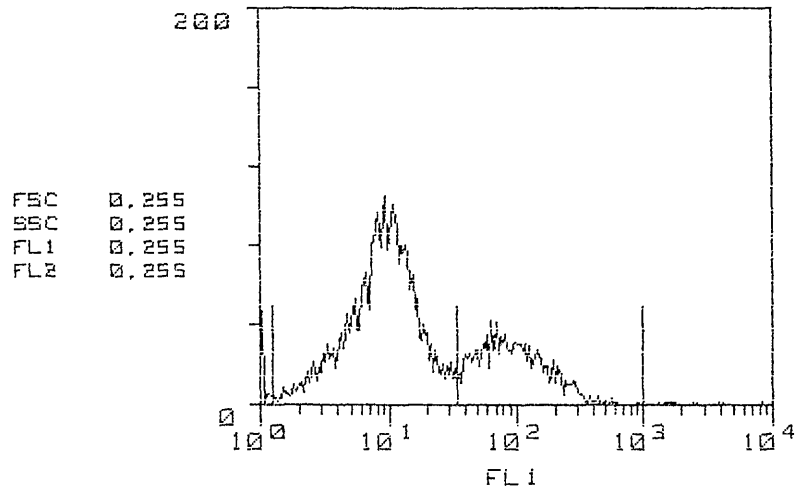
10^4
FL1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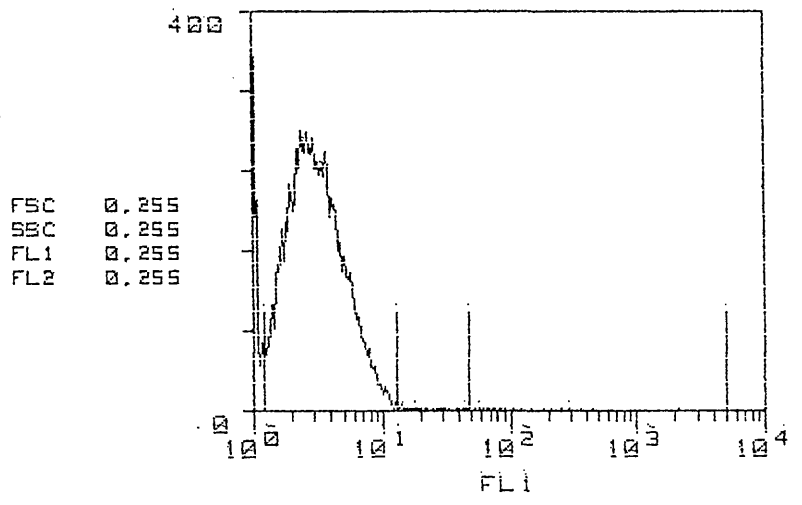
FL2 0,255
0
10^0
10^1
10^2
10^3
10^4
FL1

Figure 3.13 Flow cytometric analysis: example for Rhodamine for the reselected lineage negative population at day 7

This figure shows a representative example of Rhodamine staining for cells expanded for 7 days, then reselected for lineage negative cells. The top panel shows the negative control. The middle panel shows Rhodamine staining for cells expanded with SCF and IL-11, then reselected for lineage negative cells. The lower panel shows cells expanded with SCF, IL-11 and MIP-1α, and then reselected for lineage negative cells. In this example, the percentage of $Rhodamine^{dull}$ cells was 24.9% in the presence of SCF and IL-11 and 26.3% for SCF, IL-11 and MIP-1α.

FL1 represents fluorescence for rhodamine and the vertical markers divide the population into cells with low, intermediate and high fluorescence, working from left to right.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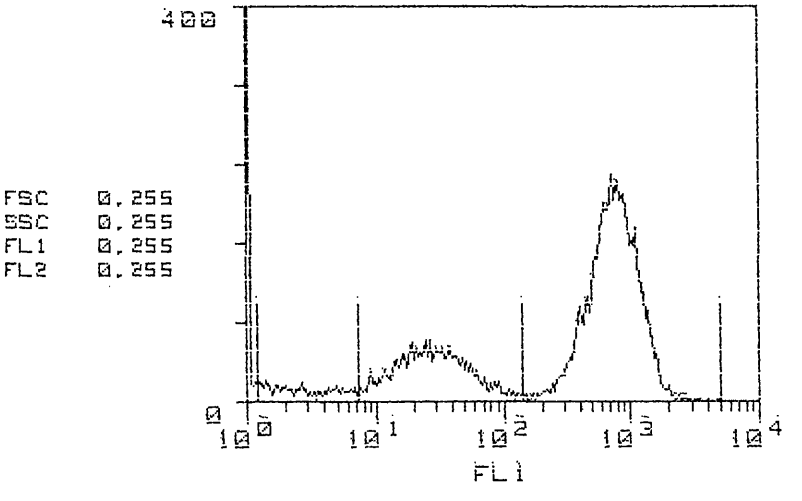
10^4
FL1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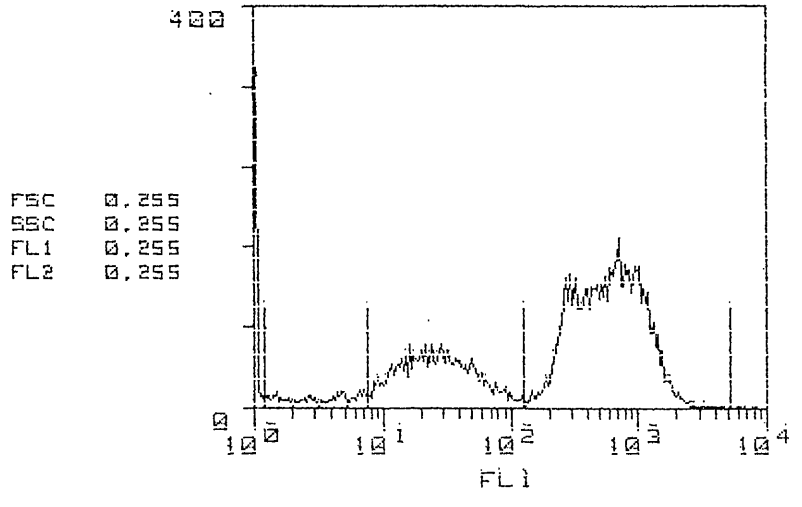
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FL1

Figure 3.14 a-g Amplification of lineage negative bone marrow cells

Normal murine bone marrow cells were enriched for lineage negative progenitors by immunomagnetic depletion as described in materials and methods. Lineage negative cells were then established in culture as shown in the protocol above. Results represent the mean (± SEM) for three consecutive experiments. The shaded columns represent results for culture with SCF, IL-11 and MIP-1α. The open columns are for SCF and IL-11.

Figure 3.14a
Increase over baseline for cell number

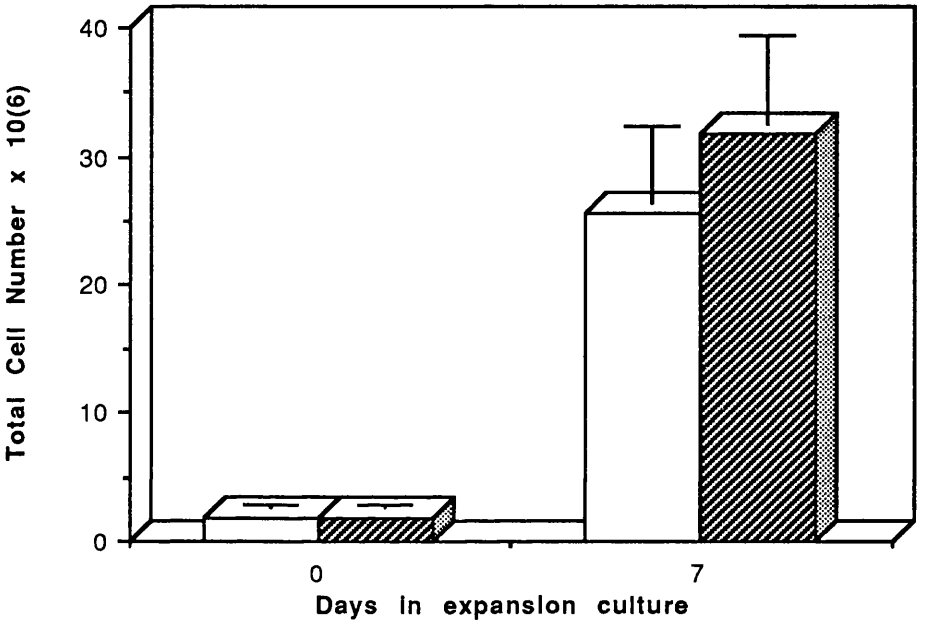


Figure 3.14b
CFU-A increase over baseline

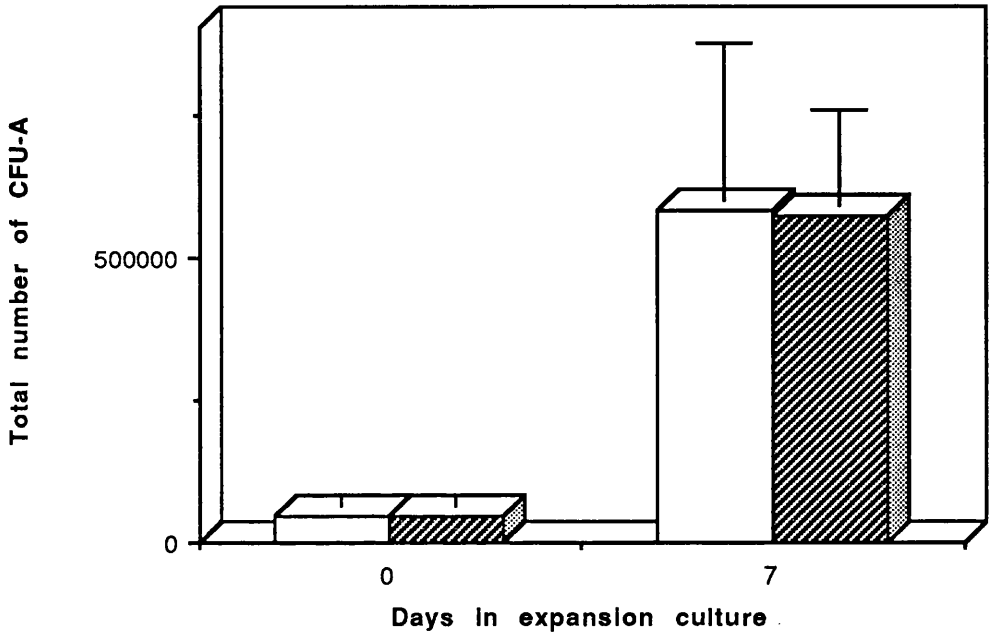


Figure 3.14c
CFU-GM increase over baseline

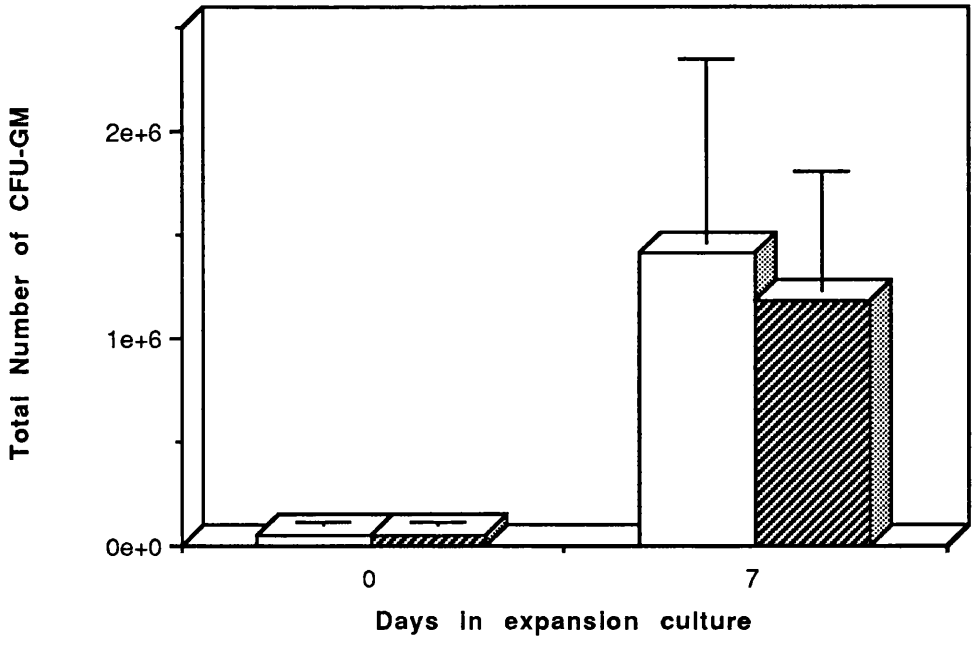


Figure 3.14d
Increase over baseline for Rhodamine dull cells

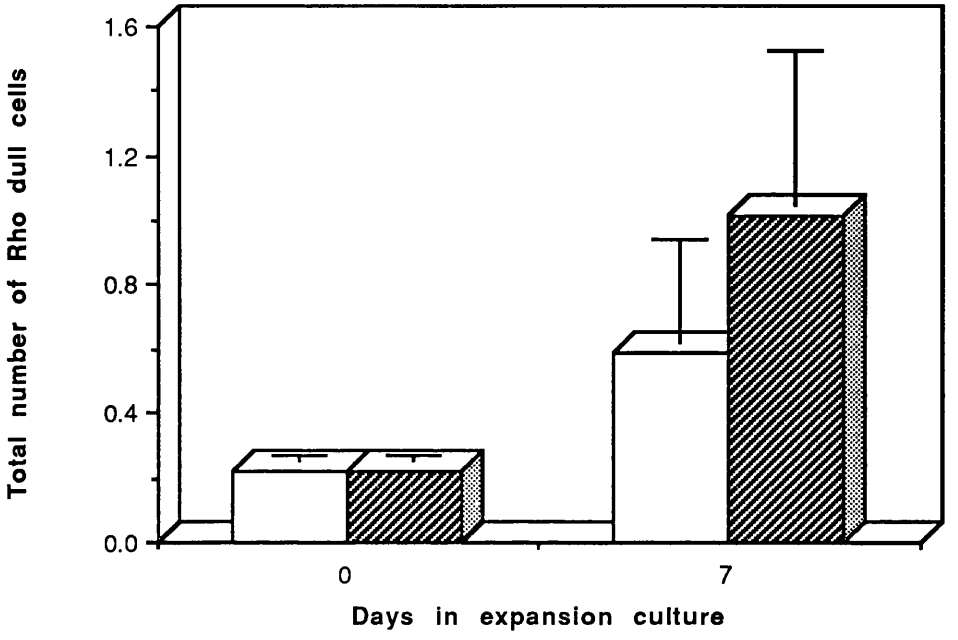


Figure 3.14e
Increase over baseline for Sca positive cells

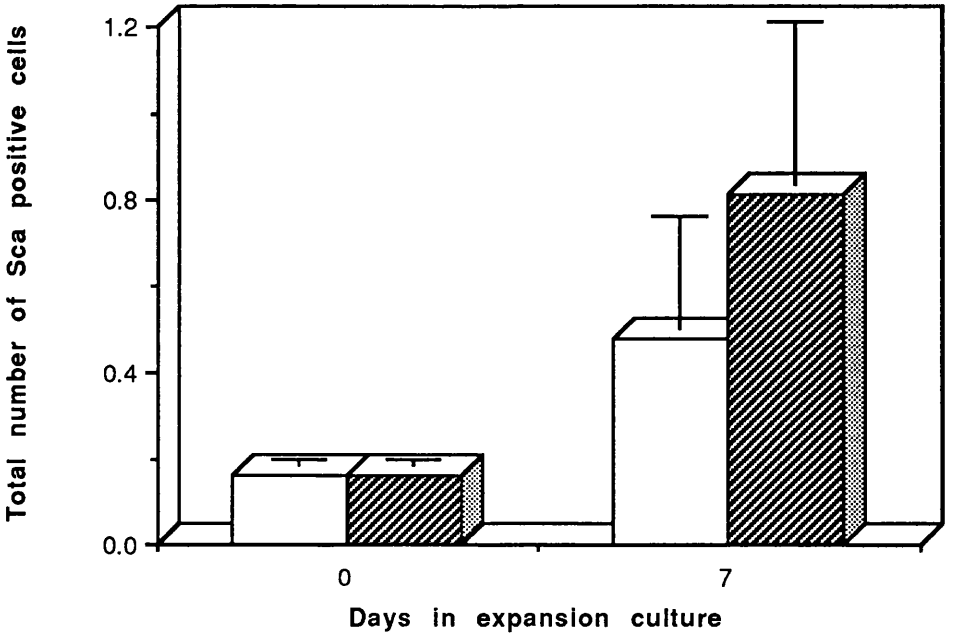


Figure 3.14f
Increase over baseline for blast cells

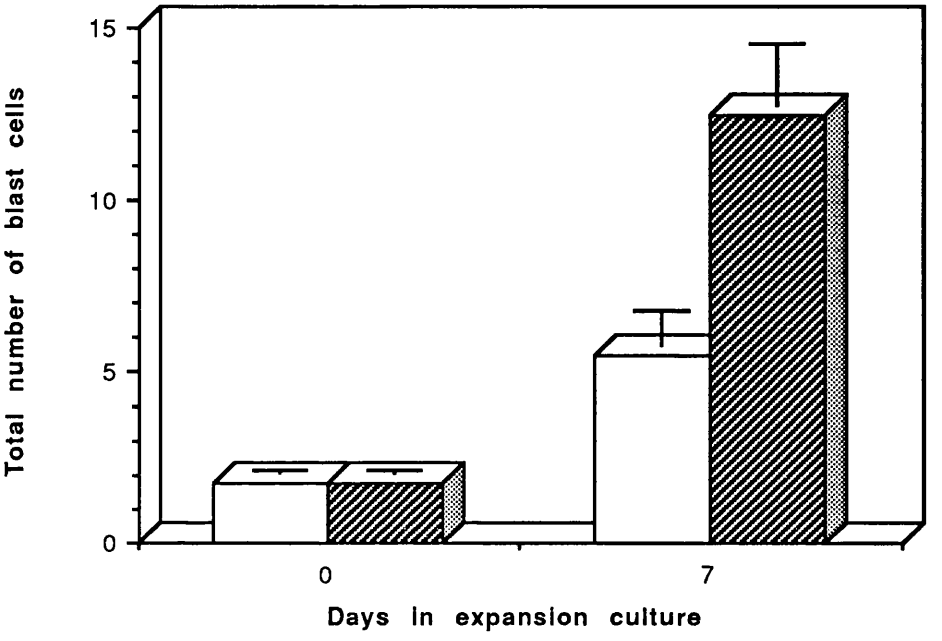
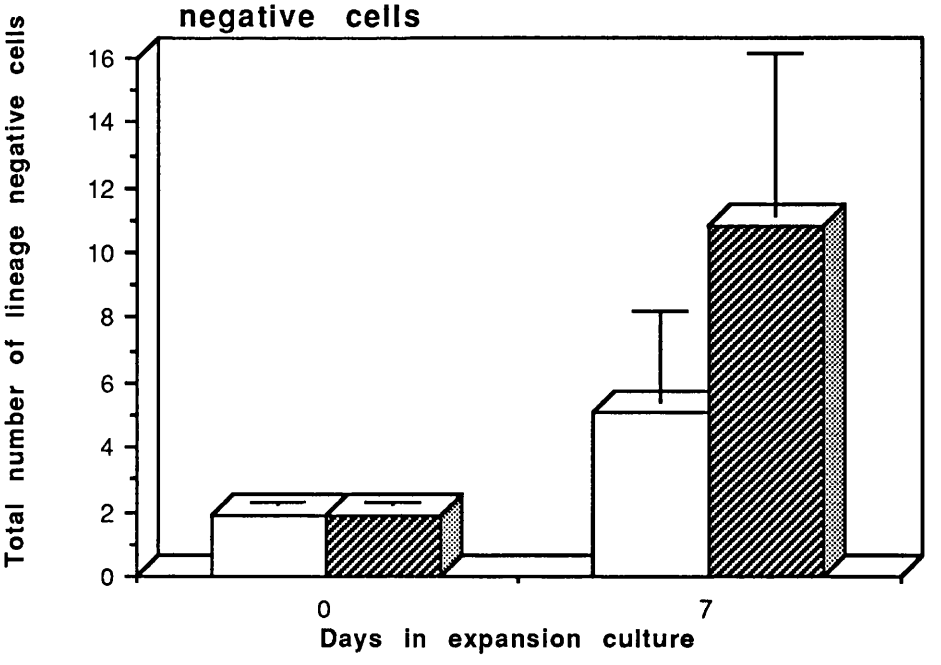


Figure 3.14g
Increase over baseline for lineage negative cells



(paired, 2-tailed t-test, p=0.03). There was no apparent difference in CFU-A or CFU-GM amplification under the two culture conditions tested (p=0.93 & 0.55 respectively). However, the four parameters used to detect more primitive progenitors (i.e. Rhodamine^{dull}, Sca+, blast morphology and lineage negative) consistently showed greater amplification (not always statistically significant) in the presence of MIP-1α (p=0.1, 0.09, 0.04, 0.1). As discussed there was marked variation between experiments. For example, having started with the same input number, the absolute number of lineage negative cells at the end of culture in the absence of MIP-1α was 3.58, 1.18 and 10.5 x 10^6 for the three replicate experiments and 8.46, 3.57 and 20.4 x 10^6 in the presence of MIP-1α for parallel cultures. Such variation makes it most unlikely that a statistical method based on these absolute numbers in such a small sample (i.e. only 3 sets of data) would prove to be statistically significant. What is of interest is the trend for greater recovery of more primitive progenitors in the presence of MIP-1α which was seen in every experiment.

The mechanisms of cytokine signal transduction for the three growth factors used in these studies are very different. SCF is the ligand for the c-kit receptor, a cell surface receptor with protein-tyrosine kinase activity. This receptor is similar to the receptors for M-CSF and PDGF. Receptors for IL-11, IL-6 and LIF share the signal transducing protein gp130 and IL-6 and G-CSF show structural homology. Members of the chemokine family including MIP-1α, MCP-1, RANTES and IL-8 are all thought to signal via seven membrane spanning receptors. In an attempt to determine if the amplification of CFU-A and CFU-GM achieved with the combination of SCF, IL-11 and MIP-1α was specific for these cytokines, or, perhaps, reflected simultaneous signalling via three very different receptors on stem and progenitor cells, cytokines which signal via similar receptors were exchanged for the standard combination.

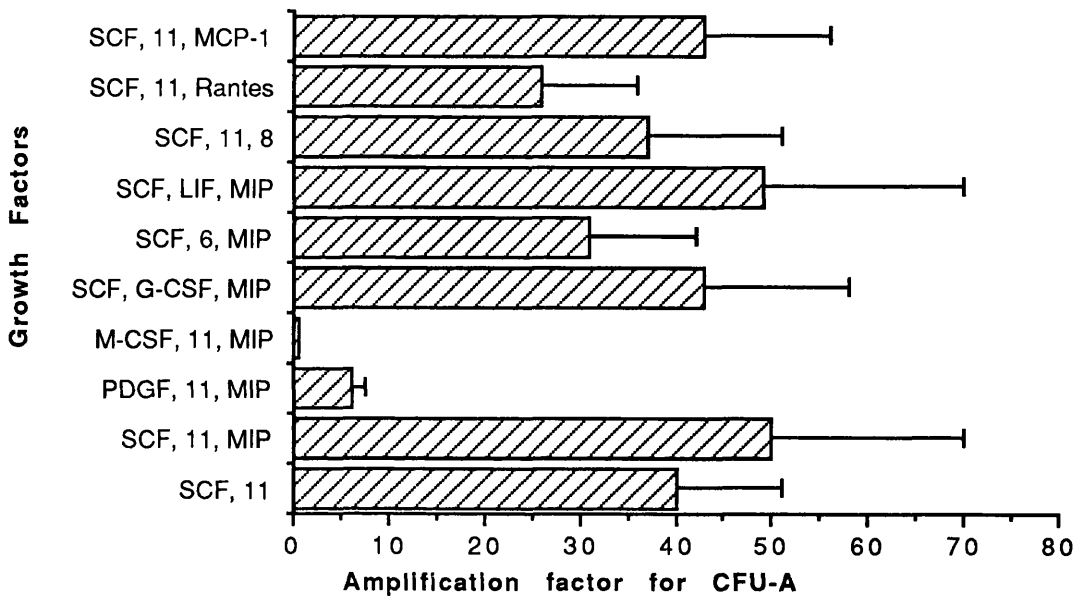
Unfractionated bone marrow was expanded under standard culture conditions for 6 days. PDGF and M-CSF were exchanged for SCF; LIF, G-CSF and IL-6 were exchanged for IL-11; and MCP-1, RANTES and IL-8 were exchanged for MIP-1α. As shown in Figure 3.15, the addition of MIP-1α to SCF and IL-11 produced only slightly increased amplification of CFU-A and no enhancement of CFU-GM expansion compared with SCF and IL-11. Exchanging MCP-1, RANTES or IL-8 for MIP-1α neither increased nor decreased the observed progenitor expansion. LIF, G-CSF and IL-6 were equivalent to IL-11 in terms of CFU-A and CFU-GM expansion. SCF, however, appeared to be essential in this combination and could neither be substituted by PDGF nor M-CSF. These findings suggest that substituting cytokines with similar signal transduction pathways is not sufficient for progenitor amplification and that SCF is unique in its ability to synergise with the other cytokines present to induce progenitor proliferation.

MIP-1α is considered to reversibly inhibit stem cell proliferation by preventing entry into the S phase of the cell cycle (Dunlop *et al.*, 1992). CFU-A and CFU-S day 12 appear to be within that part of the stem cell compartment which is a target for the actions of MIP-1α. In resting bone marrow the CFU-A compartment is generally quiescent (< 20% in S phase), but may easily be triggered to proliferate by a variety of positive stimuli. It was, therefore, of interest to determine the effect of MIP-1α on CFU-A progenitors prepared from normal marrow and the change in cycle status obtained when MIP-1α was combined with SCF and IL-11. Normal bone marrow cells were prepared in the usual manner and were cultured for 4 or 24 hours either in medium alone, or supplemented with MIP-1α . Following incubation, an Ara-C suicide assay was performed to determine the percentage of progenitors in active cell cycle. Figure 3.16 shows the results for CFU-A progenitors. After only 4 hours incubation in medium alone, the % of CFU-A

Figure 3.15 Effect of growth factor exchange

Unfractionated murine bone marrow cells were cultured for 6 days in 24 well plates as described in materials and methods. SCF, IL-11, G-CSF (20ng/ml), PDGF (10ng/ml), M-CSF (6ng/ml), IL-6 (20ng/ml), LIF (10ng/ml), IL-8 (100ng/ml), Rantes (100ng/ml) and MCP-1 (100ng/ml) were added at the start of culture only. MIP-1α was added every 48 hours from the start of culture. Amplification factors (+ SEM) are shown for CFU-A and CFU-GM progenitors. Results represent the mean of three replicate experiments.

Figure 3.15
Amplification for CFU-A: effect of growth factor exchange



Amplification of CFU-GM: effect of growth factor exchange

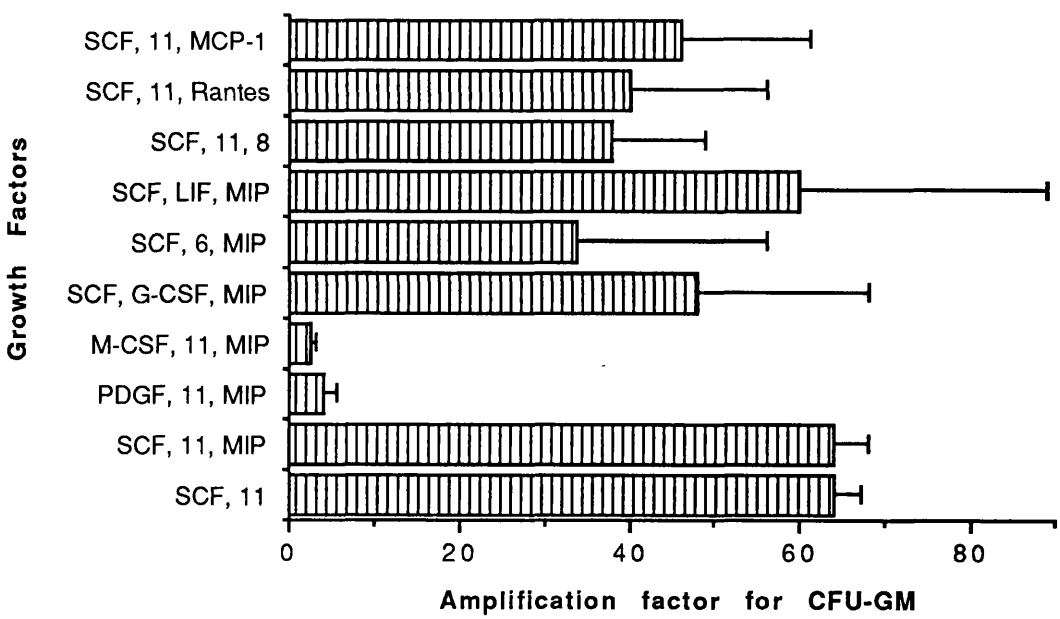
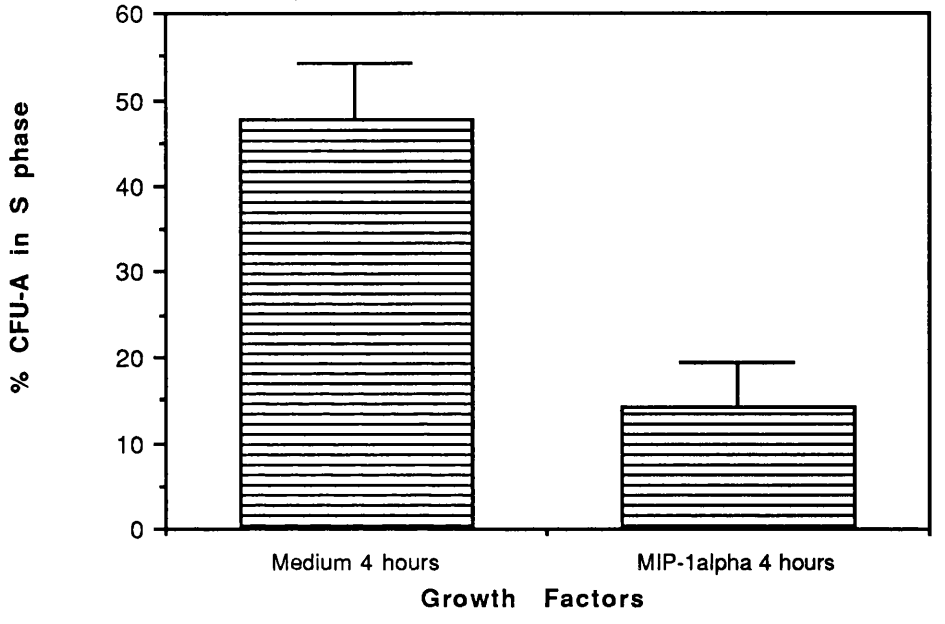


Figure 3.16 Effect of MIP-1α on cycle status of CFU-A progenitors

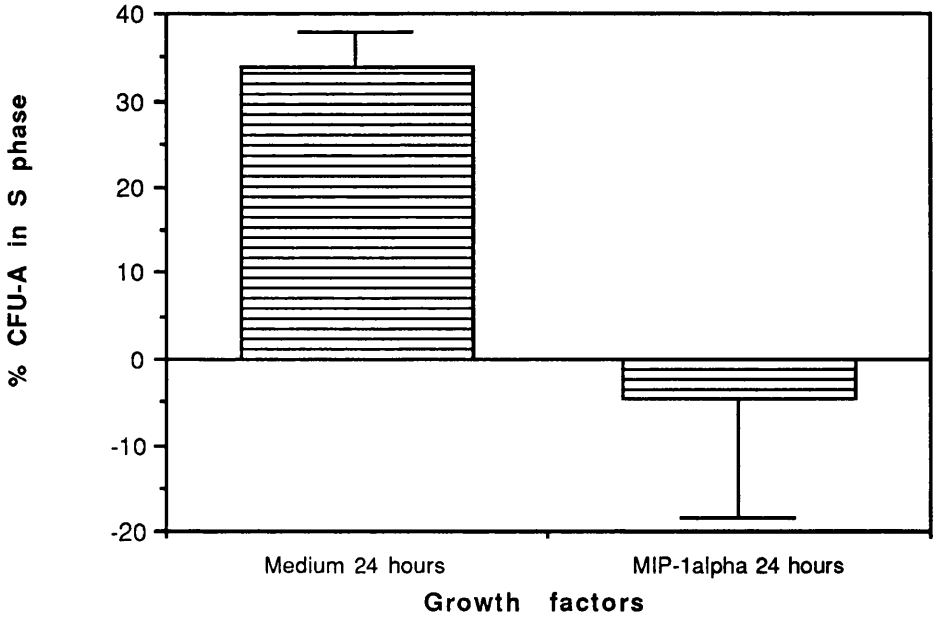
Unfractionated murine bone marrow cells were incubated for 4 and 24 hours at 37°C in Fischer's medium supplemented with DHS (10%) with and without MIP-1α. Following incubation, the cells were washed once and used in an Ara-C suicide assay, as described in Materials and Methods. After washing three times, the cells were plated into the CFU-A assay. The mean percentage of CFU-A in S-phase for four replicate experiments is shown (± SEM).

Figure 3.16

% CFU-A in S phase following incubation +/- MIP-1alpha for 4 hours:



% CFU-A in S phase following incubation +/- MIP-1alpha for 24 hours:



in S phase was approximately 50%. There are two possible explanations for this effect. The first is that factors present in DHS (which is selected for optimal growth in the CFU-A assay) may be sufficient to trigger cycling of CFU-A. The second is that, at the time this series of experiments was performed, the baseline cycle status of CFU-A for the particular batch of animals in use was greater than usual (around 20-30% in S phase), perhaps due to undetected infection. The percentage of CFU-A in active cell cycle was much less when MIP-1α was included in the medium throughout the period of incubation. Similar, but even greater inhibition was seen after a 24 hour culture period. These results suggest that in the absence of stimulatory cytokines, MIP-1α's effects are those of a classical inhibitor of stem cell proliferation.

In the next experiment, CFU-A cycle status was assessed at baseline, prior to either serum or growth factor exposure. The cells were then incubated in serum supplemented medium for 4 hours either in the presence of SCF and IL-11, or SCF, IL-11 and MIP-1α. Following incubation, cycle status was determined by Ara-C suicide assay. The results for three such replicate experiments are shown in Figure 3.17. For this series of experiments <15% of CFU-A progenitors were in S phase prior to growth factor stimulation. The percentage in cell cycle rose significantly following culture in the presence of SCF and IL-11 to > 30%. The addition of MIP-1α was able to prevent CFU-A entry into S phase, even in the presence of SCF and IL-11, at least over a 4 hour period.

This experiment was extended to time points at 24 hours, 3, 7, 10 and 14 days as shown in Table 3.2. The inhibitory effect of MIP-1α, though maintained in the face of growth factor stimulation for 4 hours, was no longer detectable by

Figure 3.17 The effect of MIP-1α on entry of CFU-A progenitor cells into cell cycle in response to growth factors

Unfractionated murine bone marrow cells were divided into three aliquots. The first was immediately used for an Ara-C suicide assay to determine the percentage of CFU-A progenitors in S-phase at baseline. The second was incubated for 4 hours in Fischer's medium supplemented with DHS (10%), SCF and IL-11. For the third, MIP-1α was added in addition to SCF and IL-11. Following 4 hours incubation, the cells were washed and then used in a suicide assay. The mean percentage of CFU-A progenitors in S-phase for the three test conditions is shown (± SEM) and represents the mean of three replicate experiments. The difference between the baseline cycle status and the response to SCF and IL-11 was statistically significant ($p=0.03$, Student's t-test, paired & 2-tailed) as was the difference between the response to SCF and IL-11 and that to SCF, IL-11 and MIP-1α ($p=0.05$).

Figure 3.17
Effect of MIP-1alpha in preventing CFU-A entry into S phase in response to growth factors

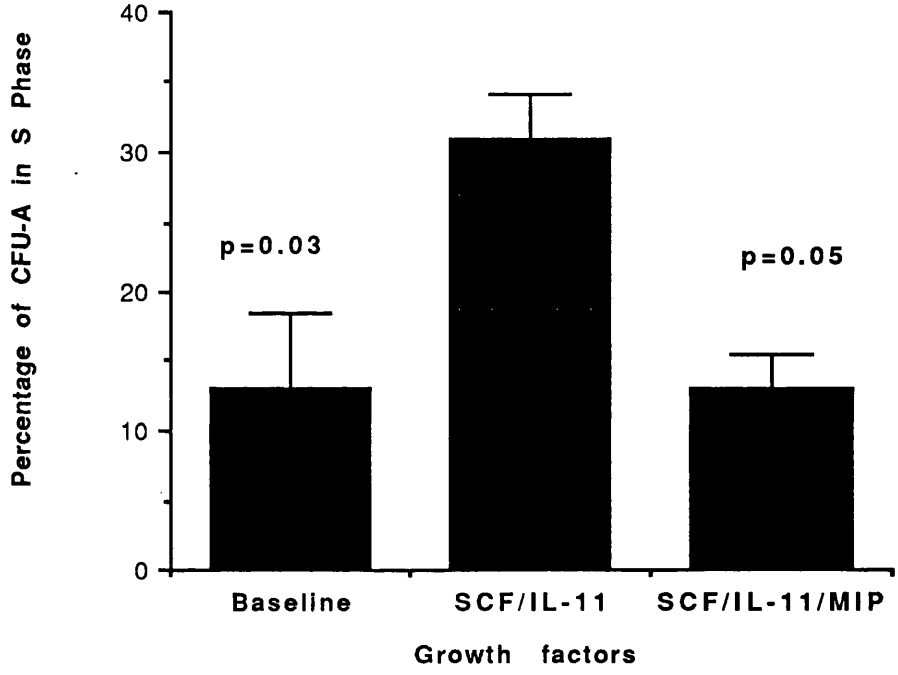


Table 3.2 A comparison of CFU-A cycle status for bone marrow cells expanded *ex vivo* with SCF/IL-11 or SCF/IL-11/MIP-1α

Unfractionated murine bone marrow cells were cultured for 14 days in 24 well plates as described in materials and methods. Baseline CFU-A cycle status was assessed by Ara-C suicide assay prior to the start of incubation. Cells were then established in cultures supplemented with either SCF and IL-11 or SCF, IL-11 and MIP-1α. SCF and IL-11 were added at the start of culture and again at the time of medium change at day 7. MIP-1α was added at the start of culture and every 48 hours. Replicate cultures were harvested and assessed by Ara-C suicide assay at 4 and 24 hours, days 3, 7, 10 and 14. Results represent the mean (± SEM) for 3 replicate experiments.

Table 3.2

Growth Factors	Baseline	4 hours	24 hours	3 days	7 days	10 days	14 days
SCF/IL-11	13 ± 5.4	31 ± 3.1	62 ± 6.8	61 ± 3	38 ± 2.5	55 ± 4	56 ± 3
SCF/IL-11/MIP-1α	13 ± 5.4	13 ± 2.5	54 ± 3.8	59 ± 6	27 ± 3.5	59 ± 5.5	60 ± 3

24 hours. Thereafter there was no difference in cycle status for cultures supplemented with SCF and IL-11, or SCF, IL-11 and MIP-1α.

Discussion

In the first experiment described in this chapter, SCF was found to promote CFU-A expansion during a 6 day incubation in serum containing medium. There has been some controversy over the ability of SCF, as a single factor, to stimulate colony formation in semi-solid agar or progenitor expansion in suspension culture. Zsebo et al showed that SCF alone promoted colony formation from total bone marrow or post 5-FU bone marrow, but not from enriched lineage negative bone marrow and concluded, therefore, that SCF did not act directly on colony forming cells (Zsebo *et al.*, 1990b). Heyworth et al investigated the role of SCF on highly purified GM-CFC, both in the presence and absence of serum, and found that SCF's effects were both direct and independent of the requirement for serum (Heyworth *et al.*, 1992). Lowry et al found that SCF used alone had negligible clonogenic capacity on 2 day post 5-FU bone marrow enriched to the level of Sca+ lineage negative progenitor cells (Lowry *et al.*, 1992). Metcalf and Nicola investigated the effects of SCF on unfractionated and on Sca+ lineage negative murine bone marrow cells. Their data strongly suggested that SCF was able to promote colony formation directly, although the effects were much less for purified cells than for unfractionated marrow (Metcalf & Nicola, 1991). De Vries et al, demonstrated direct effects for SCF on highly purified murine haemopoietic progenitor cells (de Vries *et al.*, 1991), however, Migliaccio failed to show colony formation using similarly enriched populations (Migliaccio *et al.*, 1991). Taken together, these studies suggest that the proliferative effect of SCF on highly purified progenitor cells, in the absence of serum is small, but increased levels of proliferation may be seen for unpurified cells in the presence or absence of serum. To fully clarify this issue, highly purified single cells should be

cultured under serum free conditions in the presence of SCF alone. Under such conditions, Xiao et al showed that SCF alone was unable to induce colony formation from highly purified human progenitor cells (Xiao *et al.*, 1992). In view of the above data, it is not surprising that in the experiments described above, SCF alone promoted CFU-A and CFU-GM amplification in cultures of unfractionated bone marrow in the presence of serum.

The synergistic effect of SCF and IL-11 in inducing proliferation of multipotent progenitor cells has been recognised by several investigators (Musashi *et al.*, 1991; Quesniaux *et al.*, 1992; Tsuji *et al.*, 1992). Both SCF and IL-11 are stromal cell derived cytokines which appear to have a central role in regulating haemopoiesis. SCF and IL-11, along with G-CSF and IL-6, appear to induce the entry of dormant murine haemopoietic progenitor cells into cell cycle by shortening the Go period (Musashi *et al.*, 1991). In addition, IL-11 appears to control later stages of development, promoting macrophage proliferation, megakaryocyte and erythroid colony formation (Quesniaux *et al.*, 1992). The cytokine combination of SCF and IL-11 was selected for further study firstly, because it was demonstrated to induce proliferation of CFU-A and CFU-GM progenitors cells at least as well as any other two cytokine combination and secondly, since both cytokines are stromal cell derived they are likely to have a pivotal role in the regulation of the early stages of haemopoiesis *in vivo* and may therefore be expected to interact synergistically *in vitro* in a similar manner.

In the culture conditions employed in these experiments, IL-3, either alone or in combination with other cytokines, was unable to promote progenitor expansion and even reduced the degree of expansion induced by other combinations. Morphological analysis suggested that the concentration of IL-3 used promoted terminal differentiation of progenitor cells. In view of the large number of macrophages present at the end of culture it is possible that IL-3 may, in addition, have been toxic to progenitor cells. The effect of

IL-3 appears to be highly dependent on both the concentration of IL-3 used and the presence or absence of serum in the culture medium. Ploemacher et al observed that the addition of IL-3 in high concentration to the combination of SCF and IL-11 acted in a counterproductive manner with respect to the number of colony forming cells generated and their proliferative ability (ability to produce secondary colonies) (Ploemacher *et al.*, 1993a). Since the IL-3 message is low or absent in the stromal environment under steady state conditions it is likely that IL-3 does not play a primary role in the regulation of proliferation of primitive progenitor cells. In a small number of experiments performed in our own laboratory, by titrating the concentration of IL-3, in cultures expanded with SCF and IL-11, from 50ng/ml to 0.05ng/ml, very low concentration IL-3 (0.05ng/ml) synergised with SCF and IL-11 to promote further amplification of CFU-A and CFU-GM progenitors. This effect was only reproducible under serum free conditions and in the presence of serum even 0.05ng/ml of IL-3 abolished the progenitor expansion seen with SCF and IL-11 (Holyoake *et al.*, 1994).

Although MIP-1α has been shown to have both inhibitory and stimulatory effects depending on the maturational stage of the target population, the results observed with this chemokine are somewhat surprising and difficult to interpret. It appears that when MIP-1α is added to the combination of SCF and IL-11, during the early hours of culture, progenitor proliferation (determined by Ara-C suicide assay) is inhibited, but this inhibition is overcome within 24 hours with no differences in cycle status detectable at and beyond that point. If inhibition of cell cycling were the only difference detectable, one would expect fewer cells at the end of culture in the presence of MIP-1α. However, by day 7 of culture there were consistently more cells present with a greater proportion of more primitive progenitors in the presence of MIP-1α. The likely explanation is that MIP-1α does indeed "hold up" progenitor proliferation, but at the point when this inhibition is

overcome, proliferation proceeds with less associated differentiation, accounting for both the increase in total cell number (by self-renewal) and in primitive subsets. The most convincing work demonstrating a role for MIP-1α in the maintenance of primitive progenitor cells during *ex vivo* culture has been performed by Verfaillie et al using human cells. In the long term culture conditions described, primitive human progenitor cells were cultured separated from a stromal layer by a microporous membrane. Without exogenous growth factor addition LTCIC, (the most primitive human progenitor cell detected by *in vitro* assay) fell to 50% of input numbers by week five of culture. The addition of MIP-1α alone had no effect on LTCIC maintenance. The addition of IL-3 alone resulted in exhaustion of LTCIC by week five, however, the addition of both IL-3 and MIP-1α produced full maintenance of LTCIC at input numbers for at least eight weeks (Verfaillie *et al.*, 1993). These data strongly suggest that the effect of MIP-1α observed in our own experiments is a genuine effect and as such requires further investigation.

RESULTS II

Chapter 4 Short term engraftment studies

The *in vitro* data described in chapter 3 demonstrate that SCF and IL-11 +/- MIP-1α may amplify CFU-A and CFU-GM progenitors in unfractionated bone marrow approximately 50 and 100 fold respectively. There are two possible mechanisms by which stem and progenitor cells expanded *ex vivo* may produce more rapid *in vivo* haematological recovery upon transplantation. The progenitors considered to be important for the early but "transient" engraftment following BMT include both CFU-A (equivalent to CFU-S) and probably CFU-GM (Jones *et al.*, 1990). By greatly increasing the number of these progenitors by *ex vivo* culture it was hoped that, following transplantation into a suitable model, early engraftment would be faster. Furthermore, it would be of potential clinical value if the number of cells required for rescue following myeloablation could be reduced by expanding a smaller number of "input" cells prior to transplantation. The second mechanism by which *ex vivo* culture of the cells to be transfused may accelerate haemopoietic recovery following BMT involves exposure to cytokines *ex vivo* without necessarily attempting to increase stem and progenitor cell numbers. Short term exposure (2 hours) of bone marrow cells to cytokines has been shown to modulate cell surface molecules including adhesion molecules and this may affect the rate and degree of progenitor homing to the bone marrow following transplantation (Tavassoli & Hardy, 1990; Tavassoli *et al.*, 1991; Zanjani *et al.*, 1992). This mechanism has recently been contradicted by Van der Loo et al (van der Loo & Ploemacher, 1995) and was not studied in the work described here.

To assess the engraftment potential of bone marrow cells expanded *ex vivo*, a murine bone marrow transplantation model was developed. Syngeneic female

recipient mice were given a potentially lethal dose of radiation and were then rescued using test cells from male donors which had been manipulated *ex vivo*. The dose of radiation used was sufficient to produce death from bone marrow failure if a bone marrow transplant was not performed. A single dose of radiation was tested in preliminary experiments but was shown to produce excessive non-haemopoietic toxicity. Fractionated radiation, with a minimum delay of three hours between two fractions, has been shown in previous studies to allow some repair of rapidly proliferating tissues such as the gastrointestinal tract and lung and so prevent deaths from non-haematological toxicity therefore this approach was investigated (Spangrude & Scollay, 1990).

As shown in Figure 4.1, to establish the ideal dose of radiation, a dose titration was performed in the absence of transplantation. Deaths prior to day 7 are likely to be due to non-haematological toxicity with deaths from bone marrow failure occurring between day 7 and 18 post radiation. None of the doses used in the first titration were sufficient to cause 100% mortality by day 30. In both the first and second titration a dose of 11Gy was lethal to 50% of animals. Above 11Gy the doses were lethal to 100% of animals by day 20, but occasional deaths were observed prior to day 7 and, were therefore, possibly due to non-haematological toxicity. These results, however, suggested the possibility of a good model for the intended experiments and were far more promising than with single dose radiation. The radiation dose for all the experiments described in this chapter was 12Gy, administered as 2 equal fractions exactly 3 hours apart.

The primary aim of these initial *in vivo* experiments was to assess the transient engrafting potential of stem and progenitor cells expanded *ex vivo* and to compare this with unmanipulated bone marrow cells. To allow assessment of

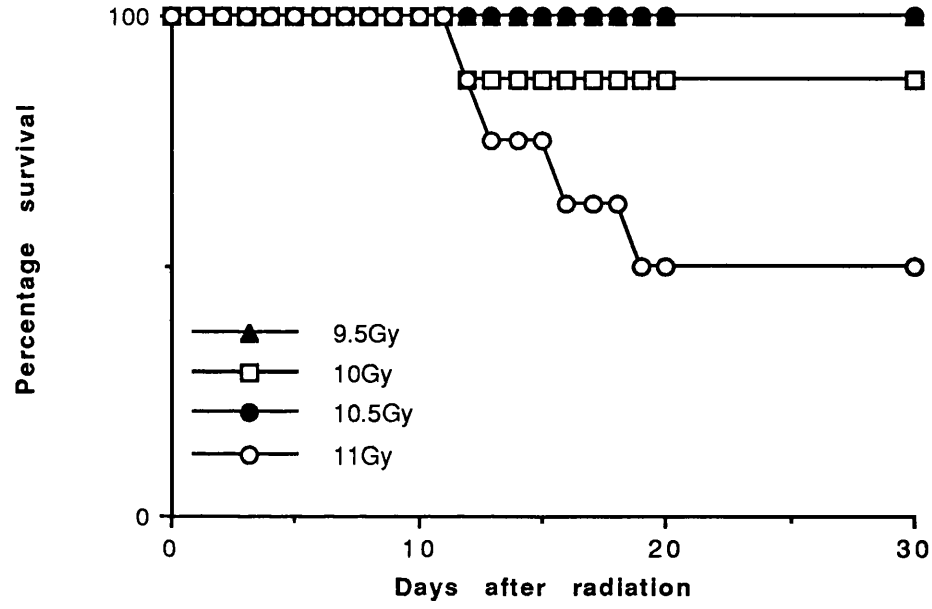
Figure 4.1 First titration of fractionated radiation in B6D2F1 mice

To establish the dose of fractionated radiation required to induce bone marrow aplasia in 100% of animals, a dose titration was carried out. Groups of 10 mice received increasing doses of radiation from 9.5 to 11Gy in two equal fractions, three hours apart. Mice were then observed for survival to day 30 following radiation. Results are shown for percentage survival against time.

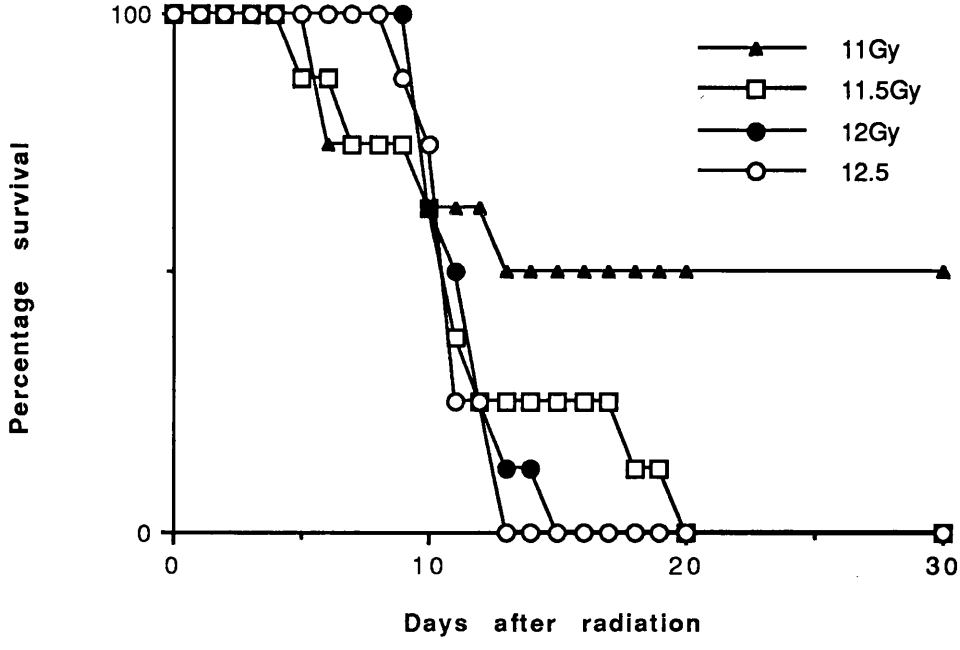
Second titration of fractionated radiation in B6D2F1 mice

Since none of the radiation doses used in the first titration was lethal to 100% of animals, a second dose titration was carried out. Groups of 10 mice received increasing doses of radiation from 11 to 12.5Gy in two equal fractions, three hours apart. Mice were then observed for survival to day 30 following radiation. Results are shown for percentage survival against time.

Figure 4.1
First titration of fractionated radiation



Second titration of fractionated radiation



the contribution of donor cells to engraftment either soon after BMT, or much later, donor cells were taken from male animals with females as recipients. The method used to detect male donor cells was Southern blotting using the Y chromosome specific probe pY353/B (Bishop *et al.*, 1985). Figure 4.2 shows the typical pattern obtained by Southern blotting of *Eco* R1 digested genomic DNA from male and female animals using this probe. Female DNA shows no hybridisation, however male DNA shows hybridisation with a consistent band of 1.5kb. Although several bands of higher molecular weight are seen, these are weaker and therefore less reliable for assessment of donor engraftment. The 1.5kb band was routinely used to look for evidence of donor cells post BMT.

Each time samples were tested for evidence of donor cells, a standard titration of male DNA diluted into female DNA was included in the gel. This was to allow an approximate quantification of the percentage of male cells present. Following hybridisation with probe pY353/B, blots were stripped and re-hybridised with a probe for GAPDH, which is not male specific and therefore could be used to assess how much DNA was loaded into each well and transferred successfully to the membrane. Figure 4.3 shows an example of the standard titration of male DNA from 100% to 0%, hybridised first with the Y specific probe and then with the GAPDH probe. The GAPDH hybridisation (showing almost equal hybridisation in every lane) confirms that for lanes in which the male signal was low, this was genuinely due to a low percentage of male donor cells on a background of female recipient cells rather than because less DNA than intended was loaded into the well or because of poor transfer to the membrane.

In Figure 4.4, the results of densitometry performed on X-rays derived from the titration described in Figure 4.3 are shown in a plot of optical density

Figure 4.2 Southern hybridisation of *Eco* R1 digested genomic blots with pY353/B

In this blot, the first two lanes show undigested male genomic DNA (10μg/lane) hybridising to the Y chromosome specific probe. The third and sixth lanes were empty. The fourth and fifth lanes were loaded with *Eco* R1 digested female genomic DNA which does not hybridise with the probe. The seventh and eighth lanes were loaded with *Eco* R1 digested male genomic DNA (10μg) which binds to the probe in a specific manner. The 1.5kb band was found to be the most reliable for assessment of male donor engraftment.

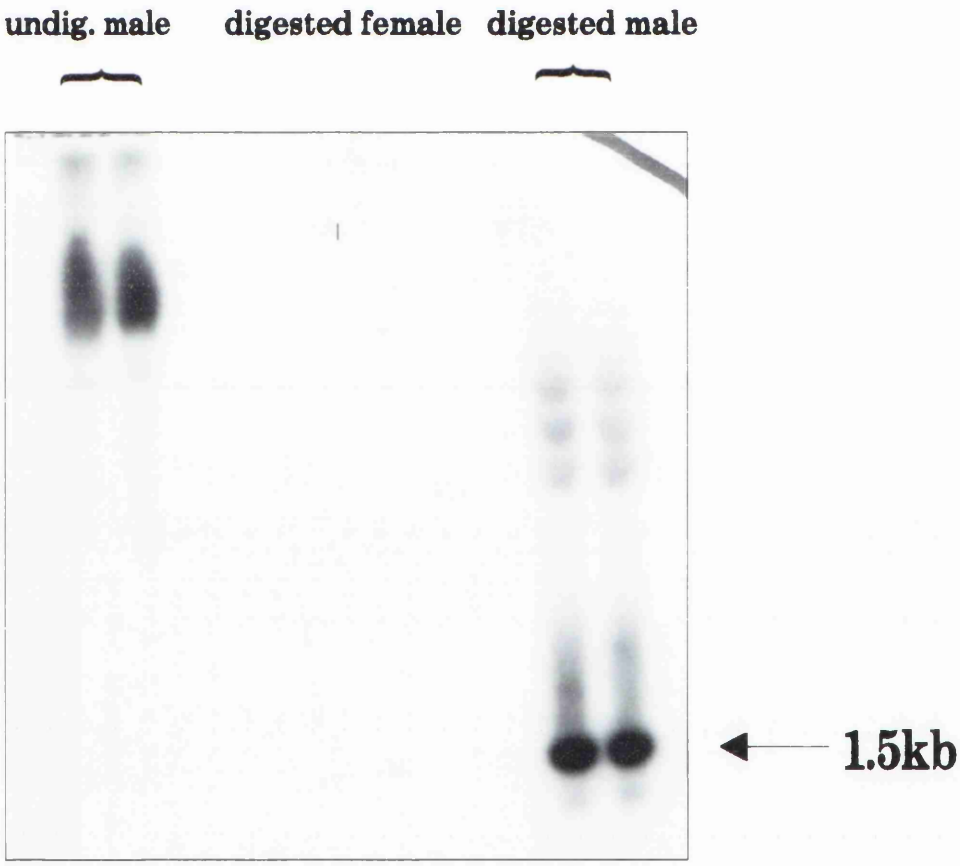

undig. male
digested female
digested male
1.5kb

Figure 4.3 Titration curve of male DNA

Male DNA was diluted with female DNA to produce a standard titration (100, 50, 25, 12.5, 6.25, 3.125, 0% male). 10μg of DNA was loaded per well. Following Southern blotting, hybridisation to the Y specific probe and X-ray exposure, the blots were stripped and re-hybridised with the GAPDH probe. The upper panel shows the titration of male DNA hybridised with the Y probe. The lower panel shows the same blot after hybridisation with GAPDH. This titration was included with all test samples to allow an estimate of the percentage donor haemopoiesis. The GAPDH hybridisation was performed to assess DNA loading from well to well (i.e. both male and female DNA would hybridise with GAPDH).

Y probe

100% 50% 25% 12.5% 6.25% 3.125% 0%

GAPDH

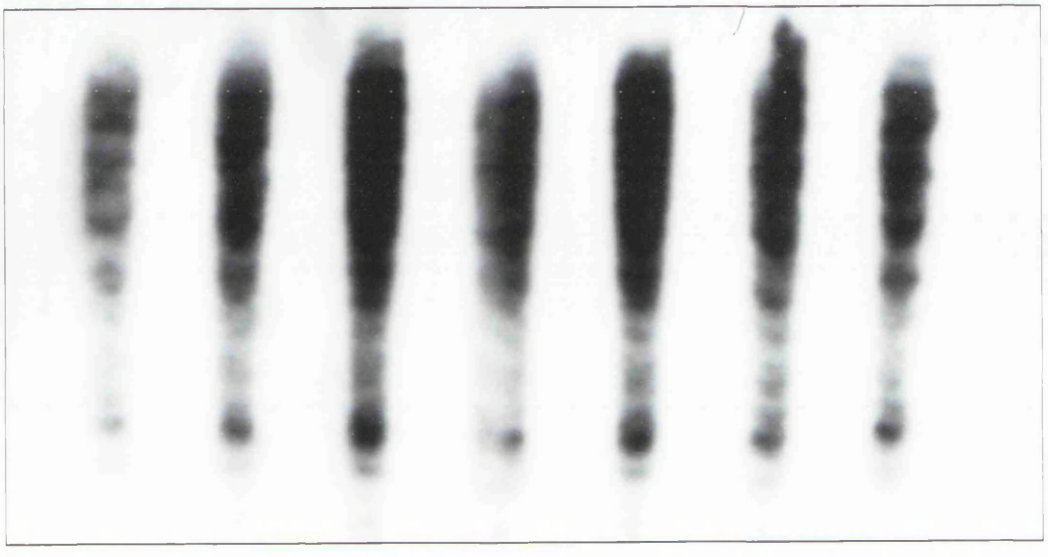
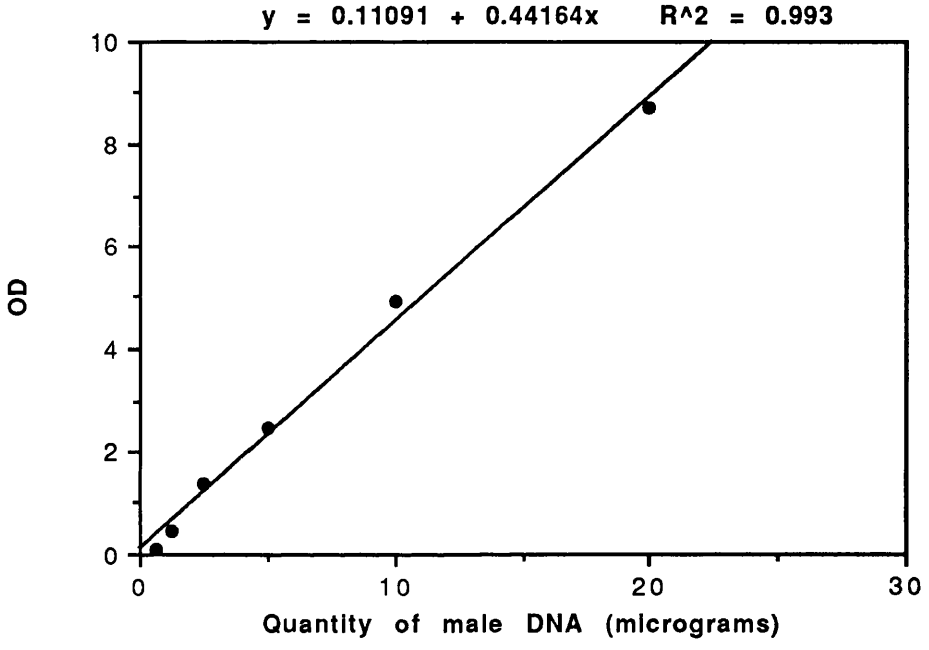


Figure 4.4 Correlation of the quantity of male DNA loaded per well with optical density by densitometry

The known quantity of male DNA loaded per well was plotted against optical density (contour OD x mm^2) measured by densitometry. As shown, the data points fell very close to a straight line with a correlation of R=0.993.

Figure 4.4


y = 0.11091 + 0.44164x R^2 = 0.993
OD
10
8
6
4
2
0
0
10
20
30
Quantity of male DNA (micrograms)

against the quantity of male DNA thought to have been loaded in each well. In this example, the correlation of optical density with quantity of DNA is excellent. The data points fall close to a straight line with a correlation coefficient of R=0.993.

The next series of experiments were designed to compare the short term (to day 30) engraftment potential of progenitor cells expanded with SCF and IL-11 +/- MIP-1α with that of unmanipulated cells. In preliminary experiments it was determined that a dose of 10^5 unmanipulated bone marrow cells per mouse was required to ensure survival of close to 100% of lethally irradiated animals (data not shown). In the experiments described in Figure 4.5, the cell dose per mouse was titrated from 2 x 10^4 to 10^3. Control BMT was with fresh unmanipulated bone marrow cells harvested on the day of BMT. For expanded populations, *ex vivo* cultures were established 6 days prior to BMT. The number of expanded cells transplanted per mouse was calculated based on the number established in culture 6 days prior to BMT rather than on the number in culture after expansion had taken place. These experiments were performed on several occasions and each time 10 animals were included in each experimental group. The percentage of animals surviving to day 30 post BMT was significantly greater, at each cell dose, following BMT with expanded compared with unmanipulated bone marrow cells. There was no difference in survival following BMT with cells cultured with SCF and IL-11 compared with SCF, IL-11 and MIP-1α. Percentage survival appeared similar following BMT with either 10, 000 unmanipulated cells or only 1, 000 expanded cells. This suggests that, at least for short term engraftment, approximately 10 fold fewer bone marrow cells are required for rescue following lethal irradiation if these cells are expanded with growth factors prior to infusion. All the animals surviving to day 30 post BMT in these experiments were observed for one year. There were no deaths later than day

Figure 4.5 Short term survival after bone marrow transplantation

Female B6D2F1 mice received fractionated radiation as detailed in materials and methods prior to tail vein injection of either medium alone (control), 2 x 10^4 / 10^4 / 5 x 10^3 or 10^3 unmanipulated fresh bone marrow cells or cells following 6 days expansion in either SCF and IL-11 or SCF, IL-11 and MIP-1α. The number of expanded cells per mouse was based on the number established in culture at day -6. Results are for survival to day 30 following BMT for 3-6 replicate experiments. In each replicate experiment there were 10 mice per experimental group.

Figure 4.5

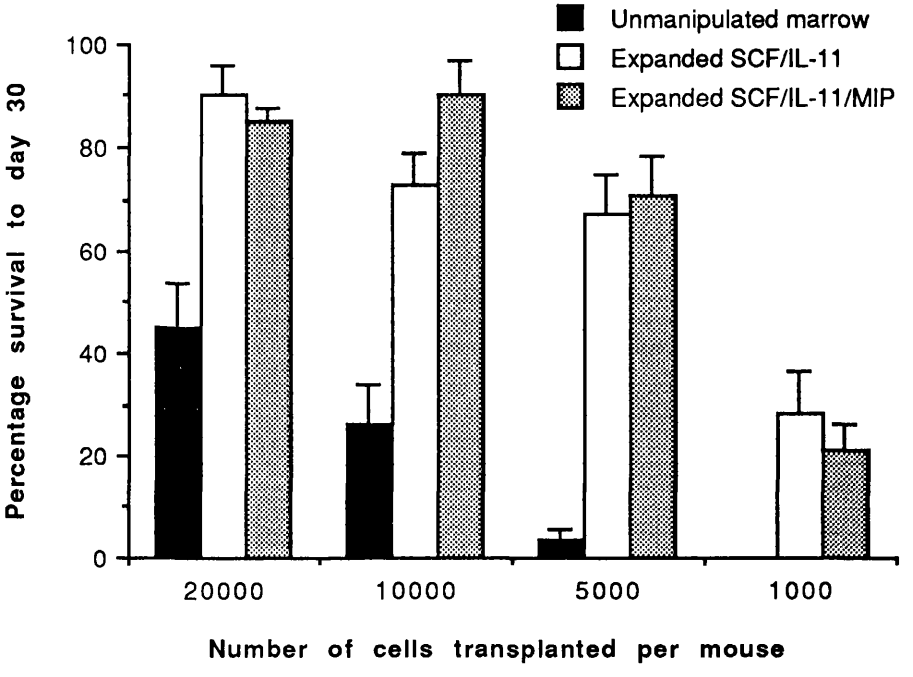

Unmanipulated marrow
Expanded SCF/IL-11
Expanded SCF/IL-11/MIP
Percentage survival to day 30
100
80
60
40
20
0
20000
10000
5000
1000
Number of cells transplanted per mouse

Figure 4.6 Assessment of donor engraftment one year after BMT

One year following transplantation of either unmanipulated or expanded bone marrow cells, surviving animals were randomly selected from the above experiments and Southern blots were performed to assess the presence of donor (male) cells in bone marrow according to the techniques described in materials and methods. The top and third panel represent results of hybridisation with the Y chromosome specific probe, the second and fourth panels results for GAPDH. In the top panel the first 6 lanes represent a titration of male DNA. The remainder of the lanes in the top panel and all of the third panel represent individual test animals. The number of cells transplanted per mouse is shown and whether the transplant was with unmanipulated bone marrow (UM), SCF and IL-11 expanded marrow (SCF/11) or SCF, IL-11 and MIP-1α expanded marrow (SCF/11/MIP) is indicated. The second and fourth panels show the results of rehybridising the blots with a probe for GAPDH which binds to both male and female DNA and, therefore, was used as a loading control.

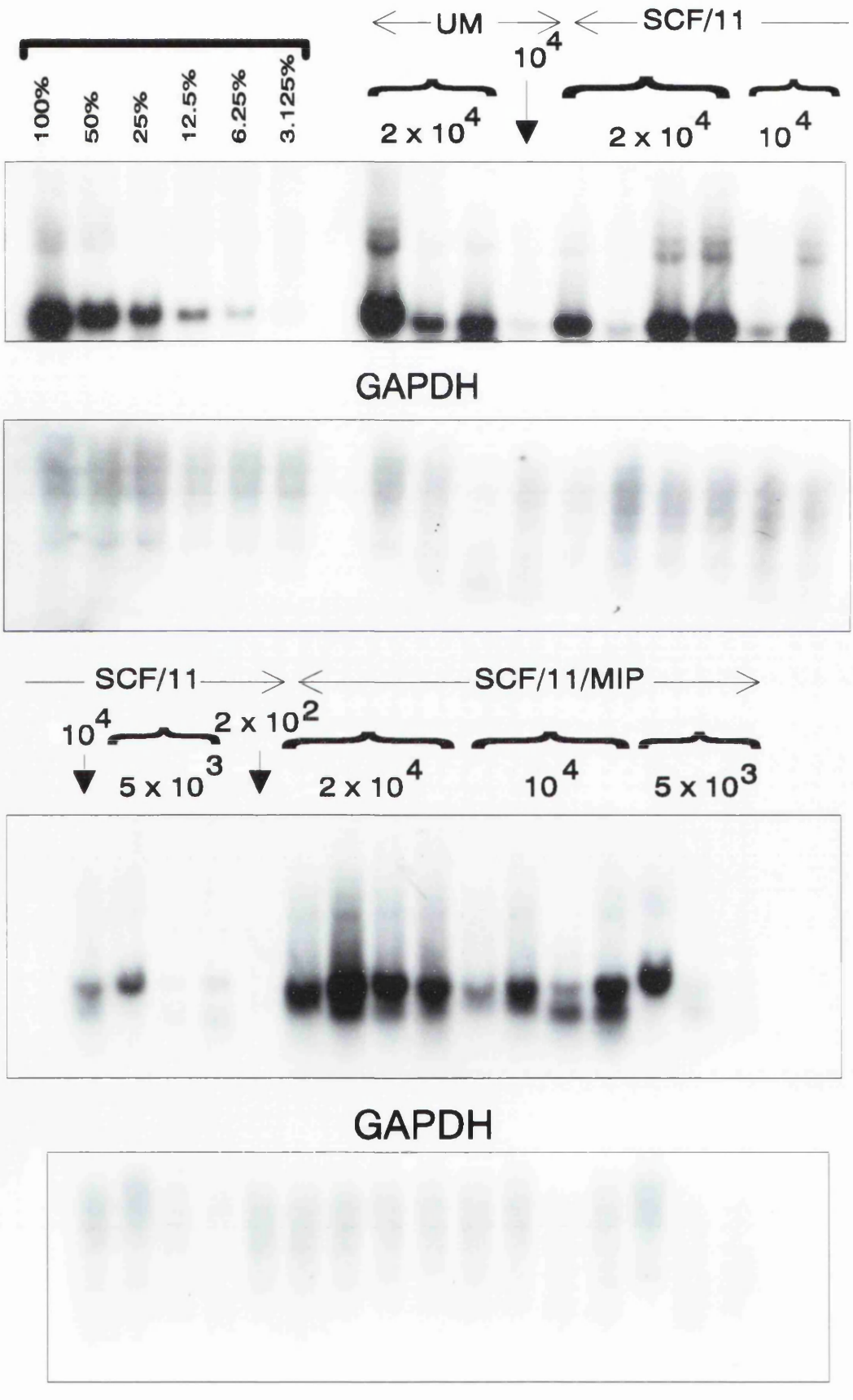

100%
50%
25%
12.5%
6.25%
3.125%
UM
SCF/11
10^4
2×10^4
2×10^4
10^4
GAPDH
SCF/11
SCF/11/MIP
10^4
2×10^2
5×10^3
2×10^4
10^4
5×10^3
GAPDH

Figure 4.7 Peripheral blood indices after bone marrow transplantation

Female B6D2F1 mice received fractionated lethal irradiation followed by tail vein injection of 5 x 10^5 fresh unmanipulated bone marrow cells, 5 x 10^5 unfractionated bone marrow cells following 6 days *ex vivo* expansion with SCF and IL-11 or 5 x 10^5 unfractionated bone marrow cells following 6 days *ex vivo* expansion with SCF, IL-11 and MIP-1α (100ng/ml added every 48 hours). At each time point, 6 animals were sacrificed from each treatment group (ie. a total of 18 animals). To enable full analysis, blood was pooled from two animals for each FBC result. Each data point therefore represents the mean for three blood samples, each of which was performed on blood pooled from two animals (Sysmex NE 8000). Results (mean SEM) are shown for a. haemoglobin, b. platelets, c. neutrophils and d. total WBC.

Figure 4.7a

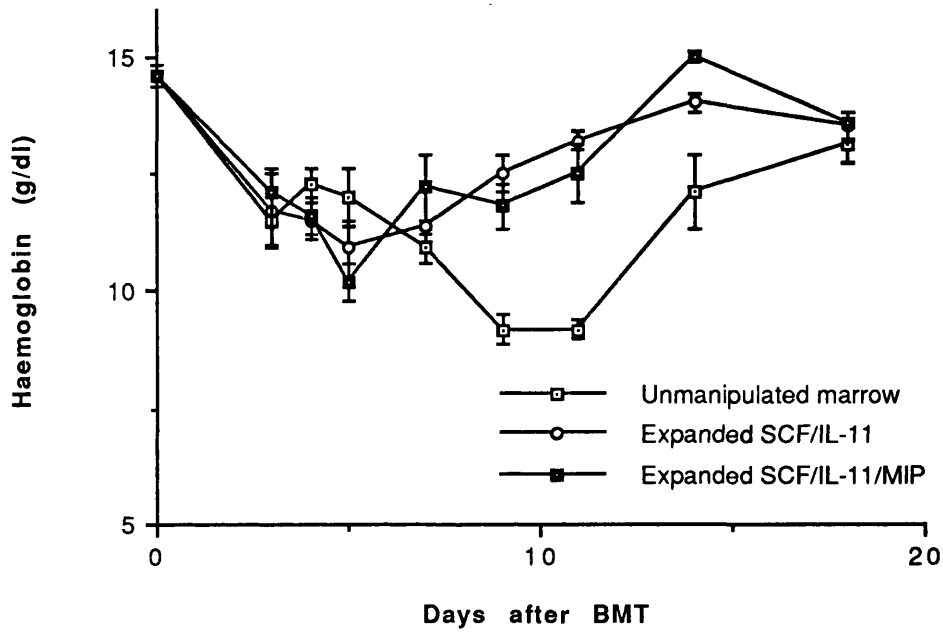


Figure 4.7b

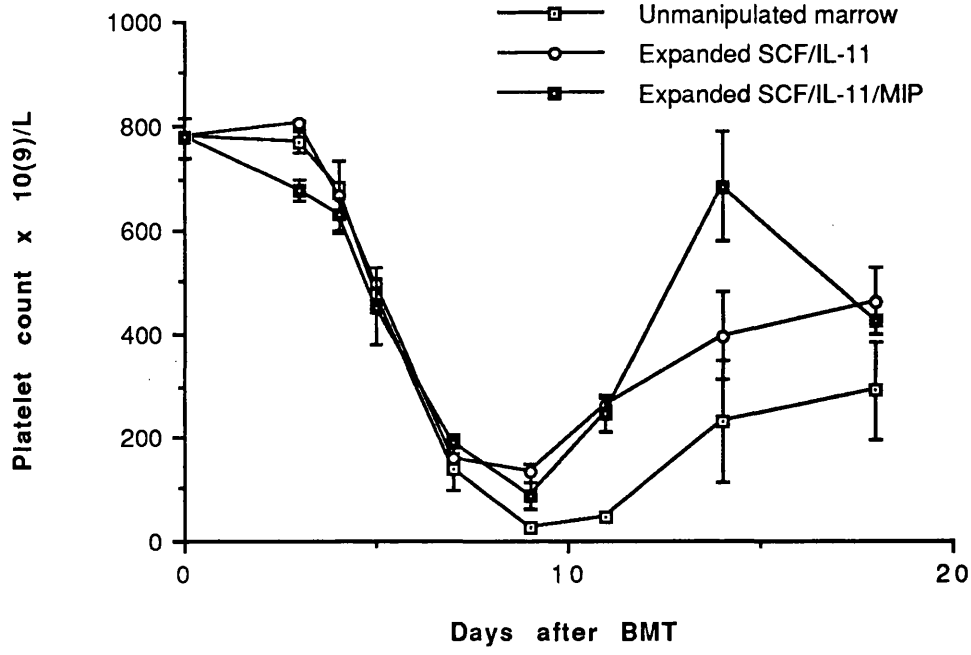


Figure 4.7c

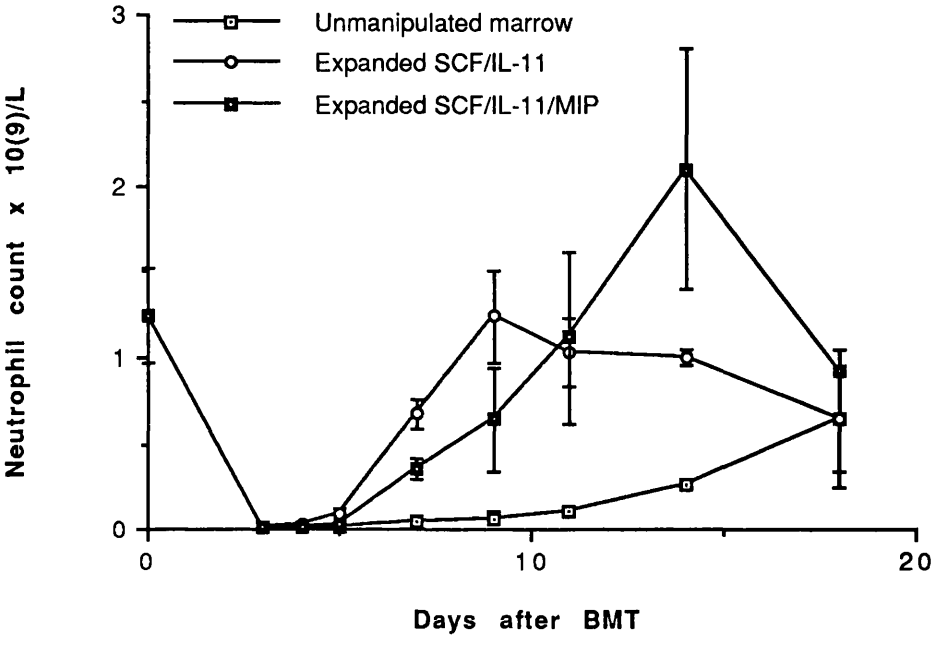
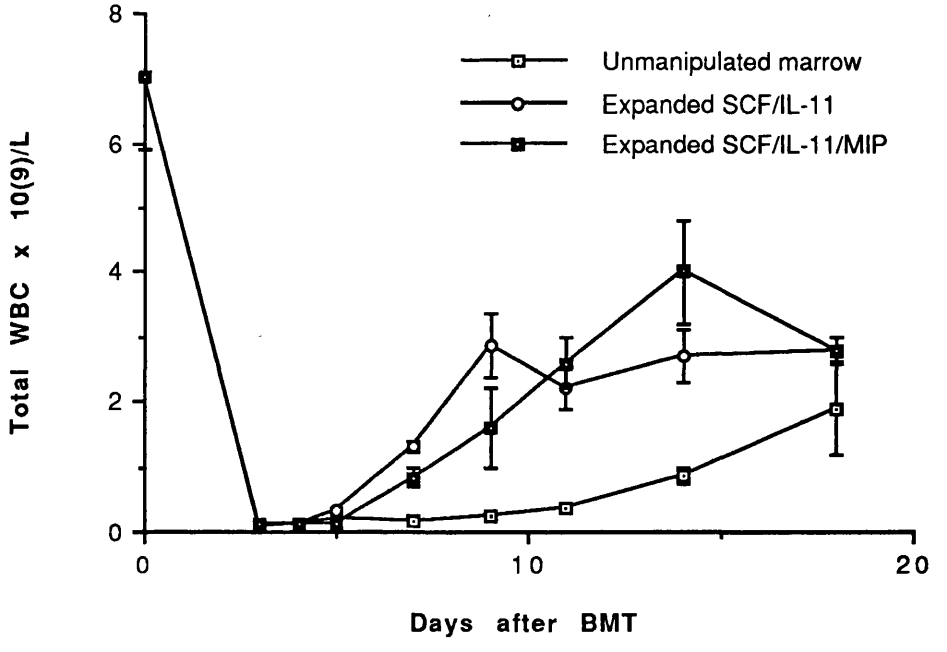


Figure 4.7d



unmanipulated bone marrow cells or cells expanded with SCF and IL-11, or SCF, IL-11 and MIP-1α. Non-transplanted, but irradiated, mice became profoundly pancytopenic and all died prior to day 18 (data not shown). The haemoglobin level from animals transplanted with either of the two expanded populations was showing signs of recovery as early as day 7 post BMT, however, for unmanipulated cells the haemoglobin level was still falling on day 7, reached a plateau and remained stable on days 9 and 11, before starting to rise (Figure 4.7a). The platelet counts fell in all experimental groups to a nadir on day 9 following transplantation. This nadir was significantly less severe for mice transplanted with cells expanded *ex vivo* with SCF and IL-11 than for unmanipulated cells ($p<0.001$). Thereafter, platelet recovery was more rapid for animals which received expanded bone marrow, although, because of the variability in platelet counts full statistical analysis was only performed for results to day 11 after BMT (Figure 4.7b, $p<0.001$ on day 11). The neutrophil count fell to zero by day 3 under all experimental conditions (Figure 4.7c). On day 5 following radiation, the mean neutrophil count was significantly higher in mice transplanted with bone marrow cells expanded *ex vivo* with SCF and IL-11 than for mice transplanted with unmanipulated bone marrow cells ($P=0.002$). Thereafter, from day 5 to day 11, the differences in neutrophil counts between animals transplanted with expanded versus unmanipulated bone marrow were highly significant and in favour of *ex vivo* expansion (Figure 4.7c, $p<0.001$ on days 7, 9 and 11). By day 14, the neutrophil counts were still higher for animals transplanted with expanded cells but this difference was no longer of statistical significance ($p=0.029$). For the above results, analysis of variance techniques (Armitage & Berry, 1987) were used to examine platelet and neutrophil counts. In each case residuals were plotted in various ways to determine a transformation which resulted in variance homogeneity and approximate normality. These plots indicated a logarithmic transformation for neutrophil counts and a square root

transformation for platelets. The transformed group means were compared at each day using the error estimate from the analysis of variance table. The p-values were adjusted using the Bonferroni method (Johnson & Wichern, 1988) to allow for multiple comparisons. In the case of the platelet counts the data for days 14 and 18 appeared markedly more variable than at other time points and these observations have not been included in the main analysis. This statistical analysis was kindly performed by Mr Jim Paul (Department of Medical Oncology, Western Infirmary, Glasgow).

It has been shown previously that for unmanipulated bone marrow there is a threshold cell and progenitor dose above which engrafment may not be further accelerated (Jones *et al.*, 1990). In the experiments described above, total cell number did not increase over two fold during *ex vivo* expansion culture and for transplantation, cell doses 10 fold over the known engraftment threshold were used. Therefore, the observed effects on both neutrophil and platelet recovery suggest that *ex vivo* culture of bone marrow cells with this cytokine combination mediated a qualitative influence on engraftment and did not reflect the small increase in the number of cells transplanted for animals given expanded cells.

Discussion

These experiments have demonstrated important advantages for the transplantation of expanded cells compared with unmanipulated cells. Since cell numbers increase only 1-2 fold over the 6 day culture period, the observed marked improvement in survival as a function of the number of cells transplanted cannot be due only to an increase in the total number of cells transplanted but must relate either to the increased concentration of CFU-A progenitors (50 fold increase over 6 days) and / or to effects on progenitor "homing". This result has important potential implications for clinical

transplantation, since, especially for autologous transplantation, there are often situations when the threshold cell number for BMT cannot be harvested either from bone marrow or by leukapheresis. In addition, if one could use only one tenth or less of a standard transplant cell dose, then the risk of tumour cell contamination would be reduced by at least one log. The first clinical study using progenitor cells expanded *ex vivo* has now been reported in which Brugger et al expanded one tenth of a standard peripheral blood progenitor cell (PBPC) product prior to infusion. The first four cases received a standard transplant dose of CD34 positive cells plus an aliquot of progenitor cells generated *ex vivo*. The following six cases received only expanded cells. All patients treated in this way experienced rapid and durable engraftment following high dose chemotherapy (Brugger *et al.*, 1995).

Following transplantation with cell doses in excess of the threshold for engraftment (i.e. optimised to ensure as rapid recovery as possible), SCF/IL-11 expanded bone marrow gave rise to significantly more rapid haematological recovery in terms of haemoglobin, platelets and neutrophils compared with unmanipulated BMT. This has previously been shown by Muench et al, using post 5-FU bone marrow and the combination of SCF and IL-1β, and, therefore, is not specific to the growth factor combination used in this work (Muench *et al.*, 1993). Previously it has been shown that transplantation with cell doses well in excess of the threshold for haemopoietic rescue cannot further hasten haematological recovery (Jones *et al.*, 1987). Therefore, it appears that cytokine exposure in some way reduces the time required for progenitors to home to the bone marrow and / or spleen and to produce functionally mature blood cells.

In these two series of experiments, we have demonstrated convincingly that transient engrafting stem cells can be expanded *in vitro* and that following their transplantation *in vivo* may favourably influence the

kinetics of engraftment. Clearly, if these results can be extended to the human system, they would have exciting clinical relevance.

RESULTS III

Chapter 5 Long Term Reconstitution

A fundamental question in haemopoiesis is whether expansion of stem and progenitor cells *ex vivo* can occur without reducing the proliferative capacity of the stem cell compartment. Although BMT with progenitor cells expanded *ex vivo* produced both more rapid haematological recovery following lethal doses of radiation and improved survival as a function of transplanted cell dose, no measure of long term engraftment had been undertaken. The capacity of stem cells to proliferate and self-renew can be studied using serial BMT. Serially transplanted bone marrow eventually fails to reconstitute lethally irradiated mice (Harrison *et al.*, 1978; Harrison & Astle, 1982; Jones *et al.*, 1989). This has been shown to be due, not to a decrease in the number of CFU-GM or CFU-S (committed progenitors and transiently engrafting stem cells), but to loss of pluripotent stem cells responsible for long term reconstitution (Jones *et al.*, 1989). The number of successful serial transplants is dependent upon both the cell dose transplanted and the interval between cell transfers, such that fewer cell passages are possible if the interval between transplants is short. This is thought to be due to dilution of LTRC by more committed progenitors early after BMT with re-establishment of the normal ratio over time. In order to obtain unambiguous results, cells of donor and recipient origin must be distinguishable.

A serial BMT experiment was therefore devised to try to repetitively stress the transplanted stem cell population and to compare unmanipulated bone marrow with SCF/IL-11 and SCF/IL-11/MIP-1α expanded bone marrow for their ability to sustain serial BMT. Serial transplantation is not a quantitative assay but could nevertheless be regarded as an indirect qualitative measurement of the most primitive stem cell. Therefore, any augmentation or reduction in the

ability of expanded marrow to sustain serial BMT compared with unmanipulated marrow would be interpreted as an indirect measure of the LTRC content of the different primary cell inoculations.

Prior to commencing the serial BMT experiment, a fractionated radiation dose titration, in the absence of BMT, was repeated to reassess the radiation sensitivity of the current animal batch. This confirmed that the dose required to cause bone marrow failure which was lethal in 100% of animals was $\geq$12.125Gy (Figure 5.1). For animals receiving the slightly higher dose (12.5Gy), there was mortality as early as day 7 which may have been caused by non-haematological toxicity. For this reason, the dose of 12.125Gy was chosen for all subsequent experiments.

The basic protocol for serial BMT is shown in Figure 5.2. Three experimental conditions were tested; unmanipulated BMT, SCF/IL-11 expanded BMT and SCF/IL-11/MIP-1α expanded BMT. In addition a group of 10 animals did not receive a cell infusion and were included as radiation only controls. The primary (1^{o}) animals were each transplanted with 5×10^5 cells (based on the number of cells used to establish expansion culture). Following 1^{o} BMT, three time points were selected for analysis. Since short term engraftment had been shown to occur significantly faster in recipients of expanded versus unmanipulated bone marrow, an early time point of one month (1/12) post BMT was first selected. At this time point the ratio of LTRC to committed progenitors might still be deranged and therefore influence the results of serial BMT. The second time point selected was at three months (3/12) following 1^{o} BMT. This should be sufficiently late post BMT for the normal balance between short and long term reconstituting stem cells to have been re-established (Jones *et al.*, 1989) and, therefore, for a true comparison between the long term reconstitution potential of unmanipulated and expanded bone

Figure 5.1 Third titration of fractionated radiation in B6D2F1 mice

Prior to commencing a serial BMT experiment the radiation dose titration was repeated. Groups of 10 mice received increasing doses of radiation from 11.75 to 12.5Gy in two equal fractions, three hours apart. Mice were then observed for survival to day 30 following radiation. Results are shown for percentage survival against time.

Figure 5.1
Third titration of fractionated radiation

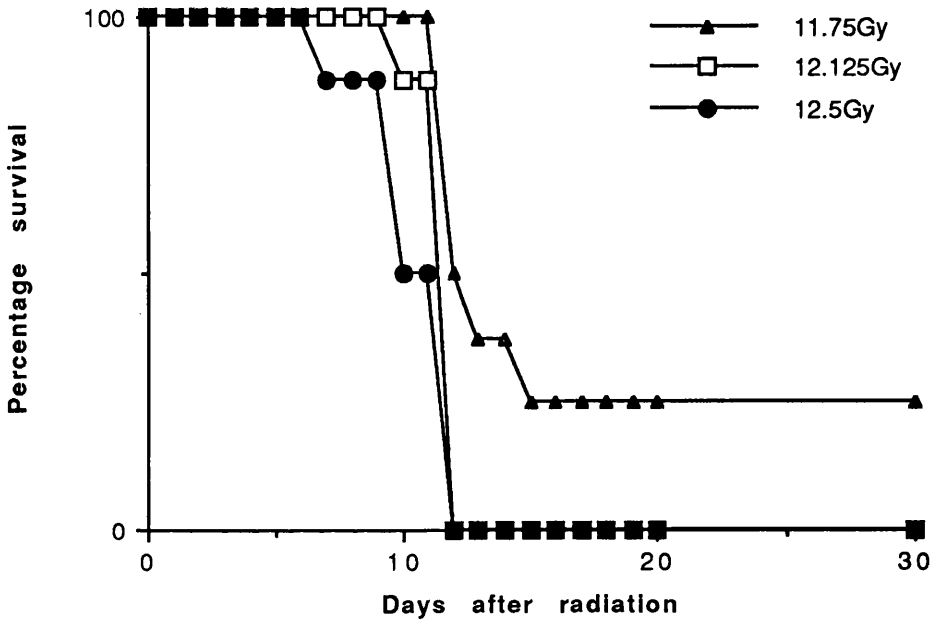
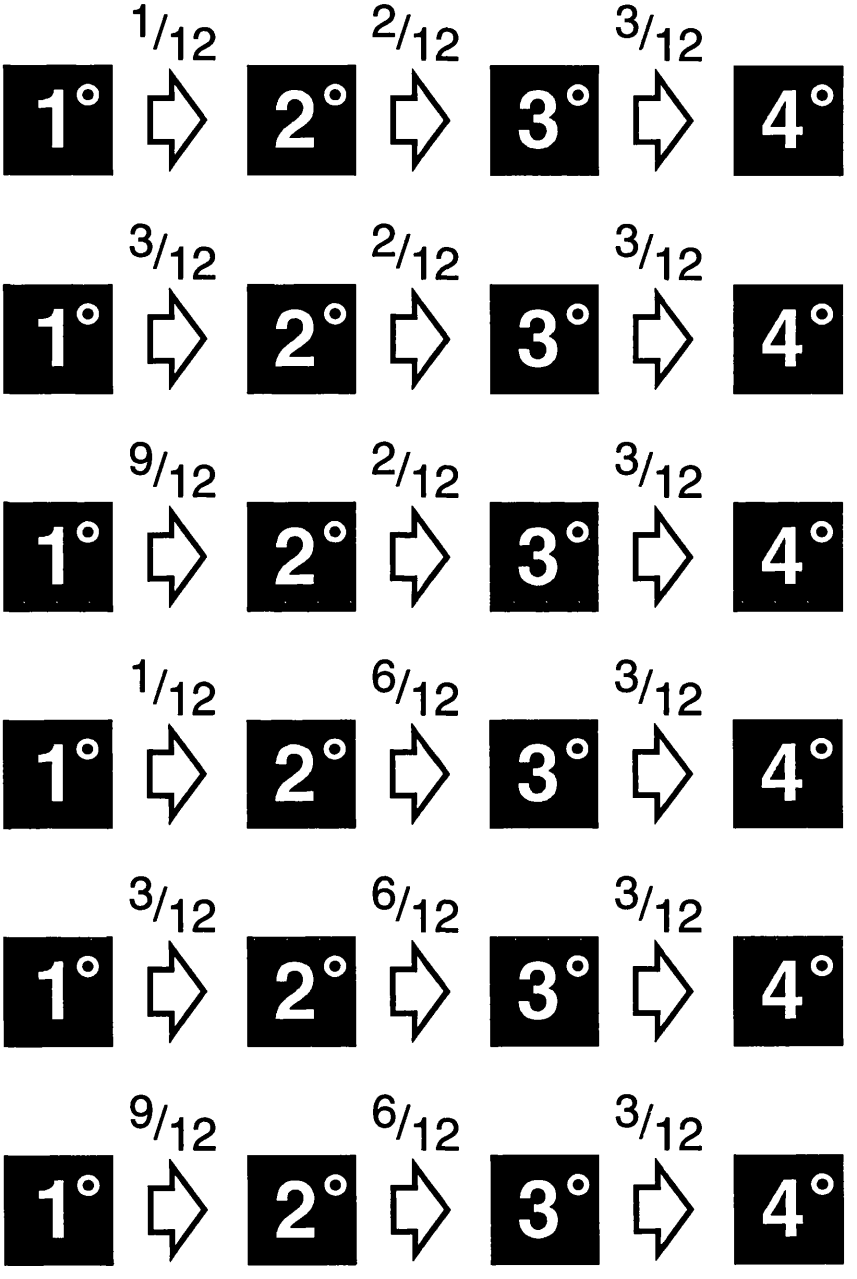


Figure 5.2 The basic scheme for serial BMT

Serial BMT was performed as described in materials and methods. Three experimental groups were established. These groups each consisted of 40 mice, transplanted with either unmanipulated bone marrow cells, bone marrow cells expanded with SCF and IL-11 or with SCF, IL-11 and MIP-1α. These mice were designated the primary (1^{o}) BMT recipients. Following 1^{o} BMT there were three time points for analysis; at one month (1/12), three months (3/12) and nine months (9/12) after BMT. At each time point 10 animals were sacrificed from the original group of 40 and used for analysis including testing the ability of pooled cells from each group to sustain serial BMT into new groups (20 mice per group) of lethally irradiated animals. These were designated as secondary (2^{o}) BMT recipients. Following 2^{o} BMT there were two time points for analysis; at two months (2/12) and six months (6/12) after BMT. At these time points 10 animals were sacrificed, analysed and marrow pooled for tertiary (3^{o}) BMT. Following 3^{o} BMT there was a single time point at three months (3/12).

Figure 5.2


1°
1/12
2°
2/12
3°
3/12
4°
1°
3/12
2°
2/12
3°
3/12
4°
1°
9/12
2°
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3/12
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2°
6/12
3°
3/12
4°

marrow to be made. Finally, a late time point at nine months (9/12) post 1^{o} BMT was selected to ensure that donor engraftment was durable with no cases of either host reconstitution or late bone marrow failure. As shown in Figure 5.2, there were two time points following 2^{o} BMT at two and six months, and one time point following 3^{o} BMT at three months. All secondary and subsequent BMT recipients each received 5×10^{6} cells for transplantation.

The full experimental design, including group identification, is shown in Figure 5.3. As a result of the large number of derivative groups of animals in this single experiment a rather cumbersome coding system was developed and adhered to throughout the study period. The initial test groups were numbered 1-4 and derivative groups were given a letter starting with A and proceeding with every alternate letter in the alphabet (e.g. A, C, E, G, I etc.) until the tertiary BMTs were completed. For quaternary BMT the groups were labelled R, S, T, U, V and W according to the chronological order of the transplant procedures. Group 1 was the radiation controls and these are not included in the Figure. Although there were three original groups, unmanipulated BMT (group 2), SCF/IL-11 expanded BMT (group 3) and SCF/IL-11/MIP-1α expanded BMT (group 4), there were three time points following 1^{o} BMT, creating nine groups (2A, 2C, 2E, 3A, 3C, 3E, 4A, 4C, 4E), and two time points following 2^{o} BMT, creating 18 groups (2G, 2I, 2K, 2M, 2O, 2Q, 3G, 3I, 3K, 3M, 3O, 3Q, 4G, 4I, 4K, 4M, 4O, 4Q). At each time point analyses were performed for FBC and number of CFU-A per femur, Southern blotting was performed to assess the contribution of donor cells and the ability to reconstitute a further batch of lethally irradiated animals (i.e. serial BMT) was tested.

Figure 5.4 shows the number of CFU-A per femur in the 1^{o} BMT recipients at the three time points chosen for analysis. At each time point three normal

Figure 5.3 The detailed scheme for serial BMT

Group 1 represented radiation only controls which did not receive a transplant. For simplicity this group has not been included in the figure. Group 2 represents animals transplanted with fresh unmanipulated bone marrow cells as a primary transplant. Group 3 represents animals transplanted with SCF and IL-11 expanded bone marrow cells. Group 4 represents animals transplanted with SCF, IL-11 and MIP-1α expanded bone marrow cells. Thereafter, each individual group was given an identifying letter e.g. 2A, 2C, 2E. Individual mice from each group were further identified by an additional number e.g. 2A1, 2A2 etc. to 2A10. If tissues were removed for analysis these were designated S for spleen, T for tibia and F for femur e.g. 2A1S, 2A1T, 2A1F etc.

Abbreviations: 1/12, one month etc. 1^{o}, primary BMT etc.

Figure 5.3

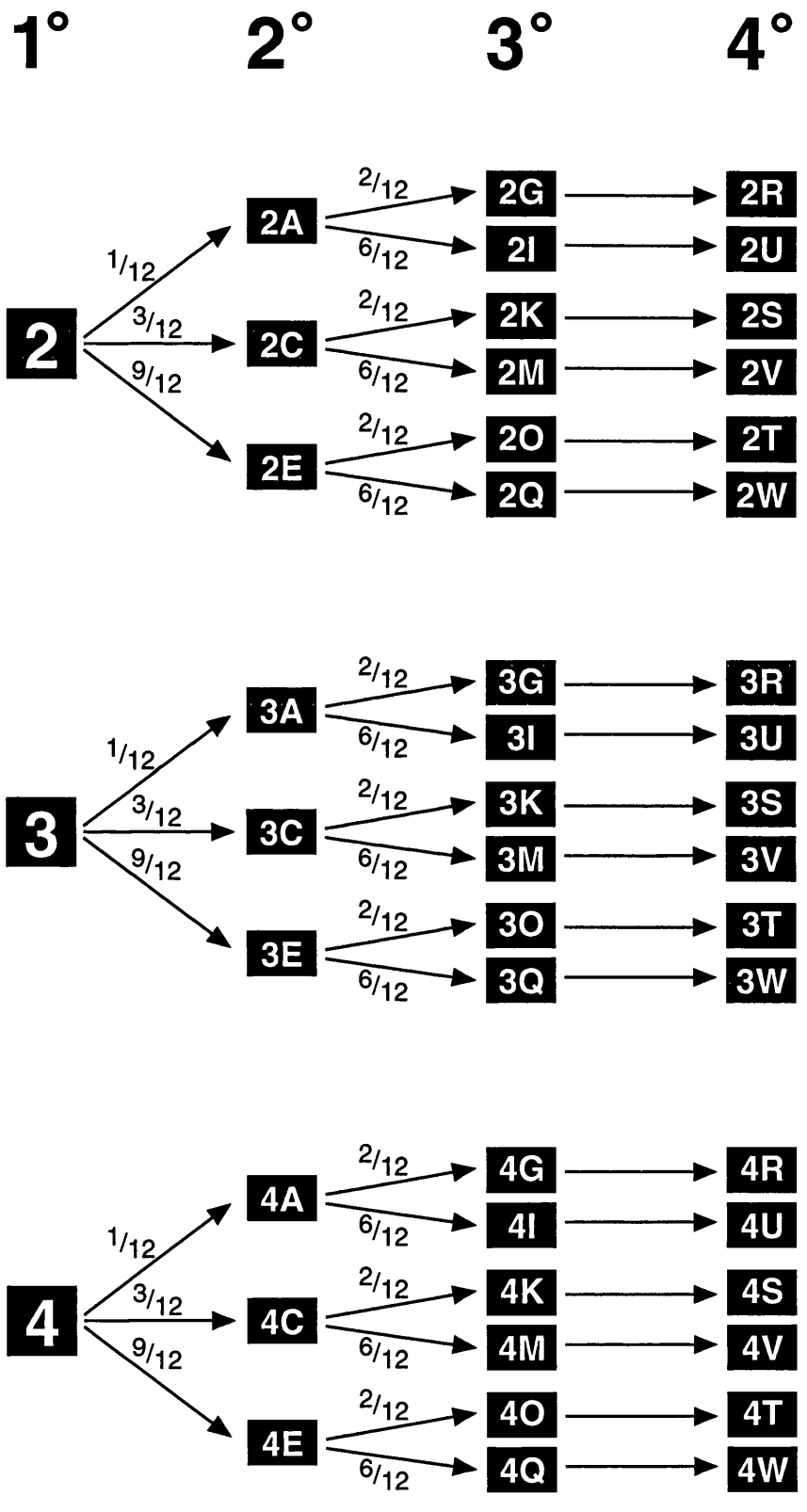
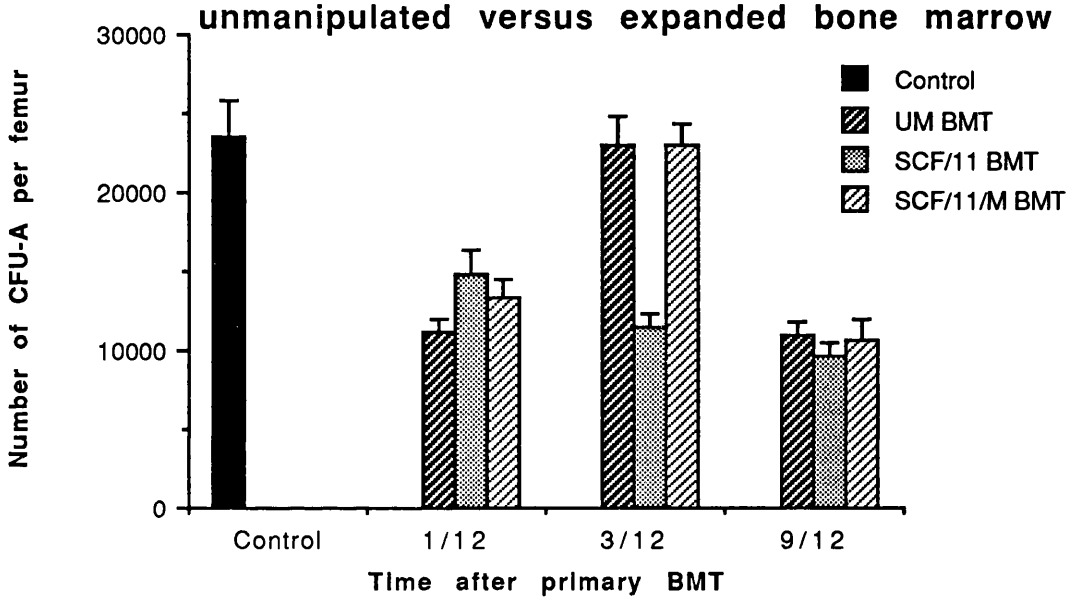

1°
2°
3°
4°
2
1/12
3/12
9/12
2A
2C
2E
2/12
6/12
2G
2I
2K
2M
2O
2Q
2R
2U
2S
2V
2T
2W
3
3A
3C
3E
3G
3I
3K
3M
3O
3Q
3R
3U
3S
3V
3T
3W
4
4A
4C
4E
4G
4I
4K
4M
4O
4Q
4R
4U
4S
4V
4T
4W

Figure 5.4 Femur CFU-A content in primary (1^{o}) BMT recipients

In the first stage of serial transplantation, three groups of lethally irradiated mice were transplanted with either unmanipulated bone marrow cells (Group 2, UM BMT), SCF and IL-11 expanded cells (Group 3, SCF/11 BMT) or SCF, IL-11 and MIP-1α expanded cells (Group 4, SCF/11/M BMT). There were three time points after 1^{o} BMT, at 1/12, 3/12 and 9/12. At these times 10 animals were sacrificed from each group and analyses were performed to measure the total CFU-A number per femur. Results represent the mean ($\pm$ SEM) CFU-A content per femur for 10 animals. Control animals were normal, having received neither radiation nor BMT.

Figure 5.4
CFU-A per femur in primary recipients of unmanipulated versus expanded bone marrow



control mice were analysed for FBC and CFU-A number per femur. The results of all control mice were then pooled. The mean result of CFU-A for all such control animals is included for each Figure. Results (except for controls) represent the mean (± SEM) for 10 animals. As shown, CFU-A numbers were reduced to approximately 50% of control numbers in 1^{o} BMT recipients at one and nine months following BMT. At these time points there was no difference between the three experimental groups. At the three month time point, CFU-A numbers were significantly higher for animals in either the unmanipulated BMT or SCF/IL-11/MIP-1α expanded BMT groups than for SCF/IL-11 expanded BMT ($p<0.001$, paired, 2-tailed t-test). Although this result was highly statistically significant and found both in the number of cells and the number of CFU-A per femur, the reason for this difference is difficult to determine. The initial tentative interpretation was that two stimulatory cytokines (i.e. SCF & IL-11) had induced differentiation of stem cells in culture, resulting in a reduction of CFU-A per femur compared with unmanipulated BMT, and that this differentiation had been prevented by the inclusion of MIP-1α such that CFU-A numbers were equivalent for either SCF/IL-11/MIP-1α expanded BMT or unmanipulated BMT. If this were the case, however, these changes would likely have persisted at the nine month time point (which they did not) and the ability of SCF/IL-11 expanded bone marrow to serially transplant would have been reduced compared with the other experimental groups. This was not seen at later time points. The alternative explanation is that, during recovery post BMT, CFU-A numbers may "over-shoot" before reaching stable numbers (Lorimore *et al.*, 1990). From the short term engraftment kinetics described in chapter 4 (Figure 4.7), this "over-shoot" would be expected to occur earliest for SCF/IL-11 expanded BMT, then SCF/IL-11/MIP-1α expanded BMT and finally for unmanipulated BMT. The three month time point may have been after the "over-shoot" had occurred and CFU-A numbers had stabilised, for SCF/IL-11 expanded BMT,

but during the "over-shoot" in the other two groups. This would explain why CFU-A numbers were still only 50% of controls at nine months following BMT, and no different in the three experimental groups.

Following 2° BMT, the nine groups of experimental animals (i.e. 2A, 3A, 4A (1/12 post 1° BMT); 2C, 3C, 4C (3/12 post 1° BMT); 2E, 3E, 4E (9/12 post 1° BMT)) were analysed for femur CFU-A content at two and six months following BMT. As shown in Figure 5.5, CFU-A were reduced compared with control animals in all experimental groups, both at two and six months post BMT and no differences were seen between the three experimental conditions (i.e. unmanipulated, SCF/IL-11 expanded and SCF/IL-11/MIP-1α expanded BMT). This suggests that, using the radiation dose described, and transplanting cell doses well in excess of threshold, CFU-A progenitors numbers did not return to control numbers even as late as six months following BMT. This has been shown previously in a serial transfer experiment performed by Jones et al (1989).

Haemopoiesis will be exclusively donor soon after lethal dose irradiation and BMT, however, unless the radiation dose is sufficient to fully ablate host bone marrow, there may be a contribution (small or large) from host marrow at later time points following BMT, especially if there are insufficient LTRC in donor marrow to maintain engraftment long term (Jones *et al.*, 1989). It was therefore important to assess the contribution by donor marrow, at both early and later time points following BMT, especially for 2° and subsequent recipients of serially transplanted bone marrow. Figure 5.6 shows the results of Southern blotting for both the Y specific probe, to detect the presence of male donor cells, and GAPDH, as a loading control, for secondary recipients of unmanipulated (group 2) versus expanded (groups 3 & 4) bone marrow analysed at two months after BMT. In all lanes with adequate DNA loading

Figure 5.5 Femur CFU-A content in secondary (2^{o}) BMT recipients

Pooled bone marrow, derived from 1^{o} BMT recipients was used to transplant lethally irradiated 2^{o} animals. These 2^{o} BMT recipients were designated **A** (derived from the 1/12 time point following 1^{o} BMT), **C** (3/12) and **E** (9/12). 10 animals from groups **A**, **C** and **E** were sacrificed at two separate time points following 2^{o} BMT, at 2/12 and 6/12. At these times analyses were performed to measure the total CFU-A number per femur. Results represent the mean ($\pm$ SEM) CFU-A content per femur for 10 animals. Control animals were normal, having received neither radiation nor BMT. Figure a. represents the 2/12 time point and b. the 6/12 time point.

Figure 5.5a
CFU-A per femur in secondary recipients of unmanipulated versus expanded bone marrow

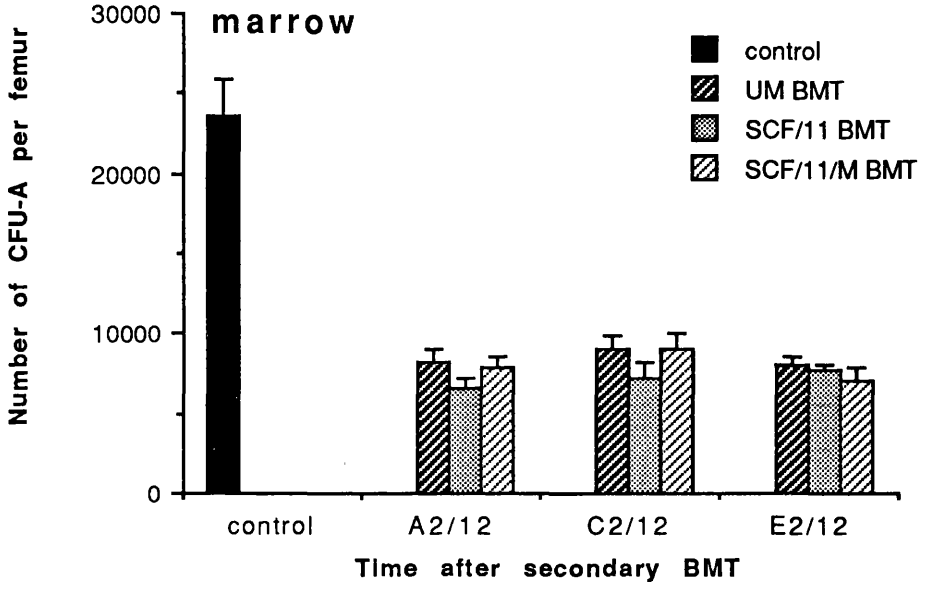


Figure 5.5b
CFU-A per femur in secondary recipients of unmanipulated versus expanded bone marrow

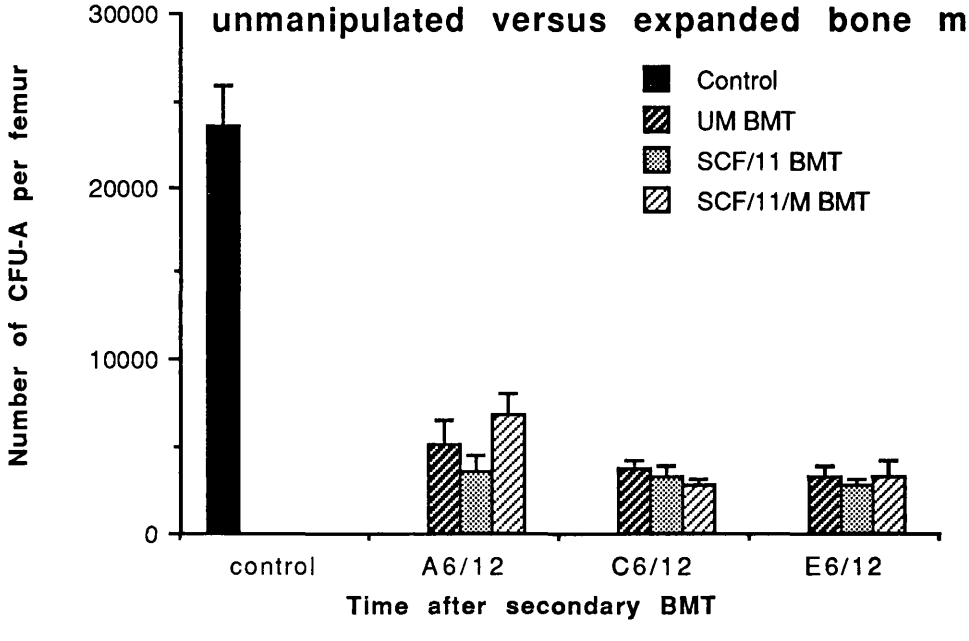
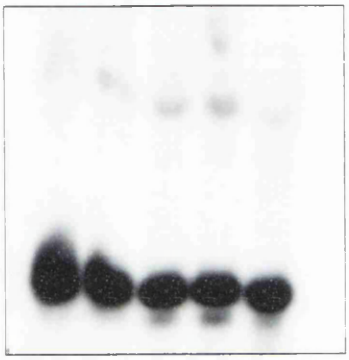


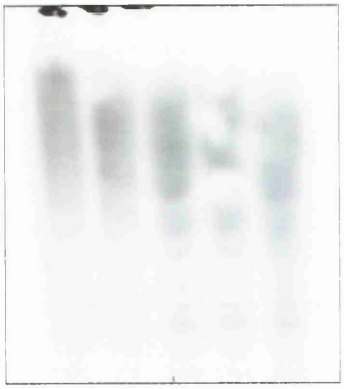
Figure 5.6 Assessment of donor engraftment in group E animals at two months following 2^{o} BMT

Genomic DNA was extracted from five randomly selected animals from each experimental group (i.e. group 2, unmanipulated BMT; group 3, SCF/IL-11 expanded BMT; group 4, SCF/IL-11/MIP-1α expanded BMT). The left hand panels represent Southern blots hybridised with the Y chromosome specific probe, the right hand panels the GAPDH loading controls.

2E2 1-5



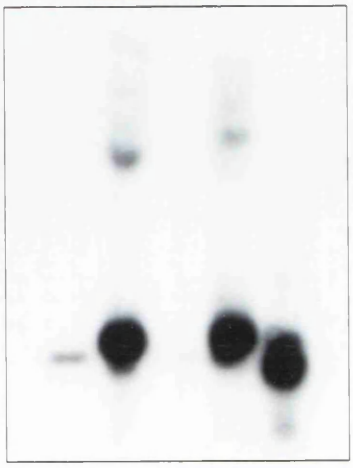
GAPDH



3E2 1-5

GAPDH

4E2 1-5



GAPDH

(all except 4E2 lanes 1 & 3) hybridisation to the Y probe produced a very strong 1.5kb band suggesting that haemopoiesis was predominantly donor in origin. A similar pattern, with evidence of donor haemopoiesis for all samples adequately loaded, is seen for groups A, C and E at 6 months following 2° BMT in Figures 5.7-5.9. These results suggest that bone marrow cells expanded *ex vivo* still contain sufficient stem cells for a proportion of haemopoiesis to be of donor origin up to six months following a second serial transplant.

Results for CFU-A per femur for 12 of the 18 groups which received BMT with marrow transplanted for a third time (i.e. 3° BMT) are shown in Figure 5.10. Please refer back to Figure 5.3 (detailed scheme for serial BMT) to clarify nomenclature. All these analyses were performed at approximately three months after 3° BMT (group G closer to 6 months). At the time of writing, groups O and Q had not been analysed. CFU-A numbers were greatly reduced compared with controls for all experimental groups, however, there appeared to be, in addition, some important differences between the groups. When the intervals between serial cell transfers were relatively short (i.e. 1/12 then 2/12 for G; 3/12 then 2/12 for K) CFU-A were almost non-existent in the femurs of mice transplanted with unmanipulated bone marrow. CFU-A numbers were greater for the groups transplanted with expanded cells. This may reflect the faster kinetics of engraftment in animals transplanted with expanded cells which allowed the ratio of committed progenitors: LTRC to return to normal earlier after BMT than for unmanipulated BMT. If this marrow was then used at an early time point for serial transplantation, stem cell numbers would be greater for expanded compared with unmanipulated marrow. When the intervals were prolonged (1/12 then 6/12 for I; 3/12 then 6/12 for M) CFU-A numbers for animals transplanted with unmanipulated bone marrow were back to the level seen for recipients of expanded BMT in

Figure 5.7 Assessment of donor engraftment in group A animals at six months following 2^{o} BMT

Genomic DNA was extracted from randomly selected animals from each experimental group (i.e. group 2, unmanipulated BMT; group 3, SCF/IL-11 expanded BMT; group 4, SCF/IL-11/MIP-1α expanded BMT). The top, third and fifth panels represent Southern blots hybridised with the Y chromosome specific probe, the second, fourth and sixth panels the GAPDH loading controls.

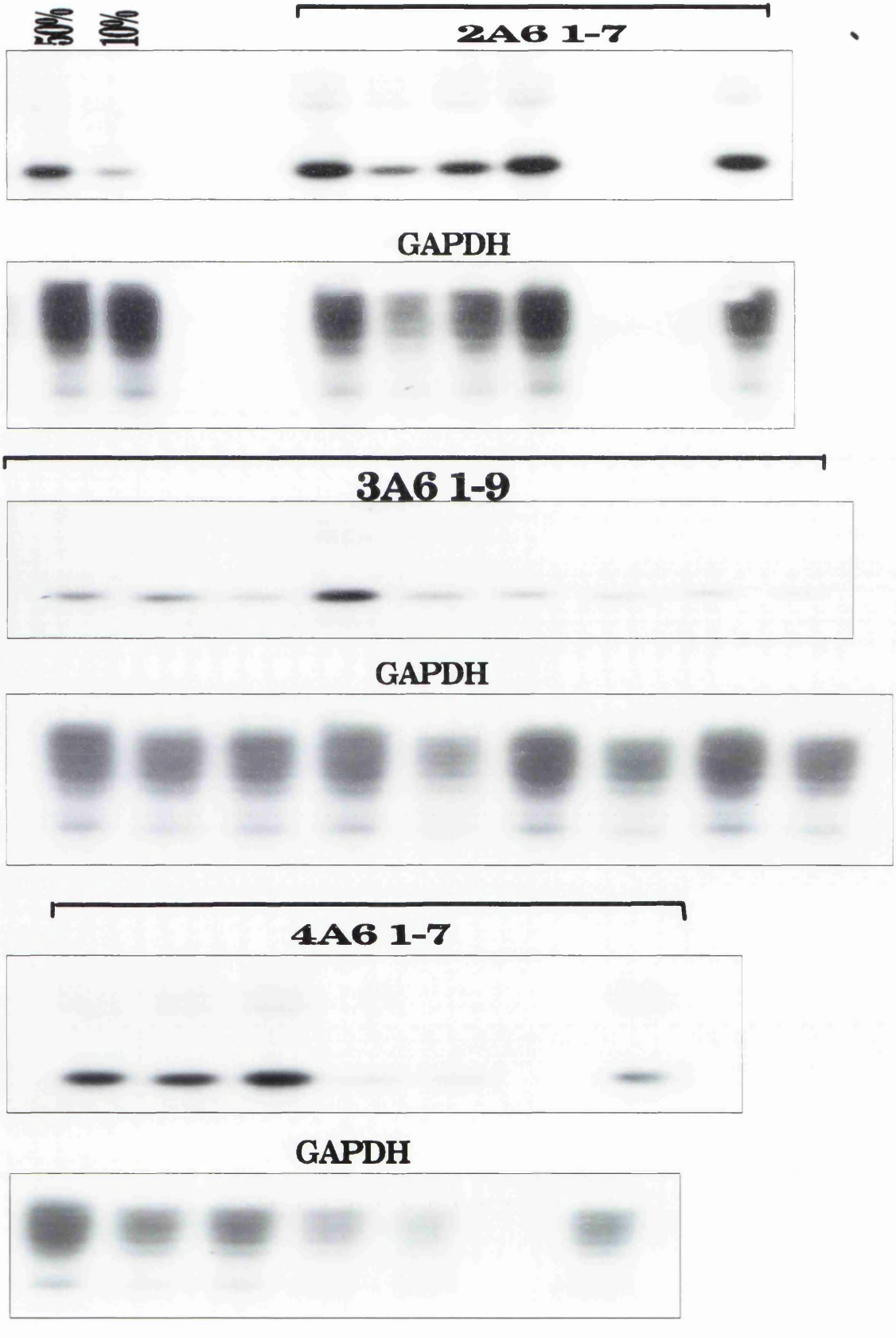

50%
10%
2A6 1-7
GAPDH
3A6 1-9
GAPDH
4A6 1-7
GAPDH

Figure 5.8 Assessment of donor engraftment in group C animals at six months following 2^{o} BMT

Genomic DNA was extracted from randomly selected animals from each experimental group (i.e. group 2, unmanipulated BMT; group 3, SCF/IL-11 expanded BMT; group 4, SCF/IL-11/MIP-1α expanded BMT). Results represent hybridisation with the Y chromosome specific probe.

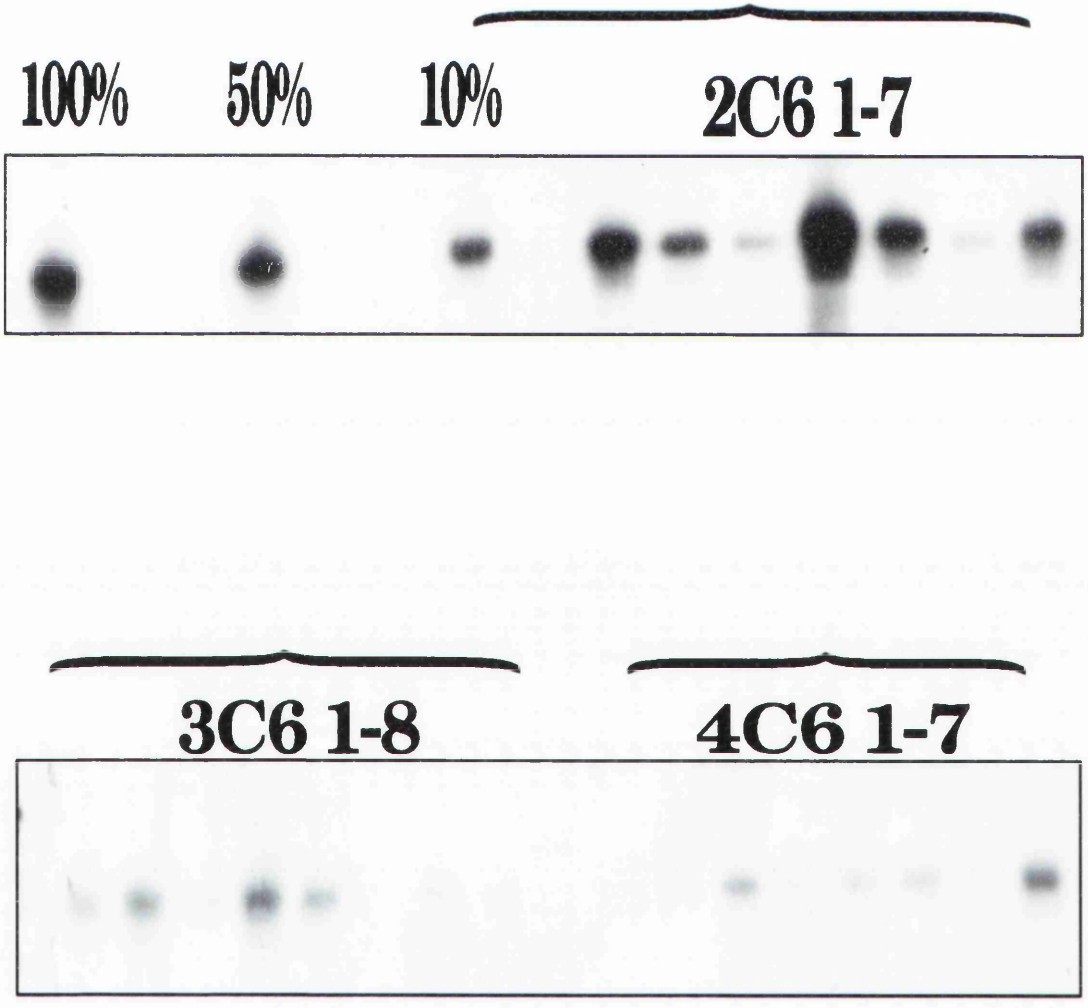
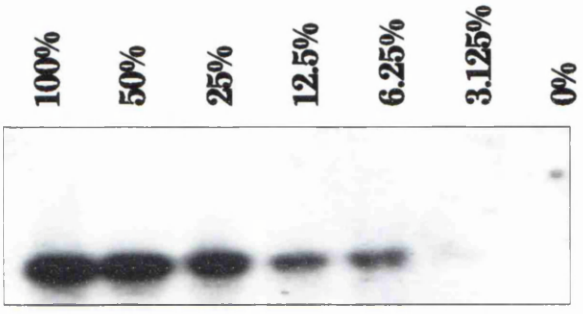
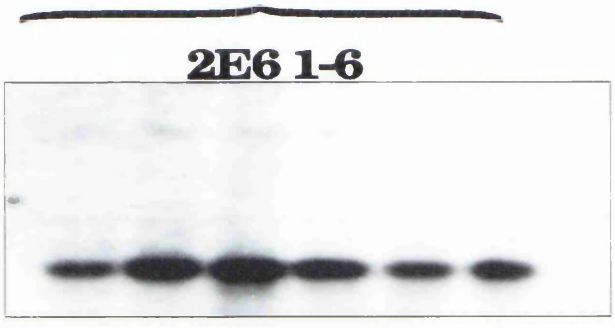
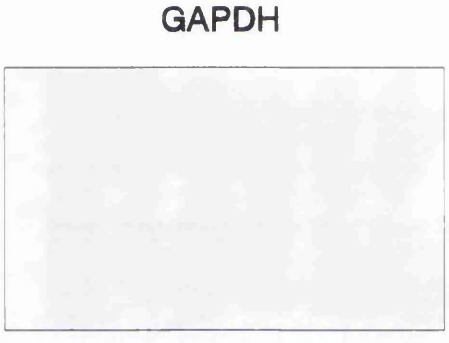

100%
50%
10%
2C6 1-7
3C6 1-8
4C6 1-7

Figure 5.9 Assessment of donor engraftment in group E animals at six months following 2^{o} BMT

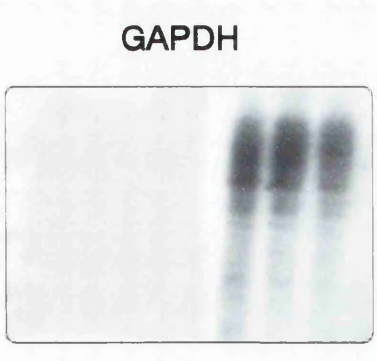
Genomic DNA was extracted from randomly selected animals from each experimental group (i.e. group 2, unmanipulated BMT; group 3, SCF/IL-11 expanded BMT; group 4, SCF/IL-11/MIP-1α expanded BMT). The top panel shows a standard titration of male DNA. The left hand panels represent Southern blots hybridised with the Y chromosome specific probe, the right hand panels the GAPDH loading controls.

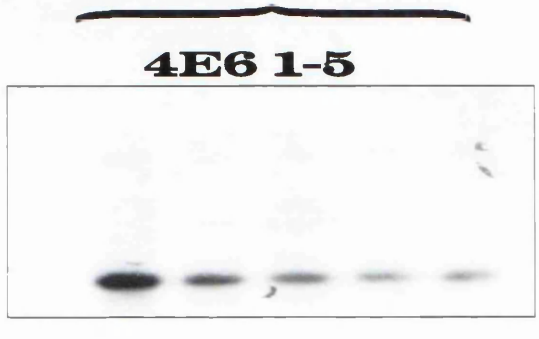

100%
50%
25%
12.5%
6.25%
3.125%
0%


2E6 1-6


GAPDH


3E6 1-6


GAPDH


4E6 1-5

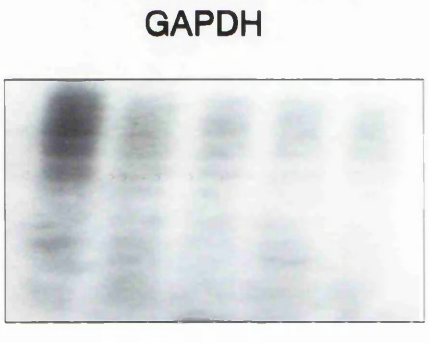
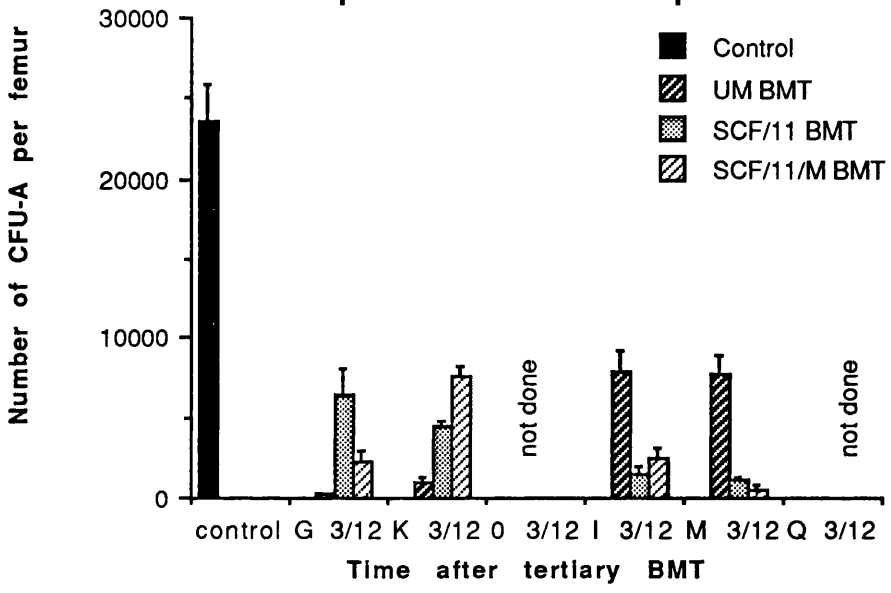

GAPDH

Figure 5.10 Femur CFU-A content in tertiary (3^{o}) BMT recipients

Pooled bone marrow, derived from 2^{o} BMT recipients was used to transplant lethally irradiated 3^{o} animals. These 3^{o} BMT recipients were designated **G** (derived from the 1/12 time point following 1^{o} BMT, and the 2/12 time point following 2^{o} BMT), **K** (3/12, 2/12), **O** (9/12, 2/12), **I** (1/12, 6/12), **M** (3/12, 6/12) and **Q** (9/12, 6/12). Groups **O** and **Q** had not been analysed at the time of writing. 10 animals from each of the groups available were sacrificed at a single time point following 3^{o} BMT, at 3/12. At this time analyses were performed to measure the total CFU-A number per femur. Results represent the mean ($\pm$ SEM) CFU-A content per femur for 10 animals. Control animals were normal, having received neither radiation nor BMT.

Figure 5.10
CFU-A per femur in tertiary recipients of unmanipulated versus expanded bone marrow



groups G and K. These changes were reflected in the survival of tertiary BMT recipients shown later in this chapter. In this case, the CFU-A numbers for recipients of expanded marrow were low (groups I and M) and, as discussed later, this may have been due to the development of leukaemia in these animals.

The results of Southern blotting to assess the contribution of male donor haemopoiesis is shown for 3° BMT recipients in Figures 5.11-5.14 (groups G, K, I, M). For group G, although the contribution of donor cells differs for individual animals, there is clear evidence that donor cells were present in every animal. Even animals in group 2G (in whom CFU-A numbers were greatly reduced) showed evidence of residual donor haemopoiesis. Approximately 3 months following 3° BMT, animals transplanted with serially passaged unmanipulated bone marrow started to die of bone marrow failure (group 2K). In Figure 5.12, animals from group 2K (in whom CFU-A numbers were also severely depleted) showed little or no evidence of residual donor haemopoiesis on Southern blotting. This blot was repeated on three occasions to confirm the result and the GAPDH hybridisation confirmed adequate DNA loading. Since these animals were dying of bone marrow failure it appears that the radiation protocol used in this study was sufficiently myeloablative to prevent early return of host haemopoiesis in the face of a failing bone marrow graft of donor cells. Both groups 3K and 4K, transplanted with expanded bone marrow, showed clear evidence of donor haemopoiesis. There were no deaths from bone marrow failure in either of these groups. For groups I and M, all animals tested showed residual donor haemopoiesis which was easily detectable by Southern blotting.

To summarise, CFU-A numbers were reduced in all recipients of 3° BMT when compared with normal controls. For serial BMT with relatively short

Figure 5.11 Assessment of donor engraftment in group G animals at three months following 3^{o} BMT

Genomic DNA was extracted from ten randomly selected animals from each experimental group (i.e. group 2, unmanipulated BMT; group 3, SCF/IL-11 expanded BMT; group 4, SCF/IL-11/MIP-1α expanded BMT). The top and third panels represent Southern blots hybridised with the Y chromosome specific probe, the second and fourth panels the GAPDH loading control.

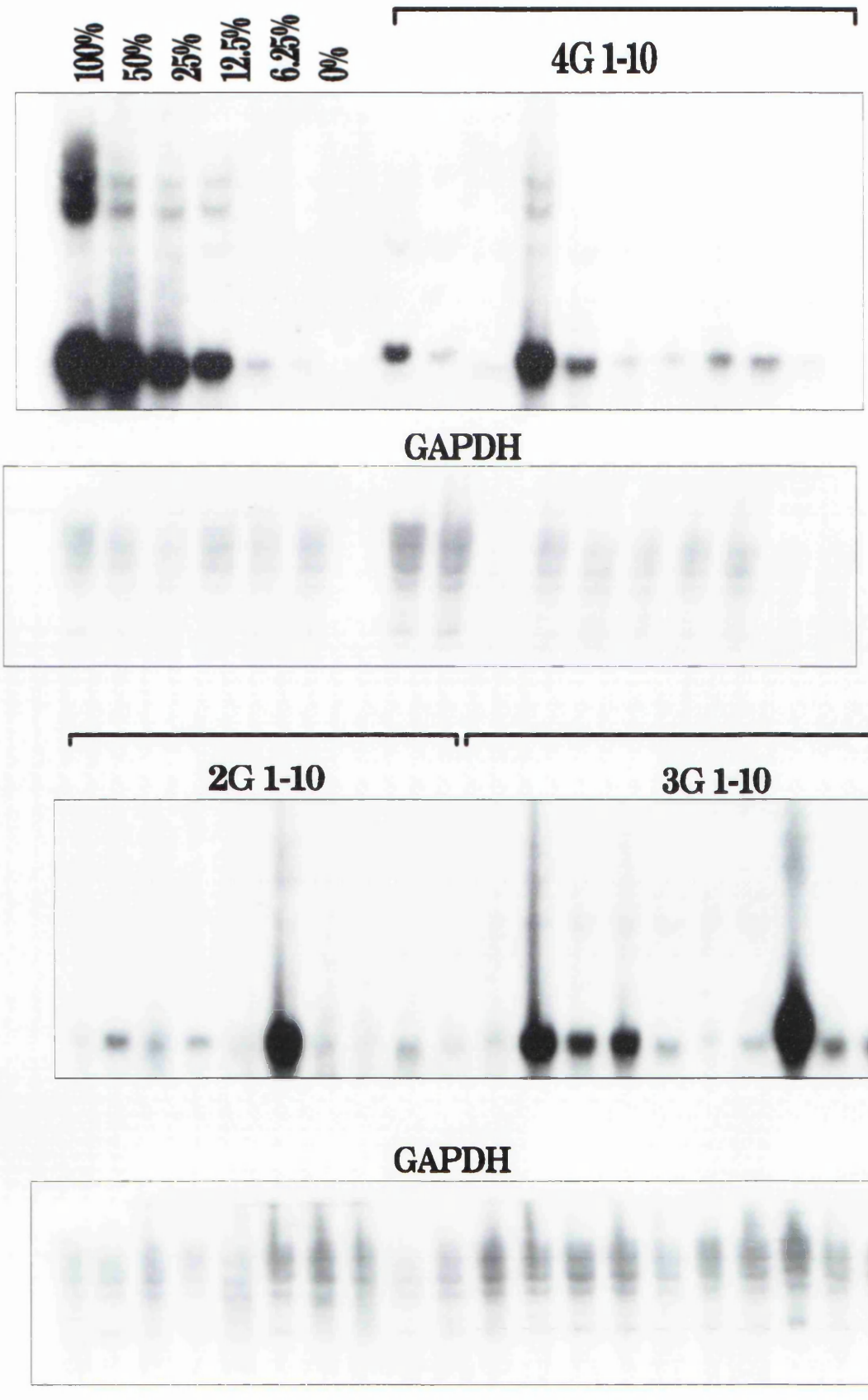

100%
50%
25%
12.5%
6.25%
0%
4G 1-10
GAPDH
2G 1-10
3G 1-10
GAPDH

Figure 5.12 Assessment of donor engraftment in group K animals at three months following 3^{o} BMT

Genomic DNA was extracted from randomly selected animals from each experimental group (i.e. group 2, unmanipulated BMT; group 3, SCF/IL-11 expanded BMT; group 4, SCF/IL-11/MIP-1α expanded BMT). The top panel shows a titration of male DNA followed by 9 individual test samples from group 2**K**. The arrows indicate hybridisation to the Y chromosome specific probe at a very low level. The third panel shows results with the Y specific probe for groups 3 and 4**K**. The second and fourth panels represent the GAPDH loading control.

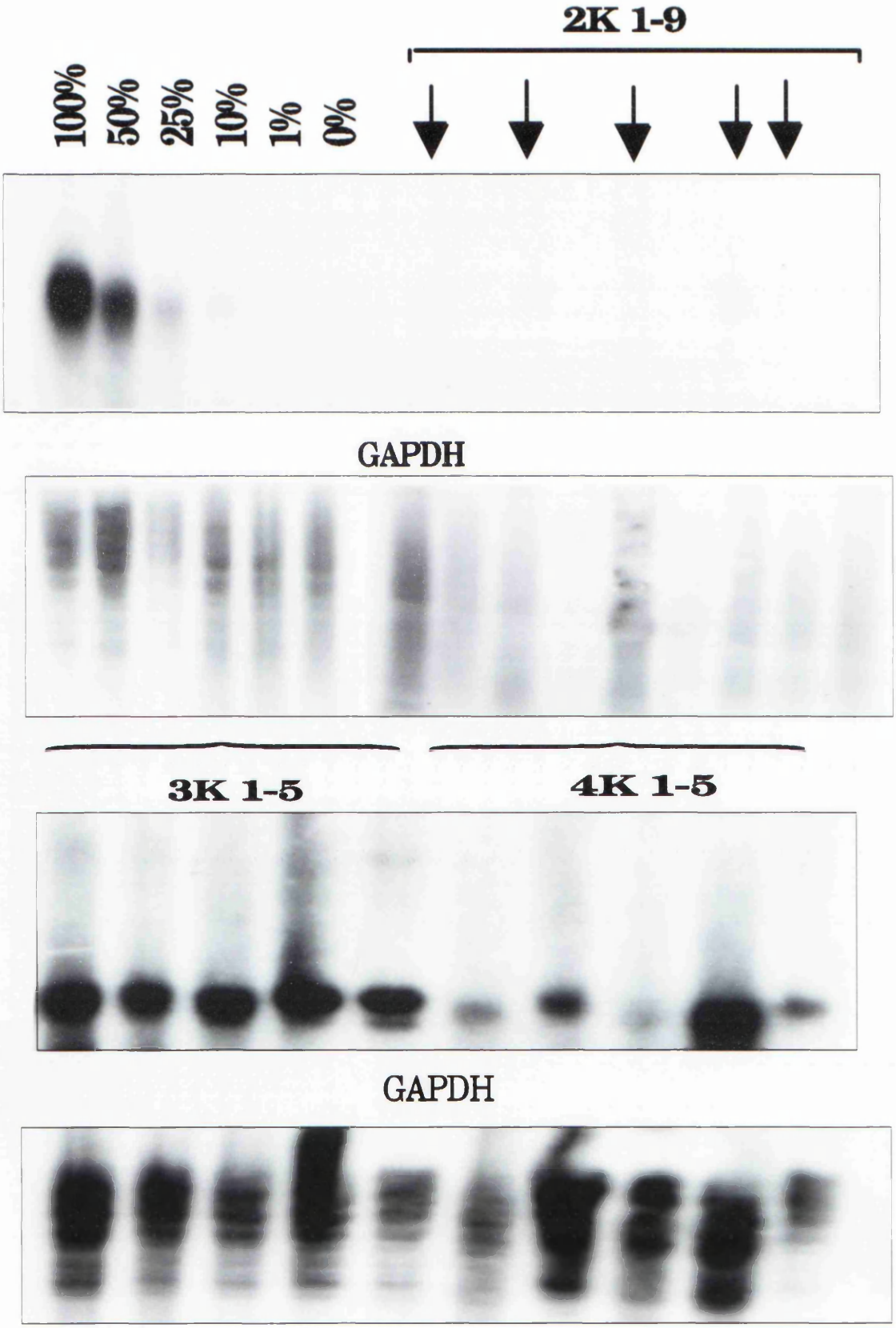
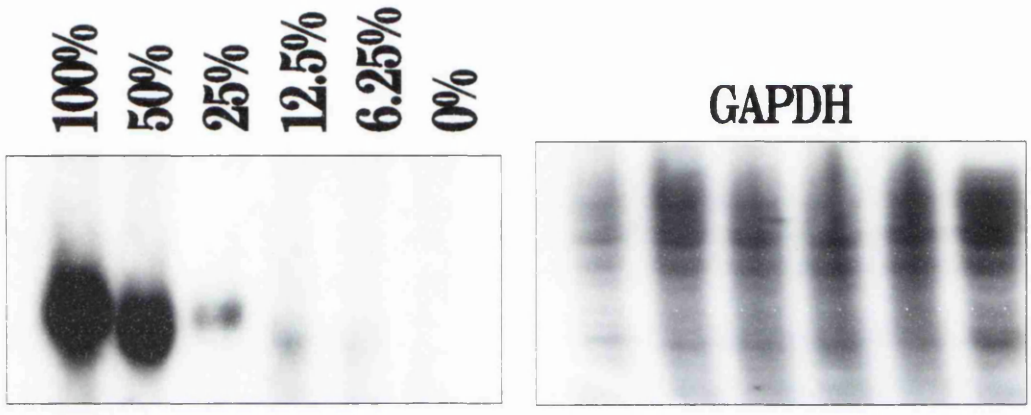
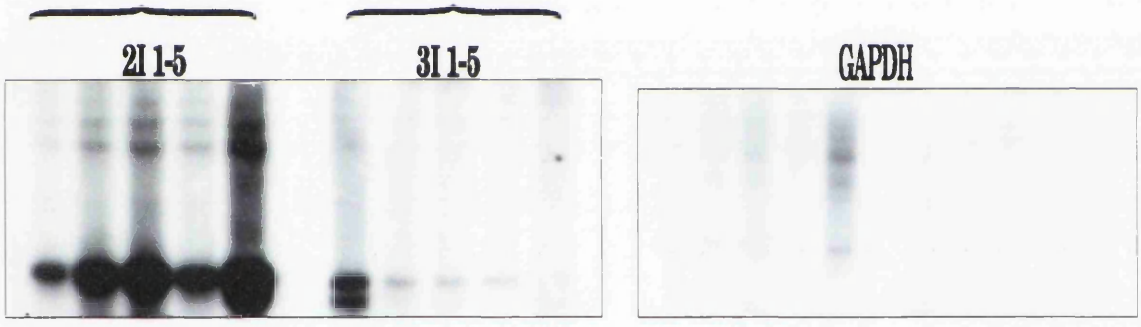

2K 1-9
100%
50%
25%
10%
1%
0%
GAPDH
3K 1-5
4K 1-5
GAPDH

Figure 5.13 Assessment of donor engraftment in group I animals at three months following 3^{o} BMT

Genomic DNA was extracted from randomly selected animals from each experimental group (i.e. group 2, unmanipulated BMT; group 3, SCF/IL-11 expanded BMT; group 4, SCF/IL-11/MIP-1α expanded BMT). The top panel shows the titration of male DNA. The left hand panels represent results for the Y specific probe, the right hand panels results for GAPDH.


100%
50%
25%
12.5%
6.25%
0%
GAPDH


2I 1-5
3I 1-5
GAPDH

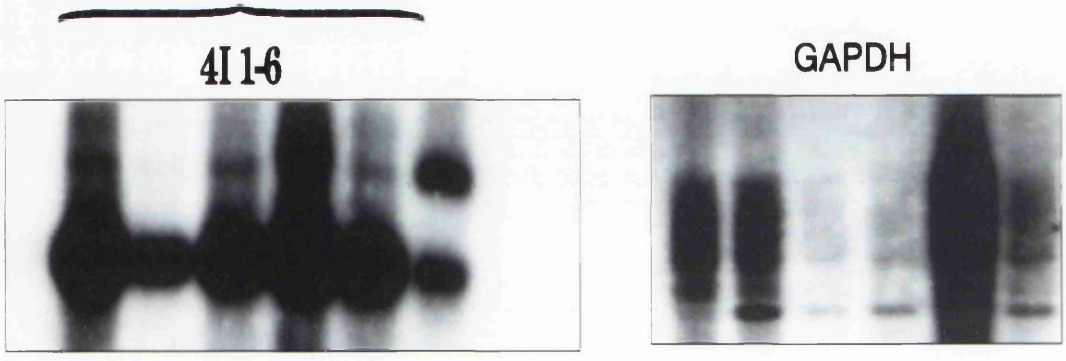
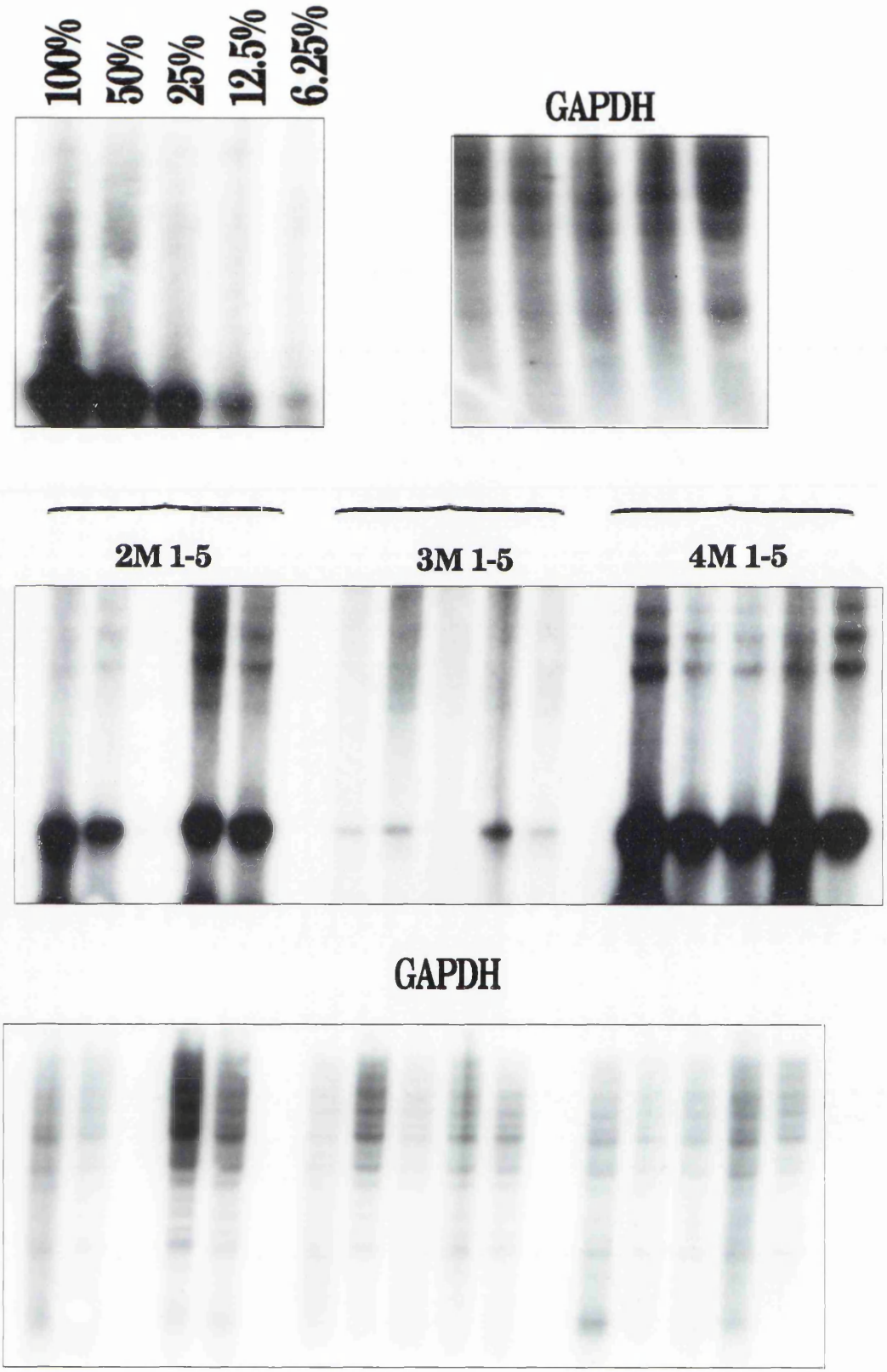

4I 1-6
GAPDH

Figure 5.14 Assessment of donor engraftment in group M animals at three months following 3^{o} BMT

Genomic DNA was extracted from five randomly selected animals from each experimental group (i.e. group 2, unmanipulated BMT; group 3, SCF/IL-11 expanded BMT; group 4, SCF/IL-11/MIP-1α expanded BMT). The top panels show the titration of male DNA and GAPDH loading control. The second panel shows results for test DNA hybridised to the Y specific probe and the third panel the corresponding GAPDH controls.


100%
50%
25%
12.5%
6.25%
GAPDH
2M 1-5
3M 1-5
4M 1-5
GAPDH

intervals between cell transfers, recipients of unmanipulated BMT showed lower CFU-A numbers compared with recipients of either SCF/IL-11 or SCF/IL-11/MIP-1α expanded BMT. With longer intervals between cell transfer, unmanipulated BMT appeared to regain the ability to sustain serial BMT and CFU-A numbers were improved for groups 2I and 2M. Except for recipients of unmanipulated bone marrow after short cell transfer intervals (i.e. 2K) there were no deaths from bone marrow failure and there was evidence of persistent donor engraftment by Southern blotting.

At each of the time points following 1^{o}, 2^{o} and 3^{o} BMT, peripheral blood was analysed for FBC. As shown in Figure 5.15 a-d, the haemoglobin level of 1^{o} BMT recipients was close to that of normal controls at each of the time points, with no differences seen between the three experimental groups. The platelet counts appeared to continue to increase towards those of normal controls, even up to nine months following BMT (Figure 5.15 b). Compared with normal controls, the total WBC (also reflected in the neutrophil count) was higher at three months following 1^{o} BMT in all three experimental groups. As was discussed regarding CFU-A numbers at three months (Figure 5.4), this difference may reflect an "over-shoot" produced during marrow regeneration following the insult of BMT. For 2^{o} BMT recipients, peripheral counts at either the two or six month time points, showed no differences between the three experimental groups (Figure 5.16). Haemoglobin, platelets and WBC were close to normal for all the analyses.

Peripheral blood counts are shown for 3^{o} BMT recipients in Figure 5.17 a-d. In all four groups analysed (G, K, I, M) mean haemoglobin levels were slightly lower for the animals transplanted with SCF/IL-11/MIP-1α expanded marrow than for the other two experimental groups (unmanipulated and SCF/IL-11 expanded BMT). In Figure 5.17 b, the mean platelet counts were

Figure 5.15 Peripheral blood values in primary (1^{o}) BMT recipients

At one month (1/12), 3/12 and 9/12 following 1^{o} BMT, 10 animals were sacrificed from each of the three experimental groups (UM BMT, SCF/11 BMT and SCF/11/M BMT). Analyses were performed on each individual animal for full blood count (FBC). Results therefore represent the mean ($\pm$ SEM) for 10 animals. Figure a. haemoglobin, b. platelets, c. white blood count (WBC) and d. neutrophils.

Figure 5.15a
Haemoglobin levels in recipients of unmanipulated versus expanded bone marrow

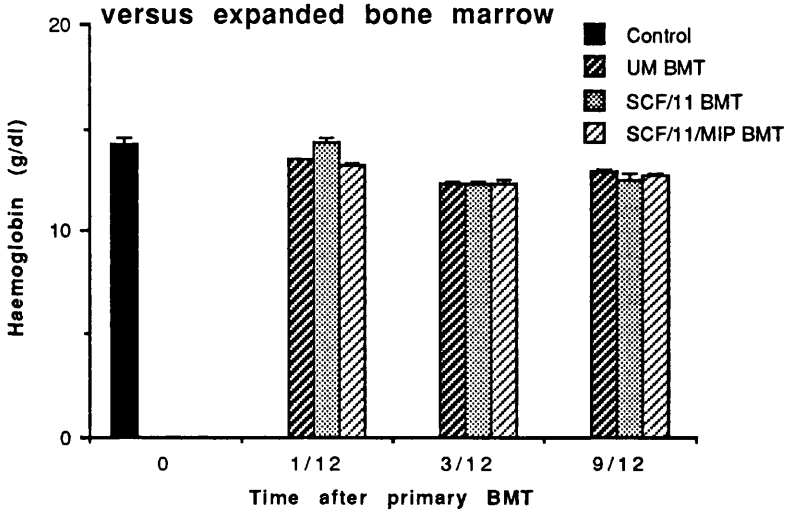


Figure 5.15b
Platelet counts in recipients of unmanipulated versus expanded bone marrow

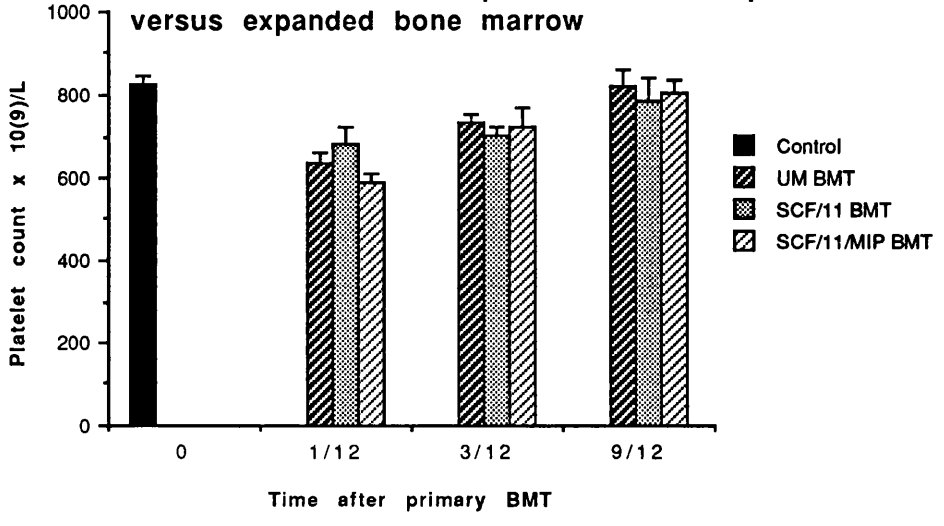


Figure 5.15c
WBC in recipients of unmanipulated versus expanded bone marrow

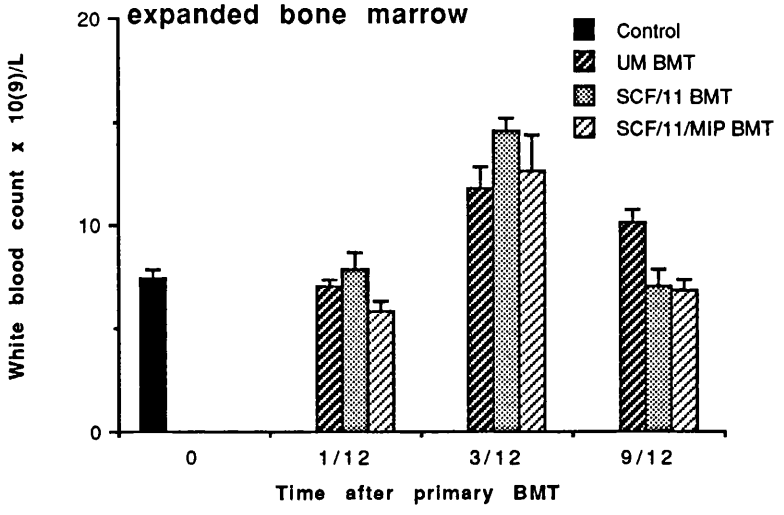


Figure 5.15d
Neutrophil counts in recipients of unmanipulated versus expanded bone marrow

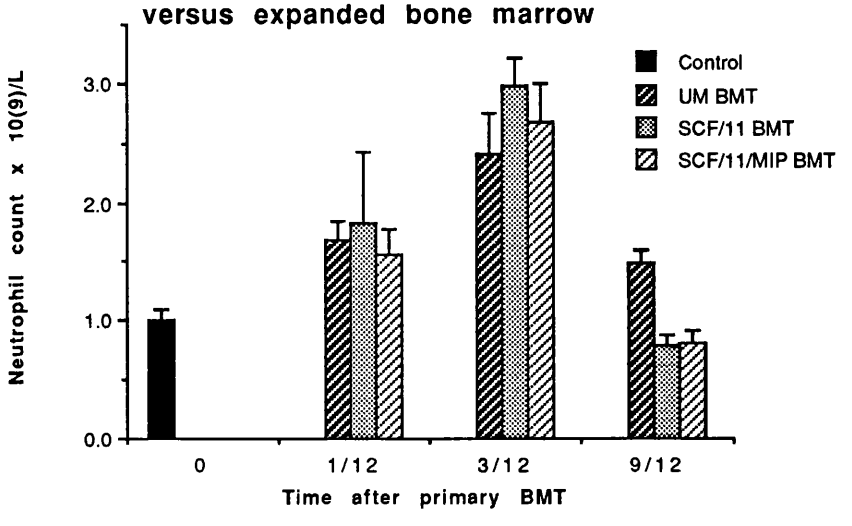


Figure 5.16 Peripheral blood values in secondary (2^{o}) BMT recipients

At 2/12 and 6/12 following 2^{o} BMT, 10 animals were sacrificed from groups **2A**, **3A**, **4A**, **2C, 3C, 4C** and **2E**, **3E**, **4E** (derived at 1/12, 3/12 and 9/12 respectively following 1^{o} BMT). FBC was performed on each individual animal. Results represent the mean ($\pm$ SEM) for 10 animals. Figure a. haemoglobin, b. platelets, c. WBC and d. neutrophils.

Figure 5.16a
Haemoglobin levels in recipients of unmanipulated versus expanded bone marrow

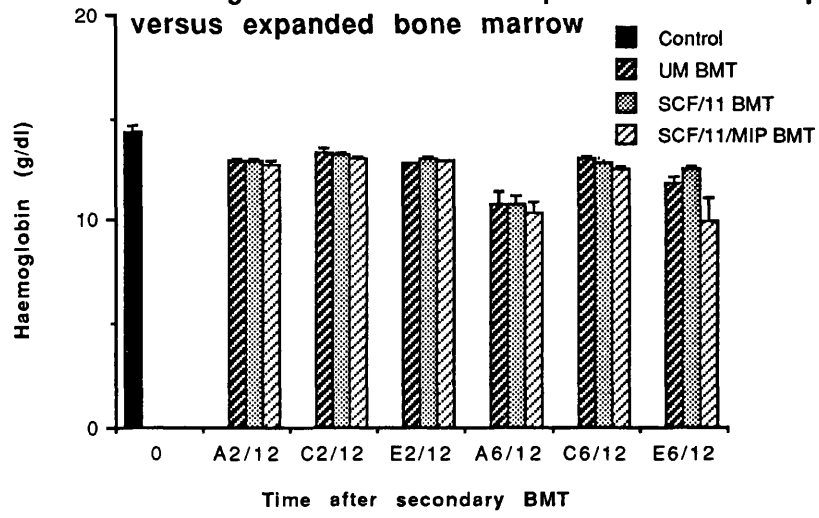


Figure 5.16b
Platelet counts in recipients of unmanipulated versus expanded bone marrow

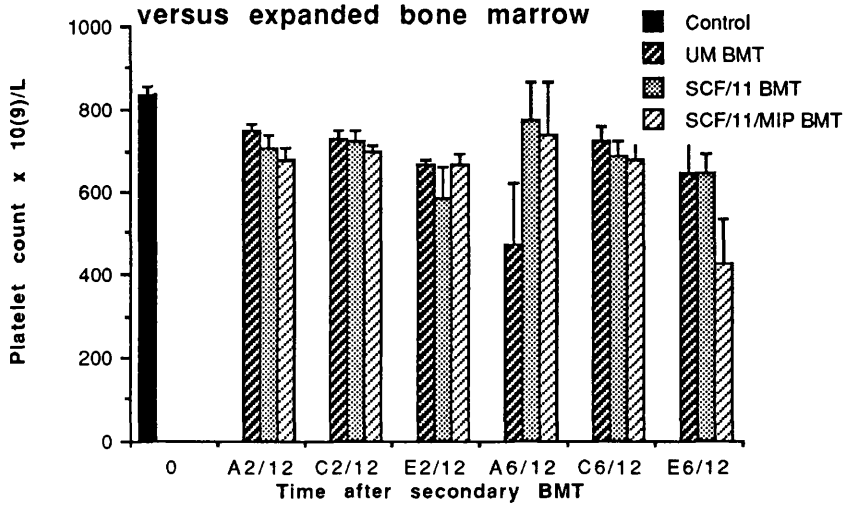


Figure 5.16c
WBC in recipients of unmanipulated versus expanded bone marrow

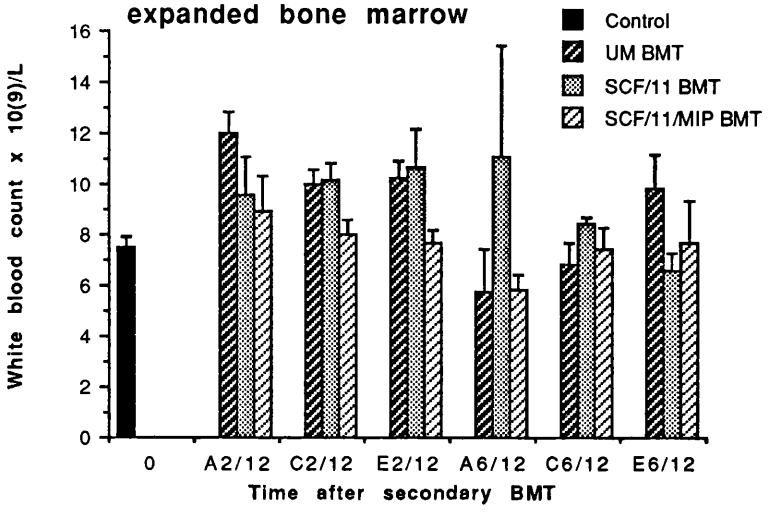


Figure 5.16d
Neutrophil counts in recipients of unmanipulated versus expanded bone marrow

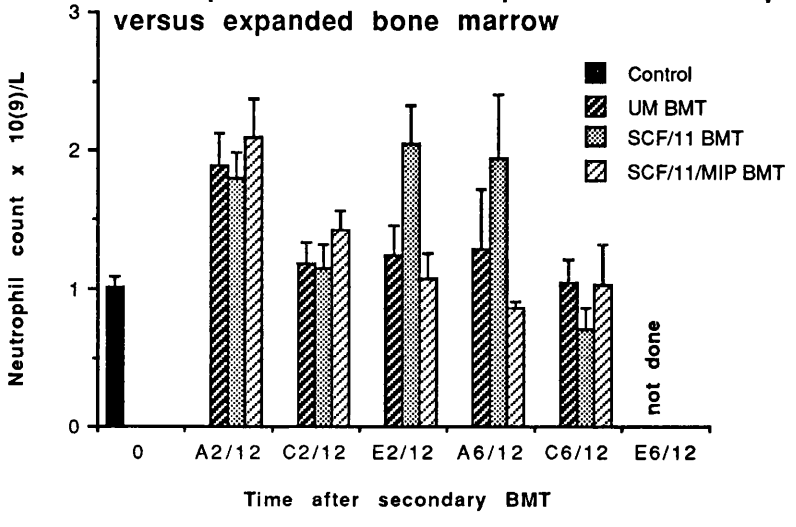


Figure 5.17 Peripheral blood values in tertiary (3^{o}) BMT recipients

At 3/12 following 3^{o} BMT, 10 animals were sacrificed from groups **2G**, **3G**, **4G**, **2K**, **3K**, **4K**, **2I**, **3I**, **4I** and **2M**, **3M**, **4M**. FBC was performed on each individual animal. Results represent the mean (± SEM) for 10 animals. Figure a. haemoglobin, b. platelets, c. WBC and d. neutrophils.

Figure 5.17a
Haemoglobin levels in recipients of unmanipulated versus expanded bone marrow

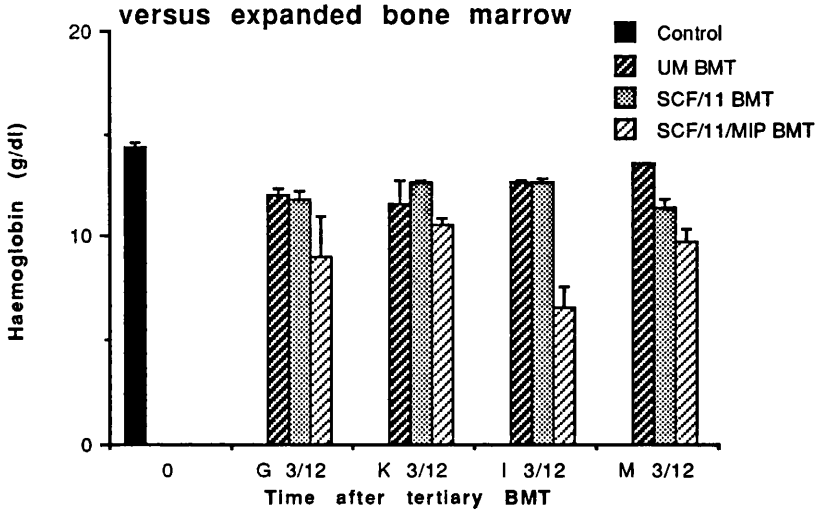


Figure 5.17b
Platelet counts in recipients of unmanipulated versus expanded bone marrow

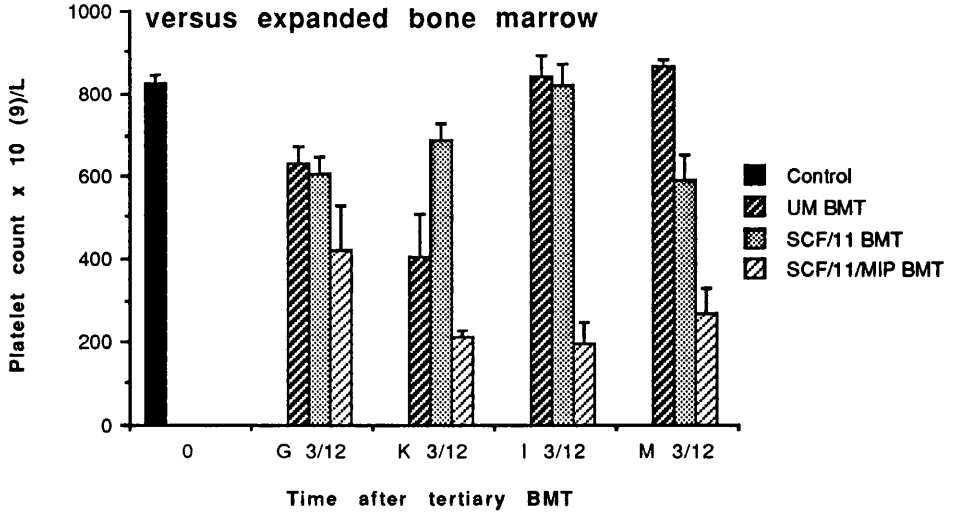


Figure 5.17c
WBC in recipients of unmanipulated versus expanded bone marrow

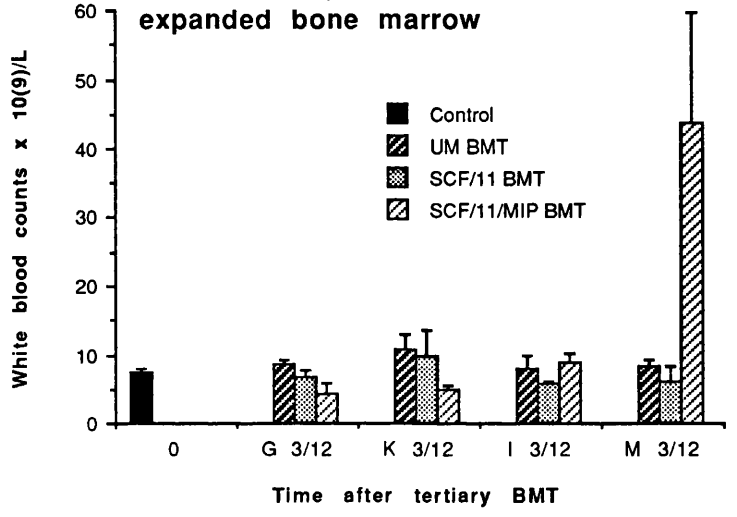
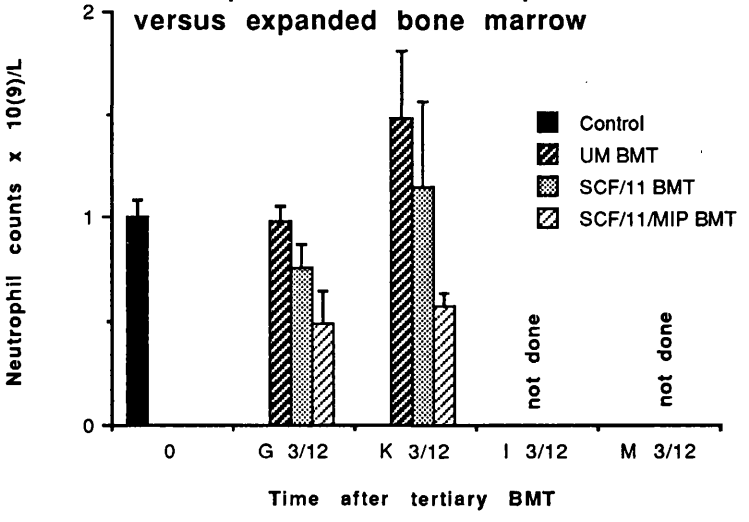


Figure 5.17d
Neutrophil counts in recipients of unmanipulated versus expanded bone marrow



far lower for the SCF/IL-11/MIP-1α BMT groups than for SCF/IL-11. The mean platelet count was also lower for the remaining 9/20 animals in group 2K (unmanipulated BMT) compared with controls. Since 11/20 animals died in group 2K prior to this analysis, it is likely that, if analysed, those animals would have had even lower platelet counts. It has already been shown that CFU-A numbers were very low in this group (Figure 5.10). The WBCs shown in Figure 5.17 c reveal a very important development in this study. Whereas the mean WBC for groups G, K and I appeared normal, the mean WBC for group 4M (SCF/IL-11/MIP-1α expanded BMT) was elevated to approximately 6 times control. The individual indices for animals in group 4M are shown in Table 5.1. At least 8 of 12 animals appeared to have developed a form of leukaemia (see chapter 6 for elaboration).

To summarise the results described so far in this chapter; it appears that unmanipulated cells used for rescue following lethal irradiation either take longer to re-establish a normal complement of LTRC compared with expanded marrow, or contain fewer LTRC than expanded marrow, because of an increase in absolute LTRC numbers during *ex vivo* expansion. When the interval between cell transfers was relatively short, unmanipulated marrow was less able to sustain serial BMT compared with expanded marrow. When the interval was increased, the ability of unmanipulated marrow to sustain serial BMT increased. This serial BMT approach, with varying time intervals between cell transfers and proceeding to quaternary BMT has been very informative and extends similar work performed by other investigators, in particular that of Muench et al (1993). Whether the abilities of unmanipulated and expanded marrow to sustain serial BMT were comparable after a longer interval was impossible to determine in these experiments, because animals transplanted with expanded marrow started to die with raised WBC, suggestive of the development of leukaemia.

Table 5.1 Peripheral blood counts of quaternary BMT recipients (Group 4M)

Full blood count results are shown individually for group 4**M** (SCF/IL-11/MIP-1α BMT). For the results for 2**M** and 3**M** refer to the previously presented histogram showing mean ($\pm$ SEM) for all three groups.

Table 5.1

Individual identification	Total WBC (x 10^9/L)	Platelets (x 10^9/L)	Haemoglobin (g/dl)
4M1	38.8	52	9.5
4M2	20.1	222	10.5
4M3	3.5	725	11.9
4M4	27.6	398	11.5
4M5	16.3	143	5.6
4M6	150.0	50	7.3
4M7	24.7	272	10.8
4M8	3.2	398	10.3
4M9	6.0	547	12.1
4M10	7.3	167	11.4
4M11	164.0	160	6.0
4M12	61.5	94	9.0

The following series of survival curves, individual FBC indices and Southern blots relate to groups of animals already described above (i.e. 3° BMT) and to 4° BMT recipients. The only group in which bone marrow was not pooled to assess its ability to sustain a fourth serial transplant was group 2K. This was because 11/20 animals in this group died of bone marrow failure and it was reasoned that a fourth serial transplant would be unsuccessful. For groups 3K and 4K, and 2G, 3G, 4G a fourth serial BMT was performed. In groups I and M, although deaths were seen in the SCF/IL-11/MIP-1α groups, these appeared to be due to leukaemia and it was, therefore, of interest to perform a fourth serial transplant to determine whether the leukaemia was transplantable.

The survival of recipients of bone marrow (3°BMT) derived at time points of one month post 1° BMT, and two months post 2° BMT (i.e. group G) is shown in Figure 5.18. Deaths early after BMT were presumed to be related to radiation toxicity, but no formal analyses were performed on these animals. One animal was killed on day 100 post BMT in the SCF/IL-11 expanded BMT group because it had developed a skin tumour. This animal was otherwise healthy. There were several deaths between days 75 and 100 in the SCF/IL-11/MIP-1α group. At the time of these deaths occurring they were presumed to be due to late bone marrow failure and no further analyses were therefore performed (see below). All three groups were sacrificed at 182 days after 3° BMT. The marrow of 10 animals from each group was pooled and used to transplant a further three groups of lethally irradiated animals (20 mice per group). The survival of these groups (2R, 3R & 4R) is shown in Figure 5.18. Mice transplanted with 5 x 10^6 serially transplanted unmanipulated bone marrow cells all died by day 25 post BMT. This timing strongly suggested that these animals were indeed dying of bone marrow failure because they were transplanted with an inadequate number of both short and LTR stem and

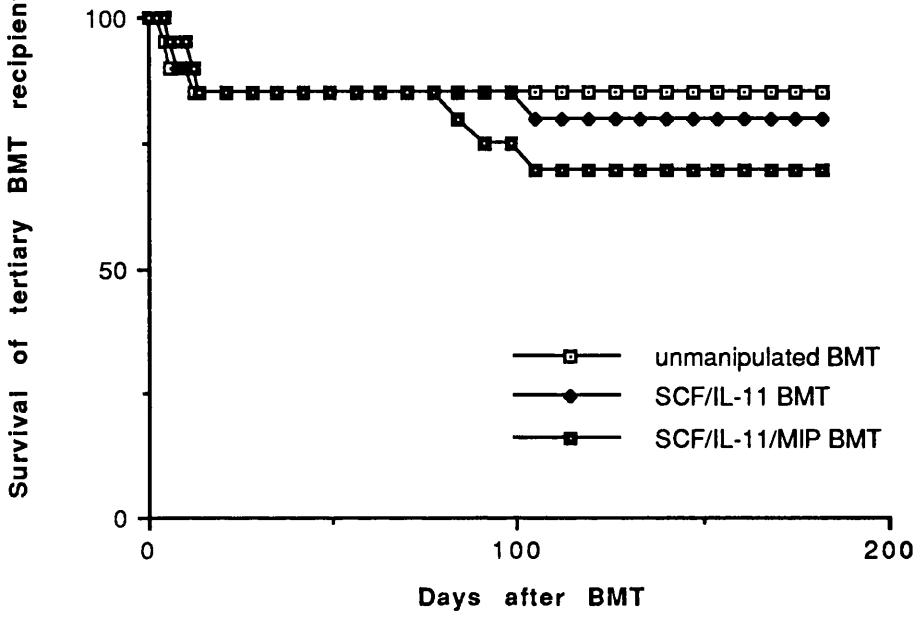
Figure 5.18 Survival curve of recipients of 3^{o} BMT (Group G)

The survival curves following BMT are shown for the three experimental groups (UM BMT, SCF/IL-11 BMT and SCF/IL-11/MIP BMT) in Group **G** (derived from the time points at 1/12 post 1^{o} and 2/12 post 2^{o} BMT). There were twenty animals in each group.

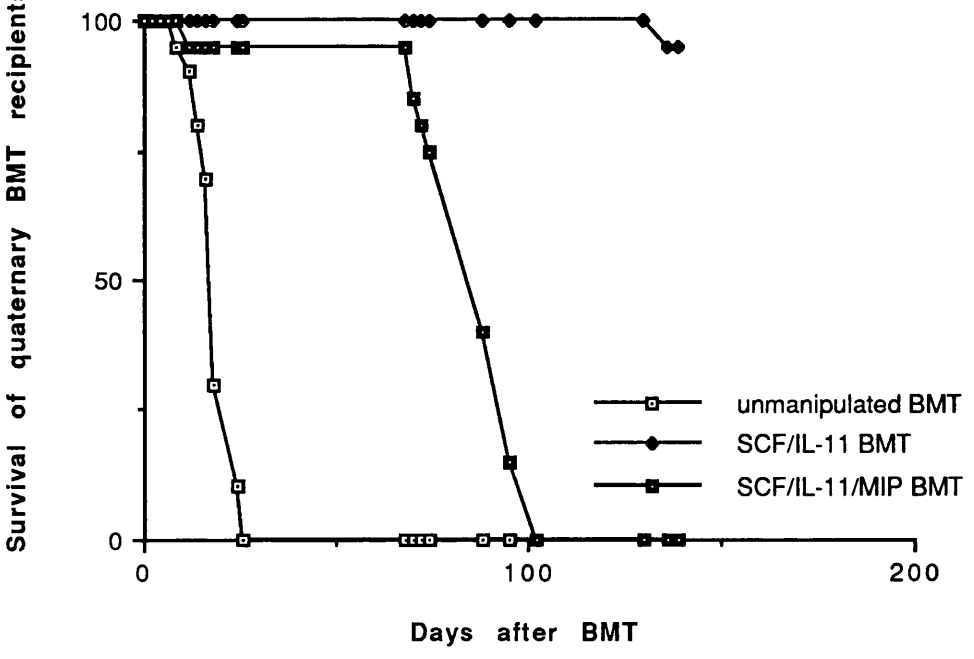
Survival curve of recipients of 4^{o} BMT (Group R)

The survival curves following BMT are shown for the three experimental groups (UM BMT, SCF/11 BMT and SCF/11/MIP BMT) in Group **R** (derived from the time points at 1/12 post 1^{o}, 2/12 post 2^{o} and 6/12 post 3^{o} BMT). There were twenty animals in each group.

Figure 5.18
Survival analysis of recipients of serially transplanted marrow: tertiary BMT (Group G)



Survival analysis of recipients of serially transplanted marrow: quaternary BMT (group R)



progenitor cells. In view of the very low CFU-A content of group G marrow (Figure 5.10) this result was not surprising. Mice transplanted with SCF/IL-11/MIP-1α expanded marrow became unwell around 75 days post BMT. This timing was identical to that for animals transplanted with SCF/IL-11/MIP-1α BMT in group G (see above), although in group G there were only a small number of deaths. This timing was very different to that of unmanipulated BMT where all the deaths occurred before day 25. Because of these interesting differences in the time after BMT until signs of malaise developed, 8/20 animals from group 4R were sacrificed and analyses performed for FBC, donor haemopoiesis and pathology. All these 8 animals were unwell at the time of sacrifice and the remaining 12/20 died. All but one animal in the SCF/IL-11 BMT group survived until 140 days after BMT when 10 of the 20 were sacrificed and analyses performed for FBC, donor haemopoiesis and immunophenotyping (see later). Table 5.2 shows the FBC results for 11 animals in group 3R (SCF/IL-11 BMT), sacrificed on days 137 (number 3R11) and 140 (3R1-10) post BMT, and 8 animals in group 4R (SCF/IL-11/MIP-1α BMT), sacrificed between days 75 and 100 post BMT. In group 3R, one animal (3R11) appeared unwell on day 137 post BMT. FBC revealed leucocytosis, thrombocytopenia and anaemia, highly suggestive of a leukaemic process. The remaining 19 animals appeared healthy at the time of analysis (day 140). The 10 animals sacrificed all had a normal FBC with no suggestion of either bone marrow failure or leukaemia. The remaining 9 animals were observed for survival. There were no further deaths in this group to day 218 post BMT. On the other hand, all 8 animals in group 4R (SCF/IL-11/MIP-1α BMT) had leucocytosis, thrombocytosis and anaemia. The remaining 12 had already died, presumably of leukaemia. The southern blot results in Figure 5.19 confirm that all the animals assessed in group 3R (SCF/IL-11 BMT) showed clear evidence of donor derived haemopoiesis. The pattern seen for group 4R suggests minimal binding of the Y specific probe to

Table 5.2 Peripheral blood counts of quaternary BMT recipients (Groups 3R and 4R)

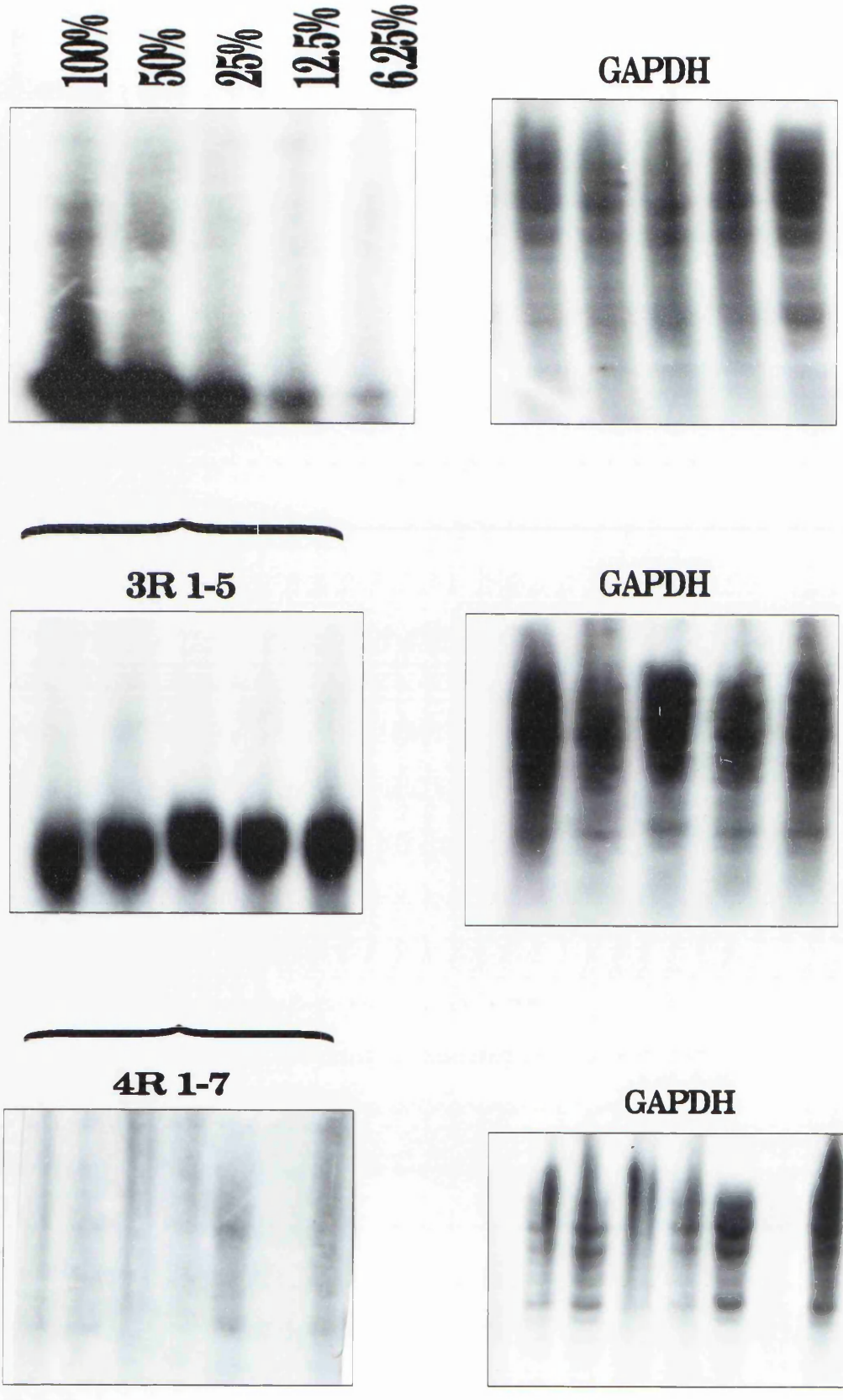
Full blood count results are shown for groups 3**R** (SCF/IL-11 BMT) and 4**R** (SCF/IL-11/MIP-1α BMT). (Group 2**R** perished soon after BMT and no analyses were performed).

Table 5.2

Individual identification	Total WBC (x 10^9/L)	Platelet count (x 10^9/L)	Haemoglobin (g/dl)
3R1	6.2	809	12.6
3R2	3.2	787	12.3
3R3	7.1	486	12.0
3R4	8.3	833	12.6
3R5	3.8	831	13.1
3R6	3.8	871	12.7
3R7	3.9	915	13.0
3R8	2.3	67	10.5
3R9	5.5	878	12.9
3R10	5.0	693	12.1
3R11	53.7	212	8.6
4R1	86.9	64	9.8
4R2	134.5	162	10.4
4R3	31.6	192	8.6
4R4	37.7	99	8.1
4R5	19.7	85	3.5
4R6	28.9	149	7.2
4R7	70.3	243	9.5
4R8	158.0	170	5.0

Figure 5.19 Assessment of donor engraftment in group R animals following 4^{o} BMT

Genomic DNA was extracted from randomly selected animals from each remaining experimental group (i.e. group 3, SCF/IL-11 BMT; group 4, SCF/IL-11/MIP BMT). Group 2**R** perished soon after 4^{o} BMT and was followed for survival only (i.e. no other analyses were performed). The top panels show the titration of male DNA and GAPDH loading control. The left hand panels represent results for the Y specific probe, the right hand panels results for GAPDH.


100%
50%
25%
12.5%
6.25%
GAPDH
3R 1-5
GAPDH
4R 1-7
GAPDH

digested DNA which had been partially degraded at the time of analysis, hence the smear, rather than clear bands. Unfortunately, all the DNA from this group was of similar quality. Referring back to the FBC results in Table 5.1 it is clear that group 4M had also developed leukaemia. In light of these results, it is likely that the small number of deaths in group 4G (SCF/IL-11/MIP-1α BMT) was also due to leukaemia rather than to bone marrow failure (see Figure 5.18).

The survival of recipients of bone marrow (3°BMT) derived at time points of three months post 1° BMT, and two months post 2° BMT (i.e. group K) is shown in Figure 5.20. Apart from a few early deaths, probably related to radiation toxicity, all three groups of animals survived until > day 75. At that point, animals in group 2K (unmanipulated BMT) began to die. The animals analysed for FBC, donor haemopoiesis and pathology, were the remaining 9 live animals. These nine animals, at the time of analysis, appeared healthy and, therefore, may not have been representative of the animals which died. These animals had normal WBC, near normal haemoglobin and reduced platelet counts. These nine animals showed no evidence (FBC & pathology) of leukaemia. It may be presumed, therefore, that the deaths seen in group 2K were due to late bone marrow failure and not to leukaemia. On day 100 post BMT, bone marrow was pooled from groups 3K (SCF/IL-11) and 4K (SCF/IL-11/MIP-1α) and used to transplant two groups of lethally irradiated animals (20 mice per group). Group 2K was not used in a fourth serial transplant because only nine animals were available which had low femur counts and therefore insufficient cells to transplant a further 20 animals. In any case since group 2K appeared to be dying of bone marrow failure a fourth transplant from this group was not likely to be successful. The survival of the recipients of marrow from groups 3K and 4K is shown in Figure 5.20 (groups 3S and 4S). Animals transplanted with SCF/IL-11/MIP-1α (group 4S)

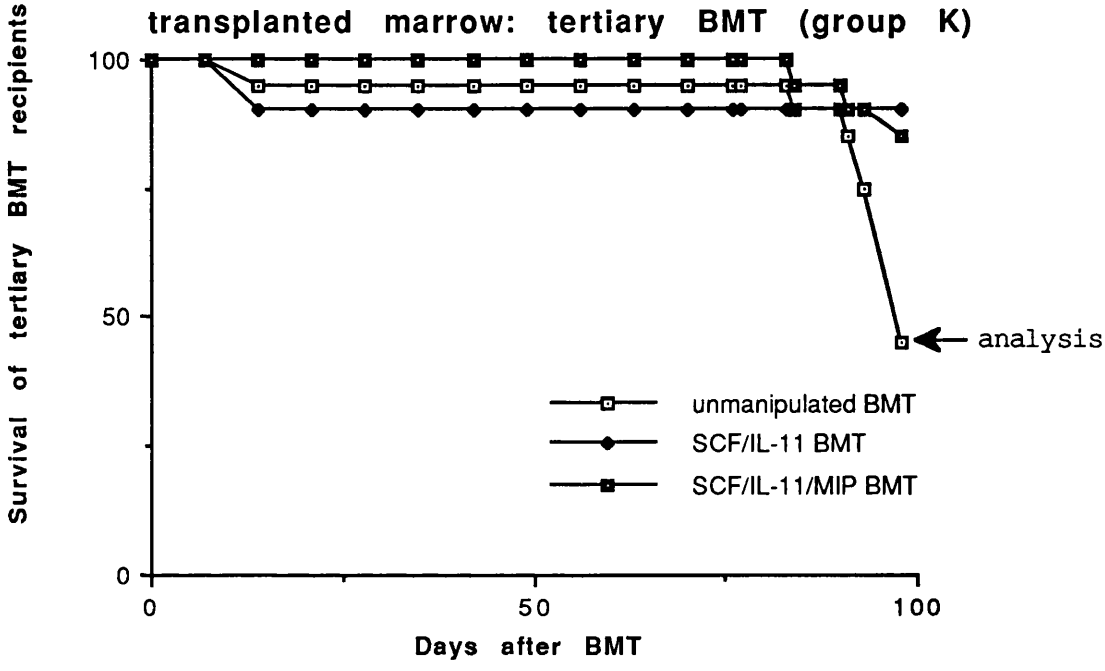
Figure 5.20 Survival curve of recipients of 3^{o} BMT (Group K)

The survival curves following BMT are shown for the three experimental groups (UM BMT, SCF/11 BMT and SCF/11/MIP BMT) in Group **K** (derived from the time points at 3/12 post 1^{o} and 2/12 post 2^{o} BMT). There were twenty animals in each group.

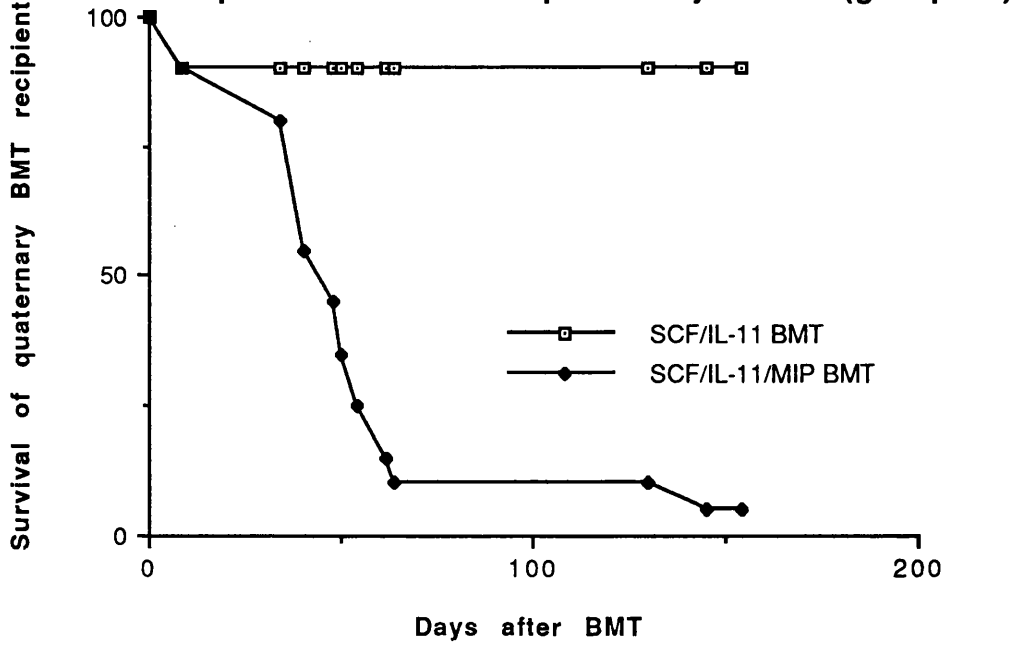
Survival curve of recipients of 4^{o} BMT (Group S)

The survival curves following BMT are shown for the two experimental groups (SCF/11 BMT and SCF/11/MIP BMT) in Group **S** (derived from the time points at 3/12 post 1^{o}, 2/12 post 2^{o} and 3/12 post 3^{o} BMT). There were twenty animals in each group. Since only 9 remaining animals were analysed from the UM BMT group in group K, there were insufficient cells to test ability to sustain a fourth serial BMT in this cohort.

Figure 5.20
Survival analysis of recipients of serially transplanted marrow: tertiary BMT (group K)



Survival analysis of recipients of serially trasplanted marrow: quaternary BMT (group S)



expanded marrow began to die around day 40 post BMT. Since animals in group 4R had all died from leukaemia or showed evidence of leukaemia at the time of analysis, it was presumed that animals in group 4S were also dying of leukaemia, but no analyses were performed. 10 animals from Group 3S were sacrificed for analysis at day 152 post BMT. These animals all appeared healthy at that time. The FBC results for these 10 animals are shown in Table 5.3. One animal had a slightly raised WBC (number 3S2) however, both the platelet count and haemoglobin were fully normal and this may have been due to intercurrent infection. Pathology revealed no evidence of leukaemia in these animals and as shown in figure 5.21, haemopoiesis was at least partially donor derived. The remaining animals in this group were alive and well at 232 days post BMT. To conclude, although full analyses were not performed on every group or on every animal within a group, it appears that animals in groups 4G, 4R and 4 S (all SCF/IL-11/MIP-1α BMT) developed leukaemia. In addition at least one animal in group 3R (SCF/IL-11 BMT) also developed leukaemia. There was no evidence of leukaemia in animals transplanted with unmanipulated BMT.

The survival of recipients of bone marrow (3^{o}BMT) derived at time points of one month post 1^{o} BMT and six months post 2^{o} BMT (i.e. group I) is shown in Figure 5.22. Once again deaths were observed in the SCF/IL-11/MIP-1α group (4I) from around 90 days post BMT. When only 6/20 animals in this group remained alive all three groups were analysed for FBC, donor haemopoiesis, pathology and ability to sustain a fourth serial BMT. FBC was also performed for three additional animals in group 4I prior to this time point. As can be seen by referring back to Figure 5.17, the FBCs were normal for groups 2I and 3I which all appeared healthy. However, the FBCs were all abnormal for group 4I. Three animals showed mild leucocytosis, all had a reduced platelet count and all were anaemic. The spleens of these animals

Table 5.3 Peripheral blood counts of quaternary BMT recipients (Group 3S)

Full blood count results are shown for group 3**S** (SCF/IL-11 BMT). Since group 2**K** was not used for a quaternary BMT there is no group 2**S** (UM BMT). Group 4**S** (SCF/IL-11/MIP-1α BMT) perished soon after BMT and no analyses were performed.

Table 5.3

Individual identification	Total WBC (x 10^9/L)	Platelets (x 10^9/L)	Haemoglobin (g/dl)
3S1	5.8	665	12.8
3S2	17.7	836	13.3
3S3	5.6	794	12.4
3S4	4.4	814	12.1
3S5	4.4	742	12.3
3S6	3.7	952	12.2
3S7	12.3	854	12.9
3S8	9.1	634	12.2
3S9	7.7	718	13.0
3S10	7.5	680	12.5

Figure 5.21 Assessment of donor engraftment in group S animals following 4^{o} BMT

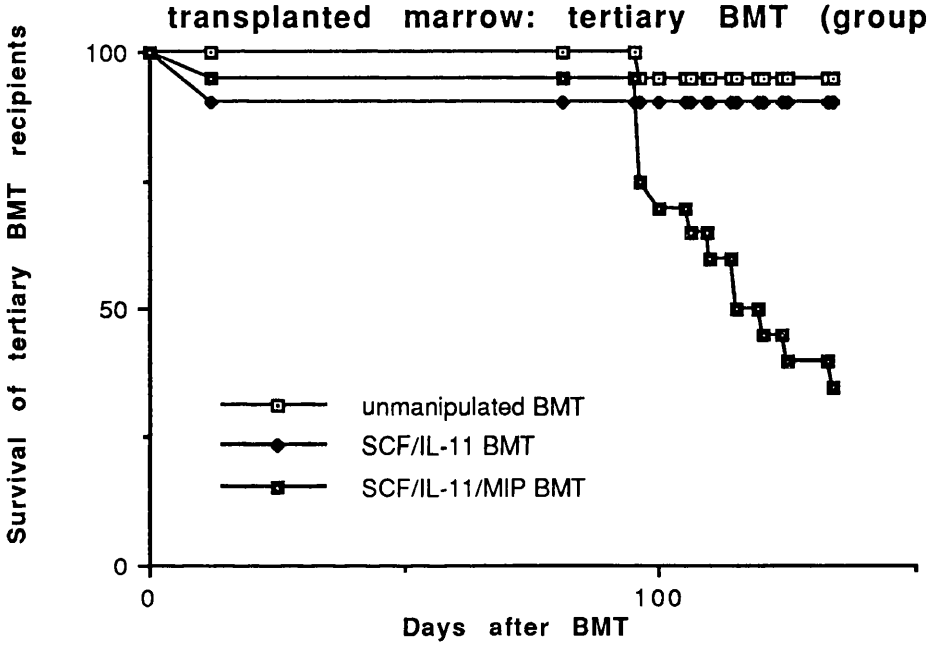
Genomic DNA was extracted from five randomly selected animals from group 3**S** (SCF/IL-11 BMT). Since some animals in group 2**K** perished during 3^{o} BMT this group was not used in a 4^{o} transplant, therefore there was no group 2**S**. Group 4**S** (SCF/IL-11/MIP-1α BMT) perished after 4^{o} BMT and was followed for survival only (no other analyses were performed). The top panels show the titration of male DNA and GAPDH loading control. The second panel shows results for group 3**S** for hybridisation with the Y probe and with GAPDH.

100%
50%
25%
12.5%
6.25%
3.125%
GAPDH
3S 1-5
GAPDH

Figure 5.22 Survival curve of recipients of 3^{o} BMT (Group I)

The survival curves following BMT are shown for the three experimental groups (UM BMT, SCF/11 BMT and SCF/11/MIP BMT) in Group **I** (derived from the time points at 1/12 post 1^{o} and 6/12 post 2^{o} BMT). There were twenty animals in each group.

Figure 5.22
Survival analysis of recipients of serially transplanted marrow: tertiary BMT (group I)



were grossly enlarged, as was the liver. At the time of writing, the animals transplanted with the pooled marrow from these three groups (4°BMT, groups 2U, 3U, 4U) were alive and well (day 40 post BMT).

The final survival curves (Figure 5.23) are for recipients of bone marrow (3°BMT and 4°BMT) derived at time points of three months post 1° BMT, six months post 2° BMT (i.e. group M) and three months post 3°BMT (i.e. group V). Ten animals from groups 2M (UM BMT) and 3M (SCF/IL-11 BMT) and all remaining animals in group 4M (SCF/IL-11/MIP-1α BMT), were sacrificed for analysis 102 days post BMT. By then, 9/20 in group 4M had died of leukaemia. 12/20 had FBC performed and the majority showed abnormalities consistent with leukaemia (Table 5.1). All showed enlargement of spleen and liver. Group 2M (UM BMT) all appeared healthy at the time of analysis, and all their FBCs were within normal limits. 8/20 in this group remained alive and well 178 days post BMT. Although group 3M (SCF/IL-11 BMT) appeared healthy at the time of analysis, one (3M7) had a very abnormal FBC (WBC 24.8 x 10^9/l, Hb. 8.4g/dl, platelets 106 x 10^9/l) and several showed a degree of pancytopenia. The remaining 8/20 animals left after the time point for analysis have since died. At the time of analysis, all three groups showed evidence of donor derived haemopoiesis in bone marrow, whether leukaemic or not (Figure 5.13). Pooled marrow from groups 2M, 3M and 4M was used to serially transplant further batches of lethally irradiated mice (15 per group). The survival curve of these animals is also seen in Figure 5.23. Since group 4M showed clear evidence of leukaemia (see FBC Table 5.1) at the time of analysis, the deaths (100% by day 20 post BMT) in group 4V were presumed to be due to leukaemia and no further analyses were performed in this group. Group 2V remained alive and well at 76 days post BMT. Group 3V began to show signs of malaise by 25 days post BMT. On day 36 post BMT the 5/15 remaining alive were sacrificed and analysed for

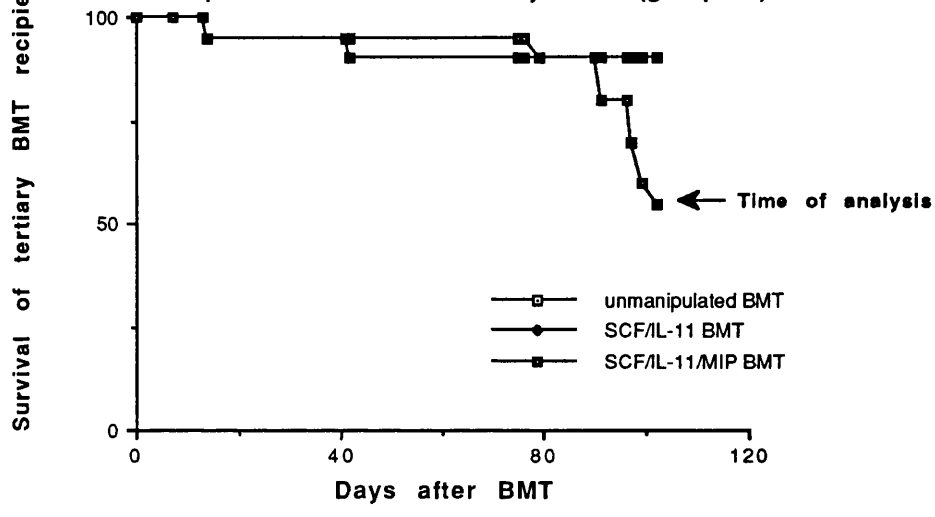
Figure 5.23 Survival curve of recipients of 3^{o} BMT (Group M)

The survival curves following BMT are shown for the three experimental groups (UM BMT, SCF/11 BMT and SCF/11/MIP BMT) in Group **M** (derived from the time points at 3/12 post 1^{o} and 6/12 post 2^{o} BMT). There were twenty animals in each group.

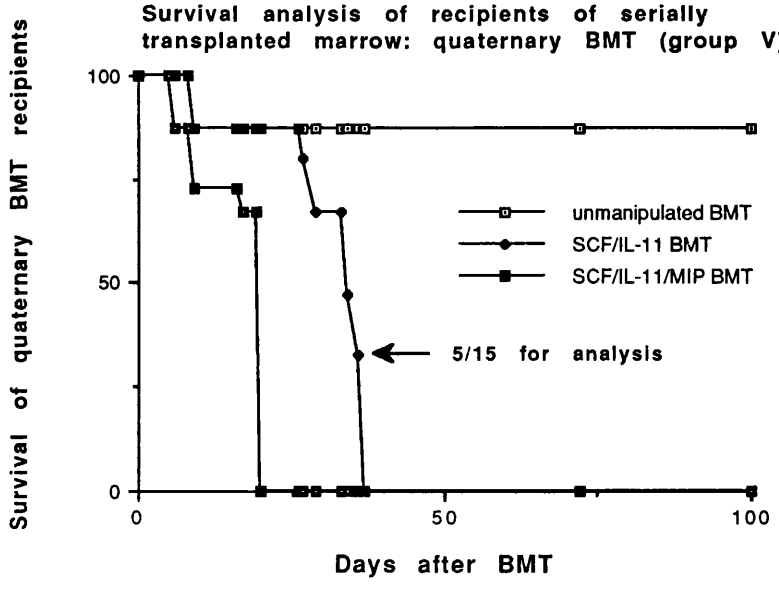
Survival curve of recipients of 4^{o} BMT (Group V)

The survival curves following BMT are shown for the three experimental groups (UM BMT, SCF/11 BMT and SCF/11/MIP BMT) in Group **V** (derived from the time points at 3/12 post 1^{o}, 6/12 post 2^{o} and 3/12 post 3^{o} BMT). There were twenty animals in each group.

Figure 5.23
Survival analysis of recipients of serially transplanted marrow : tertiary BMT (group M)



Survival analysis of recipients of serially transplanted marrow: quaternary BMT (group V)



FBC and pathology. The five individual FBCs are shown in Table 5.4. At least three animals showed significant leucocytosis, all were anaemic and severely thrombocytopenic. This result confirms that the leukaemia process seen in all groups transplanted with SCF/IL-11/MIP-1α expanded marrow also developed later in animals transplanted with SCF/IL-11 expanded marrow. During prolonged follow-up one animal from the unmanipulated BMT group (2I11) became unwell and was found to have developed leukaemia. This was the only animal transplanted with unmanipulated marrow in which this occurred.

Discussion

The results described in this chapter are fascinating but difficult to interpret. For serial transplantation with unmanipulated cells it seems clear that if the interval between transplants is short (groups G and K) then the ability to sustain serial BMT is less than if the interval is longer (groups I and M). This feature has previously been described by Jones et al and was interpreted as evidence that soon after BMT, LTRC are diluted out by committed progenitors, but by six months after BMT, the normal ratio is regained allowing further successful serial BMT (Jones *et al.*, 1989).

If this hypothesis were correct it would explain one possible mechanism underlying the improved ability of expanded bone marrow to sustain serial BMT even with short intervals between transplants. As described in Chapter 4, expanded bone marrow, used for rescue following lethal dose radiation, produced more rapid haematological recovery compared with unmanipulated cells. We can assume, therefore, that expanded marrow may also allow more rapid recovery of the ratio of LTRC to committed progenitors compared with unmanipulated material. This would explain the augmentation of the ability to serially transplant marrow seen for expanded marrow at the early time points. The ability of unmanipulated bone marrow to

Table 5.4 Peripheral blood counts of quaternary BMT recipients (Group 3V)

Full blood count results are shown for the 5 out of 15 animals in group 3V (SCF/IL-11 BMT) remaining alive at day 36 post BMT.

Table 5.4

Code	Total WBC (x 10^9/l)	Platelets (x 10^9/l)	Haemoglobin (g/dl)
3V1	23.8	10	3.3
3V2	5.8	17	6.2
3V3	111.2	57	7.9
3V4	3.8	27	10.7
3V5	25.7	25	9.0

Group 3, SCF/IL-11 BMT

V = 4^o BMT from Group M (3/12 after 1^o, 6/12 after 2^o, 3/12 after 3^o BMT)

sustain a 3° and 4° BMT was regained when the duration between the 2° and 3° BMT was increased from two months to six months (groups I and M) again in agreement with the work by Jones et al.

However, the development of leukaemia, almost exclusively in the expanded groups, suggests that the more rapid return of the normal LTRC: committed progenitor ratio post BMT cannot be the only mechanism underlying the differences between unmanipulated and expanded BMT and suggests an effect of growth factor exposure at the level of a primitive stem cell. The alternative explanation for the improved ability of expanded marrow to sustain serial BMT is that LTRC were actually increased in number during *ex vivo* expansion. If this were the case, then expanded marrow would be expected to show an improved ability to sustain serial BMT whether the interval between transplants was short or long. It was, however, impossible to compare the ability of expanded marrow to sustain serial BMT at these later time points because of the development of leukaemia. In the absence of quantitative stem cell assays (e.g. cobblestone area forming cell assay (*in vitro*) Ploemacher *et al.*, 1991) or competitive repopulation assay (*in vivo*) (Harrison, *et al.*, 1993), no conclusions can be made regarding the number of LTRC used for the 1° BMT. These experiments are now in progress, but were not completed at the time of writing.

The conclusions which can be made, with the information available, are firstly that growth factor exposure of bone marrow cells prior to transplantation appears to augment the ability to sustain serial BMT if the interval between transplants is short. Secondly, that growth factor exposure appears, in some way, to trigger the development of a leukaemia in recipient animals. This leukaemia may develop following serial BMT with unmanipulated marrow but this appears to be a very rare event (one case only). Growth factor exposure, however, appears to increase the incidence of leukaemia and the addition of MIP-1α to SCF and IL-11 appears to further

increase the incidence and reduce the latency of leukaemogenesis. The mechanism underlying the development of this leukaemia is not yet known, but is likely to relate both to the primary *ex vivo* culture and to the requirement to repopulate lethally irradiated host animals *in vivo*.

RESULTS IV

Chapter 6 Characterisation of the Leukaemia observed in Recipients of Bone MarrowTransplantation

Murine leukaemias, either arising spontaneously or induced by radiation or drugs, have been described previously and may be strain or age dependent. New Zealand Black mice spontaneously develop a B-cell lymphoproliferative disorder as they age and have been used as a murine model of B-cell chronic lymphocytic leukaemia (B-CLL) (Okada *et al.*, 1991; Marti *et al.*, 1995). In these mice, spleen weight, peritoneal cell counts, and absolute lymphocyte counts are elevated in old (>18 months) compared with young (2 months) animals. They develop lymphocytic infiltrates in the lacrymal glands, kidneys, liver and lung. There is an age dependent increase in CD5 positive B cells in blood and spleen which show oligoclonal and even clonal expansion, eventually giving rise to B-CLL (Okada *et al.*, 1991; Marti *et al.*, 1995). To our knowledge, leukaemia of this type has not been described as occurring spontaneously in the B6D2F1 strain.

Leukaemias induced by radiation are usually of the myeloid lineage and originate in the host stem cells which have themselves been exposed to radiation damage (Fennelly *et al.*, 1995). They are often associated with chromosomal changes. The leukaemia observed in this study did not occur in all groups of animals (given an identical radiation schedule), and occurred only in the later part of the serial transplantation experiment, therefore, it is most unlikely to have been related to radiation exposure. In addition, as discussed below, the leukaemia was found to be lymphoid rather than myeloid.

The leukaemia occurred almost exclusively in animals transplanted with expanded cells. This suggested that the *ex vivo* culture, by an unknown mechanism, was the initiating factor in leukaemogenesis. A range of

investigations were planned to determine if the disorder seen was truly leukaemia and if so what type of leukaemia it was. Detailed pathology was performed on normal control animals, and animals from all three experimental groups, whether the animals appeared healthy or not. Southern blotting, to detect male or female haemopoiesis, was performed as already described. Immunophenotyping was performed for both myeloid antigens (Gr-1 and c-kit) and markers typically positive in B-CLL (CD5 and surface IgM). Pooled marrow from some experimental groups was used to transplant (intraperitoneal) sub-lethally irradiated mice to determine whether the disorder was transplantable and therefore leukaemic. These mice were given only 2Gy irradiation to ensure that any deaths observed were due to leukaemia and not to bone marrow suppression. Finally, FISH studies using a Y specific marker, and cytogenetic analyses were performed.

The results of pathology from both normal and leukaemic animals are shown in Figures 6.1-9. In normal mouse peripheral blood, compared with red cells, white cells are infrequent and platelets plentiful (Figure 6.1). There are approximately equal numbers of lymphocytes and neutrophils. Eosinophils and monocytes are rarely seen and basophils are even less common. The normal neutrophils lack granulation and have a segmented nucleus (Figure 6.1). Normal lymphocytes are slightly smaller than neutrophils, with a round nucleus, little cytoplasm, no granules and no nucleoli. In the leukaemic animals, the leucocytosis consisted of a lymphocytosis. In some cases the lymphocytes appeared relatively mature, however when the disease was more advanced the cells looked more primitive. Two high power views of blood films from leukaemic animals are shown in Figure 6.2. The lymphoid cells are larger than normal with less cytoplasm. They are best described as "blast" cells.

Figure 6.1 Normal mouse blood film

High power view of a normal mouse blood film. A normal lymphocyte and neutrophil are shown. Platelets appear plentiful.

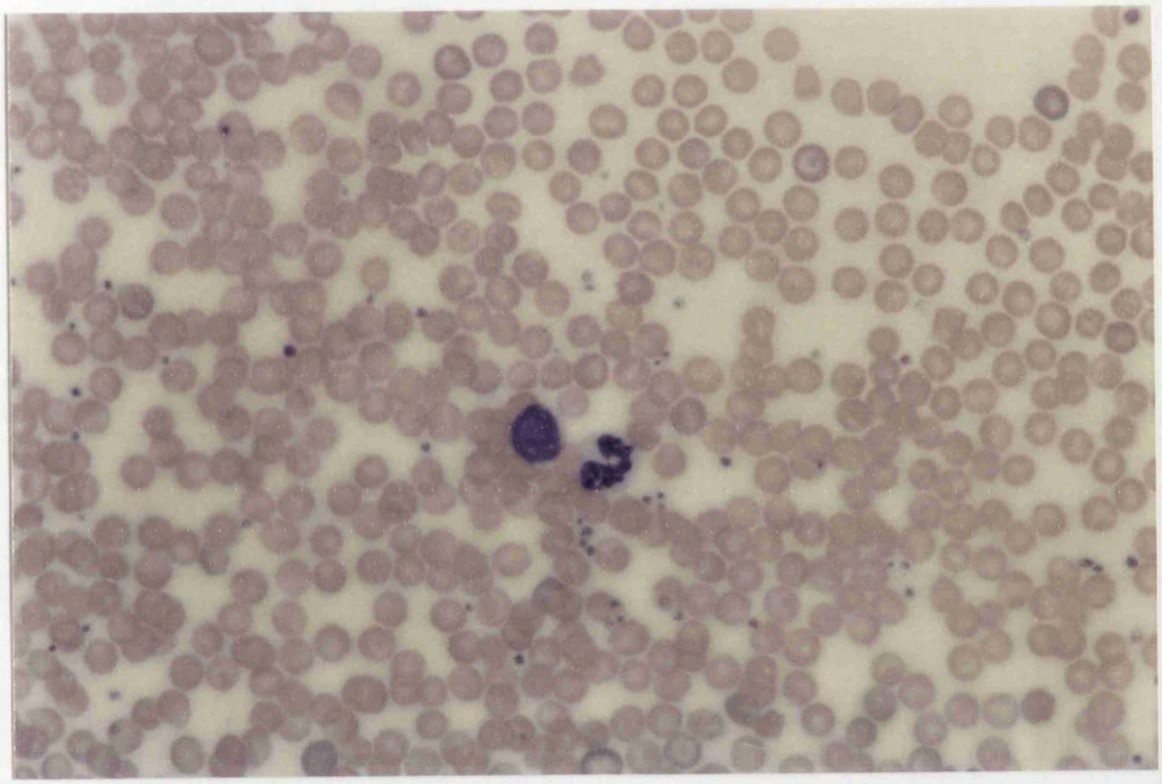
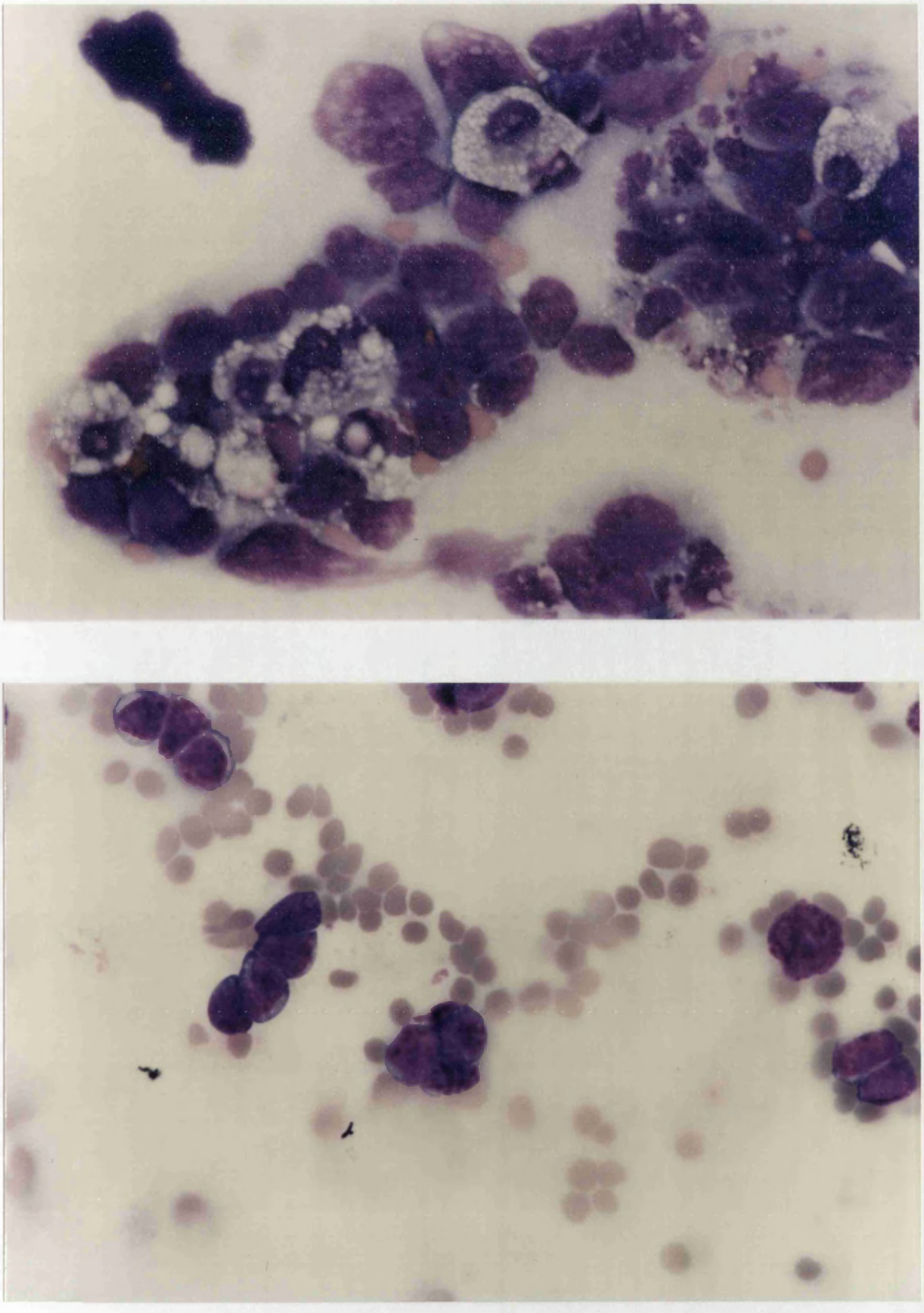


Figure 6.2 Blood films from animals with leukaemia at high power

Two high power views of the blood films from animals with lymphoproliferation. Platelets appear significantly reduced.



In the leukaemic animals, the spleens and livers were greatly enlarged. Macroscopically the spleens often appeared haemorrhagic and showed paler "follicular" areas. An example of spleen size and morphology from leukaemic mice is shown in Figure 6.3. The normal splenic architecture is completely effaced, with patchy areas of lymphoid infiltrate separated by haemorrhage. At high power, the infiltrate consists of a fairly homogeneous population of large, nucleolated lymphoid blast cells. The presence of haemorrhage is obvious. For comparison, the gross appearance of a normal sized spleen, next to two leukaemic spleens is shown.

Many of the mice examined, whether leukaemic or not, showed evidence of extramedullary haemopoiesis. This is likely to occur following lethal irradiation, with transplanted cells proliferating both in the bone marrow and in the spleen. The example shown in Figure 6.4 shows evidence of lymphoproliferation and, in addition, extramedullary haemopoiesis. The megakaryocytes are seen very clearly.

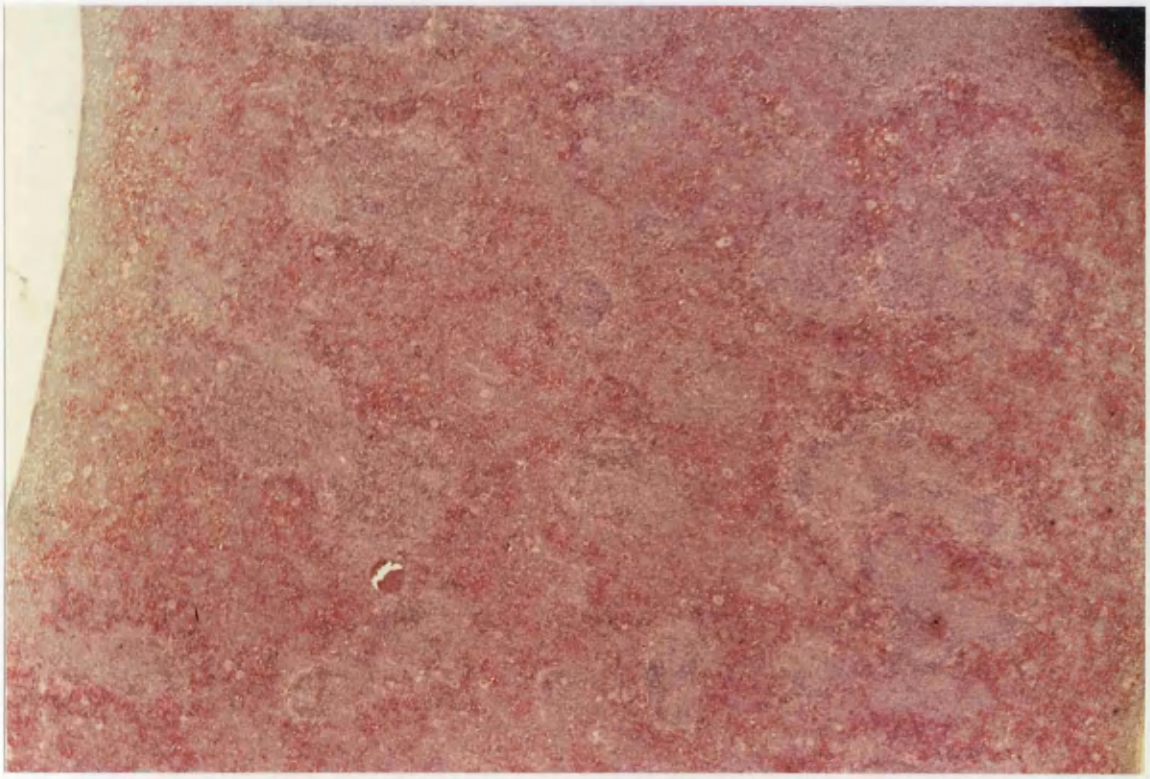
Normal bone marrow is shown in Figure 6.5. Cellularity tends to be greater in the shaft of the femur with an increase in the fat spaces towards the end as shown in this example. Bone trabeculae are seen and lymphoid cells are infrequent. In the following example (Figure 6.6), from a leukaemic animal, the bone marrow is completely infiltrated by tumour. There is little residual normal haemopoiesis although occasional megakaryocytes are present. The normal architecture, including bone trabeculae and fat spaces, has been destroyed. In some animals, intermediate stages of the disease were seen. These animals appeared healthy at the time of analysis however their bone marrow showed early infiltration with lymphoid cells and peripheral blood indices showed pancytopenia.

Figure 6.3 Splenic infiltration by leukaemia

Low power view of a spleen from an animal with lymphoproliferation. In this example the normal splenic architecture is completely destroyed. The spleen has been invaded by a lymphoid infiltrate and extensive haemorrhage is visible.

At high power the lymphoid cells appear rather pleomorphic and the haemorrhage is striking. Many of the lymphoid cells are nucleolated.

The final figure shows the gross appearances of a normal spleen next to two leukaemic spleens.



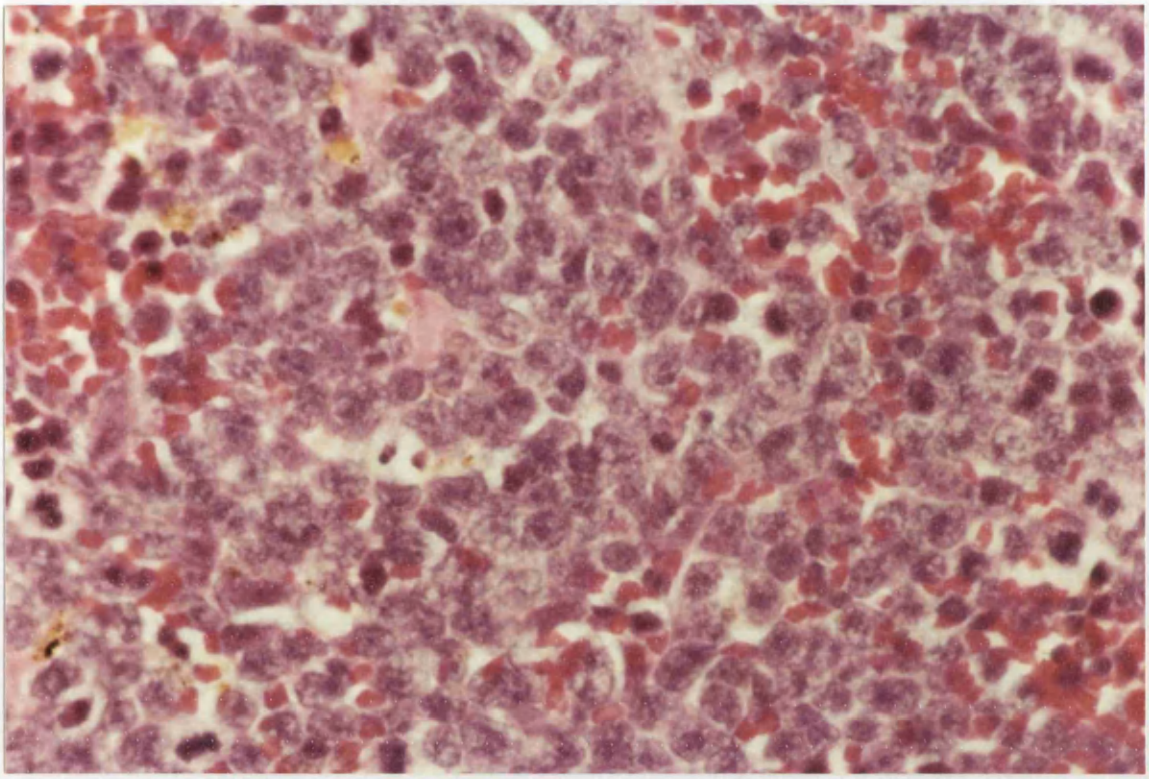


Figure 6.4 Extramedullary haemopoiesis

This example from an animal with lymphoproliferation shows evidence of extramedullary haemopoiesis. Note the presence of megakaryocytes.

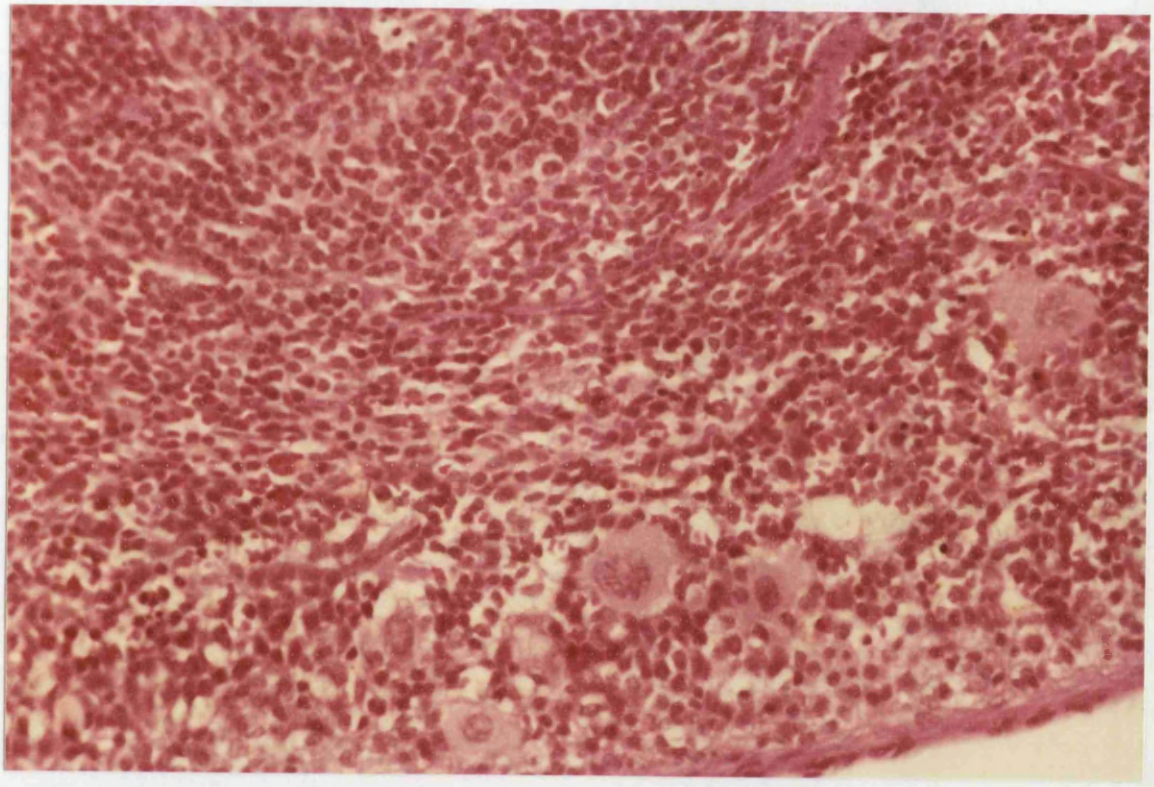


Figure 6.5 Normal bone marrow

Low power view of a normal femur. Cellularity appears normal with ample fat spaces. Bone trabeculae are present.

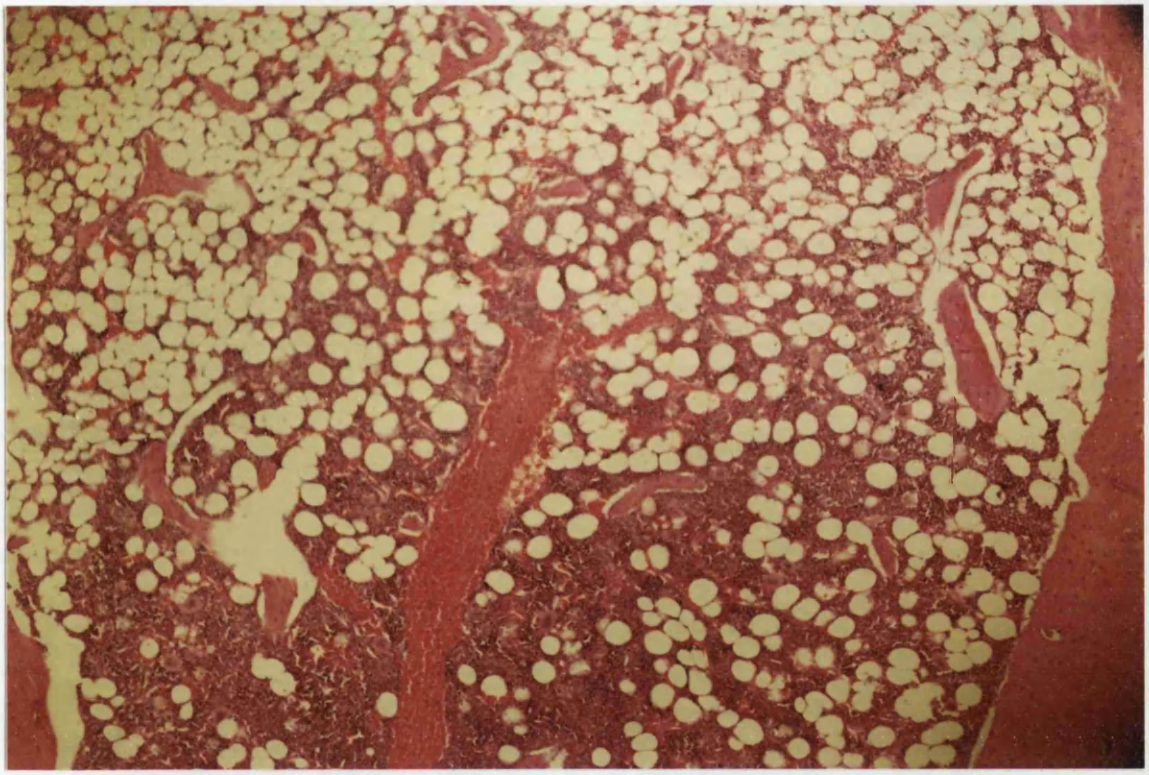
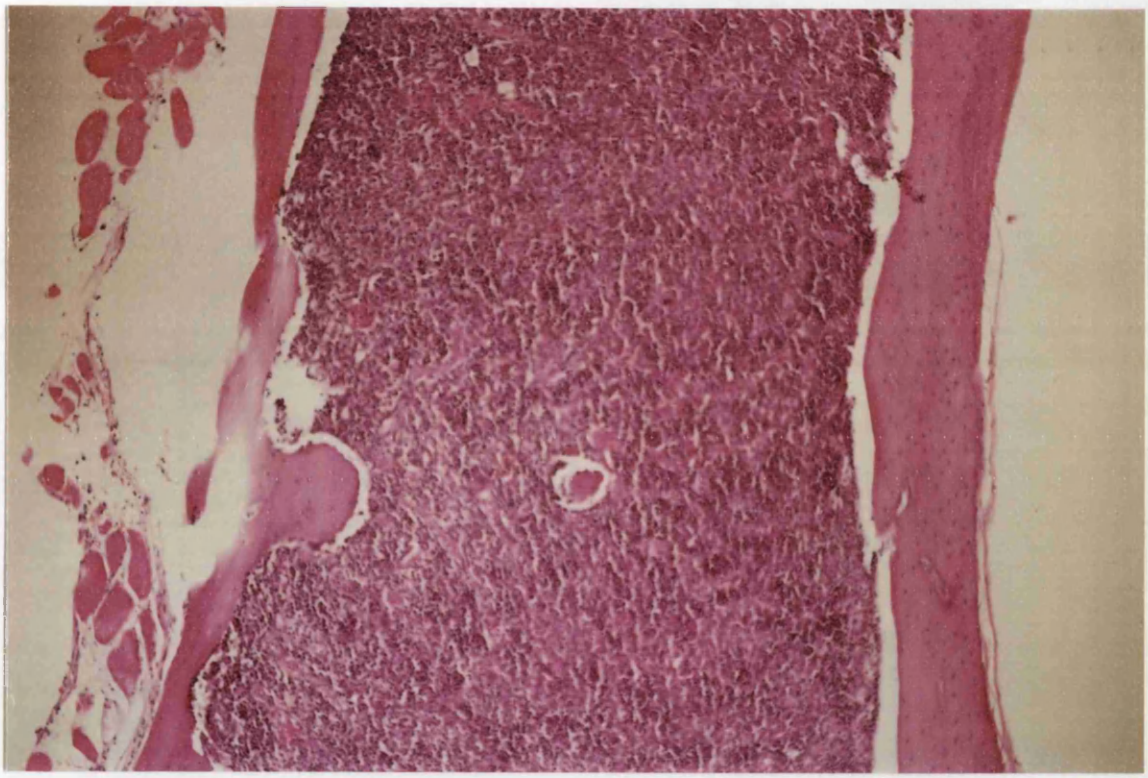


Figure 6.6 Bone marrow infiltration by leukaemia

Low power view of a femur from an animal with lymphoproliferation. Cellularity is greatly increased with few fat spaces. Bone trabeculae are reduced in number.



In some cases with splenomegaly, the spleens were cut in half and the cut surface was touched onto a slide (spleen dabs). One such preparation is shown from a leukaemic animal in Figure 6.7. In this example, the lymphoid cells are large and primitive. Many appear as smudge cells, typically seen in CLL. In intact cells there is little cytoplasm. In animals with very advanced disease, there was macroscopic evidence of infiltration of spleen, liver, lymph nodes and kidney. In Figure 6.8 a sample from an enlarged kidney is shown. In the upper part of the low power view some glomeruli remain, however, extensive tumour infiltration is shown in the lower part of the field. At high power the infiltrate is seen to consist of large lymphoid cells, similar to those seen in spleen (Figure 6.3). Finally, Figure 6.9 shows a high power view of an area found to be infiltrated by lymphoid tissue in the intestinal wall of a leukaemic mouse. This particular field shows the heterogeneous nature of the lymphoid infiltrate. There are cells of varying sizes and morphology, from small relatively mature lymphocytes, to large, nucleolated cells. Debris from dead and dying cells is also seen, re-emphasising the rapid turnover of the tumour.

As shown previously (chapter 5), in 3^{o} BMT recipients which developed leukaemia (groups 4M and 4I), evidence of residual male donor cells persisted in the bone marrow at the time of analysis. The result of Southern blotting for group M is repeated in Figure 6.10. The WBC was elevated in 8 of 12 animals in the SCF/IL-11/MIP-1α group (4M), but was still normal in the SCF/IL-11 (3M) and unmanipulated BMT (2M) groups. As shown in Figure 6.10, hybridisation to the Y specific probe was clearly demonstrated for 5 animals from group 4M confirming the persistence of male donor cells in bone marrow. It was, however, impossible to state with certainty, in these animals, whether the persistent male signal was from residual normal haemopoiesis or from leukaemic cells. Transplantation from these groups (i.e. 4M and 4I) (which were obviously leukaemic) into sublethally irradiated female animals,

Figure 6.7 Spleen dab from an animal with leukaemia

High power views of a spleen dab from an animal with lymphoproliferation.

In this preparation cellularity is greatly increased.

The majority of the cells are lymphoid blast cells with many smudge cells.

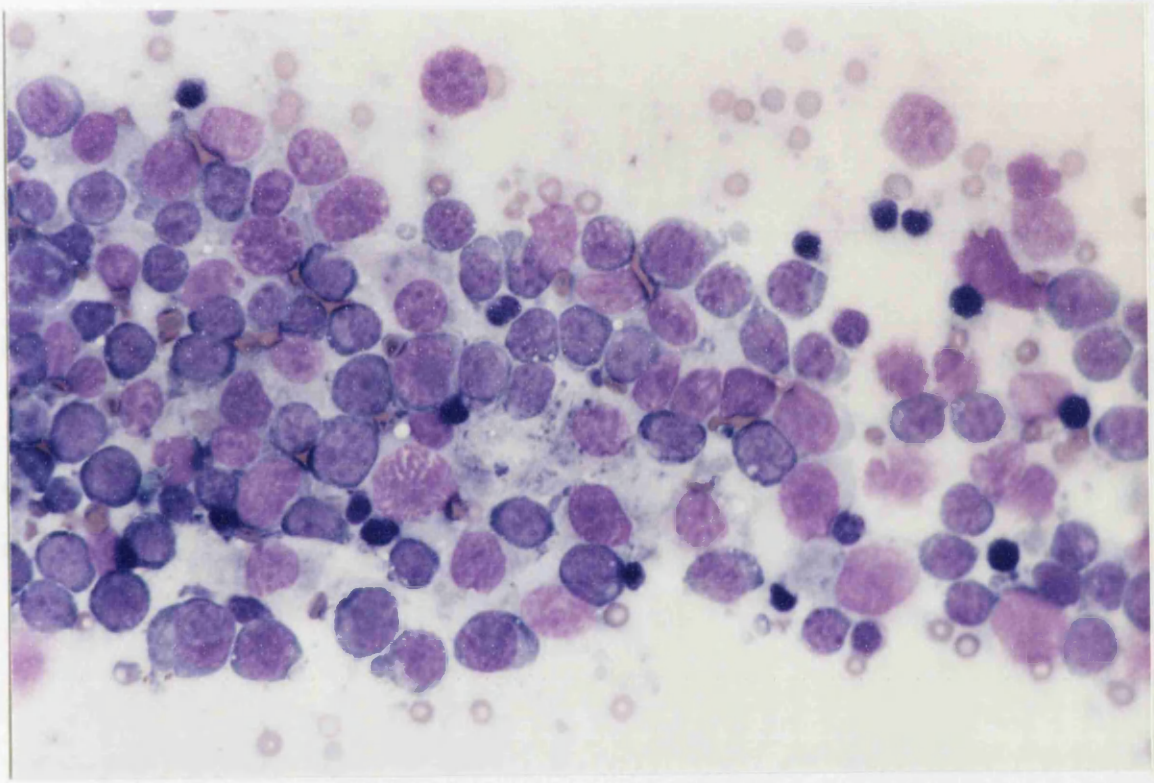


Figure 6.8 Kidney infiltration by leukaemia

Low power view of kidney from an animal with lymphoproliferation. In the upper part of the field a few remaining normal glomeruli can be seen, however, in the lower part there is evidence of lymphoid infiltration.

At high power the lymphoid cells appear pleomorphic with both small mature lymphocytes and more primitive "blasts" with nucleoli.

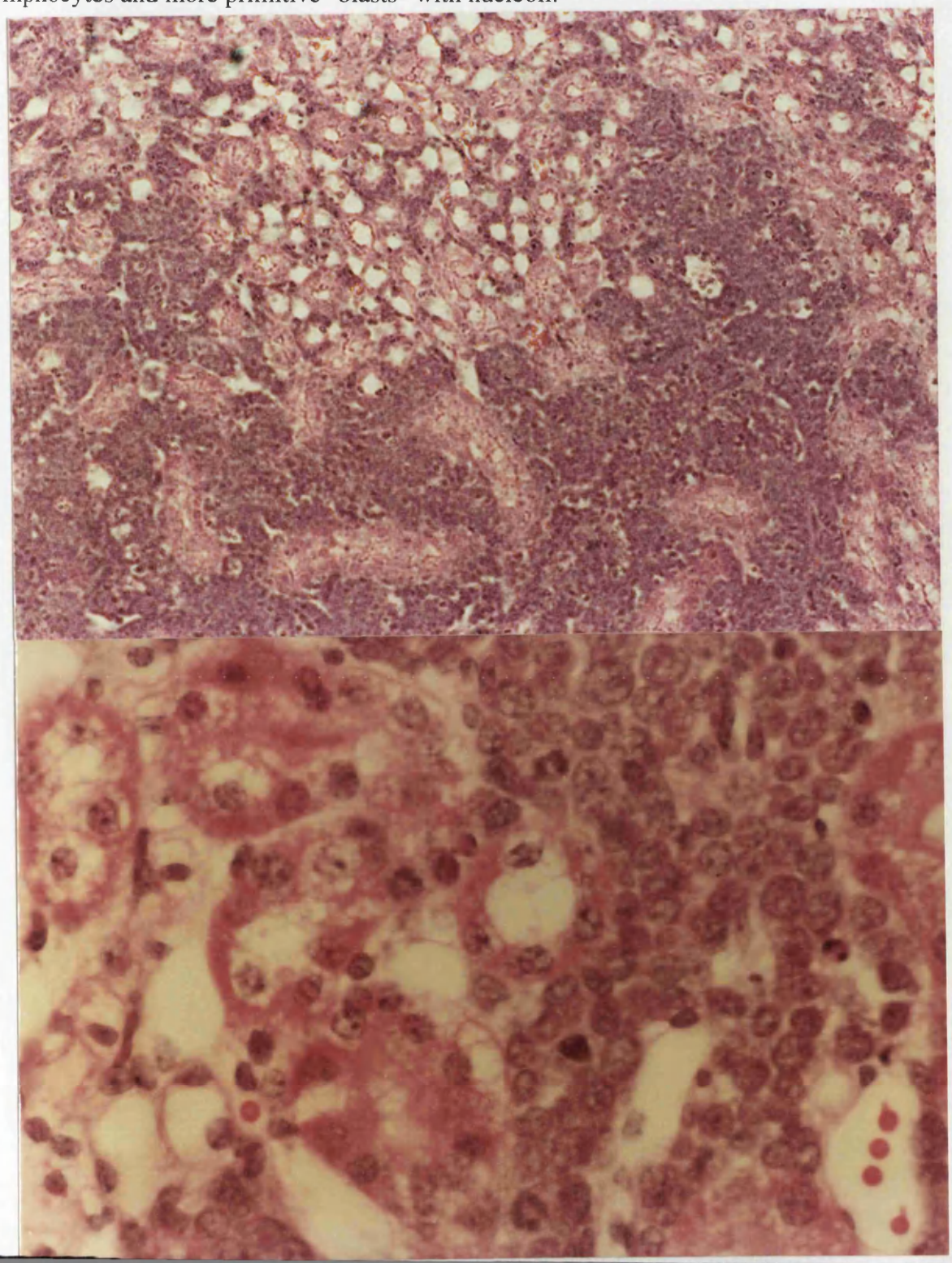


Figure 6.9 Infiltration of the intestinal wall by leukaemia

High power view of an area of lymphoproliferation in the intestine. The lymphoid cells are pleomorphic showing small mature lymphocytes and large immature lymphoblasts which are nucleolated.

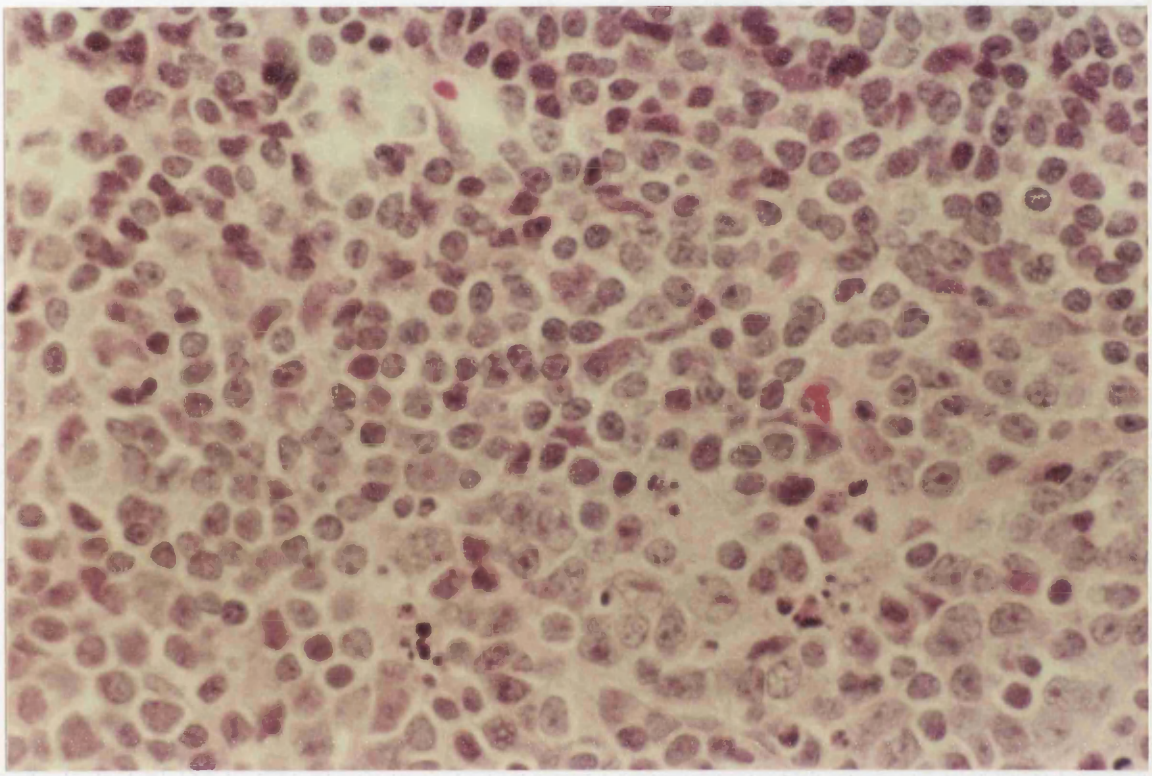
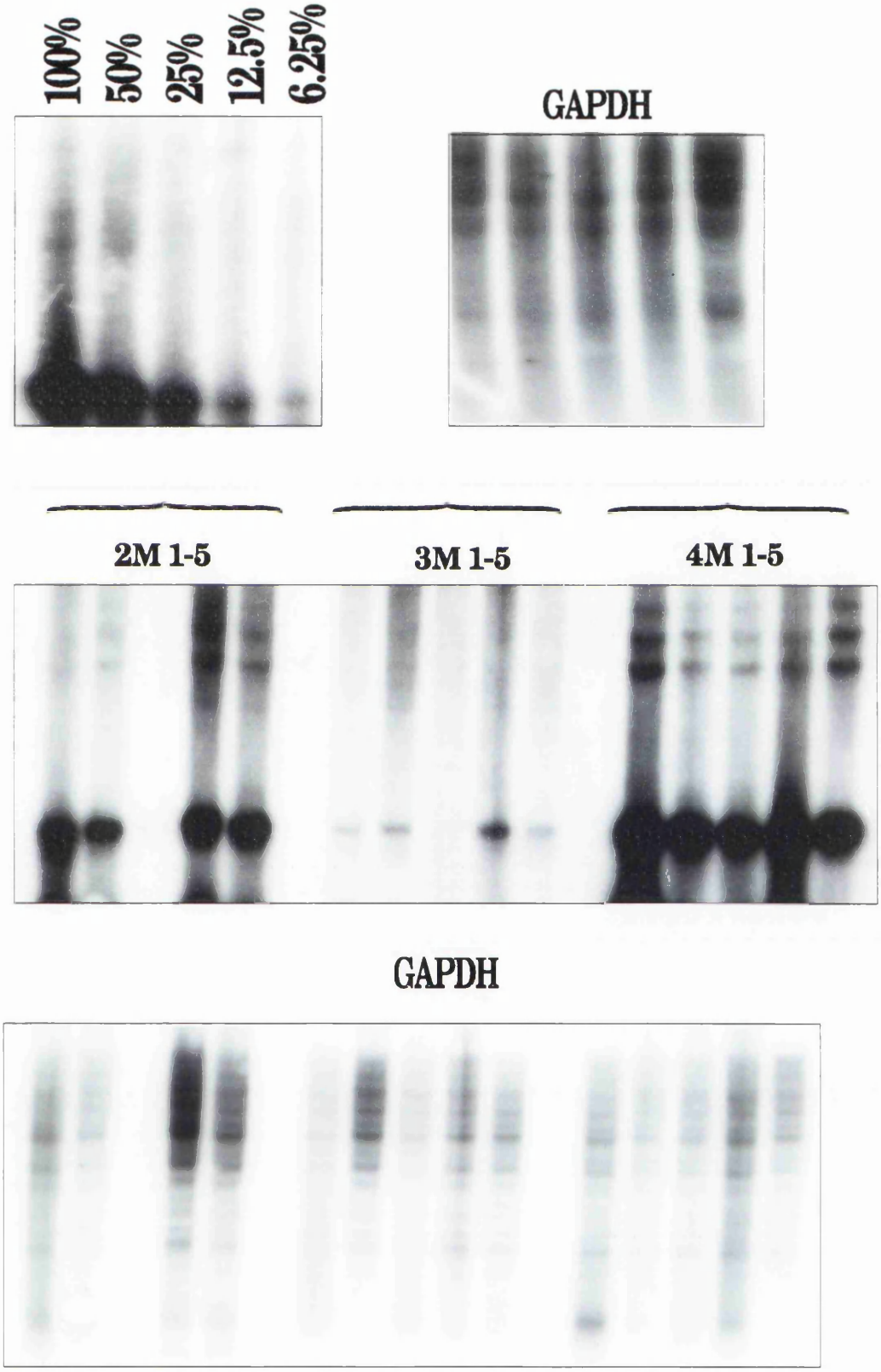


Figure 6.10 Assessment of donor engraftment in group M animals at three months following 3^{o} BMT

Genomic DNA was extracted from five randomly selected animals from each experimental group (i.e. group 2, UM BMT; group 3, SCF/IL-11 BMT; group 4, SCF/IL-11/MIP BMT). The top panels show the titration of male DNA and GAPDH loading control. The second panel shows results for test DNA hybridised to the Y specific probe and the third panel the corresponding GAPDH controls.


100%
50%
25%
12.5%
6.25%
GAPDH
2M 1-5
3M 1-5
4M 1-5
GAPDH

with Southern blotting and / or FISH, both for a Y specific probe, performed when possible on both marrow and spleen, was therefore required, to prove whether the leukaemia had arisen in cells of male or female origin. If sublethally irradiated female recipients developed a leukaemia, then the presence of only a female signal would confirm that the leukaemia was female, whereas the presence of a male signal in the majority of cells would confirm that the leukaemia was male (see later for results of these experiments). The reason for looking both at marrow and spleen is that, in mice, CD5 positive CLL usually progresses from a clonal proliferation localised in peritoneum, to the lymph nodes, spleen and finally marrow. In these animals we had already seen evidence of extramedullary haemopoiesis in the spleen, therefore it was possible that circulating peripheral leukaemic cells were coming from spleen rather than bone marrow.

Pathology of the leukaemic animals suggested that this was a lymphoid malignancy. The disease appeared to become more aggressive as it advanced, in keeping with CLL. The widespread infiltration of a variety of tissues was also suggestive of CLL. To confirm that the leukaemia was indeed CLL, immunophenotyping was performed.

CD5 positive B cells are implicated in the pathogenesis of CLL. In addition to expressing CD5, these cells express low amounts of surface immunoglobulin (IgM) (Burrows *et al.*, 1993). For this reason, immunophenotyping was performed using two markers for B cells, CD5 and IgM, to confirm that the disease was originating from CD5 positive B cells and therefore was likely to be CLL. Cells which showed double labelling for these two markers would be typical of B-cell CLL (i.e. CD5+/sIgM+). For completeness cells were also labelled for two myeloid antigens, Gr-1 and c-kit. Gr-1 is present on mature

myeloid cells of the granulocytic lineage, whereas c-kit is present on more immature myeloid stem cells.

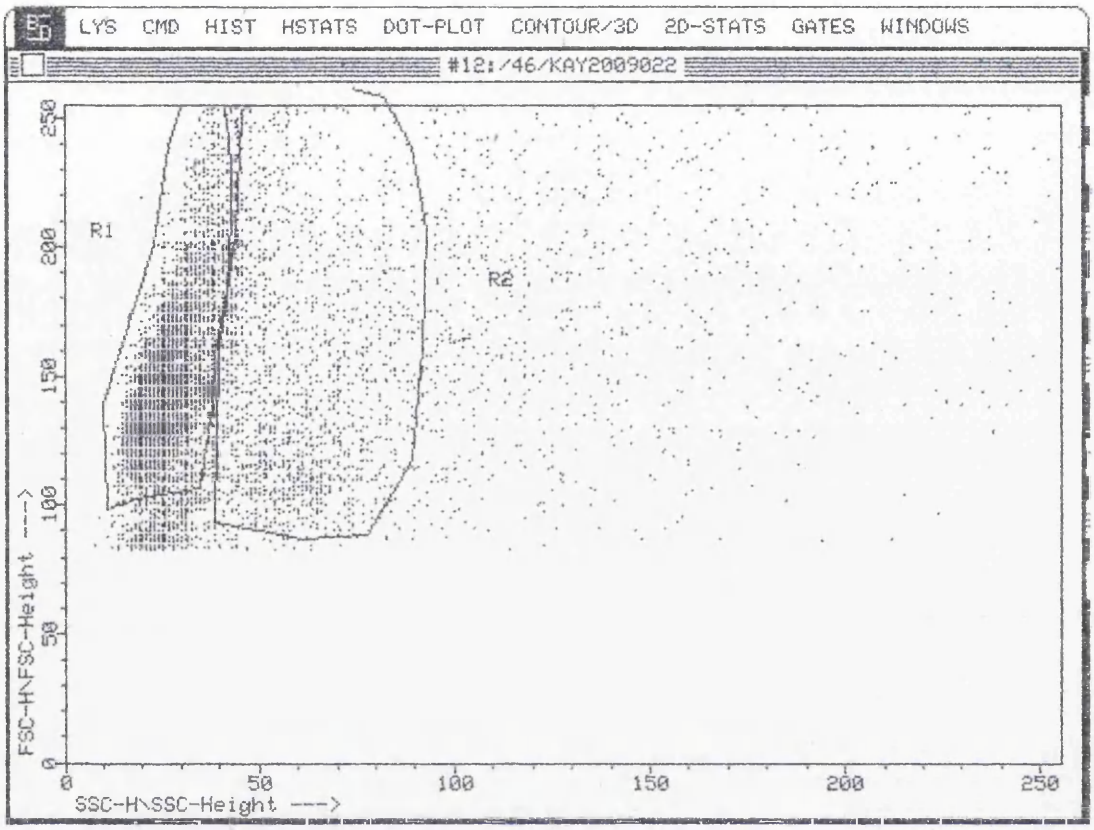
Figures 6. 11 and 6.12 show the results of immunophenotyping for the markers mentioned above. Population R1 was gated to represent lymphocytes whereas R2 represented larger more granular cells. In Figure 6.11 the cells were taken from the spleen of a normal control animal. The top line of dot plots is for population R1, the lower line for population R2. In the first panel the cells are stained with an irrelevant control antibody to allow the gates to be set accurately. The second panel shows cells labelled with both PE-CD5 and FITC-IgM. In the lymphocyte population two clear populations are seen, those which stain with CD5, which are likely to represent normal T cells and cells which stain with IgM, which are likely to represent normal B cells. Few cells show double marking for both CD5 and IgM. There is little marking for either Gr-1 or c-kit. Figure 6.12 a-c shows results for 3 animals which were developing leukaemia. In these cases, although leukaemia was definitely present, it was not at an advanced stage. For all three cases, the dot plots show a significant proportion of cells in the lymphocyte window which show double marking for both CD5 and IgM, confirming the presence of CD5+ B cells, typical of the cell type in B-cell CLL. The dot plots are very similar for all three animals presented here and have been identical in analysis of other groups with leukaemia, confirming that there was only one form of leukaemia arising under the experimental conditions used in this study.

In the previous chapter, results were presented which showed that when tertiary BMT recipients showed evidence of leukaemia, then, if their bone marrow was pooled and used to transplant further lethally irradiated animals, these would develop leukaemia and die soon after BMT (Figure 5.23). Since a radiation dose of 12.125Gy may cause death from the effects of radiation on

Figure 6.11 Flow cytometric analysis to detect CD5/IgM positive B cells

Any animals which showed serious signs of malaise were sacrificed. If either the FBC revealed abnormalities suggestive of lymphoproliferation or the spleen was found to be enlarged, the spleen was removed, half was processed for pathology and the remainder was used for antibody labelling. Cells were double labelled for CD5 and IgM and for Gr-1 and c-kit. In the top panel R1 represents the "lymphocyte population" and R2 the "granulocyte population". In the lower panel the top row is for population R1 and the lower row for population R2. The left hand results are for an irrelevant IgG antibody, the middle for CD5 and IgM and the right for Gr-1 and c-kit. In this example for a normal animal there are few if any cells which double label for CD5 and IgM.

FL1 represents fluorescence for FITC-conjugated antibodies, either IgM or c-kit whilst FL2 represents fluorescence for PE-conjugated antibodies, either CD5 or Gr-1.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R1
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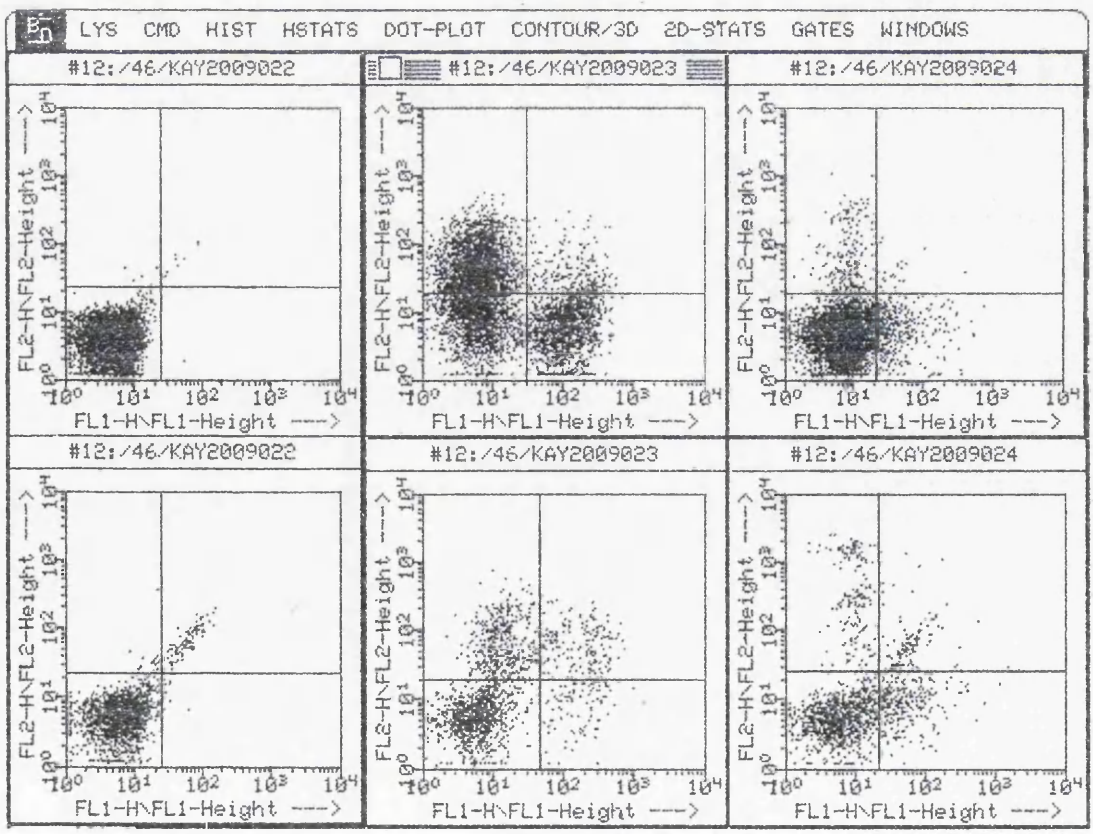
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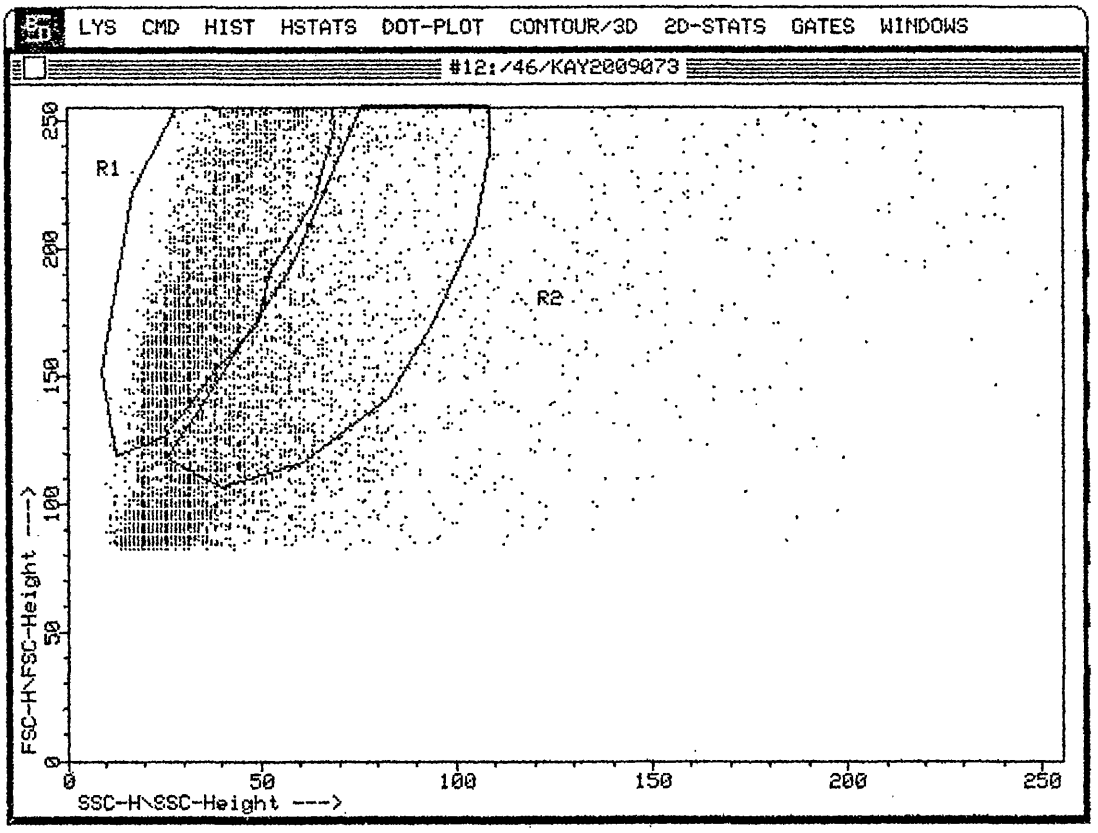
FL2-H\FL2-Height --->
FL1-H\FL1-Height --->

Figure 6.12 Flow cytometric analysis to detect CD5/IgM positive B cells

At the time of analysis this animal (group 4**I**) was found to have a greatly enlarged spleen, an elevated WBC and reduced Hb and Platelets. As shown in the figure a significant proportion of the spleen cells showed double labelling for CD5 and IgM. In this case the primary transplant was with cells expanded with SCF, IL-11 and MIP-1α.

The following two figures show results for a further two animals which showed signs of lymphoproliferation at the time of analysis. Both these cases were from group 4**I**.

FL1 represents fluorescence for FITC-conjugated antibodies, either IgM or c-kit whilst FL2 represents fluorescence for PE-conjugated antibodies, either CD5 or Gr-1.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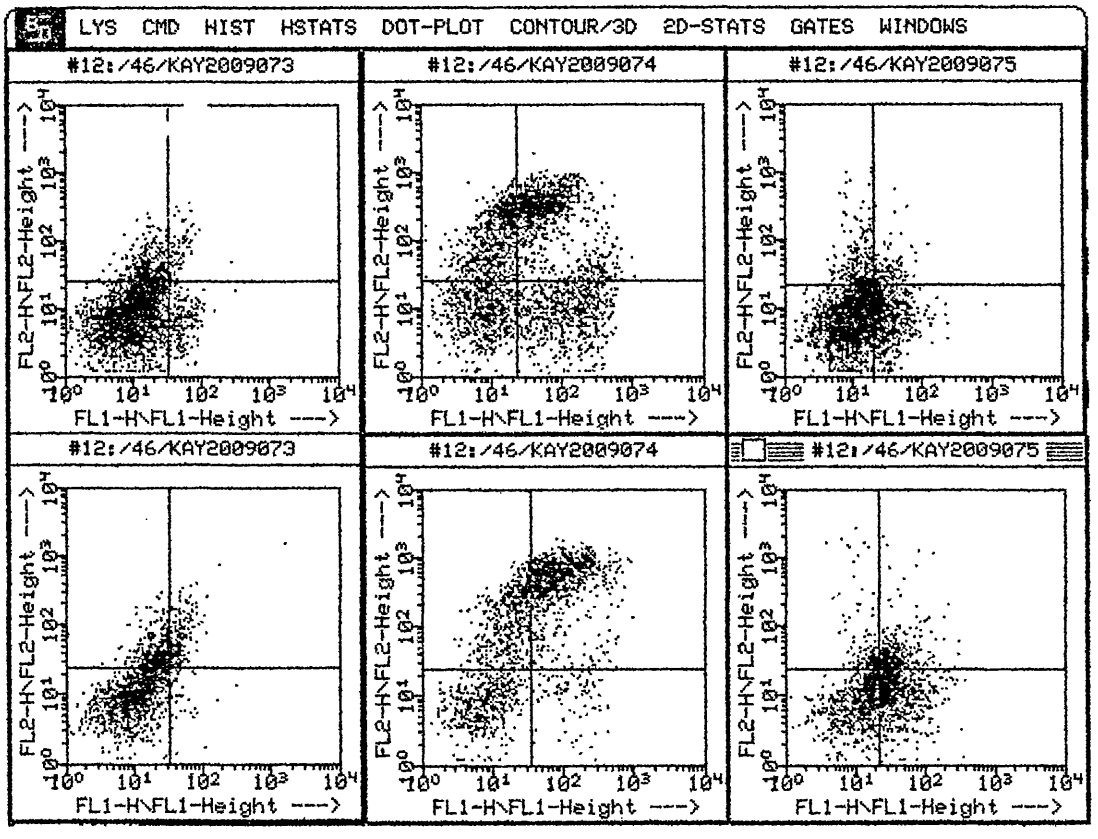
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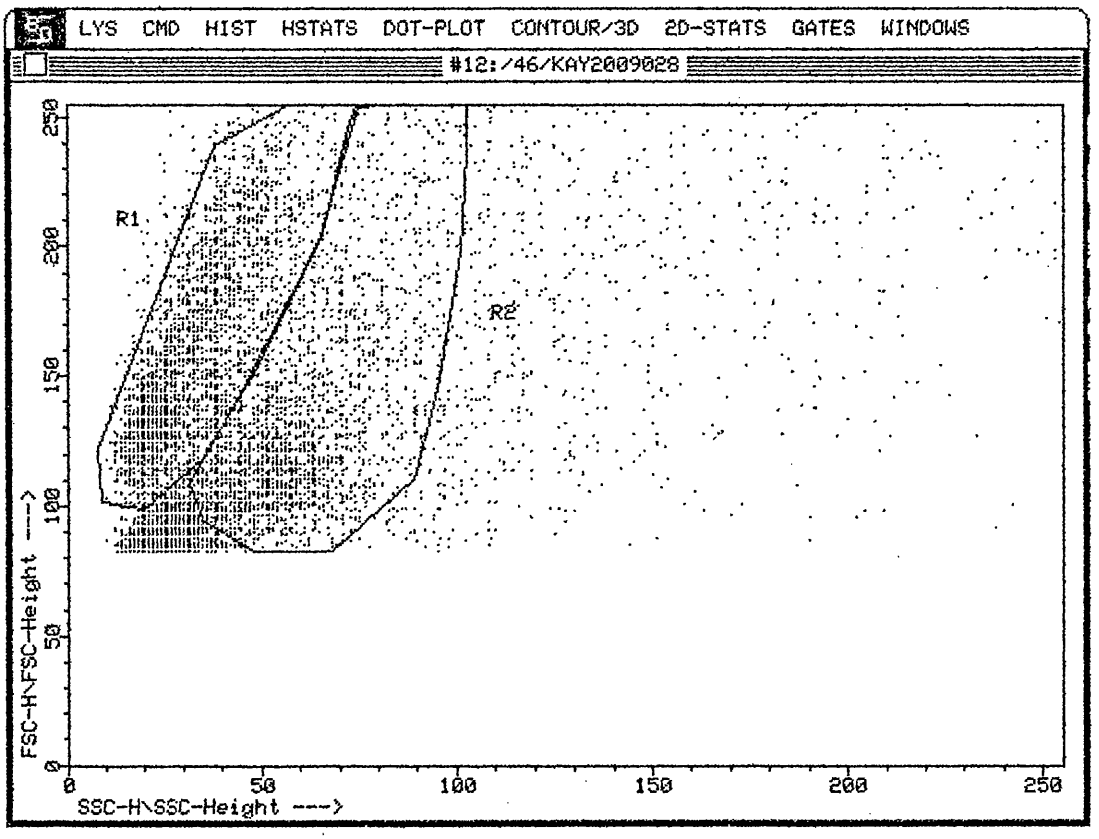
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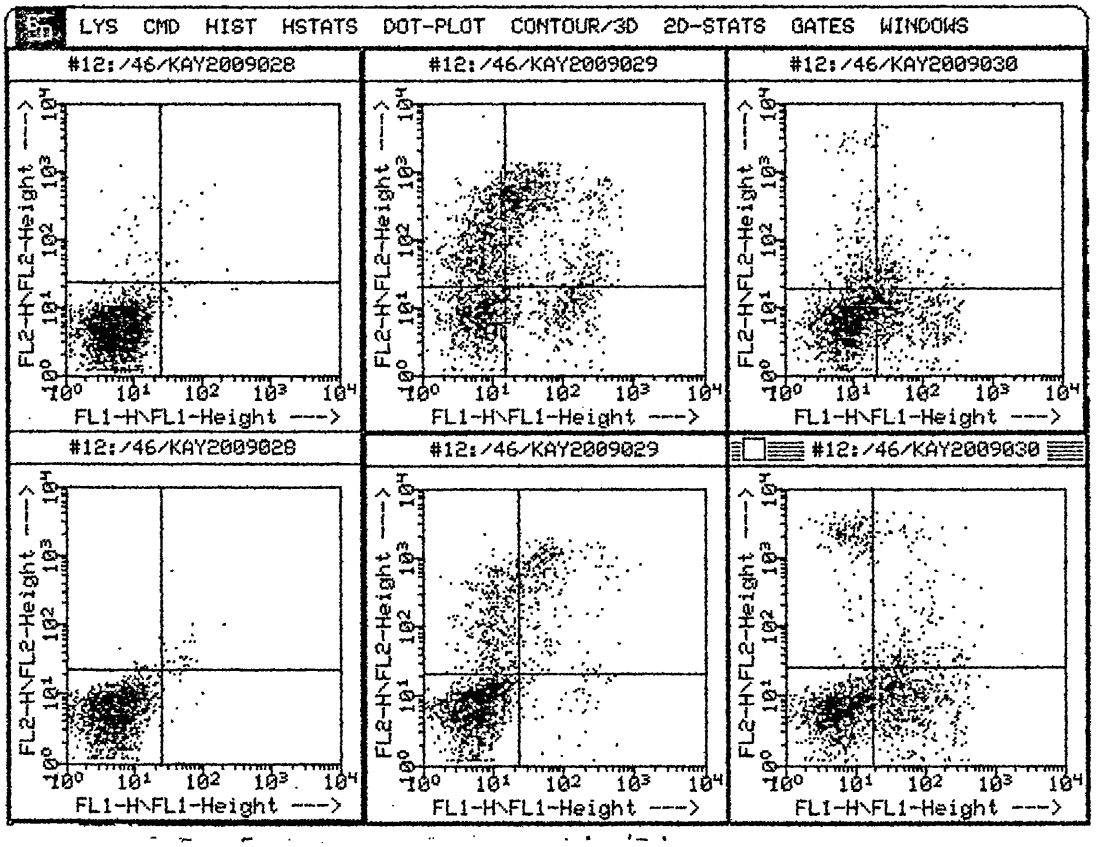
R1
R2
FSC-H\FSC-Height --->
SSC-H\SSC-Height --->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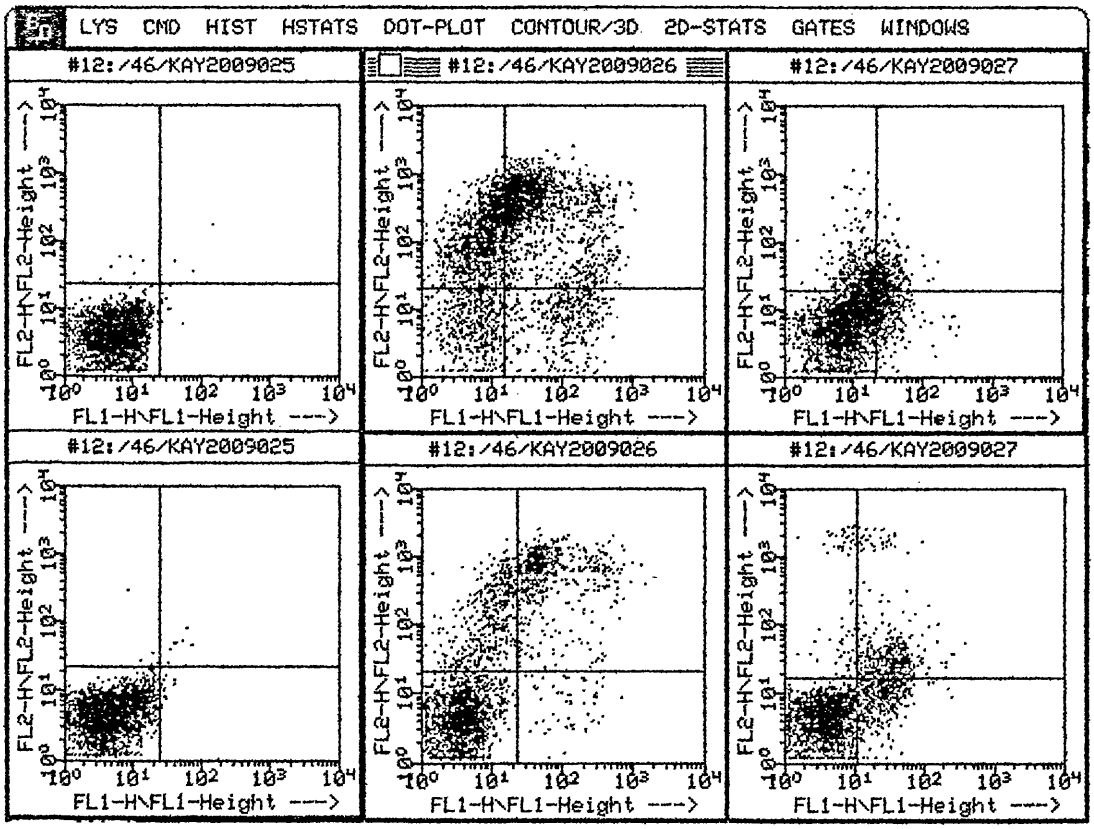
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non-haemopoietic tissues or from myelosuppression it was necessary to prove that in these animals the leukaemia had been the direct cause of death. Further transplantation experiments were therefore carried out to determine whether the leukaemia was transplantable following sublethal doses of radiation. The bone marrow cells were pooled from tertiary BMT recipients from group M and I. Mice were given only 2Gy of radiation and were then injected intraperitoneally with 10^6 cells per mouse. There were 5 animals in each group (i.e. 2MIP1-5, 3MIP1-5, 4MIP1-5 and 2IIP1-5, 3IIP1-5, 4IIP1-5, IP=intraperitoneal). Table 6.1 shows the survival of these groups of animals to 15 weeks following transplantation. The group of animals transplanted with cells pooled from group 4M (SCF/IL-11/MIP-1α expanded BMT) became unwell and 4/5 died within 4 weeks of transplantation. Since these animals received only 2Gy radiation, these deaths must have been due to the transplantation of leukaemic cells. To confirm that this was indeed the case, the remaining animal in this group, which still appeared healthy, was sacrificed and analyses were performed for pathology, FBC, FISH and immunophenotyping. These confirmed that this animal had developed B-cell CLL. At 15 weeks following transplantation, one animal in group 3MIP looked unwell and was therefore sacrificed and analyses performed for FBC, immunophenotyping, FISH, cytogenetics and Southern blotting. All remaining animals in this experiment (i.e. group 2MIP and 3MIP) were alive and well at 15 weeks after transplantation. The animals transplanted IP with pooled cells from groups 2I, 3I and 4I were well until week 15. This probably reflected the fact that the leukaemia was less advanced in groups 3I and 4I at the time of the transplant, therefore it had taken longer to appear in recipient animals. However, by 15 weeks post transplantation, 2 animals in group 4I showed obvious splenomegaly. These were sacrificed and analyses performed for FBC and Southern blotting.

Table 6.1 Demonstration that the lymphoproliferative condition was transplantable

Bone marrow was pooled from groups 2M, 3M, 4M, 2I, 3I, 4I at the time of analysis and used to inject groups of 5 animals by intraperitoneal injection. The mice were given only low dose irradiation (2Gy) to facilitate engraftment but without causing bone marrow suppression. The results show survival at 4, 10 and 15 weeks following transplantation.

Table 6.1

Code	Survival at 4 weeks (%)	Survival at 10 weeks (%)	Survival at 15 weeks (%)
2MIP (n=5)	100	100	100
3MIP (n=5)	100	100	80**
4MIP (n=5)	20*	0	0
2IIP (n=5)	100	100	100
3IIP (n=5)	100	100	100
4IIP (n=5)	100	100	60***

* At 4 weeks the remaining animal in group 4MIP showed signs of malaise and was sacrificied.
**At 15 weeks one animal in group 3MIP appeared unwell and was sacrificed
*** At 15 weeks 2 animals in group 4IIP showed signs of splenomegaly and were therefore sacrificed.
*FBC- WBC 22.1 x 10^9/L, haemoglobin13.0 g/dl, platelets 552 x 10^9/l;
**WBC 18.4, haemoglobin 10.6, platelets 239;
***WBC 57, haemoglobin 8.2, platelets 388,
WBC 11.9, haemoglobin 5.5, platelets 331
Abbreviation: IP, intraperitoneal

A number of samples from the above experiments were analysed in collaboration with the MRC Radiobiology Unit in Oxon, UK., to establish whether the leukaemia was male, therefore derived from the original cell innoculum, or female, derived from earlier BMT recipients.

Southern blotting results were available for 7 individual animals from group 4U (4^{o} serial BMT, 12.125Gy, from group 4I), one animal which developed leukaemia in group 3MIP at 15 weeks of follow-up, (2Gy radiation and IP transplant of pooled marrow from group 3M) and 2 animals from group 4IIP again at 15 weeks of follow-up. These results are shown for hybridisation to the Y specific probe, together with GAPDH loading control, in Figure 6.13. As demonstrated, haemopoiesis was either fully female or <5% male, confirming that the leukaemia, after serial passage, was female, and had, therefore, arisen in an earlier female recipient.

The animals chosen for these definitive experiments were selected because the donor animals were already showing evidence of advanced leukaemia and therefore, when transplanted into either lethally irradiated or sublethally irradiated female animals, would clearly demonstrate whether leukaemia, when it arose in recipients was male or female derived.

Results of FISH, performed by Katherine Harper (MRC, Oxon), using a Y chromosome specific probe, are shown in Table 6.2 and Figure 6.14. In these animals with advanced leukaemia, FISH confirmed that haemopoiesis was predominantly, or exclusively female. Gwyneth Watson (MRC, Oxon) kindly agreed to perform cytogenetic analyses on some representative samples from leukaemic animals. These are shown in Table 6.3 and Figure 6.15. These confirmed, firstly, the presence of 2 marker chromosomes which were

Figure 6.13 Assessment of donor engraftment in 7 selected quaternary BMT recipients (group 4U) and 3 animals transplanted with leukaemic cells following 2Gy irradiation

Genomic DNA was extracted from the bone marrow and / or spleen of selected animals (4U1-5, 12 and 13, 4IIP1 & 2 and 3MIP1). The top panel show the titration of male DNA and hybridisation of all the samples with the Y specific probe. The second panel shows results for the corresponding GAPDH loading controls.

Y titration
100%
10%
5%
2.5%
1%
0.1%
0%
4U1-5
4U12F
4U12S
4U13F
4U13S
4IIP1F
4IIP1S
4IIP2F
4IIP2S
3MIP1F
3MIP1S

Y titration
100%
10%
5%
2.5%
1%
0.1%
0%
4U1-5
4U12F
4U12S
4U13F
4U13S
4IIP1F
4IIP1S
4IIP2F
4IIP2S
3MIP1F
3MIP1S

Table 6.2 FISH results for quaternary BMT recipients and for sublethally irradiated BMT recipients

The Y chromosome paint JB16/3 and the female specific probe DXWas70 were used to label interphase or metaphase preparations as described in materials and methods. The results show the percentage of male cells scored for the Y paint, or the percentage of female cells in which 2 X chromosomes were definitely present for the female probe.

Table 6.2

Code	FISH % of cells with Y chromosome	FISH % of cells with 2X chromosomes
2V1F	>90	not done
3MIPS	0	95
4U1F	0	92
4U2F	0	not done
4U3F	0	not done
4U4F	1	83
4U5F	3	86

F=femur, S=spleen

Figure 6.14 FISH results for control male cells and for selected animals with and without leukaemia

A. This represents FISH performed with the Y specific JB16/3 paint on a metaphase preparation from a normal control male B6D2F1 mouse. A single Y chromosome is clearly seen.

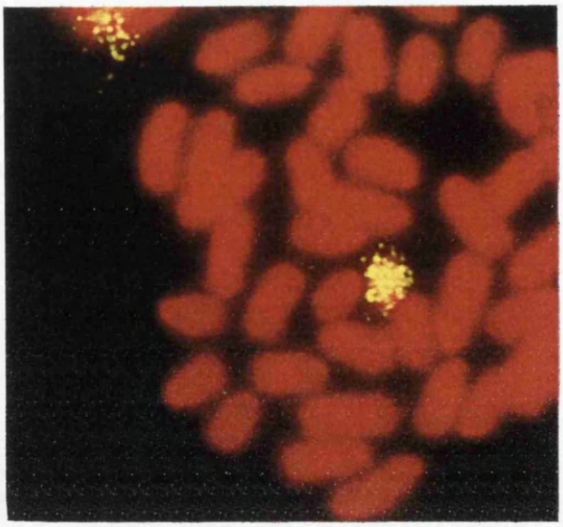
B. This represents FISH performed with JB16/3 on an interphase preparation from a non-leukaemic animal from group 3I. The majority of cells are male.

C. This represents FISH performed with JB16/3 on a metaphase preparation from a leukaemic animal. Although 40 chromosomes are present there is no Y chromosome confirming that the leukaemia must be female.

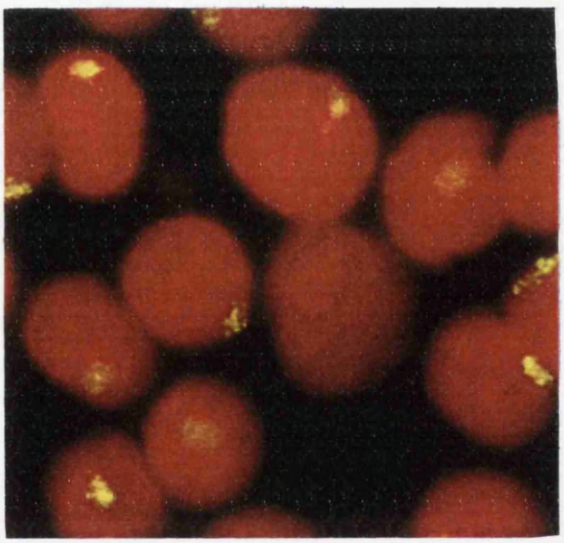
D. This represents FISH performed with the female probe DXWas70 on a metaphase preparation from a non-leukaemic animal. There is only a single X chromosome present confirming that the cell is male.

E. This represents FISH performed with DXWas70 on a metaphase preparation from a leukaemic animal. There are 2X chromosomes present, confirming that the leukaemia was female.

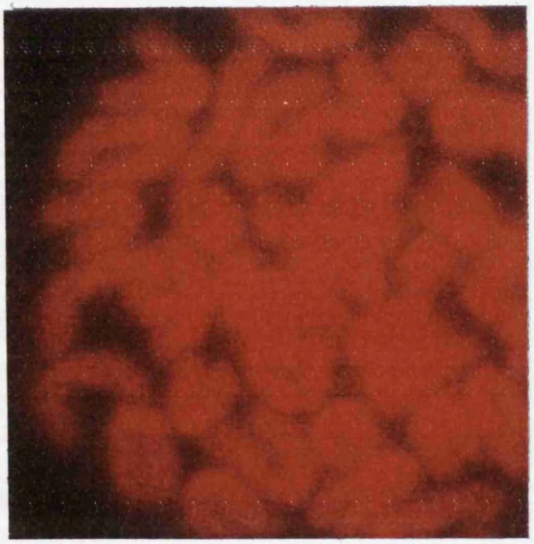
A



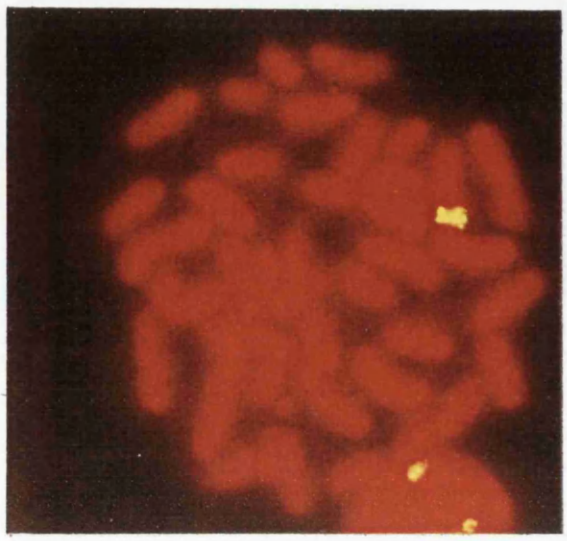
B



C



D



E

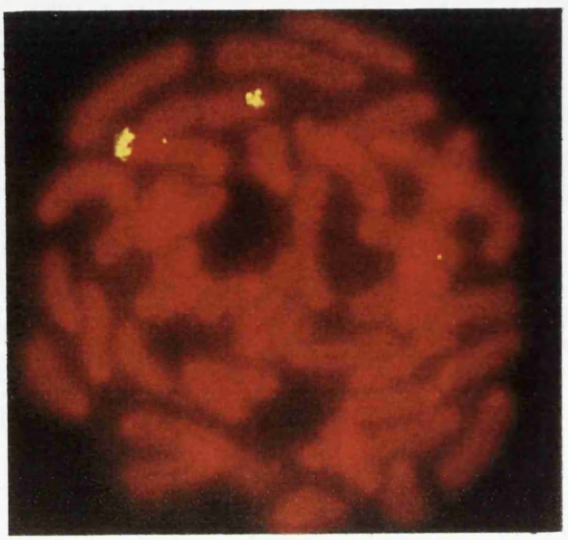


Table 6.3 Cytogenetic analysis for quaternary BMT recipients and for sublethally irradiated BMT recipients

The results shown are those for a non-leukaemic animal (2V1), an animal given 2Gy irradiation prior to intraperitoneal injection of cells pooled from group 3M and 5 animals from group 4U. The 3MIP1 animal and all 5 from group 4U showed evidence of leukaemia at the time of analysis. Results show the total number of cells scored, the number with a Y chromosome or with no Y but with either a single or 2 marker chromosomes.

Table 6.3

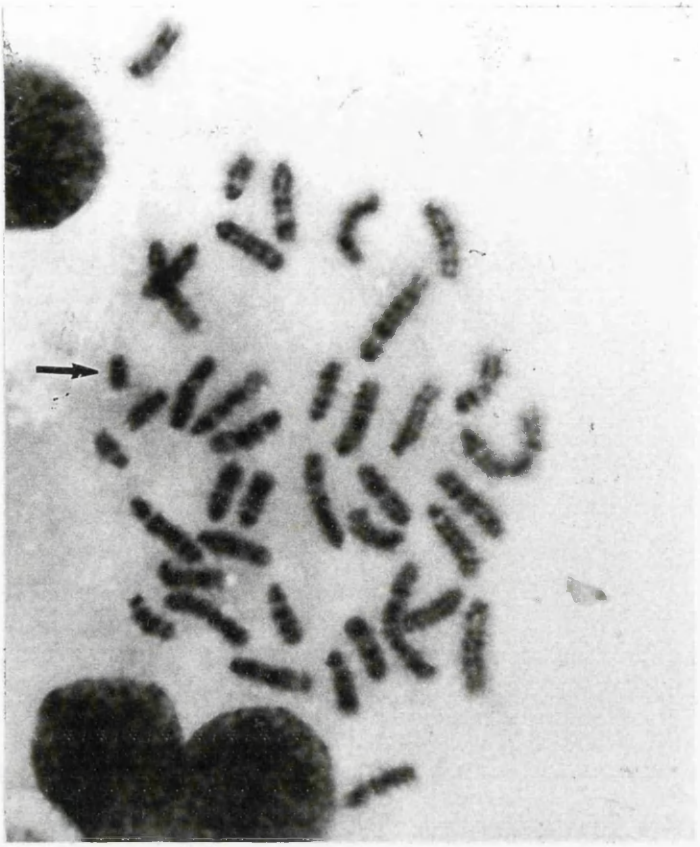
Code	Cells scored	Cells with Y chromosome	Cells with 1 marker chromosome & no Y	Cells with 2 marker chromosomes & no Y
2V1F	10	10	0	0
3MIPS	10	3	2	0
4U1F	60	1	2	41
4U2F	13	0	0	13
4U3F	15	0	0	15
4U4F	5	0	0	5
4U5F	7	0	2	5

F=femur, S=spleen

Figure 6.15 Examples of metaphase spreads showing the presence of marker chromosomes

Top: This represents a metaphase spread from a non-leukaemic animal from group 2V (2V1). There is a single Y chromosome (arrowed), showing that the cell is male, but no marker chromosomes.

Bottom: This represents a metaphase spread from a leukaemic animal (4U3). There is no Y chromosome, showing that the cell is female, but 2 marker chromosomes are present (arrowed).



identical in all animals examined and, secondly, the absence of Y chromosomes in the majority of samples examined.

These results, taken together, confirm that the leukaemia observed was CLL, arising in CD5 positive, surface IgM positive, B cells. The leukaemic cells tested were female and, therefore, the leukaemia must have arisen, at some point, in host B cells in recipient animals, rather than in the cells cultured *ex vivo*. In all animals for which full data were available, the same type of leukaemia was observed with the same type of marker chromosomes. Without clonality studies, however, it is impossible to show how many single leukaemias occurred in this study.

Discussion

In the adult mouse normal B cell populations include conventional B cells (B2 cells) and CD5 (ly-1) positive B cells (reviewed by Burrows *et al.*, 1993). CD5 positive B cells show a distinctive tissue distribution in adult mice and are most commonly found in the peritoneal cavity. Only small numbers are present in spleen and very few in lymph nodes. Progenitors of CD5 positive B cells may be found in relative abundance in fetal liver and spleen but are virtually absent from adult bone marrow. In addition, the CD5 positive B cell subset has been shown to contain cells at different stages of activation and maturation. These observations prompted speculation that CD5 positive B cells belong to a different subset of the B cell lineage than conventional B cells.

Work performed by Hayakawa et al, in 1985, supported this hypothesis (Hayakawa *et al.*, 1985). This group compared cell populations from various donor organs, from newborn and adult mice, for their ability to reconstitute particular B cell subsets when transplanted into lethally irradiated recipients. These experiments showed, that, whereas progenitor cells present

in newborn liver and spleen and bone marrow from all age groups, were able to reconstitute conventional B cells, adult bone marrow and spleen lacked the ability to reconstitute CD5 positive B cells. These cells were readily reconstituted if the transplant was with either newborn liver or cells from adult peritoneum (Hayakawa *et al.*, 1985). These findings were interpreted to suggest that B lineage progenitor cells present in adult bone marrow may give rise to conventional B cells, but not to CD5 positive B cells, and that separate progenitor cells responsible for the generation of CD5 positive B cells must be transplanted to reconstitute that lineage.

There are several other examples inconsistent with the view that a single HSC is the progenitor of all cells in the haemopoietic system relating to both erythroid differentiation, expression of fetal versus adult haemoglobin (Fantoni *et al.*, 1967) and to T cell development (Ikuta & Weissman, 1991).

Since the initial report (Hayakawa *et al.*, 1985), further studies now suggest the presence of three B cell lineages, B-1a cells (CD5 positive B cells), B-1b cells and conventional B cells (Kantor *et al.* , 1992b; Kantor & Herzenberg, 1993). According to this work, conventional B cells develop late in ontogeny and appear to be replenished from progenitors present in adult bone marrow by differentiation of non-rearranged progenitor cells; B-1a cells develop early in ontogeny and appear to maintain their numbers by self-replenishment (i.e. by division of fully mature B-1 cells); and B-1b cells, which share the property of self-replenishment with B-1a cells, may also develop from progenitors in adult bone marrow. Conventional B cells may be distinguished from B-1 cells by their anatomical localisation, functional characteristics and cell surface markers. B-1a and B-1b cells appear to be similar to each other, except that only B-1a cells express CD5. Within the limits of the experiments performed, it appeared that each of the B-1 cell populations could replenish itself but not the other (Kantor *et al.*, 1992b; Kantor & Herzenberg, 1993).

Recent cell transfer studies confirm the finding by Hayakawa that adult bone marrow contains very little progenitor activity for B-1a cells, but they do suggest that reconstitution of B-1a cells by transplanting adult bone marrow is possible, at least at a low level, (Lalor *et al.*, 1989; Stall *et al.*, 1992; Kantor *et al.* , 1992a; Kantor *et al.*, 1992b; Kantor & Herzenberg, 1993).

Considering my own data, if we assume that under normal circumstances CD5 positive B cells are present at very low frequencies in adult bone marrow, then following single and serial transfers of unmanipulated bone marrow cells, whether donor or recipient derived, this population would be under strong proliferative stress to replenish normal numbers. Under these circumstances it is likely that the resulting CD5 positive population is donor derived. However, for bone marrow cells cultured *ex vivo*, the number of CD5 positive B cells remaining viable following expansion with a cytokine combination chosen for its effects on myeloid progenitors, might be low or even non-existent, such that the host CD5 positive progenitor cells, already exposed to radiation, would receive an even greater stimulus to regenerate. These suggestions are, of course, hypothetical, but might explain the increased incidence of CD5 malignancy seen in the groups of mice transplanted with stem cells cultured *ex vivo*.

It is well recognised that B cell CLL originates from the CD5 positive B-1a population. In the vast majority of mouse strains, clonal populations of B-1a, CD5 positive B cells can be detected as the animals age (Stall *et al.*, 1988; Tarlinton *et al.*, 1988). Young mice generally show polyclonal populations but in older mice the number of clones decreases to one or two. In certain strains, however, these clones may be detected even in neonates (e.g. New Zealand Blacks) (Kantor & Herzenberg, 1993). CD5 positive B cells are responsible for production of autoantibodies and certain mouse strains are particularly susceptible to autoimmune disease. It is these strains which

develop clonal populations at the earliest age. These clones do not appear to occur at random, since there is repetitive usage of the same V_H and D elements within the clones of an individual animal (Rajewsky *et al.*, 1987; Tarlinton *et al.*, 1988). This suggests a selective force, perhaps related to antigenic stimulation. In the autoimmune models, therefore, perhaps an endogenous antigen stimulates clonal proliferation of CD5 positive, autoantibody producing, B cells.

In all mouse strains certain clones of CD5 positive B cells predominate with ageing. As such clones expand, the possibility of neoplastic transformation presumably increases. This has been demonstrated in a number of studies: Stall et al (Stall *et al.*, 1988) have shown that young New Zealand Black mice and old normal mice have detectable clones of CD5 positive B cells which may be detected by either Southern blotting or FACS analysis and tend to develop and grow rapidly upon transfer into irradiated or non-irradiated hosts. This rapid clonal proliferation may explain why serial transfer increased the incidence and aggressiveness of the leukaemia in our study. The cells invade in a characteristic pattern: peritoneum then spleen, lymph nodes and finally bone marrow. Both phenotype and growth pattern are very similar to human B cell CLL (Stall *et al.*, 1988). A further study by Fowlis et al (Fowlis *et al.*, 1989), demonstrated imbalance of CD5 positive B cell populations as early as one year of age in mice bred to be informative for two donor cell populations. Such populations occasionally produced transplantable leukaemia, shown to be CLL. The incidence of these leukaemias and the level of aggressiveness of the leukaemias were increased by serial BMT. Although the majority of the leukaemias were monoclonal there were instances of at least two clones coexisting for many months, or of a latent donor cell producing a distinct leukaemia after several serial transfers of the original tumour. Leukaemia was frequently observed if spleen cells from old, but

apparently healthy, donors were used for transplantation in young recipients (Fowlis *et al.*, 1989).

Clearly the B cell leukaemias observed in our own work were similar, if not identical, to those described above but occurred in a strain which is not know to be susceptible to B cell leukaemia. In mice transplanted with cultured cells, the possible absence of CD5 positive B cells in the donor innoculum, may have provided a stimulus for regeneration of this population in the female recipients. Since CD5 positive B cells are already more at risk of malignant transformation than conventional B cells, presumably because of the need for long lived self-replenishment, this may have been sufficient to induce leukaemia in relatively young animals. Having developed in one, or several, early recipient(s), the disease was then serially transplanted in pooled bone marrow, both increasing the incidence and the level of aggressiveness of the tumour until it eventually became detectable in the majority of animals transplanted with cultured cells. Alternatively, serial transfer of clonal populations of recipient CD5 positive B cells may have been sufficient, alone, to induce a transplantable leukaemia in 2 of the 3 experimental groups (i.e. SCF/IL-11 expanded BMT and SCF/IL-11/MIP-1α expanded BMT). Since pooling of bone marrow prior to each serial transfer would be likely to increase the incidence of leukaemia, eventually effecting all animals within the group, only 2 separate cases of leukaemia would be necessary to produce the findings observed. If this were the case, it is conceivable that the leukaemias in the two expanded groups occurred by chance and, therefore, that the *ex vivo* expansion, per se, had no influence on the generation of leukaemia.

Clearly the work presented in this chapter raises a number of important questions. It will be of interest to determine clonality of the B-cell leukaemia and to search for cytogenetic abnormalities in this leukaemia at different stages of development. It is likely that this leukaemia becomes more

aggressive as it progresses. This progression could be hastened by manipulating the experimental conditions used to produce a very useful laboratory model of leukaemogenesis. These results have important implications in a number of areas, both experimental and clinical. Stem cell expansion is regarded as having great clinical potential, both for transplantation studies and for gene transduction. If the generation of the leukaemia seen in these studies reflects an effect of stem cell expansion then this will have to be addressed.

DISCUSSION

In vitro assessment of the culture conditions required to promote CFU-A proliferation and expansion *ex vivo*

The early experimental work presented in this thesis was planned to investigate growth factor combinations which would induce expansion, *in vitro*, of multipotential progenitor cells. The availability of the CFU-A assay "in house" greatly facilitated these studies. Previous studies, performed by Pragnell and co-workers, had demonstrated that murine CFU-A progenitors were comparable to CFU-S day 12 in terms of their position within the stem cell hierarchy (Pragnell *et al.*, 1988). This *in vitro* assay, which is relatively simple to perform, was therefore used to measure stem and progenitor cells rather than the *in vivo* CFU-S assay, which is more cumbersome and expensive in terms of animal use.

The CFU-A assay was selected as the primary "read-out" for progenitor expansion because several workers had shown that CFU-S (therefore presumably CFU-A) were separable from HSC and appeared to be responsible for the early phase of haemopoietic engraftment following BMT (Jones *et al.*, 1990; Ploemacher & Brons, 1989). The experiments were planned to specifically target these "transient engrafting stem cells" to try to increase their numbers *in vitro*, and thereby to influence the kinetics of haemopoietic recovery when these expanded populations were used for transplantation.

A number of cytokines were selected for investigation. SCF had been identified only a short time before this project was initiated and the available information suggested that it was a potent physiological regulator of stem cell proliferation. Its unique ability to synergise with a range of other cytokines, *in vitro*, suggested that it would prove useful as one component for *ex vivo* expansion of stem cells. IL-11, which was cloned in 1990, was selected for

study because it appeared to have major effects on megakaryopoiesis in addition to erythropoiesis and granulopoiesis. This property was felt to be of crucial importance since an *in vitro* amplification of megakaryocytic precursors might be expected to influence platelet recovery following transplantation of the expanded cells.

In our hands, cytokine combinations involving SCF and either IL-11, IL-1β, G-CSF or IL-6, stimulated CFU-A amplification (Holyoake *et al.*, 1996). IL-1β was not selected for further study because its effects had been shown to be indirect by elaboration of other cytokines including IL-6 and G-CSF (Leary *et al.*, 1988). IL-11 was chosen, rather than either G-CSF or IL-6, both because CFU-A expansion appeared to be consistently slightly greater with IL-11 and because of its known effects on megakaryopoiesis. Over recent years, a number of investigators, including ourselves, have clearly demonstrated that CFU-S (or in our hands CFU-A) may be expanded *ex vivo* using SCF containing cytokine combinations. The choice of the second cytokine has proven to be less critical and a number of cytokines appear active in this respect including IL-11, IL-6, IL-1α, G-CSF and IL-1β (Neben *et al.*, 1994; Luskey *et al.*, 1992; de Vries *et al.*, 1991; Muench *et al.*, 1993).

Over a six day incubation period the combination of SCF and IL-11 stimulated an approximate 50 fold CFU-A amplification in cultures of unfractionated bone marrow. Even in the absence of cytokines, input CFU-A numbers were maintained over the six day culture period. The cultures were supplemented with DHS (25% v/v) which had been pre-tested to promote maximum colony growth in the CFU-A assay and may have contained low concentrations of cytokines sufficient to allow stem cell survival. Such maintenance of input CFU-A in the absence of cytokines was neither seen for cultures of highly purified Sca+ lineage negative cells in the presence of DHS nor for unfractionated bone marrow cultured under serum free conditions. These results would suggest that the combination of DHS and supporting

accessory cells was sufficient to allow survival of CFU-A progenitor cells. Whether these conditions also allowed survival of LTRC was never formally tested.

On the basis of a single experiment, the degree of CFU-A amplification was unaffected by stem cell purification. In this experiment day 2 post 5-FU bone marrow had a cloning efficiency in the CFU-A assay of 4% and amplified approximately 800 fold over 6 days in the presence of SCF and IL-11. The equivalent values for unfractionated bone marrow would be 0.2% and 50 fold. Therefore the level of enrichment for CFU-A was 20 fold and the increase in amplification potential 16 fold. This suggests firstly that the presence of accessory cells is not necessary for CFU-A amplification and secondly that accessory cells present in unfractionated bone marrow do not elaborate sufficient levels of inhibitory cytokines to prevent amplification.

Is there a role for MIP-1α?

We decided to investigate how negative regulators of stem cell proliferation might interact with stimulatory cytokines for three reasons. Firstly, there was evidence that both TGF-β and MIP-1α might act in an inhibitory or stimulatory manner depending on the culture conditions employed (Keller *et al.*, 1994; Broxmeyer *et al.*, 1990). Secondly, studies by Bodine et al suggested that although several cytokine combinations induced expansion of CFU-S in expansion cultures, in some instances this would occur at the expense of LTRC (Bodine *et al.*, 1991). In our studies we primarily wished to expand CFU-A in order to improve early engraftment post transplant and if possible we wished to achieve this aim whilst, at the same time, maintaining LTRC. If this were possible then clinical cases in whom an insufficient numbers of stem cells could be harvested might benefit from *ex vivo* expansion of their stem cells prior to transplantation. In the study by Bodine et al either IL-3 / G-CSF or IL-3 / IL-6 increased the number of both CFU-S and

LTRC over IL-3 alone. However, when all three cytokines were combined, although CFU-S expansion increased, the number of LTRC fell significantly, suggesting too great a differentiative stimulus (Bodine *et al.*, 1991). In the same report, MIP-1α, used alone, was ineffective in promoting CFU-S survival and did not alter CFU-S survival when added to IL-3 supplemented cultures. However, when MIP-1α was added to the combination of IL-3 / IL-6, CFU-S expansion fell significantly. The effect of MIP-1α was not tested with IL-3 / IL-6 / G-CSF, which had been shown to increase CFU-S but at the expense of LTRC, nor was it tested, in any combination, for effects on LTRC. In these studies cytokine combinations containing SCF were not explored and the possible role of MIP-1α was not fully elucidated. It therefore seemed logical to assess whether negative regulators, when combined with a cytokine combination known to be active in increasing CFU-A / CFU-S would affect CFU-A proliferation or the survival / expansion of LTRC. Finally, these negative regulators were selected for further study because of our group's long term interest in the control of stem cell proliferation and in MIP-1α in particular.

TGF-β and MIP-1α were assessed alone and in combination with SCF and IL-11, for effects on progenitor proliferation over 6 days expansion culture. TGF-β's effects were consistently inhibitory from the start of culture and this inhibitory effect predominated even for cultures supplemented with the stimulatory cytokines SCF and IL-11. The addition of MIP-1α alone had no effect on either cell numbers or progenitor numbers compared with medium control cultures. When added to cultures supplemented with SCF and IL-11, MIP-1α did not significantly inhibit or stimulate either the number of cells or progenitors present after six days. If any pattern was observed, the effect of MIP-1α was to increase, rather than decrease the number of progenitors present.

At the time these experiments were performed there were two factors which prompted us to continue to investigate MIP-1α in conjunction with SCF and IL-11. The first was that the cell population present at the end of culture appeared less differentiated (by morphological criteria only) in the presence of MIP-1α. The second was work presented in abstract form by Catherine Verfaillie in 1993 (Verfaillie *et al.*, 1993).

The culture system employed by Verfaillie et al differed in many ways from our own (Verfaillie *et al.*, 1993). The starting cells were from human bone marrow and were enriched to the level of CD34+ HLA-DR- (0.1-0.4% of bone marrow). These were cultured in the presence and absence of either a stromal layer or stromal derived conditioned medium. The cells were either plated in direct contact with, or separated from, the stromal layer (by a transwell insert). The cells were cultured in the absence of cytokines or in the presence of one or more stimulatory or inhibitory cytokines (e.g. IL-3, MIP-1α, TGF-β) or a combination of stimulatory and inhibitory cytokines (e.g. IL-3 and MIP-1α). The cells were cultured for 5-8 weeks before quantification of CFC and LTCIC. The main conclusion from this work was that LTCIC maintenance for 5-8 weeks required the presence of both IL-3 and MIP-1α and in addition the presence of either stroma or stroma conditioned medium (SCM). As in our own studies, TGF-β was consistently inhibitory in these experiments (Verfaillie *et al.*, 1993; Verfaillie *et al.*, 1994).

Although, as stated, these experiments differed greatly from our own, they suggested that the possible MIP-1α effect seen in our own *in vitro* experiments might be a real effect.

We next performed three consecutive experiments in which unfractionated bone marrow was cultured in the presence of SCF / IL-11 +/- MIP-1α for up to 39 days. There was no suggestion that CFC output was improved by the addition of MIP-1α or that MIP-1α prevented the cultures exhausting around day 35-40. If anything, the MIP-1α supplemented cultures

appeared less productive than the cultures without MIP-1α although this may have been because one culture of three supplemented with MIP-1α showed poor expansion from day 12 onwards.

Our next approach was to enrich day 2 post 5FU murine bone marrow for Sca+ lineage negative cells. This was performed on four occasions. These experiments proved to be expensive in terms of time and animal requirements, and produced variable levels of stem cell enrichment (204-4456 fold) and recovery (1.3-45%). This may have been the result of our inexperience in this technique. Unfortunately, although the purified Sca+ lineage negative populations were set up in expansion culture on four occasions, progenitor expansion was satisfactory in only one experiment. On this occasion, CFU-A expansion was 520 (± 130) fold in the absence of MIP-1α and 750 (± 77) fold in the presence of MIP-1α. Clearly no conclusion may be drawn from a single result. Because of our technical difficulties in purifying Sca+ lineage negative cells, no further similar experiments were performed.

Stem cell purification may have reduced our chances of demonstrating an effect of MIP-1α. Taking Verfaillie et al's finding, that stroma (or SCM) was essential for maintenance of LTCIC, into account, it is possible that by purifying stem cells we may have decreased the effect of MIP-1α in stroma free cultures, i.e. that the presence of stroma derived cells in our unfractionated bone marrow cultures may have functioned similarly, though perhaps less efficiently, to a fully formed stromal layer.

In the next series of experiments the stem cell purification strategy was simplified. A lineage negative fraction was prepared, in a single step, from normal bone marrow. It was hoped that by using this method the level of stem cell enrichment and recovery might be more reproducible than for day 2 post 5-FU marrow which was both depleted of lineage negative cells and then flow sorted. These lineage negative populations were expanded in three consecutive experiments. We reasoned that the effect of MIP-1α, if present at all, may

target only a small subset of the stem cell compartment, and therefore may be difficult to demonstrate. "Read-out" was therefore performed for cell and CFU-A / CFU-GM numbers as before, but in addition the starting cells were examined morphologically, scored for percentage of blasts and were analysed by flow cytometry for percentage of Sca+ and of Rhodamine^{dull} cells. Following a seven day incubation, "read-out" was repeated for the same parameters and the expanded population was again enriched for lineage negative cells. In all three experiments total cell number and the number of lineage negative cells recovered by the second purification step were greater in the presence of MIP-1α. In addition the expanded populations contained a greater proportion and, therefore, a greater total number of Sca+ and rhodamine^{dull} cells of blast morphology. In these experiments neither CFU-A nor CFU-GM expansion was increased in the presence of MIP-1α. This finding is difficult to explain but may suggest that MIP-1α's effect is directed at an earlier precursor that CFU-A. If this were the case CFU-A and CFU-GM would have been increased in the presence of MIP-1α, if the cultures were maintained for long enough. This had not been observed in the cultures established with unfractionated bone marrow maintained for up to 39 days (discussed above).

We therefore found it impossible to develop a simple *in vitro* assay which would demonstrate conclusively that MIP-1α was affecting progenitor expansion. However, in view of our own data, and that of Verfaillie et al, MIP-1α supplemented cultures were pursued for *in vivo* experiments.

Assessment of the short term engrafting potential of expanded populations: Comparison with unmanipulated bone marrow

In parallel with *in vitro* experiments, an *in vivo* murine model was developed to test the short term engrafting ability of expanded cells. Two forms of "read-out" were used; survival to day 30 following lethal dose radiation and rate of recovery of peripheral blood counts. It was hoped that by expanding the number of CFU-A progenitors available for transplantation, fewer starting cells would be required to rescue animals following a standard radiation protocol. This type of approach would be applicable clinically since, for a significant proportion of patients for whom autologous BMT is indicated, insufficient stem and progenitor cells can be harvested to reach the threshold which ensures rapid recovery following transplantation.

The experiments described in chapter 4 demonstrated that it was indeed possible to reduce the number of cells required for short term engraftment by a factor of 10-20 fold. Since CFU-A increased approximately 50 fold but cell number could only be reduced about 10 fold, it seemed likely that a threshold number of CFU-A progenitors was not the only requirement for transient engraftment. The CFU-A compartment is likely to be heterogeneous, comprised of both primitive and more mature multipotential progenitor cells. The expansion of CFU-A seen in these studies was probably accompanied by maturation such that the ratio of primitive to more mature cells was shifted towards more committed progenitors. If this were indeed the case these results suggested that more primitive CFU-A progenitors were also required for transient engraftment, explaining why the cell number transplanted could not be reduced by 50 fold. The number of cells required for rescue was not further reduced by the addition of MIP-1α to the combination of SCF and IL-11 suggesting that MIP-1α did not exert potent effects on maintaining the CFU-A compartment in a less differentiated state during expansion.

Similar data on short term recovery have been published by Muench et al (Muench *et al.*, 1993) and by Serrano et al (Serrano *et al.*, 1994). In the study by Muench et al SCF and IL-1β stimulated expansion of 1, 000 fold for LPP-CFC, over 100 fold for HPP-CFC and 100 fold for CFU-S starting with post 5-FU bone marrow. The number of cells required for rescue from lethal dose irradiation could be reduced by 200 fold. The difference in both the degree of progenitor expansion achieved and the ability to reduce the cell number required for rescue to such a degree, compared with our own data is most likely to be the result of pre-enrichment for stem cells by treatment of the animals with 5-FU prior to harvesting. In the study by Serrano et al day 4 post 5-FU bone marrow was expanded for 3 days with IL-3 and IL-6. Following transplantation of 5 x 10^4 cells per mouse, survival to day 30 post radiation was 68% for expanded BMT versus only 27% for non-expanded BMT .

The first clinical study to report the transplantation of progenitor cells generated *ex vivo* was reported very recently by Brugger et al (Brugger *et al.*, 1995) who applied CD34 selection to standard leukapheresis products. Following CD34 selection approximately one tenth (11 x 10^6 CD34 cells total) of a leukapheresis product was expanded *ex vivo* with a cytokine combination comprising SCF, IL-1β, IL-3, IL-6 and erythropoietin. CFU-GM progenitor cells were expanded approximately 50 fold and the few patients transplanted with expanded cells recovered peripheral blood counts as rapidly as historical controls treated with the same chemotherapy regimen. This study provided evidence that those cells responsible for transient engraftment may indeed be expanded *ex vivo* on a clinically relevant scale. One of the important applications of this type of cell manipulation would be to reduce the size of the transplant and thereby reduce the potential number of contaminating tumour cells present. The crucial question which this study did not address regarded the fate of LTRC during *ex vivo* expansion. The patients were treated

with high dose, but non-myeloablative chemotherapy and, therefore, would recover endogenous haemopoiesis within a few weeks of transplantation. In this setting there was no requirement for LTRC in the transplanted material. Whether one tenth of a leukapheresis would contain sufficient LTRC to secure long term reconstitution following myeloablative therapy is not known. The fate of LTRC in such expansion cultures cannot be addressed clinically until patients are transplanted with cells expanded *ex vivo* following truly myeloablative therapy (see later).

The next important question to address with our experiments was whether the kinetics of neutrophil and platelet recovery could be influenced by using expanded populations for rescue following radiation. For these experiments all mice were transplanted with ten fold more cells (5 x 10^5/mouse) than the minimum number required for rescue (5 x 10^4/mouse). The reason for this was based on evidence reported by Jones that the rate of engraftment correlated with the cell number transplanted until a threshold cell number was exceeded. Thereafter, further increasing the cell dose would not further hasten haemopoietic recovery (Jones *et al.*, 1987). If these data were correct then animals transplanted with expanded cells should show accelerated recovery compared with animals transplanted with a large number of unmanipulated bone marrow cells. We could then conclude that this observation was not solely due to transplanting approximately two fold more cells (total cell number increased approximately two fold over a 6 day expansion), but reflected either the progenitor content of the graft or an undetermined effect on progenitor seeding or homing.

The results of this experiment confirmed that neutrophils, platelets and haemoglobin recovered more rapidly following transplantation with expanded populations than for unmanipulated bone marrow. This difference was statistically highly significant and suggested that if a similar result could be obtained in clinical studies it would be clinically relevant. There was at least a

seven day difference in the time taken for recovery to a neutrophil count of 0.5 x 10^9/L. This occurred by day seven for mice transplanted with SCF / IL-11 expanded marrow but not until day 18 for unmanipulated cells. A similar, but less dramatic effect on neutrophil recovery was also shown by Muench et al (Muench *et al.*, 1993) and Serrano et al (Serrano *et al.*, 1994), confirming our results.

Our findings showing that transplants with expanded cells reduced the number of cells required for short term recovery following myelosuppressive therapy and that neutrophil recovery was significantly accelerated are both interesting and of potential clinical importance. However, results obtained with animal models do not necessarily mean that similar results may be possible for humans. Clinical studies are underway but to date no acceleration of neutrophil recovery has been demonstrated.

The results discussed in the preceding sections have established that those cells responsible for short term engraftment following BMT could be effectively expanded *ex vivo*. However, for a number of potential clinical applications it was also important to assess the effect that cytokine driven proliferation, in the absence of stroma, had on LTRC present in the cultures. In order to safely reduce the number of cells required for stem cell rescue following myeloablative therapy the LTRC pool would have to be expanded, or at least maintained during expansion culture, otherwise patients would be likely to experience bone marrow failure late after BMT. For gene therapy applications, it is necessary to stimulate LTRC to divide but remain pluripotent (i.e. self-renewal) to ensure efficient and long-lived gene expression.

There are a number of assays which are thought to measure LTRC. Of the available *in vitro* assay systems, the CAFC assay is likely to be the most informative since several stem cell subsets may be assessed simultaneously. Although our group did not have experience with this assay at the time of

these experiments being performed, the assay is now established "in house" and experiments are currently underway to quantify the number of stem cells in different subsets which remain after expansion either in the absence of cytokines (medium control), in the presence of SCF and IL-11, or SCF, IL-11 and MIP-1α. These experiments should provide some quantitative information regarding the maintenance of CAFC under the different culture conditions and may be sufficient to demonstrate whether MIP-1α has any effect on the maintenance of early progenitor cells under the experimental conditions used.

Assessment of the ability of expanded populations to sustain serial BMT: Comparison with unmanipulated bone marrow

The most quantitative means of measuring stem cell numbers *in vivo* is by competitive repopulation (CRA assay). This enables an accurate comparison of the long term reconstituting ability of different cell populations. In our experiments this could have been applied to unmanipulated cells and to cells expanded with SCF and IL-11 versus SCF, IL-11 and MIP-1α. The availability of mice which differ at the Ly-5 locus will facilitate the use of CRA in future studies.

For our studies serial BMT was selected to compare the long term reconstituting ability of three cells populations: unmanipulated, SCF / IL-11 expanded and SCF / IL-11 / MIP-1α expanded bone marrow. For serial BMT the donor and recipient cells must differ to allow detection of donor derived haemopoiesis at different time points following BMT. The system chosen for our studies employed male donors and female recipients. A Y chromosome specific probe was then use to perform Southern blotting on DNA samples derived from recipient animals.

Why should serial BMT be used to assess the repopulating ability of stem cell populations? Under normal circumstances bone marrow stem cells have a high proliferative capacity which may be increased by at least 10 fold

under situations requiring marrow regeneration. However, this proliferative capacity is significantly challenged when donor bone marrow cells are required to fully regenerate haemopoiesis following myeloablative doses of radiation administered to host animals (i.e. the procedure of BMT). When serial BMT is performed, the recipient animals receive a lethal dose of irradiation. At a chosen time point following the primary transplant, bone marrow cells from the primary recipient (s) are transferred to a second lethally irradiated recipient. This is then repeated until the required endpoint is reached. The reason that serial BMT may be used to compare the reconstituting ability of different cell populations is that the ability of serially transplanted marrow to repopulate irradiated hosts is limited and therefore cell populations of unknown stem cells content (expanded populations) may be compared in parallel with normal marrow. Haemopoietic cells transplanted at short intervals (8-10 weeks) have been shown, in a number of studies , to develop a progressively impaired ability to repopulate irradiated hosts (Siminovitch *et al.*, 1964; Cudkowicz *et al.*, 1964). By lengthening the interval between transfers the repopulating ability may be improved, but even with very long (6-12 months) intervals, serial transfers cannot be continued for more than 5 generations (Cudkowicz *et al.*, 1964; Harrison *et al.*, 1978; Ogden & Micklem, 1976; Siminovitch *et al.*, 1964). The exact mechanism underlying these findings is not clear. For example, the change in quality of stem cells during serial transfer could indicate that the stem cells have aged because of the number of divisions required for regeneration of haemopoiesis on multiple occasions. This would suggest that the number of divisions a stem cell can go through is limited and that the regenerative capacity of the stem cell pool is exhaustible. This limitation may however never be seen under normal circumstances since stem cell reserve in mice seems to be ample to maintain haemopoiesis throughout life. In a serial BMT experiment, as the donor cells are serially passaged, competition will arise between the fraction

of host stem cells which have survived radiation and the less competent donor stem cells, which is why markers are required to indicate the continuing presence of donor cells. The ability of the host marrow to compete will depend on the level of myeloablation caused by the radiation protocol and the quality of remaining donor cells. Eventually there will be so few stem cells remaining in the donor marrow that recipient animals will die from bone marrow failure soon after cell transfer and before host marrow is able to regenerate to any significant degree.

The use of serial transfer as a means of measuring the proliferative capacity of cell populations is, at best, inexact. It is a useful functional test of the ability of cells to reconstitute under circumstances requiring enormous stem cell reserve, but it cannot measure the number of reconstituting cells present. Although the assumption is generally made that serial transfer eventually fails because stem cells have a finite capacity for self-renewal there are a number of other possible explanations for this observation. For example, the homing behaviour of bone marrow cells which have been serially transplanted may be abnormal or stem cells which have been transplanted may never regain their normal quiescent characteristics because of changes in the microenvironment induced by radiation. Also, the signals to regenerate following BMT may shift the stem cells into differentiative rather that self-renewal divisions. If the interval between transfers is lengthened then the stem cells have more time to return to a quiescent state. The fact that leaving intervals of one year between transfers is insufficient to allow more than 5 transfers suggests that the changes to the stem cell compartment induced by BMT are in part irreversible.

In the serial transfer experiment, cell populations expanded *ex vivo* were compared with unmanipulated bone marrow. It was already realised that expanded populations had an advantage in terms of transient engrafting potential and that the ability to serially transfer was critically dependent on the

interval between transfers. I, therefore performed the second cell transfers at one, three and nine months following primary BMT to gain as much information from the experiment as possible. The interval between the second and third transplant was set at two and six months (short and long). Thereafter an interval of three months was left between transfers.

The results showed that expanded marrow had a definite advantage over fresh marrow in terms of its capacity for serial BMT, but only when the interval between transfers was short. Since the assay systems employed were not quantitative, this may have been related to a more rapid return to steady-state haemopoiesis for expanded BMT compared with unmanipulated BMT and need not imply that there were more LTRC present in the expanded transplant. Although short term haemopoietic recovery was more rapid for expanded than for unmanipulated BMT it was not clear whether this was due to an increased CFU-A concentration, to effects of growth factor exposure on progenitor homing, or to a mixture of both. Therefore, when these cells were then serially transplanted (short interval) their observed advantage in terms of ability to serially transfer may have been related to qualitative changes in transplantation ability rather than to an increase in the number of stem cells induced by the *ex vivo* expansion. For serial transfers performed after longer intervals, unmanipulated marrow regained the ability to serially transplant and at least 4 serial transfers were possible instead of 2. It would be interesting to compare unmanipulated marrow and expanded marrow for their ability to sustain serial BMT after a longer interval (i.e. 9 months). By 9 months post transplant all three experimental groups would be expected to have returned to steady state haemopoiesis. Any differences detected in the ability to serially transfer or in the proportion of donor cells at this time point would be more likely to reflect the quality of the stem cells in the original cell innoculum (i.e. fresh versus expanded). If by nine months the donor marrow had failed (related to the *ex vivo* culture) then Southern blotting would have confirmed

loss of male cells. Unfortunately I was unable to obtain data from the two experimental groups transplanted with expanded cells at the later time point because a proportion of these animals developed leukaemia.

Possible mechanisms underlying the generation of CD5 B cell leukaemia in BMT recipients

As discussed in detail at the end of chapter 6, CD5 positive B cells appear to represent an individual B cell lineage which develops and matures during ontogeny but in adult life is sustained by "self-replenishment" of mature cells. Although this is likely to be the case during steady state haemopoiesis, it is possible that, following transplantation with adult bone marrow, CD5 positive cells may be reconstituted in a limited fashion, either by differentiation of HSC, or by proliferation of the few CD5 positive cells present in the donor marrow. These possibilities have been investigated in studies using FACS sorted candidate stem cells (reviewed by Kantor & Herzenberg, 1993). In these studies, adult mouse bone marrow enriched by FACS-sorting for Thy-1^{lo}/Lin- cells was transferred into SCID mice. Conventional B cells were fully reconstituted, but in addition a small number of CD5 positive cells were also derived from the donor cells. Since the enriched population should have contained no CD5 positive B cells (B220 positive cells were <1% of the total) it seems likely that, in this instance, low numbers of CD5 positive B cells developed by differentiation of pluripotent HSC. This finding would account for the more recent data obtained from cell transfer studies which suggest that CD5 positive B cells may indeed be reconstituted to a low level from adult bone marrow (reviewed by Kantor & Herzenberg, 1993; Stall *et al.*, 1992; Lalor *et al.*, 1989; Kantor *et al.*, 1992b).

The CD5 positive B cell population, perhaps because of the requirement for "self-replenishment", appears to be at greater risk, compared with conventional B cells, of neoplastic transformation. As normal mice age

there is a tendency for clones of CD5 B cells to develop (Stall *et al.*, 1988). These clonal populations may spontaneously progress to an invasive leukaemia, but this process is accelerated by serial transfer of bone marrow populations into irradiated recipients (Fowlis *et al.*, 1989).

In our study bone marrow populations (either unmanipulated or expanded) from male mice were transplanted into lethally irradiated female recipients. After serial transfer of pooled marrow from these primary recipients a female, CD5 positive, B cell CLL developed, firstly in recipients of marrow derived from primary recipients of SCF/IL-11/MIP-1α expanded marrow, but later in recipients of SCF/IL-11 expanded marrow. In these groups, with time, eventually almost all animals developed leukaemia. In the unmanipulated BMT group only one case of CD5 positive B cell CLL occurred. Cases of advanced leukaemia were transplantable into animals treated with only 2Gy of radiation and were rapidly lethal. The mechanism underlying these observations is not clear but three hypotheses are likely.

There is substantial evidence that CD5 positive B cells are susceptible to leukaemogenesis, even before manipulation, and that these cells are present at very low numbers in normal adult bone marrow. For the first hypothesis it must be assumed that CD5 B cells may only develop by "self-replenishment", and not by differentiation of HSC. It is suggested that following transplantation with unmanipulated bone marrow, the small number of CD5 B cells present in the marrow innoculum was sufficient to regenerate the CD5 positive B cell population (albeit at a low level). Since reconstitution was only likely to be 10-20% of normal (according to previous studies) then serial transfer would further increase the number of cell divisions required (by self-replenishment) by a cell population already susceptible to leukaemogenesis. One might, therefore, expect occasional examples of leukaemia to occur (only one case in our study). Why then was the incidence of leukaemia much greater for expanded BMT? It is possible that the few CD5 positive B cells present in

adult bone marrow did not survive the *ex vivo* culture. Upon transfer then, there would be no source of replenishing CD5 positive B cells. Under these circumstances perhaps host (female) CD5 B cells would have to proliferate to replenish the CD5 B cell population. These cells would have been exposed to radiation thereby increasing further the chance of leukaemogenesis.

Alternatively, if we assume that CD5 positive B cells may be reconstituted from HSC rather than by self-replenishment, then for unmanipulated BMT, HSC present in the donor innoculum would be responsible for the low level regeneration of CD5 positive B cells following BMT. However for expanded BMT, perhaps the exposure to a combination of cytokines chosen to stimulate myelopoiesis, resulted in the HSC being triggered towards myeloid, rather than lymphoid commitment. If this were true then the donor marrow would be insufficient for reconstitution of CD5 B cells and once again the host CD5 B cells would have to proliferate and by so doing become leukaemic.

Finally, it is possible that the donor bone marrow contained insufficient CD5 B cells to regenerate the CD5 B cell population whether expanded or not. If this were so, then the host CD5 B cells would have to proliferate equally in all three experimental groups in order to replenish the population regardless of the make up of the donor cell innoculum. Although clonal populations may have been present in many or all animals due to the stress of proliferation and serial transfer, only two animals may have actually developed leukaemia (by chance in the expanded groups). When the cells were pooled and serially transplanted, eventually all animals in those two groups developed leukaemia. The reason that the SCF/IL-11/MIP-1α group developed leukaemia earlier than the SCF/IL-11 group may be simply that the animal in that group which developed the primary tumour did so at an earlier time point than in the SCF/IL-11 group (i.e. by chance).

The development of leukaemia in this study has generated a number of important questions. For example, does unmanipulated bone marrow or expanded bone marrow contain detectable CD5 positive B cells? This may be a difficult question to answer. Since, if present at all, these cells are likely to occur at a very low frequency they would be difficult to detect by flow cytometry. Practically, this would be complicated by CD5 expression by normal T cells. If a B cell population was first selected on the basis of B220 and IgM expression this could then be labelled for CD5. Even so, these cells may be too infrequent to be readily identified. Since T cells express CD5 it may be difficult to separate a "clean" enough population of B cells to perform RT-PCR for CD5. The chances of detecting a signal from small numbers of contaminating T cells must be high.

A further question is whether CD5 B cell reconstitution following BMT is of donor or host origin. This could be simply determined by using donor and recipient mice with a detectable marker (e.g. Ly-5.1/5.2, male / female). Following BMT, recipient spleen, peritoneal fluid, lymph nodes and bone marrow could be harvested, flow sorted for CD5 positive B cells and then examined for the relevant markers.

The next question would be, if the CD5 B cell population following BMT is donor derived does it develop by "self-replenishment" of CD5 B cells or by differentiation of HSC? This could be addressed by transplanting highly purified cells which were completely depleted in CD5 B cells and looking for donor markers in the recipients post BMT. If both total bone marrow and highly purified marrow were able to reconstitute donor derived CD5 B cells this would suggest differentiation from HSC, whereas, if only total bone marrow reconstituted CD5 B cells, then "self-replenishment" would be the more likely explanation.

A crucial question however, is whether this type of experiment can be repeated with the same result. In a repeat experiment it would be useful to

reduce the interval between transfers (to try to speed the process of leukaemogenesis) and to avoid pooling of the cells used for subsequent transfers. In this way we could determine how often individual leukaemias arose and whether they occurred predominantly in the expanded BMT groups. Additional information could be obtained by performing clonality studies on the BMT recipients at varying time points after serial transfer and to look for cytogenetic abnormalities. This should provide information on how many clones contributed to leukaemogenesis.

Finally, if the first hypothesis is correct and expanded marrow can be shown to be depleted in CD5 B cells could the leukaemia be prevented by co-transplanting expanded marrow with a rich source of CD5 B cells (e.g. from peritoneum) thereby removing the signal for host CD5 B cells to proliferate? This experiment would be relatively easy to perform and would be of key interest.

Clinical Application of Stem/Progenitor Cell Expansion

Over the last few years, attempts have been made to expand human haemopoietic progenitor cells *ex vivo* with a view to transplantation *in vivo* (Brugger *et al.*, 1993; Haylock *et al.*, 1992; Henschler *et al.*, 1994; Shapiro *et al.*, 1994). There are several potential clinical applications for stem cell expansion. For autologous stem cell rescue, it may be possible to reduce the harvesting requirements and, therefore, the likelihood of tumour cell contamination. Expanded progenitor cells may be used to ameliorate pancytopenia post transplant, or to cover multiple courses of high dose chemotherapy and so prevent cumulative myelosuppression. Finally, culture conditions may be developed which could be applied to gene transduction protocols.

Most investigators initiate stem cell expansion with CD34 cells purified by positive selection since these cells appear to be superior to

unselected material (Moore, 1995). In addition, up to a 4.0 log depletion of contaminating tumour cells can be obtained using CD34 selected starting populations (Shpall *et al.*, 1994). A recent study suggested that any remaining tumour cells do not increase in number during *ex vivo* expansion, and this may represent a further purging effect (Brugger *et al.*, 1995).

The first clinical studies using progenitor cells expanded *ex vivo* have now been reported, two only in abstract form (Alcorn *et al.*, 1996; Brugger *et al.*, 1995; Champlin *et al.*, 1995; Zimmerman *et al.*, 1995). In the first of two studies performed by our group (these separate studies are not included in this thesis), we established the safety and feasibility of transfusing CD34 selected cells which had been selected from cryopreserved peripheral blood progenitor cells (PBPC) and then expanded *ex vivo* with a combination of five cytokines. Ten patients received $\geq 20 \times 10^4$CFU-GM/kg unmanipulated PBPC (i.e. a dose sufficient for stem cell rescue in the event that the expanded cells failed to mediate engraftment) and in addition an aliquot of expanded cells (range 33-279 $\times 10^4$ CFU-GM/kg). There were no toxic effects from the progenitor cells which had been generated *ex vivo* but no acceleration of either neutrophil or platelet recovery was seen (Alcorn *et al.*, 1996).

In the study by Brugger et al, in a small number of patients, stem cell rescue was performed using only expanded cells, without additional unmanipulated cells. One tenth of a standard PBPC product was expanded with cytokines *ex vivo* prior to infusion. Six patients were transplanted with expanded cells only, and five of six experienced rapid engraftment following high dose, but non-myeloablative, chemotherapy.The remaining patient died prior to the anticipated time of engraftment. This study was unable to answer the question of whether sufficient LTRC were present in one tenth of a PBPC collection either before or after *ex vivo* culture since the patients did not receive myeloablative therapy and would be expected to recover endogenous haemopoiesis within a few weeks of chemotherapy.

In the second study performed by our group, the primary objective was to determine whether CD34 cells expanded *ex vivo* would secure both short and long term engraftment following fully myeloablative chemo/radiotherapy (Holyoake *et al.*, 1996). To our knowledge, this is the first report testing the ability of progenitor cells generated *ex vivo* to sustain haemopoiesis following myeloablative regimens. Only four cases were treated. All four cases experienced delayed haematological recovery and were therefore given unmanipulated PBPC "back-up" to secure engraftment.

The results of this study were not favourable yet were of considerable clinical importance. *In vitro* data had suggested that LTCIC may be maintained during *ex vivo* expansion culture, but this was the first clinical study to test the engraftment potential of expanded cells following fully myeloablative conditioning. Although *in vivo* studies in murine models are used to prove the presence of LTRC in cell infusions, such studies are not possible clinically. There is, therefore, no evidence to suggest that maintenance of LTCIC signifies the presence of cells capable of long term reconstitution. The preparative regimen employed in the study by Brugger et al (Brugger *et al.*, 1995), although intensive, included neither total body irradiation nor melphalan / busulphan (used in our study). Despite re-infusing sub-optimal CFU-GM doses, they were still able to demonstrate prompt recovery of blood counts, not significantly different from historical controls. In our study, all four patients received fully myeloablative conditioning regimens. Although, by our standards, the first case received an inadequate CFU-GM dose for transplantation, this dose was comparable to some of the cases treated in the study by Brugger et al. This case showed no evidence of neutrophil engraftment. The other three cases did show early neutrophil engraftment but this was not maintained. A biphasic pattern of neutrophil recovery was seen in three of the four cases suggesting that the early recovery was mediated by committed progenitor cells present in the *ex vivo* expanded

material, while late recovery was provided by the unmanipulated PBPC given as "back-up" in every case. This provides strong evidence that, under the culture conditions used in our study, stem cells expanded *ex vivo* may not contain sufficient LTRC to ensure engraftment following myeloablative conditioning. This pattern may not have been observed for the cases reported by Brugger et al since the early phase of neutrophil engraftment and the subsequent fall in neutrophils may have overlapped with endogenous neutrophil recovery from the high dose, but non-myeloablative, chemotherapy used in their study. Alternatively, the culture conditions employed in the two studies may have been sufficiently disparate, to produce progenitor cells with a significantly different profile of myeloid lineage commitment.

Clearly much more work is required before the clinical potential of stem cell expansion may be realised. To optimise recovery of LTRC it may prove necessary to expand stem cells in the presence of both positive and negative regulators of stem cell proliferation and supporting stroma.

The work described in this thesis has provided invaluable information regarding the *in vitro* requirements for haemopoietic stem cell survival. The data obtained from animal studies has given us the framework on which to develop clinical studies. In parallel with clinical development we now hope to investigate in more detail the factors which are crucial for stem cell maintenance and expansion *ex vivo*.

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