THE CONTROL OF NORMAL AND LEUKAEMIC HUMAN CELLS BY THE COLONY-STIMULATING

FACTORS

Dr. C. G. Begley

AMENDMENTS

EXAMINER 1: Nil required

EXAMINER 2:

1. The examiner is correct in noting that the size of clones derived from primary human leukaemic cells was small compared with clones derived from a morphologically-equivalent population of normal cells. He therefore suggests that it is not valid to conclude that leukaemic cells are CSF-responsive. There was however a dose-related increase in the number of leukaemic clones in cultures stimulated by increasing concentrations of CSF. Furthermore as presented in the thesis, all myeloid leukaemic samples studied demonstrated specific binding of ^{125}I G-CSF. The presence of specific receptors for CSF and a dose-related CSF response in terms of number of leukaemic cells stimulated, argue strongly for these cells being CSF-responsive.

These studies using purified GM-CSF and G-CSF failed however to provide an explanation for the limited clonal proliferation and often transient nature of clones generated in vitro by leukaemic cells. The demonstration that CSF receptor expression and responsiveness to CSF's are broadly within normal limits has emphasized the need for further studies to establish the cellular mechanisms in leukaemic cells responsible for the characteristically abnormal proliferative pattern exhibited in vitro and for the dominance expressed by a leukaemic clone over pre-existing normal progenitors in vivo. Exploration of the mechanisms of internalization and intracellular processing and degradation of the CSF's within leukaemic cells may help in understanding how the programming of leukaemic cells differs from equivalent normal populations.

2. The examiner is incorrect in stating that evidence of binding of ^{125}I G-CSF did not always correlate with G-CSF-stimulated proliferation. All samples that displayed ^{125}I G-CSF binding demonstrated G-CSF-stimulated proliferation in cultures. The results shown in Table 8.3 and 8.4 document the ability of ^{125}I G-CSF to bind to all myeloid leukaemic samples studied

(in contrast to lymphoid leukaemias; Table 8.5). These Tables also show the ability of purified G-CSF to stimulate the clonal proliferation of myeloid leukaemic samples but as stated in the legends, results are only shown for day 7 and 14 timepoints. A proportion of leukaemic samples displayed only transient clonal proliferation in vitro (Fig. 7.1, Table 8.1). As stated on page 221, 6 of 20 myeloid leukaemias would have been regarded as showing no evidence of clonal proliferation had they been examined at the day 7 timepoint alone.

The question of G-CSF-stimulated differentiation in primary leukaemic cells was very difficult to address. While there was evidence of morphological differentiation in leukaemic cells stimulated by G-CSF, the real issue was whether G-CSF was able to decrease the self-renewal potential of leukaemic cells. Experiments to examine this question with primary leukaemic samples were unsuccessful (as outlined on page 240) and studies were therefore performed using the human leukaemic cell line HL60.

3. The examiner notes that it is odd that CSF stimulated both the proliferation of HL60 cells and ultimately terminal differentiation. The coupling of CSF-stimulated proliferation and differentiation is a consistent observation for both normal and leukaemic murine cells (see review, Metcalf 1984) and as documented in the thesis, normal and leukaemic human cells. A similar coupling of differentiation-proliferation can also be observed when differentiation is induced by chemical agents. As discussed on pages 266 and 281, although CSF stimulates proliferation and apparently paradoxically differentiation, there is evidence that the balance between these two opposing actions of CSF can be influenced in favour of differentiation or proliferation by the concentration of CSF (Metcalf, 1980; Johnson, 1983; Li and Johnson, 1984). The coupling of these two responses suggests that the intracellular programming of a cell is such that differentiation can not occur without proliferation (except at a particular stage of differentiation where e.g. metamyelocytes (post-mitotic cells) differentiate to mature neutrophils without proliferation).

4. The examiner questions whether CSF enhances the survival of mature cells in vivo in a manner similar to that demonstrated in vitro (Chapter 9). This seems a very likely possibility and makes good teleological sense. It is not likely however that such a mechanism is the sole explanation for the observed responses in experimental animals injected with recombinant CSF's. In these animals, the increase in numbers of mature cells is accompanied by increased mitotic figures (e.g. in peritoneal macrophages) and increased numbers of progenitor cells. These observations argue for a proliferative action of CSF in vivo.

THE CONTROL OF NORMAL AND LEUKAEMIC HUMAN CELLS

BY THE COLONY STIMULATING FACTORS

By

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A Thesis Submitted for the Degree of Doctor of Philosophy

in the University of Melbourne

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TABLE OF CONTENTS   Page

CHAPTER 2 METHODS

PREFACE

In accordance with the regulations regarding the degree of Doctor of Philosophy in the University of Melbourne, the author has conducted 90% of the work described in this thesis. This thesis is less than 100,000 words in length, exclusive of tables, bibliographies and appendices.

The author wishes to acknowledge the contribution in collaborative projects of:

Dr. N. Nicola for supplying partially purified preparations of CSF-α, CSF-β and purified murine G-CSF, and for performing the experiments using radio-iodinated G-CSF.

Dr. A.W. Burgess for providing purified murine GM-CSF and M-CSF.

Dr. R. Cutler for providing purified Multi-CSF.

Dr. G.R. Johnson for providing HPCM and erythropoietin and for performing the experiment on erythroid enhancement stimulated by rHGM-CSF (Chapter 3).

Dr. C.J. Sanderson for providing purified EDF and the hybridoma supernatant with EDF-activity.

Dr. E. Wang (Genetics Institute) and Dr. J. DeLamarter (Biogen) for providing rHGM-CSF.

Dr. A.F. Lopez for providing monoclonal antibodies WEM-G1, WEM-G11 and NIMP-R10.

Dr. G.F. Burns for providing monoclonal antibodies 25E11 and 9.1C3.

SEVERAL OF THE STUDIES REPORTED IN THE THESIS HAVE BEEN PUBLISHED OR SUBMITTED FOR PUBLICATION

1. BEGLEY, C.G., Lopez, A.F., Vadas, M.A., Metcalf, D. (1986). The clonal proliferation in vitro of enriched populations of human promyelocytes and myelocytes. Blood 65: 951-958.

2. Lopez, A.F., BEGLEY, C.G., Andrews, P., Butterworth, A., Vadas, M.A. (1985). Identification of a human granulocyte functional antigen (GFA-2) involved in antibody-dependent cell mediated cytotoxicity and phagocytosis. J. Immunol. 134: 3969-3977.

3. Metcalf, D., BEGLEY, C.G., Nicola, N.A. (1985). The proliferative effects of human GM-CSF-α and β and murine G-CSF in microwell cultures of fractionated human marrow cells. Leuk. Res. 9: 521-527.

4. Nicola, N.A., BEGLEY, C.G., Metcalf, D. (1985). Identification of the human analogue of a regulator that induces differentiation in murine leukaemic cells. Nature 314: 625-628.

5. Metcalf, D., Nicola, N.A., BEGLEY, C.G. (1985). The colony stimulating factors and myeloid leukemia. In: "Leukemia 1985. UCLA Symposium on Molecular and Cellular Biology. New Series. Vol. 28" Eds. R.P. Gale and D.W. Golde. Alan R. Liss, New York.

6. Metcalf, D., BEGLEY, C.G., Johnson, G.R., Nicola, N.A., Vadas, M.A., Lopez, A.F., Williamson, D.J., Wong, G.G., Clark, S.C., Wang, E.A. (1986) Biological properties in vitro of a recombinant human granulocyte-macrophage colony stimulating factor. Blood (in press).

7. BEGLEY, C.G., Metcalf, D., Lopez, A.F., Nicola, N.A. (1985). Fractionated populations of normal human marrow cells respond to both human colony stimulating factors with granulocyte-macrophage activity. Exp. Hematol. 13: 956-962.

8. Lopez, A.F., BEGLEY, C.G., Williamson, D.J., Warren, D.J., Vadas, M.A., Sanderson, C.J. (1986). Murine eosinophil differentiation factor: an eosinophil specific colony-stimulating factor with activity on human cells. J. Exp. Med. (in press).

9. BEGLEY, C.G., Lopez, A.F., Nicola, N.A., Warren, D.J., Sanderson, C.J. (1986). Purified colony-stimulating factors enhance the survival in vitro of mature human neutrophils and eosinophils in a lineage specific manner: a rapid sensitive microassay for colony stimulating factors. Blood (in press).

10. Burns, G.F., Cosgrove, L., Triglia, T., Lopez, A.F., Werkmeister, J.A., BEGLEY, C.G., Haddad, A.P., d'Apice, A.J.F., Vadas, M.A., Cawley, J.C. The IIb-IIIa glycoprotein complex which mediates platelet aggregation is directly implicated in leukocyte adhesion. (Submitted).

ADDITIONAL STUDIES NOT REPORTED IN THE THESIS

1. Burns, G.F., Triglia, T., Werkmeister, J.A., BEGLEY, C.G., Boyd, A.W. (1985). TLiSA1, a human T lineage-specific activation antigen involved in the differentiation of cytotoxic T lymphocytes and anomolous killer cells from their precursors. J. Exp. Med. 161: 1063-1078.

2. Burns, G.F., BEGLEY, C.G., Mackay, I.R., Triglia, T., Werkmeister, J.A. Supernatural killers. Immunol. Today (in press).

3. Johnson, G.R. and BEGLEY, C.G. Transfusion-dependent
    β-thalassemia: in vitro characterization of peripheral blood
    multipotential and committed progenitor cells. (Submitted).

4. Metcalf, D., BEGLEY, C.G., Johnson, G.R., Nicola, N.A., Lopez,
    A.F., Williamson, D.J. (1986). Effects of purified bacterially-
    synthesized murine Multi-CSF (IL-3) on hemopoiesis in normal
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I owe more than I can say to my wife Merrin for her assistance and understanding and to my mother for her constant support.

ABBREVIATIONS

AML	:	Acute myeloid leukaemia
BFU-E	:	Burst forming unit-erythroid
BPA	:	Burst promoting activity
cDNA	:	Complementary-deoxyribonucleic acid
CFC	:	Colony-forming cell
CFU-S	:	Colony-forming unit - spleen
CFU-E	:	Colony-forming unit - erythroid
CLL	:	Chronic lymphatic leukaemia
CM	:	Centimeter
CML	:	Chronic myeloid leukaemia
CMML	:	Chronic myelomonocytic leukaemia
CSF	:	Colony stimulating factor
CSF-α	:	Colony stimulating factor derived from human sources that preferentially stimulates day 14 CFC
CSF-β	:	Colony stimulating factor from human sources that preferentially stimulates day 7 CFC
DME	:	Dulbecco's modified Eagle's medium
EBSS	:	Eisen's Balanced Salt Solution
EDF	:	Eosinophil differentiation factor
Eo-CFC	:	Eosinophil colony-forming cells
Eo-CSF	:	Eosinophil-CSF
EPA	:	Erythroid Potentiating Activity
EPO	:	Erythropoietin
FACS	:	Fluorescence activated cell sorter

Fc	:	Fc region of immunoglobulin
FCS	:	Foetal calf serum
FITC	:	Fluorescein isothiocyanate
g	:	Units of gravity
G-CSF	:	Granulocyte-colony-stimulating factor
GCT-CM	:	Giant cell tumour conditioned medium
GM	:	Granulocyte-macrophage
GM-CFC	:	Granulocyte-macrophage colony-forming cell
GM-CSF	:	Granulocyte-macrophage colony-stimulating factor
HPCM	:	Human placental conditioned medium
HT-PBS-azide	:	Human tonicity phosphate buffered salt solution with sodium azide
Ig	:	Immunoglobulin
IMDM	:	Iscove's modified Dulbecco's medium
LCM	:	Leucocyte conditioned medium
LIA	:	Leukaemia inhibitory activity
M	:	Monocyte/macrophage
M-CSF	:	Macrophage-colony-stimulating factor
mg	:	Milligram
Min.	:	Minute
ML	:	Millilitre
MLCM	:	Mouse lung conditioned medium
Multi-CFC	:	Multipotential-colony-forming cell
Multi-CSF	:	Multipotential-colony-stimulating factor
MW	:	Molecular weight
ng	:	nanogram
NK	:	Natural killer cell

nm	:	Nanometer
PBS	:	Phosphate buffered saline
Ph^1	:	Philadelphia chromosome
PHA	:	Phytohaemagglutinin
PHA-LCM	:	Phytohaemagglutinin stimulated leukocyte conditioned medium
pg	:	picogram
PWM	:	Pokeweed mitogen
RBC	:	Red blood cell
rHGM-CSF	:	Recombinant human granulocyte-macrophage-CSF.
RNA	:	Ribonucleic acid
RPMI	:	RPMI 1640 culture medium from Gibco
SCM	:	Pokeweed mitogen stimulated mouse spleen conditioned medium
SE	:	Standard error of the mean
T	:	Thymus
TdT	:	Terminal deoxynucleotidyl transferase
U/5637-CM	:	Bladder carcinoma cell (U/5637) conditioned medium
µg	:	Microgram
v/v	:	The volume of one solution as a percentage of the total volume

SUMMARY

To examine the control of human haemopoietic cells by the haemo-
poietic hormones (the Colony Stimulating Factors, CSF's) both puri-
fied factors and purified cell populations are required.

Studies were initially performed to characterize the action of
purified murine and purified and partially-purified human CSF's on
human marrow cells. Of the purified murine CSF's, G-CSF was active
on human bone marrow cells. The murine molecule Eosinophil Differ-
entiation Factor (EDF) was shown to an Eosinophil-CSF with activity
on murine and human cells. Recombinant human GM-CSF (rHGM-CSF) was
shown to possess all the biological activities of the partially-
purified native molecule CSF-α. Both the glycosylated and non-glyco-
sylated preparations of rHGM-CSF showed equivalent biological activity
in vitro.

Subsequent studies were performed to purify and characterize a
population of normal committed-granulocyte progenitor cells and the
action of CSF's on these cells was examined. Normal human promyelo-
cytes and myelocytes were obtained using the monoclonal antibody
WEM-G11 and the fluorescence activated cell sorter. These cells
demonstrated transient, CSF-stimulated proliferation in vitro and
generated neutrophilic clones of less than 40 cells in size (clusters).
These cells were stimulated to proliferate by human CSF-α, CSF-β
and murine G-CSF. Clone transfer experiments documented the ability
of clones initiated by one CSF (α or β) to proliferate when trans-
ferred to cultures stimulated by the other CSF. The cross-species
activity of human CSF-β and murine G-CSF, and the ability of CSF-β
to compete with radio-iodinated murine G-CSF for binding-sites on
murine (and human) cells suggested that CSF-β was the human equival-
ent of murine G-CSF.

A comparative study of leukaemic promyelocytes demonstrated that fractionated promyelocytes-myelocytes from patients with chronic myeloid leukaemia also showed transient clonal proliferation in vitro and responded to CSF-α, CSF-β and murine G-CSF. Promyelocytes from the blood of these patients generated clones of only two cells in CSF-unstimulated cultures. This behaviour was mimicked when normal promyelocytes-myelocytes were pulse-stimulated by CSF for 45 min.

Leukaemic cells from patients with acute myeloid leukaemia also demonstrated CSF-stimulated proliferation in vitro and were responsive to CSF-α, CSF-β, rHGM-CSF and murine G-CSF. There was however considerable heterogeneity in the CSF-responsiveness of these cells. Differentiation-induction by CSF in leukaemic cells was examined using the human leukaemic cell line HL60. When stimulated by CSF, these cells showed increased expression of myeloid surface antigens (as monitored by three monoclonal antibodies) and decreased numbers of clonogenic cells. In some experiments the number of clonogenic cells was reduced to zero.

In an attempt to establish a sensitive micro-assay for CSF, two target cell populations were examined. Normal promyelocytes-myelocytes displayed CSF-stimulated proliferation in a micro-assay system but this was not associated with a heightened sensitivity to CSF. The survival of mature human neutrophils and eosinophils in vitro was however shown to be enhanced by CSF's in a "lineage-specific" manner and this assay was between 10^2-10^3 times more sensitive to CSF than agar cultures.

These studies demonstrated that the Colony Stimulating Factors enhanced survival, stimulated proliferation and stimulated differentiation-commitment of normal and leukaemic human cells.

OUTLINE OF THIS THESIS

Chapter 1 contains a review of the cellular basis and control of haemopoiesis with discussion of the concepts of stem cells, committed progenitor cells and the regulation of haemopoiesis by the glycoprotein molecules, the Colony Stimulating Factors (CSF's). The myeloid leukaemias are also discussed as an example of disordered haemopoiesis.

Chapter 2 contains a detailed description of the methods used in this study.

Chapter 3 examines the action of purified and partially purified murine and human CSF's on normal human progenitor cells. The properties of murine Eosinophil Differentiation Factor and a recombinant human Granulocyte-Macrophage CSF are discussed in detail.

The experiments described in Chapter 4 used monoclonal antibodies in combination with the cell sorter to fractionate normal human marrow cells. Results are presented for four interesting monoclonal antibodies. One antibody, WEM-G11 identifies a population of normal promyelocytes-myelocytes and the proliferation of these cells in agar and liquid cultures (Chapter 5) and their responsiveness to purified murine and partially-purified human CSF's is examined (Chapter 6). In Chapter 7 the behaviour of equivalent populations of leukaemic cells and their response to CSF is examined and compared with normal promyelocytes-myelocytes. These studies are extended in Chapter 8 to include other leukaemic cell populations. The CSF-responsiveness of these cells and in particular the action of CSF to stimulate differentiation-committment in human leukaemic cells is discussed.

Chapter 9 examines the CSF-responsiveness of mature human granulocytes and documents the heightened sensitivity of these cells to CSF compared with progenitor cells.

An overall discussion of these studies with possible directions of future studies is presented in Chapter 10.

CHAPTER 1

THE CELLULAR BASIS AND CONTROL OF HAEMOPOIESIS

1.1 GENERAL INTRODUCTION

Blood is a complex mixture of mature cells that subserve highly specialized functions. These cells include red blood cells, neutrophil polymorphonuclear cells, eosinophil polymorphonuclear cells, monocytes, platelets and lymphocytes. While monocytes and lymphocytes can proliferate in response to appropriate stimuli, the other mature cells are non-dividing and have a finite lifespan.

The haemopoietic system, like other tissues which are called upon to continually produce a population of mature cells with limited lifespan, is organized in an hierarchical manner. The most primitive cells (stem cells) are also the least frequent cells. Stem cells have several important properties that include multipotentiality (i.e. the ability to give rise to cells of more than one lineage), the ability to self-renew (thus producing stem cells with identical or very similar properties), and the capacity for many cell divisions. Because of the low frequency of these cells and the difficulty involved in obtaining homogenous populations of stem cells, these cells are the most poorly understood.

Stem cells give rise to committed progenitor cells (see Figure 1.1) which have a lower proliferative capacity, can only give rise to cells in a limited number of lineages and have limited capacity for self-replicative divisions. Committed progenitor cells give rise to cells with reduced or no proliferative potential and ultimately a fully-differentiated, functionally mature cell is generated. These later stages of differentiation can occur in the absence of cell division.

Cells with proliferative potential can only be clearly identified on the basis of functional studies which demand expression of the ability to proliferate. In such studies of haemopoiesis in rodents
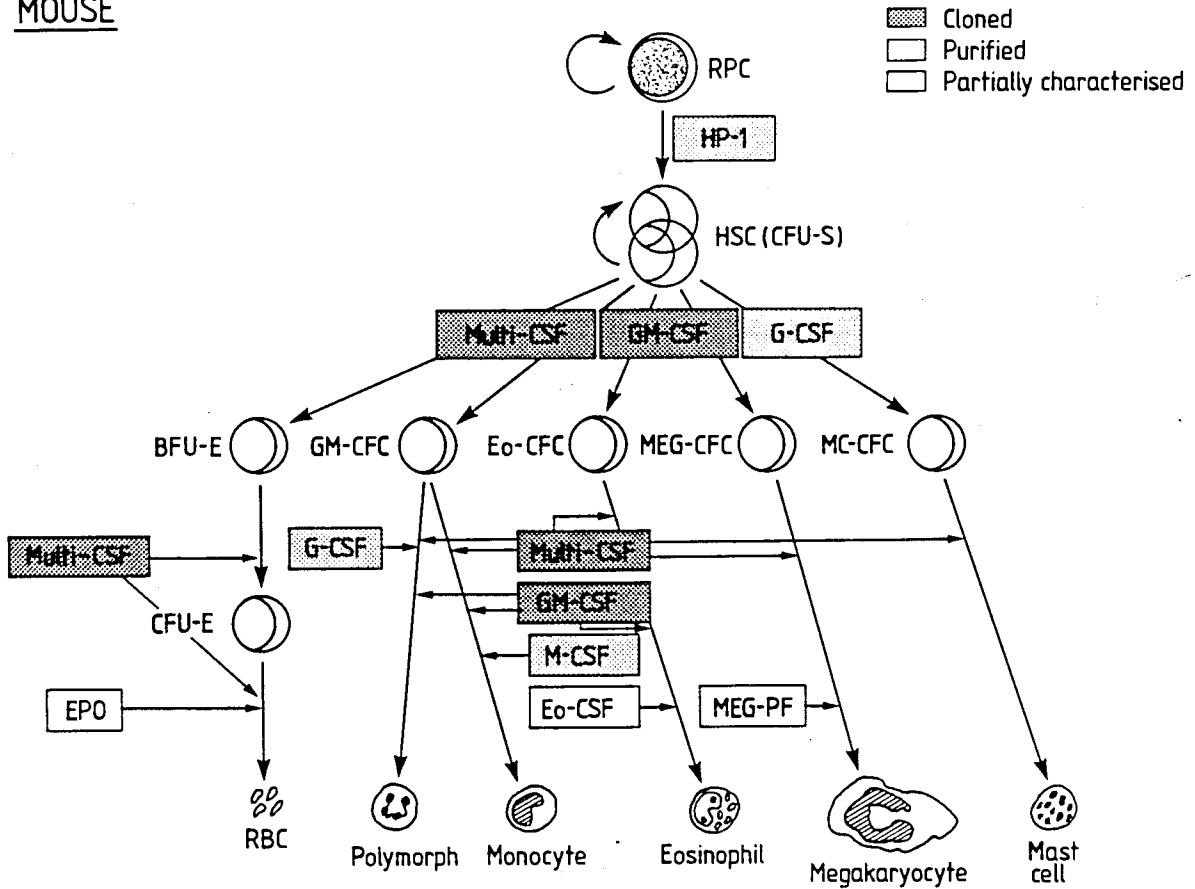
Fig. 1   A diagram representing the haemopoietic cells and their
regulatory factors as determined for murine haemopoiesis.
The most primitive stem cells (repopulating haemopoietic
cell, RPC) are not influenced by the colony stimulating
factors (CSF's) and are capable of extensive self-renewal.
These stem cells are probably stimulated by haemopoietin 1
(HP-1). Other stem cells (HSC) give rise to spleen colonies
in irradiated mice (CFU-S) and multipotential colonies
in vitro. These cells in the mouse are stimulated by Multi-
CSF, GM-CSF and G-CSF (in decreasing order of activity on
such cells). Late acting lineage-specific molecules-
erythropoietin (Epo), eosinophil-CSF (Eo-CSF) and megakaryo-
cyte potentiating factors (Meg-PF) have been described
but are generally less well characterised. The committed
progenitor cells (colony forming cells, CFC) are the
earliest cells committed to the indicated cell lineage.
In the erythroid lineage, BFU-E are the early and CFU-E
the late erythroid-committed cells (After Nicola and
Metcalf, 1985a).

stem cells are identified by their ability to give rise to a nodule
(colony) of cells in the spleen of an irradiated animal (Till and
McCulloch, 1961). Therefore, this stem cell has been a CFU-S
(Colony-Forming-Unit-Spleen) (Figure 1.1). The committed progenitor
cells are referred to as Colony-Forming-Cells (CFC's) because of
their ability to generate colonies of daughter cells in semi-solid
culture medium such as agar (Bradley and Metcalf, 1966; Pluznik and
Sachs, 1965) or methylcellulose (Iscove et al, 1974). The lineage
to which a progenitor is committed is also only recognized in retro-
spect by examining the morphology of cells present in a particular
colony. A prefix or suffix is used to denote a particular lineage,
thus Granulocyte-Macrophage-CFC (GM-CFC), Eosinophil-CFC (Eo-CFC)
or for the erythroid lineage Burst-Forming-Unit-Erythroid (BFU-E),
Colony-Forming-Unit-Erythroid (CFU-E).

The proliferation and functional activation of haemopoietic
cells in vitro is under the control of a family of glycoprotein
hormones called Colony-Stimulating Factors (CSF's). These molecules
are named because of their ability to stimulate progenitor cells to
give rise to colonies in vitro. Some of the CSF's display specific-
ity for particular lineages and this is indicated by a prefix, thus
Granulocyte-CSF (G-CSF), Macrophage-CSF (M-CSF). One murine molecule
is named Multipotential-CSF (Multi-CSF) to indicate a broad spectrum
of activity on all haemopoietic lineages including some activity on
stem cells, however the human analogue of this molecule is not clear
either in terms of biological activity or nucleotide homology.
Recently a human-derived mouse-active molecule has been purified
that is active on stem cells but has no activity on committed progen-
itor cells (Haemopoietin 1) and such a molecule may only represent
one of a family of stem cell-growth factors.

Of the molecules controlling haemopoiesis in vitro only four have been clearly implicated as being of relevance in vivo. Erythropoietin (primarily a differentiative-stimulus of the erythroid lineage and indubitably of relevance in vivo), Granulocyte-Macrophage-CSF (GM-CSF), G-CSF and M-CSF can be demonstrated in the circulation or urine of rodents or man, thus suggesting a possible role in physiological (and pathological) states. The other molecules are not detected in the circulation, suggesting that their role in vivo might be, predictably for stem cell-active factors, a local one.

This review will consider in detail the concepts of stem cells, committed progenitor cells, the colony stimulating factors and their cells of origin, and the relevance of these studies to pathological states (the myeloid leukaemias). Brief specific reviews of relevant topics are also included in the introduction to each chapter.

1.2 HAEMOPOIETIC CELL POPULATIONS AND THEIR REGULATORS

1.2.1 Stem Cells

The assay for the small proportion of bone marrow cells with the capacity for extensive proliferation and differentiation into multiple lineages was developed by Till and McCulloch (1961). The assay determines the number of haemopoietic colonies on the spleen surface 7-14 days after bone marrow transplantation into irradiated recipients. Each colony originates from a single stem cell (CFU-S) which has extensive capacity for self-renewal (Siminovitch et al, 1963). Colonies may contain cells of all haemopoietic lineages, including lymphocytes (Wu et al, 1968) and are derived from undifferentiated blast cells (Goldschneider et al, 1980).

The factors that initiate stem cell division and determine whether such a division will be "self-renewing" or "differentiative" remain unclear. This problem has been extensively reviewed (Quesenberry

and Levitt, 1979; Till and McCulloch, 1980; Ogawa et al, 1983). It has been proposed that the most primitive multipotential haemopoietic stem cells reside in a limited number of "niches" within the bone marrow (Schofield, 1978). True self-renewal would only occur in the most primitive cells and only in the environment of the bone marrow "niche". This model explains both the apparent immortality of stem cells (Lajtha and Schofield, 1971; Harrison, 1973) and the "hierachy" of stem cells (Rosendaal et al, 1976, 1979; Hodgson and Bradley, 1979; Hodgson et al, 1982; Magli et al, 1981). Some evidence for a cellular "niche" regulating stem cell development comes from studies of long-term bone marrow cultures (Dexter et al, 1977; Bentley and Foidart, 1980; Bentley et al, 1982) in which a stromal layer is required for maintenance of CFU-S in vitro.

An alternative view is that stem cell commitment is under control of CFU-S-modulating factors, which remain poorly defined. The best characterized haemopoietic factors are the colony stimulating factors (CSF's), however, the action of these molecules on stem cells is limited and may be restricted to multipotential cells. Other factors acting on stem cell populations are poorly characterized but include stimulatory regulators (Lord et al, 1977; Lowenberg and Dicke, 1977; Cerny et al, 1975) and inhibitory regulators (Lord et al, 1976; Frindel and Guigon, 1977) and the proliferative status of CFU-S is apparently determined by the relative levels of these regulators (Toksoz et al, 1980).

Recently a human-derived, murine-active factor has been purified. This molecule, haemopoietin 1 is active on murine stem cells but has no CSF activity. No activity of this molecule on human cells has been described (Jubinsky and Stanley, 1985) although being of human origin it is tempting to speculate that this molecule would subserve

a similar function in man. Unfortunately it will prove difficult to test the action of this molecule on human stem cells because clearly the murine CFU-S assay system is not applicable to human stem cells. The cross-species reactivity of haemopoietin 1 is intriguing and suggests the possible importance of this molecule because of its strong conservation through evolution. Further characterization of stem cell-active factors will continue to be limited by the difficult-ies involved in obtaining purified populations of stem cells and the cumbersome assay systems that are required. However in this regard the use of murine bone marrow cells following treatment with 5-fluorouracil has proven to be useful in providing an enriched population of primitive haemopoietic cells (Bradley and Hodgson, 1979; Hodgson and Bradley 1979; Iscove et al, 1985).

      1.2.1.1. Multipotential Cells. Both murine (Johnson and Metcalf, 1977) and human (Fauser and Messner, 1979) multipotential colonies can be grown in vitro. Colonies of murine origin contain "mature" CFU-S capable of a limited number of self-renewing divisions in vitro (Metcalf et al, 1978; Humphries et al, 1981).

The growth of human mixed colonies was first reported using α-medium, 30% foetal calf serum, PHA-LCM and erythropoietin in methyl-cellulose (Fauser and Messner, 1979). Subsequent modifications of this system have included Iscove's Modified Dulbecco's medium with 5×10^{-5}M 2-mercaptoethanol (Ash et al, 1981); addition of bovine serum albumin, (Hara et al, 1981) sodium selenite (Fitchen et al, 1982) and fibrinogen clot cultures without erythropoietin but with aplastic anaemia serum (Nishihira and Kigasawa, 1981).

Evidence for clonality of human multipotential colonies is indirect but is provided by the linearity of colony growth when vary-ing numbers of cells are cultured, (Ash et al, 1981; Hara et al, 1980)

sedimentation velocity studies (Fauser and Messner, 1978) and co-culture of cells from males and females (Fauser and Messner, 1978; Hara et al, 1980) and G-6P-D isoenzyme studies (Powell et al, 1982). All these studies have been severely limited by culture conditions in which the total number of colonies per culture frequently exceeds several hundreds. Colony overlap can occur with as few as 30 colonies per culture (Singer et al, 1979a) and thus estimates of the frequency of human multipotential colonies and evidence for their clonal origin must be interpreted with caution. Further studies are required utilizing either more stringent culture criteria or purified populations of cells to examine these questions more closely. All studies to date have probably over-estimated the frequency of multipotential colonies.

The cells giving rise to human multipotential colonies (Multi-CFC) appear to be non-cycling under normal steady-state conditions. This is in contrast to normal BFU-E or GM-CFC and to Multi-CFC in situations of active marrow regeneration (Fauser and Messner, 1979a). Multi-CFC are unable to be separated from BFU-E or GM-CFC on the basis of sedimentation velocity (Fauser and Messner, 1978) however differences in bouyant density have been observed (Francis et al, 1983). As with GM-CFC (Winchester et al, 1977; Cline and Billing, 1977; Janossy et al, 1978; Fitchen et al, 1980) and BFU-E (Moore et al, 1980; Winchester et al, 1978; Robinson et al, 1981), Multi-CFC express HLA-AB and Ia-like antigens (Fitchen et al, 1982; Lu et al, 1983). The similarities with progenitor cells have precluded purification of Multi-CFC.

The frequency of each haemopoietic lineage present in multi-potential colonies has not been adequately determined. In two studies, all colonies were found to contain both erythroid cells and neutrophils, monocytes were present in approximately 40% of

colonies, megakaryocytes in 20% and eosinophils in 5% of colonies
(Messner and Fauser, 1980; Ash et al, 1981). T-lymphocytes have been
reported (Messner et al, 1981) but probably reflect the use of over-
crowded cultures especially as this observation has not been confirmed
by other workers. The existence of colony-forming cells with only
erythroid and eosinophilic potential has also been demonstrated
(Nakahata et al, 1982).

Although human multipotential colonies consist of cells of more
than one lineage and thus display one characteristic of stem cells
(that of multipotentiality), human Multi-CFC bear little relationship
to murine CFU-S. Human Multi-CFC possess almost no self-renewal
capacity (Messner and Fauser, 1980; Ash et al, 1981) and thus represent
relatively "mature" cells in the hierachy of stem cells (Rosendaal
et al, 1976, 1979; Hodgson and Bradley, 1979; Magli et al, 1981). A
more primitive human stem cell has been reported by Nakahata and
Ogawa (1982). These cells have considerable self-renewal potential
and generate colonies of undifferentiated blast cells or multi-
potential colonies (but without T-lymphocytes). Such cells have been
detected in cord blood but not adult blood or marrow.

Study of the ontogeny of haemopoiesis in the human foetus showed
that Multi-CFC (and GM-CFC and BFU-E) populate the foetal liver
throughout 12-23 weeks of gestation. The bone marrow becomes popul-
ated at 15-16 weeks and the spleen at 19 weeks (Hann et al, 1983).

Using similar culture conditions as those described for normal
cells, growth of multipotential colonies has been examined in poly-
cythaemia rubra vera (Fauser and Messner, 1981), chronic myeloid
leukaemia (Hara et al, 1981; Lepine and Messner, 1983) and is reduced
in aplastic anaemia (Hara et al, 1980).

1.2.2 <u>Committed Granulocyte-Macrophage Progenitor Cells</u>

Colonies of neutrophils, monocytes and eosinophils (clones of greater than 40 cells), can be grown from human blood or bone marrow cells when cells are stimulated by peripheral blood cell underlayers (Iscove et al, 1971; Chervenick and Boggs, 1971) or human placental conditioned medium (Dresch et al, 1977). If cultures are examined after 7 days of culture, most colonies are neutrophil in type whereas after 14 days, colonies of neutrophils, macrophages and eosinophils are present. Using sedimentation velocity techniques (Johnson et al, 1977; Inoue and Ottenbreit, 1978; Jacobsen et al, 1978; Miller et al, 1978), lectin binding (Morstyn et al, 1980; Nicola et al, 1980) or monoclonal antibodies (Strauss et al, 1984; Skubitz et al, 1983a; Young and Hwang-Chen, 1981; Andrews et al, 1983). It is possible to separate progenitor cells forming colonies after 7 days of culture from those forming colonies after 14 days of culture. These and other experiments suggest that the day 7 colony-forming cells are the progeny of day 14 progenitor cells (Dresch et al, 1979; Jacobsen et al, 1979; Moore et al, 1980). Murine and human progenitor cells have been identified as undifferentiated blast cells (Morstyn et al, 1980; Nicola et al, 1981; Beverley et al, 1980; Griffin et al, 1982). The immediate progeny of progenitor cells appear to be cluster-forming cells (generating clones of less than 40 cells) and at least some of these cells also appear to have the morphology of undifferentiated blast cells (Morstyn et al, 1980; Nicola et al, 1981; Griffin et al, 1982). Studies using fractionated populations of marrow cells demonstrated an apparent inability of cells such as promyelocytes and myelocytes to proliferate in vitro (Griffin et al, 1981; Ball and Fanger, 1983) although in vivo labelling studies using tritiated thymidine showed that such cells are clearly capable

of proliferation (Warner and Athens, 1964). The apparent inability of these cells to proliferate clonally in agar may be due either to an intrinsic inadequacy of the culture system or to a deficiency in culture-scoring techniques currently employed.

The survival of progenitor cells in vitro is dependent on the presence of colony-stimulating factor (CSF). Fewer than 5% of murine marrow granulocyte-macrophage progenitor cells are able to survive more than 24 hours in the absence of CSF (Metcalf, 1970; Lin and Stewart, 1974). Transfer of colonies initiated by CSF to cultures lacking CSF leads to cessation of colony growth and death of colony cells (Metcalf and Foster, 1967; Paran and Sachs, 1968). Similar studies with human marrow cells have documented the requirement for CSF for cell survival (Morstyn et al, 1981). The levels of CSF required for cell survival are lower than those required for proliferation (Burgess et al, 1982).

The proliferation of murine and human progenitor cells is dependent on stimulation by CSF. CSF can influence both entry into the cell cycle (Moore and Williams, 1973; Tushinski and Stanley, 1983) and duration of cell cycle time (Metcalf and Moore, 1973; Metcalf, 1980). Few cells are even able to complete a cell cycle in progress at the time of CSF-withdrawal (Metcalf, 1980). The concentration of CSF can hasten the onset of clonal proliferation in vitro (Metcalf, 1970) although the asynchronous onset of clonal proliferation remains unexplained but apparently unrelated to cell cycle at the time the culture is initiated (Metcalf, 1972). CSF concentration can also determine to what extent the proliferative-potential of a progenitor cell is expressed in vitro (Metcalf, 1980). The action of CSF to determine differentiation commitment has been reviewed by Metcalf (1984, 1985, 1985a) and has significant implications for leukaemic

cells. This action of CSF on normal and leukaemic cells will be discussed in the context of acute myeloid leukaemia.

Despite the requirement for CSF for both progenitor cell survival and proliferation, continued proliferation following withdrawal of CSF has been observed for murine cells (Metcalf and Burgess, 1982) with up to 30% of cells proliferating following transfer to cultures lacking CSF (Metcalf and Merchav, 1982). Likewise, 30% of human clones continued to proliferate following CSF-withdrawal (Morstyn et al, 1981). These observations have not been adequately explained, although one trivial explanation may be the carry-over of CSF in the agar droplet during clone transfer experiments.

1.2.2.1   Regulators of the Granulocyte-Macrophage Lineage:

Murine Colony-Stimulating Factors (CSF's). More is known about regulator control of the granulocyte-macrophage lineage than any other. This area has been extensively reviewed by Metcalf (1977, 1984, 1985, 1985a, 1986).

Four molecules stimulate proliferation and differentiation of murine granulocyte-macrophage progenitor cells in vitro. These molecules have been purified and shown to be active at concentrations of 10^{-11}M to 10^{-12}M (Burgess et al, 1977; Stanley and Heard, 1977; Nicola et al, 1983; Ihle et al, 1983). Granulocyte-macrophage CSF (GM-CSF) stimulates mixed granulocyte-macrophage colonies as well as pure granulocyte and pure macrophage colonies. It also stimulates pure eosinophil colonies (Burgess et al, 1977; Johnson and Metcalf, 1980). Granulocyte CSF (G-CSF) stimulates primarily granulocyte colonies (90% or greater) in cultures of murine bone marrow cells and has a unique ability to induce differentiation and suppress self renewal of murine myelomonocytic leukaemia cells (Nicola et al, 1983). Macrophage CSF (M-CSF) stimulates predominantly pure macrophage colonies (Stanley and Heard, 1977).

Multi-CSF is active upon all these progenitor cell populations but is also active on erythroid, megakaryocyte and mast cell lineages. Multi-CSF also stimulates multipotential colony formation (Ihle et al, 1983; Clark-Lewis et al, 1984; Cutler et al, 1985). The genes encoding GM-CSF and Multi-CSF have been cloned (Yokota et al, 1984; Fung et al, 1984; Gough et al, 1984) and expressed in mammalian cells and E.coli.

All these molecules (with the exception of M-CSF) are glyco-proteins composed of a single polypeptide chain with an apparent molecular weight 23,000-28,000. M-CSF is a glycoprotein with a molecular weight of approximately 70,000 and consists of two disulphide subunits of similar size.

Antisera have been raised in rabbits against M-CSF (Shadduck and Metcalf, 1975; Stanley et al, 1976) and Multi-CSF (Ihle et al, 1982). The antiserum raised against M-CSF does not cross-react with GM-CSF, G-CSF or Multi-CSF (Shadduck and Metcalf, 1975; Stanley, 1979; Burgess and Metcalf, 1980).

All 4 molecules have been radiolabelled with retention of full biological activity. There are approximately 73,000 receptors for M-CSF present per cell for adherent peritoneal macrophages, and these receptors are restricted to macrophages, their precursors and macro-phage cell lines (Stanley and Guilbert, 1981; Guilbert and Stanley, 1980). This receptor has been identified as the proto-onc gene c-fms (Sherr et al, 1985). Receptors for G-CSF are essentially restricted to the granulocyte-lineage and the frequency of receptors increases with differentiation (Nicola and Metcalf, 1985). With the exception of M-CSF, there are relatively few receptors present on responding cells (hundreds to a few thousand) for each CSF. Cellular binding characteristics are similar for the 4 CSF's; binding to cells is

rapid, dissociation is extremely slow and receptor-binding is completely specific (Nicola and Metcalf, 1984; Palaszynski and Ihle, 1984; Walker and Burgess, 1985). There is, however apparent down-regulation of unoccupied CSF receptors by other CSF-receptor complexes that is organised in an hierarchical manner (Walker et al, 1985).

In summary, 4 actions have been established for the purified murine CSF's. These are (1) the requirement for CSF for the survival of progenitor cells in vitro; (2) the requirement for CSF for cell division, with few cells even able to complete the cell-cycle in progress of the time of CSF withdrawal; (3) differentiation commitment and (4) activation of mature cell function.

1.2.2.2 <u>Human Colony-Stimulating Factors</u>. Three types of human CSF have been defined. The M-CSF class, present in human urine, is characterised by its low activity in stimulating human bone marrow progenitor cells in contrast to its relatively high activity in stimulating macrophage colonies from mouse bone marrow progenitor cells (Wu and Yunis, 1980; Das et al, 1981). This molecule has sequence, structural and binding homology with murine M-CSF (Das et al, 1980; Motoyoshi et al, 1982) and a cDNA for this molecule has been molecularly cloned (Kawasaki et al, 1985).

Two other human CSF's with activity on human granulocyte-macrophage progenitor cells have been identified. These have been termed CSF-α and CSF-β and can be separated from each other by hydrophobic chromatography on phenyl-Sepharose (Nicola et al, 1979). They have been identified in conditioned media obtained from placenta, monocytes and the Giant Cell Tumour (GCT) cell line (Nicola et al, 1979; Abboud et al, 1981; Vadas et al, 1984). CSF-α stimulates granulocyte, macrophage and eosinophil colony formation from human bone marrow cells (Nicola et al, 1979; Morstyn et al, 1981) and functionally activates mature neutrophils and eosinophils (Vadas et al, 1984). This is

probably the molecule purified by Lusis et al (1981; 1982). In contrast, CSF-β stimulates predominantly granulocyte colony formation and activates mature neutrophils but has no action on eosinophil progenitor cells nor on mature eosinophils.

Recently a human CSF has been molecularly cloned and shows approximately 60% amino acid sequence homology with murine GM-CSF (Wong et al, 1985; Lee et al, 1985; Cantrell et al, 1985). The same CSF has been purified from the human Mo-cell line (Gasson et al, 1984) and was shown to be active on mature human neutrophils (Weisbart et al, 1985). Other activities of this molecule have not been examined and its relationship to the previously described CSF-α and CSF-β is not clear. However, based on the amino acid homology with murine GM-CSF, a similar spectrum of activities on human progenitor cells might be predicted.

The human equivalent of murine G-CSF also remains to be clearly identified. Murine G-CSF stimulates the functional activity of mature human neutrophils but not eosinophils (Lopez et al, 1983) and thus has selectively of action similar to human CSF-β, however its action on human progenitor cells has not been examined. Furthermore, analogous to murine G-CSF, human CSF-β but not CSF-α will induce differentiation in murine WEHI-3B myelomonocytic leukaemia cells (Metcalf, 1983).

There is no evidence at present for a human molecule analogous to murine Multi-CSF. Recently a molecule described as human pluripoietin has been purified from the conditioned medium of the bladder carcinoma cell line U/5637 (Welte et al, 1985). It is most likely, however, that this molecule is CSF-β based on its ability to induce differentiation in murine WEHI-3B cells and its inability to stimulate eosinophil colony formation from human bone marrow cells.

Other sources of human regulators have been described (Walasek et al, 1976; Shah et al, 1977; Price et al, 1975; Wang et al, 1985; Kimura et al, 1979; Ruppert et al, 1983) but have been inadequately characterized either in terms of biological or physicochemical characteristics to allow comparison with the molecules described above or to allow definition of a separate class of regulator.

1.2.3 Committed Eosinophil Progenitor Cells

A role for a T-lymphocyte-derived humoral factor regulating the eosinophil response was first demonstrated in vivo (Basten and Beeson, 1970). The growth of eosinophil colonies in vitro was stimulated by mitogen-stimulated lymphocyte-conditioned medium (Chervenick and Boggs, 1971; Metcalf et al, 1974). Kinetic analysis of eosinophil production in vivo (Mahmoud et al, 1977) and in vitro (Bartelmez et al, 1980, 1980a) suggested that two regulators might be involved in a two-stage process of eosinophil production.

The early descriptions of murine Eo-CSF (Burgess et al, 1980) are now ascribable entirely to Multi-CSF (Cutler et al, 1985). In addition, however, a human-active Eo-CSF has been demonstrated in mouse SCM (Metcalf et al, 1983). This activity is able to be separated from Multi-CSF and is also produced by mouse T-lymphocyte clones (Kelso and Metcalf, 1985). This molecule is described as having no proliferative effects on mouse haemopoietic cells. The molecule is a glycoprotein with a molecular weight of about 35,000 (Metcalf et al, 1983).

A murine eosinophil differentiation factor (EDF) has also been reported. This molecule stimulates proliferation of eosinophils in liquid culture systems but does not stimulate colony formation in

agar (Sanderson et al, 1985). It is produced by a mouse T-cell hybridoma without detectable GM-CSF, Multi-CSF or interferon. This molecule has been highly purified (Sanderson et al, 1985a) and its relationship to the human-active Eo-CSF is unknown.

It is possible that the requirement for additional regulators in these culture systems is met by the plasma or serum used, as considerable variation exists in the ability of different batches of human plasma to support eosinophil colony growth (Johnson and Metcalf, 1980). A putative "eosinophilopoietin" has been described that stimulates the terminal maturation stages of eosinophils in vivo (Mahmoud et al, 1977) and in vitro (Bartelmez et al, 1980a) giving maximal numbers of eosinophils by day 2, but this molecule(s) remains poorly characterized.

Human sources of an Eo-CSF activity include conditioned media from placenta (Nicola et al, 1979) and the GCT-monocytic cell line (Abboud et al, 1981). These molecules have molecular weights of approximately 30,000 and cannot be separated from associated GM-CSF activity (CSF-α).

1.2.4 Committed Erythroid Progenitor Cells

An analysis of the kinetics of developing erythroid colonies (Gregory, 1976; Iscove, 1977) and the physical properties of the cells giving rise to these colonies (Heath et al, 1976; Gregory and Eaves, 1978) provided evidence that the two subpopulations of progenitor cells were under the influence of two regulators: the more primitive committed progenitor (BFU-E) being under the influence of one regulator and giving rise to a more mature progenitor population (CFU-E) which generates haemoglobinised erythroid cells under the influence of a

second regulator. Only the second regulator (erythropoietin) is required to generate haemoglobinised colonies from CFU-E while both regulators are required for haemoglobinised colonies from BFU-E.

A wide variety of names have been adopted for the activity stimulating the differentiation of BFU-E to CFU-E but one of the most common is "Burst Promoting Activity" (BPA) (Iscove, 1978). This activity is a property of three murine CSF's (i.e. Multi-CSF, GM-CSF and G-CSF) and there is no evidence for a distinct murine molecule, BPA or Erythroid-CSF (see Metcalf, 1984).

The differentiation of CFU-E to form haemoglobinised erythroid cells is regulated by erythropoietin in all mammalian sytems (Iscove, 1977). Erythropoietin is active across species (Krantz and Jacobson, 1970) and erythropoietin from sheep plasma (Goldwasser and Kung, 1971) and human urine (Miyake et al, 1977) stimulate mouse (and human) CFU-E.

Factors which stimulate the differentiation of human BFU-E to CFU-E have been described in medium conditioned by the Mo-cell line (Gauwerky et al, 1982), placenta (Hoang et al, 1983) and human leuco-cytes (Hoang et al, 1983). All have apparent molecular weights of 45,000 and are not associated with GM-CSF activity (Lusis and Golde, 1981; Hoang et al, 1983). This erythroid potentiating activity (EPA) for human BFU-E has been purified from Mo-cell conditioned medium (Westbrook et al, 1984) and is without GM-CSF activity. The gene encoding this molecule has been molecularly cloned (Gasson et al, 1985). The murine equivalent of human EPA is not clear.

Human EPA has recently been shown to be identical to a protein "tissue inhibitor of metalloproteinases" which complexes with metallo-proteinases and prevents the destruction of collagen (Docherty et al, 1985). In the context of the poor biological assays for EPA

and the absence of a murine equivalent for this molecule, this raises
the possibility that the action of this protein is completely non-
specific in enhancing erythroid colony formation in vitro.

Erythroid potentiating (or Burst promoting) activity has also
been described for the purified recombinant human GM-CSF. While
Emerson et al. (1985) claim to show a direct action of this molecule
on erythroid progenitor cells, their assay system is non-linear with
poor colony growth below 100 cells per ml. This suggests that the
action of GM-CSF is via an accessory cell and is consistent with
the observations of others suggesting an indirect action on erythroid
progenitors (Metcalf et al, 1985).

1.2.5. Committed Megakaryocyte Progenitor Cells

Megakaryocyte colonies can be grown in vitro from both murine
(Metcalf et al, 1975) and human (Vainchenker et al, 1979) bone marrow
cells. There are two subpopulations of progenitor cells (Williams
and Jackson, 1978) and differentiation of megakaryocytes appears to
be a two-stage process under the control of two regulators (Williams
and Levine, 1982). No exclusive megakaryocyte-CSF has been detected
from mouse sources, but both Multi-CSF and GM-CSF can stimulate
megakaryocyte progenitors (Cutler et al, 1985; Metcalf et al, 1980).
A putative megakaryocyte-CSF has been purified from human plasma
(Hoffman et al, 1985) however neither the specificity nor the
direct action of this molecule on megakaryocyte-progenitors has
been demonstrated because of the high cell numbers used in the
culture system.

The requirement for a second factor in culture systems may be
met by the serum or plasma used in murine and human cultures (Metcalf
et al, 1975; Messner et al, 1982; Solberg et al, 1985). The regulator

for megakaryocyte maturation has been called thrombopoietin (for both human and murine systems) (Williams et al, 1981). Three sources of thrombopoietin have been examined: human embryonic kidney cells (McDonald et al, 1975), plasma of thrombocytopenic rabbits (Evatt et al, 1979) and mouse peritoneal macrophage conditioned-medium (Williams et al, 1982).

1.2.6. Mature Cells: Stimulation by CSF

The CSF's have an important role in stimulating functional activities of mature cells. Murine M-CSF has been shown, not only to stimulate proliferation of macrophages (Stanley et al, 1976) but also production and release of interleukin 1 (Moore et al, 1980a), prostaglandin (Kurland et al, 1979), arachidonic acid (Ziboh et al, 1982), interferon (Fleit and Rabinovitch, 1981) and plasminogen activator (Hamilton et al, 1980). M-CSF also stimulates the ability of macrophages to kill tumour cells (Wing et al, 1982). GM-CSF can stimulate some of these functions as well as the ability of macrophages to kill Leishmania parasites (Handman and Burgess, 1979). Multi-CSF is also active on mature macrophages (Crapper et al, 1985).

GM-CSF stimulates RNA and protein synthesis in mature granulocytes (Burgess and Metcalf, 1977a; Stanley and Burgess, 1983). Both GM-CSF and G-CSF stimulate the ability of polymorphs to exhibit antibody-dependent cytotoxicity for tumour cells while M-CSF has no such action (Lopez et al, 1983). Similarly, antibody-dependent cytotoxicity exhibited by human neutrophils is stimulated by both CSF-α and CSF-β (Vadas et al, 1983), while eosinophils are not stimulated by CSF-β. CSF-α increases autofluorescence, superoxide production and protein synthesis of eosinophils (Vadas et al, 1983). Killing of tumour cells (Vadas et al, 1983a) and Schistosomula parasites (Dessein et al, 1982) by eosinophils is also enhanced. Two studies (Stanley

and Burgess, 1983; Vadas et al, 1983) have also suggested that CSF may have a role in enhancing survival of mature cells, similar to the action on progenitor cells (Morstyn et al, 1981; Metcalf and Merchav, 1982) although no formal studies have been performed to examine this question.

1.3 CELLULAR SOURCES OF HAEMOPOIETIC REGULATORS

1.3.1. Colony Stimulating Factors

The growth of granulocyte-macrophage colonies in vitro can be stimulated by numerous body tissues or organ extracts (Bradley et al, 1971; Sheridan and Stanley, 1971) and GM-CSF synthesized by different murine organs has been shown to have the same biochemical properties (Nicola et al, 1979). The actual cells capable of synthesizing CSF have not been completely determined. Mitogen-stimulated T-lymphocytes can synthesize Multi-CSF, GM-CSF and human-active Eo-CSF (McNiell, 1973; Parker and Metcalf, 1974; Burgess et al, 1980; Clark-Lewis and Schrader, 1981; Kelso and Metcalf, 1985; Cutler et al, 1985). Similarly, mitogen-stimulated human T-lymphocytes are able to synthesize GM-CSF (Cline and Golde, 1974; Prival et al, 1974; Shah et al, 1977). This activity has been variously ascribed to the T-lymphocyte helper population (Verma et al, 1983), the T-lymphocyte suppressor population (Chikkappa and Phillips, 1984) and both helper and suppressor populations (Hesketh et al, 1984). Both murine-helper and suppressor T-lymphocyte clones can synthesize CSF (Kelso and Metcalf, 1985).

Initial studies suggested monocytes were the cells producing CSF (Moore and Williams, 1972; Chervenick and Lo Buglio, 1972; Golde and Cline, 1972; Moore et al, 1973a) however it has been suggested that instead, monocytes produce a factor with a powerful enhancing effect on CSF production by lymphocytes (Bagby et al, 1981). Mono-

cytes are also capable of producing inhibitory factors (To et al, 1983) and have been clearly shown to produce murine G-CSF (Metcalf and Nicola, 1985).

Fibroblast cell lines produce M-CSF (Stanley and Heard, 1977; Austin et al, 1971) and freshly isolated fibroblasts have been described as a source of GM-CSF (Koury and Pragnell, 1982). Mouse bone marrow adherent-cell-derived fibroblast cell lines produce M-CSF (Cronkite et al, 1982; Lanotte et al, 1982) although stromal cells may also synthesize GM-CSF (Chan and Metcalf, 1972). Stromal cell lines have been reported with activities consistent with production of Multi-CSF (Kodama et al, 1982) and possibly haemopoietin-1 (Li and Johnson, 1985). Human fibroblasts (and endothelial cells) synthesize CSF and this ability is markedly enhanced by a factor produced by monocytes (Bagby et al, 1983; 1983a).

Numerous neoplastic cell lines produce CSF's. The murine myelomonocytic leukemia WEHI-3B produces Multi-CSF (Warner et al, 1969; Ihle et al, 1982; Clark-Lewis et al, 1984) as does a T-cell hybridoma (Schrader et al, 1982). Human neoplastic lines producing CSF include the Mo-T-lymphocyte cell line (Golde et al, 1978), monocyte-like tumour lines (Di Persio et al, 1978), the bladder cancer cell line, U/5637 (Svet-Moldavsky et al, 1983), pancreatic carcinoma cells (Wu et al, 1979) and lung cancer cells (Asano et al, 1977).

1.3.2 Burst Promoting Activity

The question of a specific molecule capable of enhancing the formation of BFU-E in vitro has remained confused, primarily because of the difficulty in obtaining pure populations of human erythroid progenitor cells. In the murine system, purified Multi-CSF (IL-3), GM-CSF and G-CSF all have burst promoting activity (Metcalf, 1984), however in the human system no such activity has been shown for a

purified molecule. Sources of human burst promoting activity (BPA) or erythroid potentiating activity (EPA) include aplastic anaemia serum (Nissen et al, 1979), conditioned medium from human leucocytes or kidney (Aye, 1977), the Mo-T cell leukaemic cell line (Golde et al, 1980), the monocyte cell line U/937 (Ascensao et al, 1981), the GCT-tumour cell line (Abboud et al, 1980) and some T cell leukaemia-lymphoma cell lines (Hamburger, 1980; Kubota et al, 1982; Tarella et al, 1982; Froom et al, 1985).

Controversy exists regarding the cells which produce enhanced proliferation of BFU-E in vitro. Whilst some workers have demonstrated little dependence of either human marrow (Lipton et al, 1980; Linch et al, 1982; Levitt et al, 1985) or peripheral blood BFU-E (Torok-Storb et al, 1980; Nomdedeu et al, 1980; Zuckerman 1981; Levitt et al, 1985) on T lymphocytes, others have shown that T cells are required for growth of peripheral blood BFU-E (Nathan et al, 1978; Mangan and Desforges, 1980; Kanamura et al, 1980; Mangan et al, 1982). Both T cells of the helper and cytotoxic phenotypes are capable of BPA synthesis (Haq et al, 1983; Wisniewski et al, 1985). Similarly, monocytes (Eaves and Eaves, 1978; Gordon et al, 1980; Zuckerman, 1980; Zuckerman et al, 1983; Levitt et al, 1985), large granular lymphocytes (Pistoia et al, 1985) and a population of non-adherent, radioresistant, E receptor-negative cells (Meytes et al, 1979; Linch et al, 1985) which probably represent natural killer cells, all have BFU-E enhancing activities. Moreover, monocytes and lymphocytes may interact synergistically (Zuckerman, 1981; Reid et al, 1981). Both T-cell and monocyte derived BPA (provided by crude conditioned medium) is reported to act directly on highly puri-fied erythroid progenitor cells (Linch and Nathan, 1984) although the technical problems involved in identifying one human erythroid

colony in a microwell containing 2×10^5 sheep red cells in methyl-cellulose are considerable. CFU-E are also reported to be dependent on BPA for optimal growth (Golde et al, 1980; Linch et al, 1985). Evidence also exists for suppression of human erythroid colony growth in a genetically restricted manner through HLA-DR antigen recognition by a subset of OKT8 positive, HLA-DR positive T cells (Torok-Storb and Hansen, 1982; Lipton et al, 1983).

1.3.2.1 Natural Killer (NK) Cells. The role of natural killer (NK) cells as modulators of haemopoiesis remains confused. NK cells appear to interact with normal bone marrow cells (Hansson et al, 1981) and can destroy these cells in vivo (Riccardi et al, 1981). They react with the human leukaemic cell line K562 (Lozzio and Lozzio, 1975) which has erythroid features (Tonkonow et al, 1982). In the mouse, NK cells are implicated as effectors of genetically determined resistance to haemopoietic grafts with activity against at least a proportion of immature bone marrow precursors (Kiessling et al, 1977; Lotzova and Savary, 1977; Cudkowicz and Hochman, 1979; Gallagher et al, 1980; Bloom, 1982; Warner and Dennert, 1982). In man high levels of NK cell activity prior to, and early NK cell activity following bone marrow transplantation are associated with the development of graft-versus-host disease (Lopez et al, 1979; Livnat et al, 1980; Lopez et al, 1980; Dokhelar et al, 1981). Some workers have demonstrated that lymphocytes with NK characteristics are able to inhibit myeloid (Hansson et al, 1982; Barr and Stevens, 1982; Spitzer and Verma, 1982; Bacigalupo et al, 1981; Morris et al, 1980) and erythroid (Degliantoni et al, 1985; Mangan et al, 1984) colony formation in vitro. Inhibition may be mediated by a soluble factor (Degliantoni et al, 1985). On the other hand, others have demonstrated enhancement of BFU-E derived from both blood (Meytes

et al, 1979; Mangan et al, 1982, 1984; Pistoia et al, 1985) and bone

marrow (Mangan et al, 1984; Linch et al, 1985). This enhancement is

variably reported in relation to the degree of enhancement observed

with other T cell populations (Mangan et al, 1984; Pistoia et al,

1985). Evidence exists for suppression of NK cells by bone marrow

monocytes (Uchida, 1984) and an interaction between NK cells and

monocytes has been suggested to be important in the modulation of

haemopoiesis (Pistoia et al, 1985).

The relevance of these studies to haemopoiesis in vivo is

uncertain, particularly as the number of NK cells in the bone marrow

is small (Abo and Baulch, 1981), their activity is low (Lotzova et

al, 1979; Targan et al, 1980), and the in vitro studies mentioned

above have used NK to blood/bone marrow cell ratios of at least

0.5:1. However there is evidence for a possible role of such cells

in patients with pure red cell aplasia associated with chronic

lymphatic leukaemia (Nagasawa et al, 1981; Mangan et al, 1982a),

aplastic anaemia (Bacigalupo et al, 1980) and in patients with NK

lymphocytosis with neutropenia (Bom-Van Noorloos et al, 1980;

Pandolfi et al, 1982).

1.4 MYELOID LEUKAEMIA

Based upon their natural history, the leukaemias can be divided

into two major categories, acute or chronic leukaemia. These then

can be subdivided according to morphological characteristics into

leukaemias of myeloid or lymphoid cells. Only the myeloid leukaemias

will be considered here.

1.4.1 Acute Myeloid Leukaemia

The major clinical features of acute myeloid leukaemia (AML)

are tiredness, lethargy, fever, pallor, easy bruising or bleeding

and infections. The major haematological features are a profound

reduction in normal haemopoietic elements with bone marrow replacement by leukaemic blast cells. Results of treatment are poor with few patients surviving 5 years, regardless of differences in therapy (Medical Research Council, 1979; Rai et al, 1981) suggesting that ultimately the outcome is determined by the biological nature of the disease. These features are common to AML of various morphological subtypes, although for example patients with acute promyelocytic leukaemia (AML M3; FAB Classification) (Bennett et al, 1976) are particularly prone to haemorrhagic episodes due to hypofibrinogenaemia (Hirsh et al, 1967).

A variety of karyotypic abnormalities have been described in AML (Rowley, 1980) with the most constant being the 15:17 translocation of acute promyelocytic leukaemia (AML M3) (Van den Berghe et al, 1974). The proto-oncogene, c-fes maps on q25-26 of chromosome 15 and thus may be implicated in this disease (Harper et al, 1983).

The clonal nature of AML has been demonstrated by karyotypic and glucose-6-phosphate dehydrogenase (G6PD) analyses. G6PD studies demonstrated the expected double enzyme pattern in somatic fibroblasts but a single isoenzyme in the leukaemic blast cells (Wiggans et al, 1978). In some patients with AML, the leukaemic clone may also involve other lineages (Blackstock and Garson, 1974; Fialkow et al, 1981) although the typical AML clone appears to be restricted to the granulocyte-macrophage lineage (Fialkow, 1982). These results suggest either that some AML clones arise in unipotential cells (e.g. CFC) or that associated with leukaemic transformation some multipotential stem cells lose their multipotentiality.

1.4.2 Chronic Myeloid Leukaemia

Chronic myeloid leukaemia (CML) is characterised by a slow onset and a course lasting several years, usually terminating in a phase of acute transformation (see review Champlin and Golde, 1985).

During the chronic phase there is myeloid hyperplasia with mature forms predominating in both blood and bone marrow. The disease usually transforms into an illness which resembles AML, but transformation into ALL can be recognised morphologically or using anti-ALL serum (Janossy et al, 1978a), terminal deoxytransferase content (Marks et al, 1978) or heightened sensitivity of lymphoblasts to vincristine and prednisolone (Canellos et al, 1971). Bifocal transformation with clones having different cytogenetic markers has been described (Olinici et al, 1978).

Karyotypic and G6PD analyses have demonstrated this disease to be clonal in nature, with a dominant granulocyte-macrophage population but with erythroid cells, eosinophils, megakaryocytes, B lymphocytes but not marrow fibroblasts, originating from the same cell (Whang et al, 1963; Rastrick et al, 1968; Lawler, 1977; Fialkow et al, 1967, 1977, 1978; Koeffler et al, 1980; Maniatis et al, 1969; Golde et al, 1977).

In 95% of patients with CML the abnormal clone can be identified by the presence of the Philadelphia chromosome (Nowell and Hungerford, 1960) but Philadelphia chromosome negative CML is described (Fialkow et al, 1980). The Philadelphia chromosome is a reciprocal translocation between chromosomes 9 and 22 (Rowley, 1973). Chromosome 22 carries the proto-oncogene c-sis in its distal translocated protion (Swan et al, 1982) and in the translocation, the proto-oncogene c-abl is transposed from the tip of chromosome 9 to chromosome 22 (De Klein et al, 1982; Heisterkamp et al, 1982; Dalla-Favera et al, 1982; Groffen et al, 1983). The proto-oncogene c-sis is not transcribed (Gale and Canaani, 1984). The breakpoints on chromosome 9 are scattered over a wide region (Heisterkamp et al, 1983; Heisterkamp et al, 1985) and the translocation may even occur between chromosome

22 and other chromosomes (Verma and Dosik, 1980). The region on chromosome 22 involved in the translocation is denoted the bcr (breakpoint cluster region) because the chromosome 22 breakpoints fall within a 5.8-kilobase-pair segment (Groffen et al, 1984). A novel abl messenger RNA is detected in CML cells, derived from the Philadelphia chromosome (Gale and Canaani, 1984; Collins et al, 1984) and a new abl polypeptide with an altered amino-terminal segment is present (Konopka et al, 1984, 1985). These result from a fusion of the translocated c-abl to the bcr gene (Shtivelman et al, 1985; Heisterkamp et al, 1985), although the relationship between this new gene product and the development of CML is uncertain.

1.4.3 In Vitro Growth Characteristics

1.4.3.1 Acute Myeloid Leukaemia. The growth of AML cells in vitro is highly abnormal with one of four growth patterns being observed. There may be complete absence of in vitro proliferation; formation of small clusters; formation of large clusters or formation of colonies and clusters but with an abnormally high cluster to colony ratio. The frequency of clonogenic cells varies greatly; the proliferating cells are of abnormally light buoyant density and the clones generated may show little or bizarre maturation (Moore et al, 1973; 1974; Spitzer et al, 1976; Dicke et al, 1983; 1983a). The growth pattern exhibited by AML cells does not correlate with the morphological subtype and appears to be variably correlated with clinical course (Moore et al, 1974; Spitzer et al, 1976a, Elias and Greenberg, 1977; Hörnsten et al, 1977; Vincent et al, 1977; Keating et al, 1980; Beran et al, 1980; Gustavsson et al, 1981; Jehn et al, 1983).

During complete remission the growth characteristics of the cultured cells return to normal (Moore et al, 1973; Spitzer et al, 1977) however many of these apparently normal colony cells react

with antisera specific for human cancer cell nucleoli (Dicke et al,
1983; Davis et al, 1983) and in some patients, remission marrow cells
remain positive with leukaemia specific antiserum (Malcolm et al,
1983). These results suggest that in some patients during complete
remission, leukaemia-derived cells may exhibit normal or near normal
differentiation.

Techniques have been described that allow a proportion of AML
cells to generate colonies of blast cells in cultures stimulated by
PHA or PHA-LCM (Dicke et al, 1976; Buick et al, 1977; Park et al,
1980; Lowenberg et al, 1980; Marie et al, 1982). These colonies
attain maximum size between 5 and 7 days of culture and thereafter
quickly degenerate. The colonies are able to be recloned and again
generate blast colonies (Buick et al, 1979; McCulloch et al, 1981).
Exponential growth can be maintained in long-term liquid cultures
(Nara and McCulloch, 1985) and the cell with self-renewal properties
has an immature immunological phenotype (Griffin et al, 1983a; Wouters
and Lowenberg, 1984). The property of self-renewal correlates with
clinical response (McCulloch et al, 1981, 1982, 1982a). These condi-
tions however may detect only a subset of clonogenic leukaemic cells
(Swart et al, 1982).

The in vitro growth characteristics of the preleukaemic or
smouldering leukaemic states are very similar to those of the acute
myeloid leukaemias. These conditions have a reduced frequency of
GM-CFC, increased numbers of abortive myeloid clusters with defective
maturation and increased numbers of abnormally light density GM-CFC
(Milner et al, 1977; Faille et al, 1978; Berthier et al, 1979;
Verma et al, 1979; Greenberg and Mara, 1979; Spitzer et al, 1979;
Senn and Pinkerton, 1972; Greenberg et al, 1971). The GM-CFC derived
from patients with preleukaemic states may show a heightened sensit-
ivity to agents such as retinoic acid (Bailey-Wood et al, 1985).

The numbers of BFU-E and CFU-E are decreased (Koeffler et al, 1978; Chui and Clark, 1982; May et al, 1985) and circulating blast cell progenitors (which are absent from normal individuals) are detectable (Senn et al, 1982). In contrast, diseases with low propensity to leukaemic evolution have normal in vitro growth characteristics (Moore et al, 1973; Greenberg et al, 1976, 1981). The clinical outcome in the preleukemic syndromes correlates with in vitro marrow growth characteristics (see review Greenberg, 1983). Similarly when studied in liquid culture considerable overlap exists between the growth patterns of AML and the preleukaemic states, with increased in vitro proliferation and decreased maturation being associated with progressive disease (Golde and Cline, 1973; Elias and Greenberg, 1977; Koeffler and Golde, 1978).

1.4.3.2 Chronic Myeloid Leukaemia. In CML there is a marked elevation in the levels of progenitor and mature cells. The granulocyte, monocyte, erythroid, eosinophil and multi-CFC progenitor cells are increased in both blood and bone marrow (Moore et al, 1973; Goldman et al, 1974; Moberg et al, 1974; Berthier et al, 1977; Leong et al, 1979; Eaves and Eaves, 1979; Goldman et al, 1980; Hara et al, 1981). Cells derived from these progenitor cells contain the Philadelphia chromosome (Chevernick et al, 1971; Moore and Metcalf, 1973; Aye et al, 1973; Singer et al, 1979; McCarthy et al, 1980; Goto et al, 1982; Carbonell et al, 1983; Denegri et al, 1978; Dube et al, 1984). The cell forming granulocyte-macrophage colonies has been identified as the myeloblast and close to 100% of these cells are clonogenic in agar cultures (Moore et al, 1973; Metcalf et al, 1978; Griffin et al, 1982). The in vitro growth chracteristics may change to those of acute myeloid leukaemia at the time of acute transformation (Moore et al, 1973; Moore, 1977) with the new clone showing a growth advantage over the original CML clone (Lowenberg et al, 1985).

In CML there is evidence for normal haemopoietic populations co-existing with the Philadelphia-chromosome positive clone. In primary cultures (Carbonell et al, 1983) and in long-term cultures (Eaves et al, 1983; Dube et al, 1984) Philadelphia-chromosome negative progenitor cells can be identified. Intensive chemotherapy has been followed by marrow regeneration with Philadelphia-chromosome negative cells (Smalley et al, 1977; Goto et al, 1982; Dube et al, 1984a) and a similar phenomenon can be observed with leukaemic cells treated in vitro (Degliantoni et al, 1985a).

1.4.4 Leukaemic Cell Differentiation

Myeloid leukaemic cells display a range of surface molecules which include insulin receptors (Chen et al, 1983), low density lipo-protein receptors (Vitols et al, 1984; Ho et al, 1978) and a myriad of antigens detected by monoclonal antibodies.

Many leukaemias display lineage specific markers as assessed by histochemical or immunological techniques (Greaves, 1982; Schlossmann et al, 1976; Seligmann et al, 1981) and may also display normal functional activity (Van Furth et al, 1983) including produc-tion of CSF by monocytes of leukaemic origin (Golde et al, 1974; Goldman et al, 1976; Bianchi Scarra et al, 1981). Some fresh leukaemic cells and cell lines display markers of different lineages (Fioritoni et al, 1980; Marie et al, 1978; Smith et al, 1983; Bettelheim et al, 1985) and clinically such leukaemias are associated with a poor prognosis (McCulloch et al, 1984; Smith et al, 1983). This is in contrast to lymphoid malignancies where lineage specificity is main-tained and leukaemic cells appear to be "arrested" at some particular stage of normal differentiation (Greaves, 1982; Seligmann et al, 1981), although exceptions are recorded (Bettelheim et al, 1982).

When freshly derived human leukaemic cells are cultured in vitro, they may acquire histochemical or antigenic markers of mature cells (Pegoraro et al, 1980; Marie et al, 1981; Breitman et al, 1981; Fibach and Rachmilewitz, 1981; Ozawa et al, 1983; Koeffler et al, 1980a; Honma et al, 1983; Boyd and Sullivan, 1984; Flynn et al, 1983; Shkolnik et al, 1985; Koeffler et al, 1985; Touw and Lowenberg, 1985). These changes are variable, correlate poorly with changes observed in normal cells and may involve markers of different lineages (Marie et al, 1981a; Ferrero et al, 1983a; Touw and Lowenberg, 1985; Paietta et al, 1985). Both murine and human leukaemic cells differentiate following treatment with some cytotoxic agents (Sachs, 1978; Hayashi et al, 1979; Sugiyama et al, 1979; Lotem and Sachs, 1980; Griffin et al, 1982a).

1.4.5 Inhibitory Influences of Leukaemic Cells

Although initial experiments failed to detect inhibition of normal progenitor cells by leukaemic cells, (Greenberg et al, 1971; Robinson et al, 1971; Goldman et al, 1976) others have demonstrated suppression by leukaemic cells (Morris et al, 1975; Chiyoda et al, 1975, 1976; Knudtzon and Mortensen, 1976; Gordon et al, 1978; Spitzer et al, 1981) and this suppression may be mediated via several mechanisms. Leukaemic cells produce a factor inhibiting normal but not leukaemic cells (Broxmeyer et al, 1978a, 1979). This leukaemia-associated inhibitory activity (LIA) is a high molecular weight acidic isoferritin (Broxmeyer et al, 1981). Lactoferrin, present in the secondary granules of neutrophils, decreases production of CSF by a specific subset of monocytes (HLA-DR-positive monocytes). Polymorphs derived from CML clones have lower levels of lactoferrin than normal cells and furthermore, monocytes derived from CML clones are less sensitive to lactoferrin-inhibition than normal cells (Broxmeyer et al, 1977, 1978b, 1979, 1980). Prostaglandin E, generated by mono-

cytes, inhibits growth of leukaemic myeloid precursors to a much lesser degree than it does normal GM-CFC (Pelus et al, 1980; Aglietta et al, 1980).

The role of stromal cells may also be important in modulation of leukaemic and normal populations. Stromal cells modulate self generation in haemopoietic cells (Zipori, 1981; Miyanomae et al, 1982) and the frequency of marrow fibroblast colony-forming cells is abnormally low in AML (Kaneko et al, 1982; Nagao et al, 1983) as is their capacity to produce CSF (Greenberg et al, 1981), with evidence for suppression of marrow fibroblast colony-forming cells by leukaemic cells (Nagao et al, 1983a).

1.4.6 Colony Stimulating Factors and Myeloid Leukaemia

This subject has been extensively reviewed by Metcalf (1971, 1977, 1980a, 1984) and Greenberg (1981). Human leukaemic cells are absolutely dependent upon CSF for in vitro proliferation, although differences in response are observed with different crude sources of CSF (Francis et al, 1979, 1980; Dicke et al, 1976; Buick et al, 1979; Swart et al, 1982). Murine G-CSF (MGI-2) is unique among the CSF's because of its profound ability to induce differentiation in murine leukaemic cells (Lotem et al, 1980; Burgess and Metcalf, 1980a; Metcalf, 1979, 1980b; Metcalf and Nicola, 1982). In some situations, differentiation induction may be enhanced as a result of an inter-action between chemical or cytotoxic agents and CSF's (Okabe et al, 1977, 1977a, 1979; Hayashi et al, 1979; Lotem and Sachs, 1979; Falk and Sachs, 1980). Induced differentiation and reduced clonogenicity of murine leukaemic cells can be translated into an in vivo survival advantage for the animal (Lotem and Sachs, 1981; Metcalf, 1984).

In human leukaemia, serum and urine CSF levels are normal or elevated, with increased levels detectable during episodes of infec-tion (Metcalf et al, 1971; Metcalf, 1981, 1983). However CSF produc-

tion in the marrow is low in some patients with AML (Greenberg et al, 1978) with low numbers of fibroblast colony forming cells (Kaneko et al, 1982; Nagao et al, 1983) and suppression by the leukaemic cells of fibroblast colony formation (Nagao et al, 1983a), suggesting that CSF levels may be subnormal in the vicinity of the leukaemic cells thus allowing proliferation and survival without capacity for suppression of self-renewal of leukaemic cells (Metcalf, 1984).

An alternative hypothesis is that leukaemic cells remain blocked in an undifferentiated state because of their inability, compared with normal cells, to synthesize G-CSF following appropriate stimulation (Sachs, 1982). McCulloch has postulated that leukaemic cells represent a novel abnormal population consisting of components of normal differentiation assembled abnormally. These cells may either self-renew or undergo terminal divisions with loss of proliferative potential (McCulloch, 1979; 1983). Despite these controversies, studies on murine leukaemic cells WEHI-3B, have clearly shown that G-CSF is able to enforce differentiation in these cells and suppress self-generation (Metcalf and Nicola, 1982). A subline of WEHI-3B that fails to respond to G-CSF in this manner, lacks receptors for G-CSF (Nicola and Metcalf, 1984). These observations are clearly of possible potential relevance to human leukaemias. The demonstration that murine WEHI-3B leukaemia cells can be forced to differentiate by the normal regulator G-CSF is analogous to the response of normal murine progenitor cells (Nicola et al, 1983) and raises the possibility that human leukaemic cells might remain responsive to similar haemopoietic regulators. However, the human analogue of murine G-CSF remains to be identified, receptors for such a molecule have not been demonstrated on human leukaemic cells and the functional relevance of such a receptor is undetermined. Some of these questions will be addressed in this thesis.

1.5 AIM OF THIS THESIS

The aim of this thesis was to study the role of the Colony Stimulating Factors (CSF's) in the regulation of normal and leukaemic haemopoiesis.

Purified murine granulocyte-macrophage-active CSF's and purified and partially-purified human CSF's were available, but to characterize the action of these molecules on human cells required a highly purified population of committed granulocyte-macrophage progenitor cells. While techniques using the bulk separation of cells by density separation or velocity sedimentation have had limited success, the use of lectin or monoclonal antibody probes combined with the fluorescence activated cell sorter have achieved partial separation of cell populations. This technique was therefore employed. It was hoped that the study of the action of CSF on normal populations of committed-progenitor cells would allow comparison with the action of CSF on human leukaemic cells.

It was of particular interest to examine the action, if any of murine G-CSF on human cells. This molecule is a profound inducer of differentiation in murine leukaemic cells and it was therefore of importance to determine its action on normal and leukaemic human cells and/or to characterize a candidate human equivalent of this molecule.

Hopefully these studies will contribute to the further purification of sequential cells in each haemopoietic lineage and to a further understanding of the control of normal and leukaemic haemopoiesis.

CHAPTER 2

METHODS

2.1 SOURCES OF BLOOD AND MARROW

Marrow cells were obtained from rib segments and sternal curettings removed at surgery from patients with no haematological abnormality. The rib segments were from patients undergoing thoracic surgery usually for excision of a carcinoma of the lung or drainage of an empyema. Sternal curettings were obtained from patients undergoing cardiac surgery. Marrow aspirates were obtained from patients with non-leukaemic disorders when the aspirates were required for clinical assessment of the patient. Leukaemic marrow and blood specimens were obtained from patients with acute and chronic myeloid leukaemia when these specimens were required for the clinical management of the patient. Normal blood specimens were obtained from healthy volunteers in the laboratory. All samples were taken with the prior informed consent of patients and volunteers. These investigations were performed after review and approval by the Ethics Committee of The Walter and Eliza Hall Institute.

2.2 MEDIA

2.2.1 Eisen's Balanced Salt Solution (EBSS)

The ingredients for this medium were Trizma base 0.6g/litre, Na_2HPO_4 (anhydrous) 0.21 g/litre, $MgSO_4$ (anhydrous) 0.12 g/litre, NaCl 7.0 g/litre, $CaCl_2$ 0.22 g/litre, KCl 0.45 g/litre, Phenol red 0.02 g/litre. Four separate solutions were prepared in 200 ml of distilled water. Solution 1: Trizma base, Na_2HPO_4, Phenol red; Solution 2: NaCl and KCl; Solution 3: $MgSO_4$; Solution 4: $CaCl_2$. The pH of solution 1 was adjusted to pH 7.3 with concentrated HCl. The solutions were mixed and distilled water was added to give a final volume of 1.0 litre. The solution was passed through a millipore filter prior to use (0.22 μ, Millipore Corp., Bedford, Mass., U.S.A.).

2.2.2 <u>Dulbecco's Modified Eagle's Medium (Single Strength)</u>
<u>for Liquid Culture (DME)</u>

The ingredients were Dulbecco's modified Eagle's medium H6,
instant tissue culture powder H16 (Grand Island Biological Company,
New York), 2 x 10 litre packets, 68 g $NaHCO_3$, 1.2 g penicillin G,
2 g streptomycin (antibiotics were from Glaxo, Melbourne). The
medium was prepared by dissolving the ingredients in a final volume
of 20 litres in double distilled water, adjusting the pH to 6.8 to
7.0 with carbon dioxide gas and passing it through a 0.22 μ millipore
filter membrane. This medium was used in all experiments requiring
the culture of haemopoietic cells (except HL60 cells) in a liquid
medium.

2.2.3 <u>Dulbecco's Modified Eagle's Medium (Double Strength)</u>
<u>for Agar Culture</u>

The ingredients were 2 x 10 litre packets of Dulbecco's modified
Eagle's medium (as above), 98 g $NaHCO_3$, 0.4 g L-asparagine, 1.38 g
penicillin G, 1.5 g streptomycin, 30 ml DEAE Dextran (50 mg/ml)
(Pharmacia South Seas, Sydney). It was prepared by dissolving the
DME in 6 litres of distilled water, the $NaHCO_3$ in 1.8 litres of
distilled water, the L-asparagine in 60 ml of distilled water, and
the penicillin and streptomycin in 19 ml of distilled water. The
dextran was then added and the pH was adjusted by passing CO_2 gas
through the medium. This medium was used in all agar studies, except
for the growth of erythroid colonies and was mixed 3:2:5 (v/v) with
FCS and 0.6% of agar prior to culture.

2.2.4 <u>Iscove's Modified Dulbecco's Medium (Double Strength)</u>
<u>for Agar Cultures</u>

The ingredients were 2 x 1 litre packets of instant tissue
culture medium powder (Gibco, New York, U.S.A.), 9.8 g $NaHCO_3$, 0.4 g

L-asparagine, 120 mg penicillin G, 200 mg streptomycin, 3 ml DEAE Dextran 50 mg/ml, 11.8 μl 2-mercaptoethanol. All components were dissolved in 780 ml double distilled water before being sterilised by passage through a 0.22 μ millipore filter membrane. This medium was used in all studies of erythroid colony formation and was mixed 3:2:5 (v/v) with FCS and 0.6% of agar prior to culture.

2.2.5 RPMI-1640

Twenty litres of RPMI-1640 contained 2 x 10 litre packets of instant tissue culture medium powder (Gibco, New York, U.S.A.), 40 g $NaHCO_3$, 11.7 g NaCl, 2.2 g sodium pyruvate, 1.2 g penicillin G and 2 g streptomycin.

The tissue culture medium powder was dissolved with stirring in ten litres of double distilled water and the remaining ingredients were dissolved in five litres double distilled water. Four ml of concentrated HCl was added and then the RPMI gassed with CO_2 to approximately pH 7.0 before being filtered with a 0.22 μ millipore filter. This medium was used in all liquid cultures of HL60 cells.

2.2.6 Human Tonicity Phosphate Buffered Salt Solution With Azide (HT-PBS-Azide)

To prepare 1 litre of HT-PBS, 2.85 g of $Na_2HPO_4.2H_2O$, 0.625 g of $NaH_2PO_4.2H_2O$ and 7 g NaCl were made up to 1 litre in distilled water (0.02M phosphate, pH 7.3). To this was added sodium azide (0.01% v/v). This buffer was used for all experiments in which cells were labelled with antibodies for analysis by the FACS.

2.3 AGAR CULTURE ASSAY FOR PROGENITOR CELLS

2.3.1 Cell Preparation

Human marrow aspirate samples were collected in tubes containing 200 units of heparin to prevent clotting. Samples were diluted in 3 ml of Eisen's Balanced Salt Solution (EBSS) and the cells were dispersed by gentle pipetting with a 1.0 ml pipette.

Any clumps were allowed to settle out. The supernatant containing the cells was transferred to another tube. The marrow cell suspension was centrifuged at 400 g for 7 min. The buffy coat containing nucleated white cells was carefully removed and resuspended in fresh EBSS.

Human marrow cells were also obtained from rib fragments and sternal curettings. Ribs were transported to the laboratory on the day of excision and were broken into fragments with bone cutters. The marrow was then flushed out of the shaft with EBSS using an 18 gauge needle and 10 ml syringe. Sternal curettings were collected within 1 hour of removal and cells were extracted by gentle pipetting with 15 ml of EBSS. Cells were transferred to sterile centrifuge tubes, clumps were removed and a single cell suspension was produced as described above. The cell suspension was centrifuged at 400 g for 7 min. The layer of fat floating on top of the suspension after centrifugation was discarded and the cells were carefully removed and resuspended in fresh EBSS. For most experiments, cells were prefractionated using density separation (as described below).

2.3.1.1 Density Separation. To obtain cells of density <1.077 g/cm^3, blood or marrow cells in 10 ml of EBSS were layered onto 10 ml of Ficoll-Paque (Pharmacia, Uppsala) and centrifuged at 400 g for 20 min. at room temperature. The interface cells were transferred to a second tube and washed two times in EBSS prior to culture.

2.3.1.2 Adherent Cell Removal. In some experiments adherent cells were removed by plastic-adherence. 1 x 10^7 cells were added to 20 ml DME and 20% (v/v) FCS and placed in 25 cm^2 tissue culture flask (Corning, New York, U.S.A.). Cells were incubated at

37°C in a fully humidified atmosphere of 10% CO_2 in air for 1 hour. The flask was then gently shaken and the non-adherent cells removed and counted.

2.3.1.3 Incubation with Monoclonal Antibody 9.1C3. Monoclonal antibody 9.1C3 has been described in detail elsewhere (Burns et al, 1984) and was raised by immunizing mice with human large granular lymphocytes. Spleen cells were then fused with the NS-1 line. In some experiments, cells were incubated with the antibody for 15 min. at room temperature. An equal aliquot of cells (approx. 200 µl) was also incubated with DME and 10% FCS prior to culture in agar (and served as control cultures).

2.3.2 Preparation of Human-Derived Stimulatory Factors

2.3.2.1 Human Placental Conditioned Medium (HPCM)

Human placentas were obtained from the Royal Women's Hospital, Melbourne. An incision was made through the outer placental membranes and a flap of placenta was lifted up. From inside this incision, small sterile pieces (1 cm^3) were obtained. These pieces were incubated in 20 ml RPMI-1640 with 5% foetal calf serum for 7 days at 37°C in CO_2 as previously described (Burgess et al, 1977a). This material was active in stimulating the formation of human colonies.

2.3.2.2 Bladder Carcinoma-Cell Conditioned Medium (U/5637-CM)

Bladder carcinoma cells (U/5637 cells) (Svet-Moldavsky et al, 1983) were cultured in DME and 10% fetal calf serum at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Cells were cultured at a density of 1-2 x 10^6 cells per ml and the conditioned medium was harvested after 7 days. This material was active in stimulating the formation of human colonies.

2.3.2.3 CSF-α and CSF-β

The U/5637-conditioned medium was separated into two stimulatory fractions referred to as CSF-α and CSF-β. This was done using hydro-

phobic chromatography on a Phenyl-Sepharose column or Blue Sepharose CL-6B, Pharmacia, Uppsala, Sweden. This technique has been previously described for separation of CSF-α and CSF-β from HPCM (Nicola et al, 1979).

2.3.2.4 Recombinant Human GM-CSF (rHGM-CSF)

Recombinant human GM-CSF was provided by Dr. G.G. Wong (Genetics Institute, Boston). The details of molecular cloning and expression have been described elsewhere (Wong et al, 1985, 1985a). A cDNA for human-active GM-CSF was cloned from a library prepared from the human Mo T-leukaemia cell-line, and the cDNA was expressed in monkey COS cells. This material was used for studies on normal human bone marrow cells and mature human neutrophils and eosinophils.

rHGM-CSF was also provided by Biogen, Geneva. The cDNA for this material was isolated on the basis of nucleotide homology with murine GM-CSF (Gough et al, 1984) and expressed in an E.coli expression system. This material was used for studies on normal human bone marrow cells, leukaemic cells and HL60 cells. For all CSF preparations 50 Units of CSF was that amount giving a 50% maximal response.

2.3.2.5 Human Erythropoietin

Erythropoietin was prepared from the urine of anaemic patients who were homozygous for β-thalassaemia but had not been transfused for many years. The urine was concentrated and then dialyzed against double distilled water. Protein was lypholized and fractionated by phenyl-Sepharose chromatography and further purified by ethanol (75% v/v) precipitation as previously described (Cutler et al, 1985a). This material was provided by Dr. R. Cutler and Dr. G.R. Johnson.

2.3.3 Preparation of Murine-Derived Stimulatory Factors

2.3.3.1 Mouse Lung Conditioned Medium (MLCM) and Purified

GM-CSF, G-CSF. Mouse lung conditioned medium
was prepared by removing the lungs from C57BL mice, washing the lungs
in EBSS and culturing them for 48 hours at 37°C in 10% CO_2 in serum-
free DME in sterile Falcon tubes (17 x 100 mm). The mouse lung condi-
tioned medium was filtered through a double layer of cotton gauze.
Murine GM-CSF was then purified as previously described (Burgess et
al, 1977) and was supplied by Dr. A.W. Burgess (Ludwig Institute,
Melbourne) and purified G-CSF (Nicola et al, 1983) was provided by
Dr. N.A. Nicola.

2.3.3.2 Spleen Conditioned Medium (SCM) and Purified

Multi-CSF. Mouse spleen conditioned medium was
prepared by removing spleens from BALB/c mice and preparing a single
cell suspension by passage through a fine wire sieve followed by
repetitive pipetting. Conditioned medium was prepared in 500 ml
batches in Ehrlenmeyer flasks by culturing spleen cells at 2 x 10^6
cells per ml in RPMI-1620 containing 5% (v/v) heat-inactivated human
plasma, 0.3% (v/v) pokeweed mitogen (Gibco, New York, U.S.A.) and 5
x 10^{-5}M 2-mercaptoethanol (Calbiochem, Sydney). Cultures were incub-
ated for seven days at 37°C in a fully humidified atmosphere of 10%
CO_2 in air. Medium was harvested by passage through a 0.45 μ milli-
pore filter. Multi-CSF was purified (Cutler et al, 1985) and provided
by Dr. R. Cutler.

2.3.3.3 L-Cell Conditioned Medium and Purified M-CSF.

Conditioned medium was harvested from confluent cultures of mouse
L-cells grown in DME and 5% (v/v) fetal calf serum at 37°C in a fully
humidified atmosphere of 10% CO_2 in air. Purified M-CSF was provided
by Dr. A.W. Burgess (Ludwig Institute, Melbourne) as previously
described (Stanley and Heard, 1977).

2.3.3.4 <u>Murine Hybridoma Supernatant and Purified EDF</u>.
Supernatant from a murine T-cell-hybridoma and purified Eosinophil
Differentiation Factor (EDF) were provided by Dr. C.J. Sanderson
(Mill Hill, London). The isolation of the mouse hybrid cells
(NIMP-TH1) and the purification of EDF have been described in detail
(Sanderson et al, 1985).

2.3.4 <u>Agar Culture</u>

Cultures were prepared in 35 mm Petri dishes using a final volume
of culture medium of 1 ml (Metcalf, 1977). A final concentration
of 0.3% agar was used. This was achieved by mixing in a tube or
Ehrlenmeyer flask equal volumes of double strength tissue culture
fluid (DME), FCS and 0.6% agar. Sufficient cells were added to the
mixture to produce the final cell concentration required. The cell
suspension in the agar medium was pipetted in 1 ml volumes to the
culture dishes. Each culture dish contained 0.1 ml of the stimulus.
The cell suspension in agar medium was then mixed and allowed to gel.
After gelling, cultures were transferred to an incubator operating at
37°C with a fully humidified atmosphere of 10% CO_2 in air (or 5% CO_2
in air for culture of erythroid colonies). All cultures were performed
using a biohazard hood. The formula for the double strength medium
(Dulbecco's modified Eagle's medium) was described previosly. This
medium was supplemented with 40% foetal calf serum. The batches of
foetal calf serum (FCS) used were Flow Laboratories, N.S.W., Australia,
Batches 29101941, 29101998 (for culture of GM-progenitors) and Common-
wealth Serum Laboratories, Melbourne, Australia, foetal calf serum
Batches 328-3 (for culture of erythroid progenitors) and 358-3 (for
culture of HL60 cells). These batches of foetal calf serum were pre-
tested and selected because they were found to support maximal colony

cell growth (both highest number of colonies and the highest number of cells in each colony). Between 20-40 different batches of FCS were screened for each batch of FCS selected.

The techniques for growing human granulocyte, macrophage and eosinophil colony-forming cells have been previously described in detail (Metcalf, 1977, 1984).

The basic culture technique was modified for special requirements. The cell number cultured varied from 1,000 to 200,000 cells per dish.

2.3.4.1 Scoring of Agar Cultures. Mouse cultures were socred after 7 days of incubation. Human cultures were scored at varying times throughout the culture period (as indicated in particular experiments). An Olympus dissection microscope was used and replicate (usually quadruplicate) cultures were examined. Colonies (aggregates of >40 cells) and clusters (2-40 cells) were counted at 7 days and at 14 days. A total clone count was made at earlier timepoints. It was found that human eosinophil colonies were usually tight and pigmented brown, however not all tight colonies were comprised of eosinophils, so murine and human eosinophil colonies were always identified using specific stains. Two techniques were used, either all the colonies in an agar dish were stained using a whole plate staining technique or 45 sequential colonies were picked off and stained.

2.3.4.2 Whole Plate Staining of Colonies in Agar. One ml of 2.5% glutaraldehyde (TAAB Laboratories, Reading, England) was added to the agar culture dish and it was allowed to stand overnight with the lid on. The dish was then submerged in water and the agar gel was carefully eased out of the dish with a small spatula. The agar gel was then transferred to a glass slide (76 x 32 mm) and a

hardened ashless Whatman filter paper (5.5 cm diameter, No.540) gently placed on the disc to promote uniform drying. After drying overnight, the filter paper was removed by soaking the slide in water for 3 min. The dehydrated flattened and fixed agar discs were stained with Luxol fast blue (60 min) (Luxol fast blue MBS, G.T. Gurr, High Wycombe, Bucks, dissolved in 70% ethanol saturated with urea), then washed for 20 min. and finally counterstained with Mayer's haematoxylin for 5 min. and washed for 10 min. The slides were mounted in DPX mounting medium and covered with a coverslip.

2.3.4.3 <u>Staining of Picked Off Colonies</u>. A fine Pasteur pipette was used to pick off 45 intact sequential colonies from the cultures with as little as possible surrounding agar. Each colony was placed onto a glass slide.

The colonies were allowed to dry and stained for 20 min. with Luxol fast blue and counterstained with Mayer's haematoxylin.

2.3.4.4 <u>Clone Transfer Studies</u>. Developing clones of cells after 2 to 5 days (as specified in each experiment) were gently picked off using a fine tipped Pasteur pipette and carefully transferred to a second culture dish. Each dish was numbered and on its undersurface a wax circle was drawn to identify the position to which the clone of cells was transferred. The recipient dishes contained 1 ml of agar medium which had been allowed to gel. Transfers were performed in a laminar flow hood. Care was taken to transfer as little surrounding agar as possible (<10 µl).

The clones were usually initiated in one stimulus and then transferred to a second stimulus or to no stimulus.

An estimate was made of the cells in each clone immediately after transfer. After a further incubation period of 3-11 days, the clones clones were picked off and transferred to a microscope slide. They were stained with Luxol fast blue and haematoxylin.

Then the number of cells in each clone was carefully counted and
the clones were typed as granulocyte, granulocyte-macrophage or
eosinophil. The contamination rate with fungi or bacteria was less
than 1%.

       2.3.4.5 Plate Mapping Studies. Cultures were mapped using
tissue culture dishes that had been marked to allow accurate realign-
ment on a numbered reference grid. The location and size of all
clones of 2 or more cells were recorded in sample areas of the culture
and mapping was repeated at intervals during the 14 day observation
period.

       2.3.4.6 Clone-Size Studies. The number of cells per clone
was determined by counting cell numbers in a minimum of 100 consecu-
tive clones from fixed, stained cultures using 500x magnification.

       2.3.4.7 Mouse Bone Marrow Agar Cultures. Cultures were
performed in 35mm plastic petri dishes as described previously
(Metcalf, 1984). 75,000 C57BL/6 femoral bone marrow cells were
cultured in 1 ml of 0.3% agar (Difco Laboratories, Detroit, Mich.,
U.S.A.) in DME containing 20% FCS. In each case, 0.1 ml of test
material was added to quadruplicate culture dishes before the addi-
tion of the cell suspension in agar medium. Cultures were incubated
at 37°C in a fully humidified atmosphere of 10% CO_2 in air, and
colonies containing more than 50 cells were scored on day 7 of culture
using a dissecting microscope.

       2.3.4.8 Mouse Foetal Liver Agar Cultures. Cultures were
performed in 35mm plastic petri dishes. 20,000-30,000 12-day (day
of detection of plugs = day 0) CBA foetal liver cells were cultured
in 1 ml of 0.3% agar in DME containing 20% FCS. In each case, 0.1 ml
of test material was added to quadruplicate culture dishes before
the addition of the cell suspension in agar medium. Cultures were
incubated at 37°C in a fully humidified atmosphere of 10% CO_2 in

air, and colonies containing more than 50 cells were scored on day 7 of culture using a dissecting microscope.

2.3.4.9 Delayed Addition of Multi-CSF. Replicate cultures of mouse bone marrow and foetal liver cells were initiated using rHGM-CSF. After 2 days of culture 400 units of Multi-CSF was added to cultures. All additions were performed in a laminar flow hood.

2.4 LIQUID CULTURE ASSAYS

2.4.1 Human Leukaemic Cells

2.4.1.1 HL60 Cells. HL60 cells were obtained from Dr. R.C. Gallo (National Cancer Institutes, Bethesda, Md.). The cells were recloned in agar and one of these clones was used in these studies. Cells were grown in 25 cm^2 tissue culture flasks (Corning, New York, U.S.A.) with RPMI-1640 medium and 20% (v/v) preselected FCS. Cultures were split and fresh medium added weekly. Cells were cultured at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Experiments utilizing liquid cultures contained initial cell concentrations of 20,000-25,000 cells per ml. After 1 week in culture, cells were harvested and cell counts, morphological, surface antigen and clonogenic analyses were performed. An aliquot of cells (50 µl-200 µl) was also placed in 1 ml of fresh medium for a second week of culture. This was repeated at the end of the second week of culture. Cultures were either unstimulated or stimulated by CSF and were performed using flat bottom, 24-well tissue culture plates (3.5 ml Linbro, Flow Laboratories, Virginia).

2.4.1.2 Fresh Leukaemic Cells. Leukaemic cells were cultured in DME and 20% (v/v) FCS at an initial cell density of 10^6 cells per ml. Cells were harvested at varying timepoints and cell counts, morphological, surface antigen and clonogenic analyses performed. Cultures were either unstimulated or stimulated by CSF and were cultured at 37°C in a fully humidified atmosphere of 10% CO_2 in air.

Flat bottom, 24-well tissue culture plates were employed (3.5 ml, Linbro, Flow Laboratories, Virginia).

2.4.1.3 <u>Cell Counts and Differential Cell Counts</u>. Cell counts were performed by taking an aliquot of cells (usually 0.1 ml) mixed with eosin (0.9 ml) and viable nucleated cells were counted in a haemocytometer. A further aliquot of cells was resuspended in 0.2 ml EBSS and 10% FCS and centrifuged onto alcohol cleaned slides in a Shandon-Elliot cytocentrifuge (1,500 rpm, 10 min.).

After drying in air, slides were stained in May-Grünwald's stain (BDH Chemicals Ltd., Poole, England) (0.6 gm per 100 ml methanol) and counterstained with 4% (v/v) Giemsa in water. The slides were then mounted in DPX and differential counts performed with an oil immersion objective, counting a minimum of 200 cells.

The cells were classified into:

(a)   Blast cells - early cells with a large nucleus, nucleoli and basophilic agranular cytoplasm (myeloblasts were included in this category).

(b)   Promyelocytes and myelocytes - these were larger than the blast cells and contained azurophilic granules.

(c)   Metamyelocytes and mature granulocytes.

(d)   Lymphocytes.

(e)   Monocytes.

(f)   Nucleated red blood cells.

(g)   Plasma cells.

(h)   Mature eosinophils.

A third aliquot of cells was cultured in agar cultures stimulated by CSF. Cultures were counted at 7 and 14 days for colonies and clusters.

2.4.1.4 <u>Labelling of Cells With Monoclonal Antibodies</u>.
Monoclonal antibody WEM-G11 has been described elsewhere (Lopez et
al, 1985). Both NIMP-R10 and WEM-G11 were obtained as a result of
cell fusion between spleen cells from a BALB/c mouse (hyperimmunized
with human neutrophils) and the mouse myeloma line Sp2/0. The anti-
bodies were purified from ascites fluid by precipitation with 40%
saturated ammonium sulphate followed by ion-exchange chromatography
on DEAE-Sephacel (Pharmacia, Uppsala, Sweden). $F(ab')_2$ fragments of
antibody WEM-G11 were prepared as previously described (Lamoyi and
Nisonoff, 1983) and were directly conjugated to fluorescein isothio-
cyanate (FITC) (Goding, 1976). Both antibodies bound to mature
neutrophils and WEM-G11 also bound to mature eosinophils. Both anti-
bodies were provided by Dr. A.F. Lopez (The Walter and Eliza Hall
Institute).

Antibody 25E11 was provided by Dr. G. Burns (The Walter and
Eliza Hall Institute) and was raised by immunizing BALB/c mice with
human mononuclear cells and fusing spleen cells with the NS-1 cell
line. The antibody was purified on a protein A-Sepharose column.

An aliquot of cells (approximately 10^5 cells) was incubated
with antibody at 4°C for 30 min. Cells were then spun through an
underlayer of FCS at 4°C to remove unbound antibody. A second incuba-
tion (30 min. at 4°C) with FITC-conjugated sheep-anti-mouse antibody
(Silenus Laboratories, Australia) was performed for antibodies that
were not directly FITC-conjugated. Cells were spun through an under-
layer of FCS at 4°C and resuspended in HT-PBS-azide. Propidium-iodide
(1 μg/ml) (Calbiochem., California) was added to all cell suspensions
prior to analysis by the FACS II.

2.4.2 Microwell Cultures

All cultures were performed using 60-well Lux 5260 HLA tissue culture plates (Miles Laboratories, Naperville, IL.). Each well contained 10 µl of DME and 20% (v/v) FCS. Volumes of 5 µl of serial dilutions of CSF-containing preparations or purified CSF's were placed in microwells prior to the addition of the target cells in culture medium. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Eosin (5 µl) was added to cultures 15 min. prior to cell counts being performed.

2.4.2.1 Proliferation of Fractionated Normal Human Marrow

Cells. 200 normal marrow cells (fractionated using the fluorescence-activated cell sorter and monoclonal antibody WEM-G11) were cultured per microwell (described above). After 2, 5 and 8 days of incubation, direct cell counts were performed on all wells using an inverted microscope. For morphological analysis, cultured cells were removed from wells using a fine Pasteur pipette and cytocentrifuge preparations made and stained with May-Grünwald and 4% Giemsa.

2.4.2.2 Survival of Normal Human Neutrophils and Eosino-

phils: Cell Preparation and Culture. The preparation of purified blood neutrophils and eosinophils has been described in detail (Vadas et al, 1985). Red blood cells were sedimented from freshly drawn heparinized blood using 2 ml of dextran (Dextran 150,000, Sigma Chemicals, St. Louis, Mo.) per 10 ml blood for 30 min. at 37°C. The leukocyte-rich supernatant was collected and washed twice with DME and 10% FCS. Cells were then resuspended and layered onto a discontinuous Metrizamide gradient.

A stock solution of 30% Metrizamide (Nyegaard, Oslo, Norway) in Tyrode's gel/DNase was prepared and diluted with Tyrode's gel/DNase to solutions of 18-25% (w/v). Gradients were prepared by

carefully layering 2 ml volumes of decreasing densities of Metriza-
mide into a 15 ml conical centrifuge tube (Falcon Plastics, Stansen,
Melbourne). Cells ($5-10 \times 10^7$) were layered on top in DME, 10% FCS/
DNase and the tubes spun at 1,200g for 45 min. Cells were then
collected from each interface, counted and cytocentrifuge slides
prepared for staining with May-Grünwald and 4% Giemsa. Neutrophil
preparations were >98% pure and eosinophil preparations >95% pure.
In some experiments contaminating red cells were removed from eosino-
phil preparations by hypotonic lysis in distilled water for 20 sec.
This procedure did not alter the subsequent survival of eosinophils
in control experiments.

Cells were cultured either in CSF-stimulated or unstimulated
cultures at 37°C in a humidified atmosphere of 10% CO_2 in air.
Viable direct cell counts were performed on replicate wells at varying
time intervals using an inverted microscope and eosin-exclusion as
the measure of cell viability.

2.4.2.3 Factor-Dependent Murine Cell Lines. Microwell
assays using the continuous haemopoietic cell lines 32D cl.3
(provided by Dr. J. Greenberger, Boston) and FCD-P1 (supplied by
Dr. T.M. Dexter, Manchester) were performed in microtitre trays
using 200 cells per well (as described above). Viable cell counts
were performed (using an inverted microscope) after 48 hours of
incubation.

2.5 CELL SEPARATION USING THE FACS II

2.5.1 Cell Preparation for Cell Sorting

2.5.1.1 Isolation of Cells From Bone Marrow. Marrow cells
were removed by flushing marrow samples with EBSS. Cells were washed
and layered on Ficoll-Paque. Interface cells were washed and resus-
pended in DME and 10% FCS.

2.5.1.2 <u>Cell Fixation</u>. In some experiments cells were fixed prior to staining with monoclonal antibodies. This was performed by resuspending cells in 1% formaldehyde, 5 mM sodium azide and 2% glucose.

2.5.1.3 <u>Labelling of Cells With Monoclonal Antibodies</u>.
Monoclonal antibodies WEM-G1 and WEM-G11 have been described elsewhere (Lopez and Vadas, 1985; Lopez et al, 1985). Both were obtained after the fusion of BALB/c spleen cells (of a mouse immunized with human neutrophils) with the Sp2/O cell line. Antibodies were purified from ascites by precipitation with 40% saturated ammonium sulphate followed by ion-exchange chromatography. WEM-G1 and $F(ab')_2$ fragments of WEM-G11 were conjugated to FITC (Goding, 1976). Both antibodies were provided by Dr. A.F. Lopez.

Antibody HTF-1 was provided by Dr. F.R. Rickles (Connecticut, U.S.A.) and was raised by immunizing mice with human brain tissue factor and fusing the spleen cells with NS-1 cell line. Antibody was purified from pristane-induced ascites by precipitation with 50% saturated ammonium sulphate followed by euglobulin precipitation and dialysis in HT-PBS.

Antibody 25E11 was provided by Dr. G. Burns. $F(ab')_2$ fragments were prepared (Lamoyi and Nisonoff, 1983) and conjugated to FITC (Goding, 1976).

The cell suspension (described above, 0.5 ml of 3×10^7 cells) was incubated with the antibody at 4°C for 30 min. (50 µl of 1:100 dilution). Cells were then spun through an underlayer of FCS at 4°C to remove unbound antibody. A second incubation (30 min. at 4°C) with FITC-conjugated sheep-anti-mouse antibody (Silenius Laboratories, Australia) (50 µl of 1:20 dilution) was performed for antibodies that were not FITC-conjugated. Cells were spun through

an underlayer of FCS at 4°C to remove unbound antibody then resus-
pended at a final concentration of 5×10^6 cells per ml in HT-PBS-
Azide for cell sorting.

2.5.2 Conditions for Cell Sorting on the Basis of Light

Scatter Intensity and Fluorescein Fluorescence

The cell sorter used was a Becton Dickinson FACS II that had
been modified to allow sorting on the basis of 3 simultaneously
measured optical parameters.

The low angle light scatter intensity detected for each cell
was that light emerging from the saline stream within a cone half
angle 15° with axis in the direction of the laser beam. The high
angle light scatter cone had a half angle of about 20° and axis at
90° to the laser beam. These parameters were referred to as low
angle (or 0°) light scatter intensity and high angle (or 90°) light
scatter intensity respectively.

The instrument was calibrated each day with 1.83 µl diameter
fluorescent standard beads (Polysciences Inc., Warrington PA 18976,
Catalogue No. 9847).

The 0° gain was adjusted such that the 0° scatter peak of human
lymphocytes was at channel 120. The polymorphonuclear cell peak
then appeared at about channel 160. A neutral density filter (Ditric
Optic ND 10) was inserted in front of the 0° light scatter detector
and a 2.5 neutral density filter inserted in front of the photomulti-
plier tube (EMI No. 9798) for detecting 90° scattered light.

The light scatter and fluorescent properties of normal and
leukaemic cells after labelling with fluorescent monoclonal anti-
bodies were investigated.

2.5.2.1 Handling of Cells During Sorting. Cells were

prepared (as described previously) and after labelling were taken

to the sorter on ice (5 to 10^6 cells/ml in HT-PBS-Azide). The cell
sorting rate routinely used was 2,000 to 3,500 cells/sec. During a
long sorting procedure, the sorting was interrupted at hourly inter-
vals and the cells remaining in the tube were resuspended as they
tended to settle. The cells in the chosen collection windows were
deflected into sterile siliconized glass tubes.

These tubes were filled with 50% FCS, 50% (v/v) DME to 1 cm
from the top. When removing these from the FACS, a sterile lid was
put on top and cells washed from the sides of the tube using a
sterile pipette. This improved cell yield.

Cells were centrifuged at 400 g for 20 min, the supernatant
discarded and the cells resuspended in DME and 10% FCS. The yield
of cells was routinely 50% of the cells sorted by the FACS. The
yield was increased when more cells were collected in any one tube.

2.5.2.2 Cell Counting, Differentials and Agar Cultures.
An aliquot of 10 µl of the sorted cells was mixed with 10 µl of eosin
and viable nucleated cells counted in a haemocytometer. A further
aliquot of cells was centrifuged onto microscope slides for morpho-
logical examination and a third aliquot of cells was cultured in
agar cultures (as described).

2.5.2.3 Pulse-Stimulation of Fractionated Cells. An equal
aliquot of cells was placed in sterile centrifuge tubes (approx.
50 µl) and varying concentrations of CSF-α or CSF-β added. The volume
was made up to 200 µl by the addition of DME and 10% FCS. Cells
were incubated for 45 min. at 37°C in a fully humidified atmosphere
of 10% C)$_2$ in air. Cells were then spun through an underlayer of
FCS (5 ml) and all the supernatant was removed. DME and 10% FCS
(200 µl) was used to resuspend the cells which were then cultured
in CSF-stimulated and unstimulated agar cultures. This washing
procedure involved dilution of the initial CSF by at least 10^4X.

CHAPTER 3

CHARACTERIZATION OF HUMAN-ACTIVE COLONY STIMULATING FACTORS

3.1 INTRODUCTION

The granulocyte-macrophage colony stimulating factors (CSF's) are specific glycoproteins that control the production and functional activity of granulocytes and macrophages. Four murine granulocyte-macrophage CSF's (reviewed Metcalf, 1984, 1985, 1985a) have been purified to homogeneity and the genes encoding two of these molecules cloned and expressed (Gough et al, 1984; Fung et al, 1984; Yokota et al, 1984). While granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF) are relatively "lineage specific" in their action, GM-CSF and Multipotential-CSF (Multi-CSF) are both active on neutrophils, macrophages and eosinophils (as well as other lineages). There is, however no evidence for a murine eosinophil-CSF (Eo-CSF) although by analogy with the neutrophil and macrophage lineages its existence was predicted. An eosinophil differentiation factor (EDF) has been purified from a murine hybridoma supernatant (Sanderson et al, 1985) and although this molecule did not stimulate murine eosinophil colonies in agar it was clearly a proliferative stimulus for eosino-phils in liquid cultures. As such it was an excellent candidate for the murine Eo-CSF with the apparent inactivity in agar cultures possibly being explained by suboptimal culture conditions. Further evidence for an Eo-CSF was provided by experiments demonstrating that conditioned media from lectin-stimulated murine spleen cells (SCM) and murine T cell clones stimulated only the proliferation of human eosinophil colonies (Metcalf et al, 1983; Kelso and Metcalf, 1985). For these reasons, EDF was obtained and its activity on mouse and human cells examined in agar cultures.

Several crude sources of human CSF's have been described. The studies described here were performed using human placental conditioned medium (HPCM) and medium conditioned by the cell line U/5637 (Nicola et al, 1978; Svet-Moldavsky et al, 1983). Two human CSF's

with granulocyte-macrophage activity have been identified in HPCM
(Nicola et al, 1979) and Giant-Cell-Tumour (GCT)-conditioned medium
(Abboud et al, 1981), and are separable based on differences in
hydrophobicity. CSF-α (the first eluting species from phenyl-
sepharose chromatograms) stimulates granulocyte, macrophage and
eosinophil colonies while CSF-β does not stimulate eosinophil
colonies (Nicola et al, 1979). The nature of the CSF(s) present
in U/5637-conditioned medium (U/5637-CM) was not known.

Towards the end of these studies, a cDNA for human-active GM-CSF
was cloned from a library prepared from the human Mo T-leukemia cell
line. This cDNA showed 70% homology in the nucleotide sequence of
the protein coding region with murine GM-CSF cDNA (Wong et al, 1985).
GM-CSF with the same N-terminal amino acid sequence was independently
purified from Mo-conditioned medium (Wong et al, 1985a) and shown
to stimulate granulocyte-macrophage colony formation by human cells.
The recombinant GM-CSF cDNA was expressed in Monkey COS cells, the
protein purified and provided for determination of biological
activities. Recombinant human GM-CSF was also obtained from an E.coli
expression system by Biogen, Geneva and provided for characterization
of biological activities.

3.2 RESULTS

3.2.1 Preparations of Native Human Colony Stimulating Factors

Figure 3.1A shows a titration curve for normal human bone marrow
cells stimulated by HPCM and U/5637-CM. Both CSF preparations stimul-
ated the same number of progenitor cells within the two subsets of
granulocyte-macrophage progenitors identified at days 7 and 14 of
culture. These two subsets of human granulocyte-macrophage progenitors
can be clearly separated based on differences in sedimentation velocity

(Johnson et al, 1977; Inoue and Ottenbreit, 1978; Miller et al, 1978) and lectin binding characteristics (Morstyn et al, 1980; Nicola et al, 1980).

The morphology of colonies stimulated by HPCM and U/5637-CM is shown in Table 3.1. Both CSF preparations stimulated primarily neutrophil colonies at the day 7 timepoint while at day 14 the colony morphology was neutrophil, macrophage and eosinophil in type. The proportion of colonies stimulated by each CSF preparation was very similar.

The two CSF's in human placental conditioned medium active on human granulocyte and macrophage progenitor cells have been previously separated on the basis of differences in hydrophobicity (Nicola et al, 1979). The earlier eluting species (CSF-α) stimulated the formation of neutrophilic granulocyte, macrophage and eosinophilic granulocyte colonies with colony formation peaking by day 14 of culture, while the later eluting species (CSF-β) stimulated almost exclusively the formation of neutrophilic granulocyte colonies - colony formation peaking by day 5-7 of culture.

This technique was used to examine the U/5637-CM and confirmed the presence of two CSF's with activity on human cells (Figure 3.2). The first eluting species (CSF-α) stimulated primarily day 14 colony formation while the second species stimulated primarily day 7 colony formation (CSF-β). CSF-β fractions active on human marrow cells also stimulated granulocyte colony formation by murine marrow cells and induced differentiation in murine myelomonocytic leukaemic WEHI-3B D^+ cells whereas CSF-α fractions had no such activities. The low frequency, small size and maturity of the murine granulocytic colonies stimulated by CSF-β were typical of colonies stimulated by G-CSF (Metcalf and Nicola, 1983).
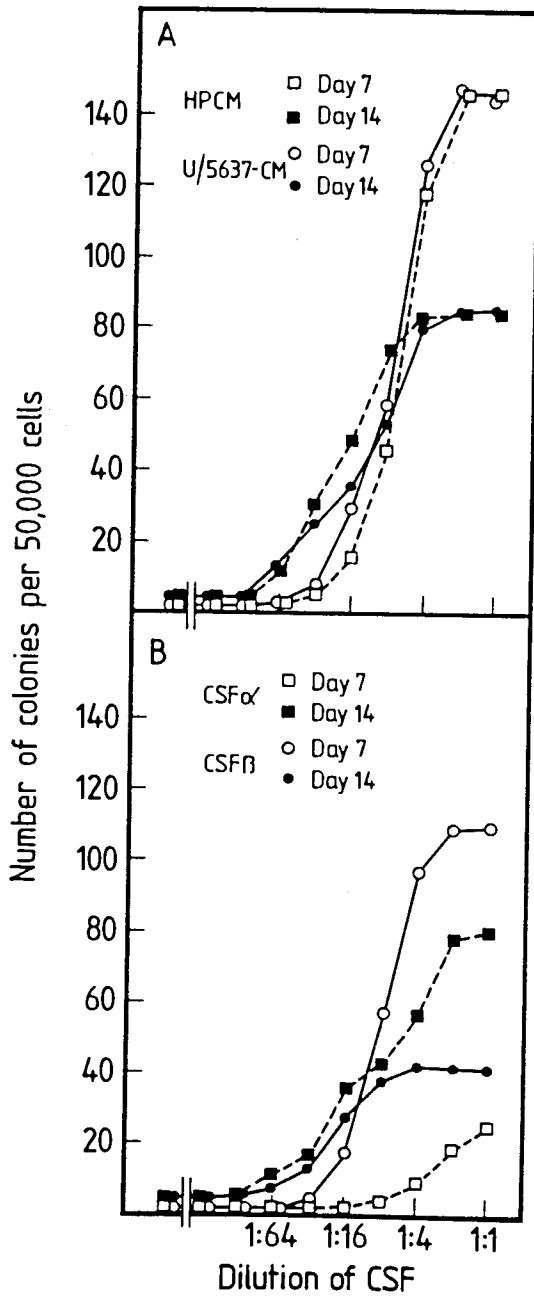
Fig. 3.1 Dose-response curves for HPCM and U/5637-CM-stimulated colony formation of normal human bone marrow cells (upper panel) and CSF-α and CSF-β-stimulated colony formation (lower panel). CSF-α and CSF-β were obtained from U/5637-CM (as described). 3×10^4 cells were cultured per ml and replicate cultures examined at days 7 and 14 of culture.

TABLE 3.1. Morphology of Normal Human Bone Marrow Colonies Stimulated by HPCM,

U/5637, CSF-α and CSF-β

Stimulus	Percentage of Colonies							
	Day 7				Day 14			
	G	GM	M	Eosin.	G	GM	M	Eosin.
HPCM	83	12	5	0	41	16	36	7
U/5637-CM	74	19	7	0	40	12	34	14
CSF-α	76	10	14	0	37	11	40	12
CSF-β	81	15	4	0	78	10	12	0

5×10^4 human marrow cells were cultured for 7 or 14 days. Stimuli used were HPCM, U/5637-CM and CSF-α and CSF-β obtained from U/5637-CM fractionated using phenyl-sepharose chromatography.

G = neutrophilic granulocyte, GM = neutrophilic granulocyte-monocyte, M = monocyte, Eosin. = Eosinophil.
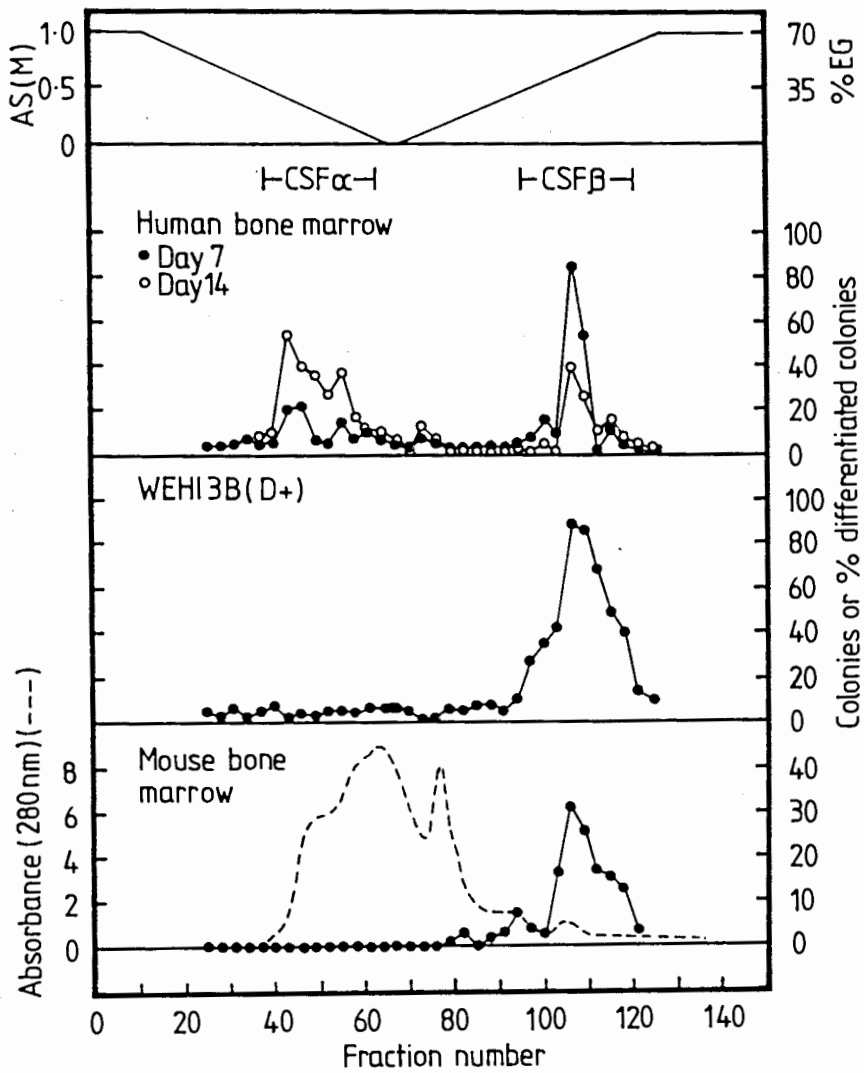
Fig. 3.2 Separation of human U/5637-CM on phenyl-Sepharose CL-4B.
Aliquots of each fraction were assayed for their ability
to stimulate colony formation from normal human marrow
cells (50,000 cells/plate) and colonies were scored at
both day 7 and 14 of culture (second panel), their ability
to induce differentiation in colonies formed by murine
myelomonocytic leukaemic cells (WEHI-3B D⁺) (300 cells/
plate) after 7 days of culture (third panel) and their
ability to stimulate colony formation by normal murine
C57BL/6 bone marrow cells (75,000 cells/plate) after
7 days of culture (fourth panel). Also shown in the
fourth panel is the absorbance of fractions at 280 nm
(dashed line). Abbreviations are AS, ammonium sulphate
concentration; %EG, percentage by volume of ethylene
glycol in water.

Titration curves for the U/5637-CSF-α and CSF-β preparations are shown in Figure 3.1B and the total number of colonies stimulated by unfractionated HPCM and U/5637-CM is shown for comparison (Figure 3.1A). Both preparations demonstrated apparently maximal numbers of stimulated colonies without high-dose inhibition of colony numbers. The preferential stimulation by CSF-α and CSF-β of day 14 and day 7 colony formation respectively is again demonstrated.

The morphology of colonies stimulated by U/5637-CSF-α and CSF-β is shown in Table 3.1. The colony morphology stimulated by U/5637-CM is also shown for comparison. Both CSF-α and CSF-β stimulated primarily neutrophil colonies following 7 days of incubation. After 14 days of culture, CSF-α stimulated neutrophil, macrophage and eosinophil colonies while CSF-β did not stimulate eosinophil colonies. These results were completely consistent with the previously described actions of these molecules when obtained from HPCM (Nicola et al, 1979; Morstyn et al, 1981) however the two molecules were more widely separated, and showed less variation between different preparations fractionated from different batches of U/5637-CM when compared with different batches of HPCM. For these reasons, U/5637-CM was used for all preparations of CSF-α and CSF-β.

3.2.2 Murine CSF's Active on Human Cells

The action of purified murine CSF's on human bone marrow cells was examined. As shown in Figure 3.3, murine G-CSF stimulated the formation of day 7 colonies comparable to the number stimulated by CSF-β. These colonies were 94% neutrophil in type. Few day 14 colonies were stimulated by G-CSF and these were only neutrophil colonies. Murine M-CSF, GM-CSF and Multi-CSF were inactive on normal human bone marrow cells (see also Chapters 5 and 6).
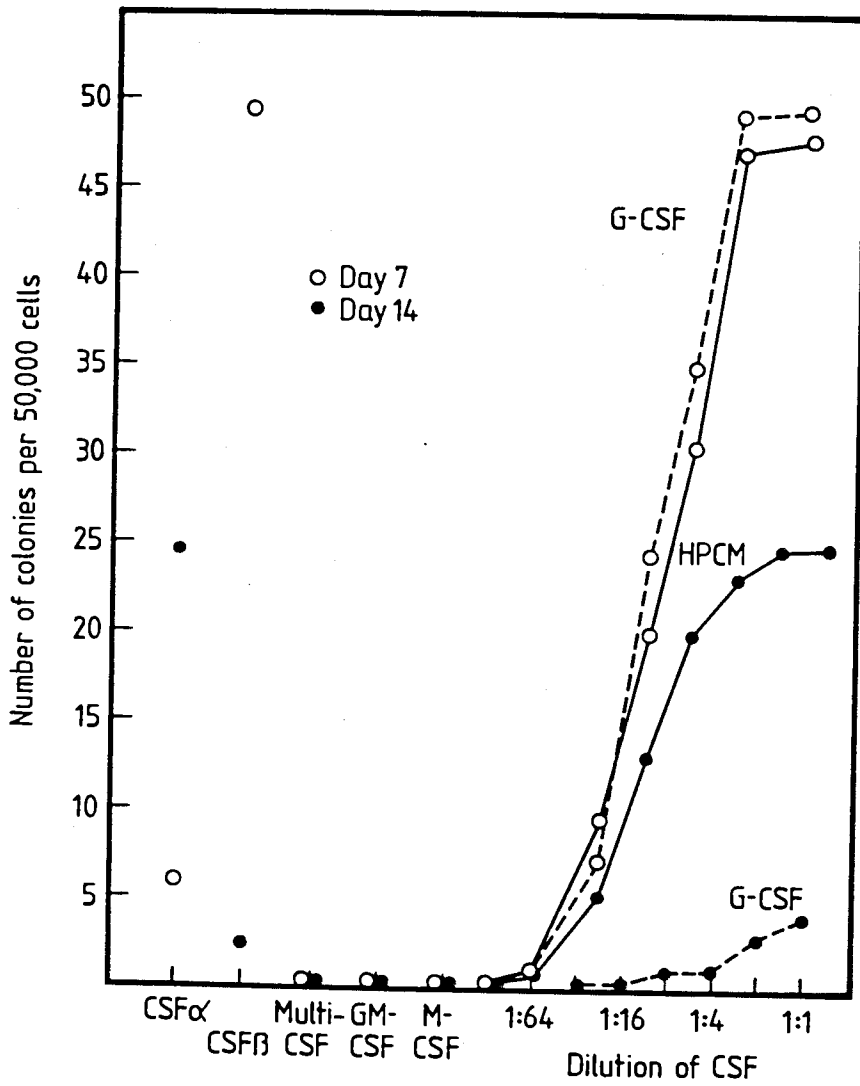
Fig. 3.3 Dose-response curve for murine G-CSF stimulated colony

formation from normal human bone marrow cells. 3×10^4

cells were cultured per ml and stimulated by murine

G-CSF, GM-CSF, M-CSF and Multi-CSF. Human CSF-α and

CSF-β stimulated colony formation is also shown.

3.2.2.1 Murine Eosinophil Differentiation Factor:

a Murine CSF. Eosinophil differentiation factor (EDF) has previously been shown to stimulate mature, functionally active eosinophils in liquid cultures of murine bone marrow (Sanderson et al, 1985). It was therefore of interest to determine whether EDF could stimulate the clonal proliferation and differentiation of murine eosinophil progenitors in semi-solid agar cultures, particularly since this had not been observed with EDF or the human-active Eo-CSF present in SCM (Metcalf et al, 1983). Small numbers of eosinophil colonies were demonstrable in 7-day cultures of mouse bone marrow and foetal liver cells (Table 3.2). In all but one experiment, in which crude conditioned medium was used (Table 3.1, experiment 4), these were the only colonies stimulated by EDF although the expected proportions of neutrophil, neutrophil-macrophage and macrophage colonies were stimulated by mouse spleen conditioned medium (SCM). However, EDF stimulated significant numbers of neutrophil, neutrophil-macrophage and macrophage clusters (3-49 cells per clone) in all bone marrow and foetal liver samples suggesting that it also had some activity on these lineages. Notwithstanding a degree of cross-reactivity, these data indicated that EDF was a murine Eo-CSF.

3.2.2.2 Murine EDF (Eo-CSF) Stimulates Human Eosinophil
Colonies

Initial experiments examined the ability of the murine hybridoma supernatant to stimulate human bone marrow cells to form colonies in agar. This material stimulated day 14 colony formation but did not stimulate day 7 colony formation. The absolute number of colonies was less than that stimulated by CSF-α or HPCM (Table 3.3, experiments 1-3), but was similar to the numbers of colonies stimulated by murine SCM. Subsequent experiments were carried out using purified material

TABLE 3.2. Morphology of Mouse Colonies Stimulated by Eosinophil Differentiation Factor

Experiment Number	Cells	Stimulus	Mean Number of Colonies	Type of Colony (Percentage)			
				Eosinophil	Neutrophil	Neutrophil-Macrophage	Macrophage
1	Bone Marrow	SCM	135	4	20	41	35
		HS	2	100	0	0	0
2	Bone Marrow	SCM	135	2	5	44	49
		HS	1	100	0	0	0
3	Bone Marrow	SCM	135	2	5	47	46
		EDF	2	100	0	0	0
4	Foetal Liver	SCM	83	2	2	36	60
		HS	4	84	11	0	5
5	Foetal Liver	SCM	85	1	1	36	62
		EDF	4	100	0	0	0
6	Foetal Liver	SCM	102	7	9	43	41
		EDF	4	100	0	0	0

Quadruplicate plates containing 7.5×10^4 C57BL bone marrow or 3×10^4 CBA foetal liver cells were incubated for 7 days with SCM, the hybridoma supernatant (HS) or purified EDF. After counting the number of colonies on each plate, they were fixed and stained to allow morphological identification. The results are expressed as mean number of colonies per plate.

TABLE 3.3. Morphology of Human Bone Marrow Colonies Stimulated by Eosinophil Differentiation Factor

Experiment Number	Stimulus	Total No. of colonies	Type of Colony (Percentage)				Calculated No. of Eosinophil colonies
			Eosinophil	Neutrophil	Neutrophil-macrophage	Macro-phage	
1	HS	13	100	0	0	0	13
	CSF-α	36	39	0	44	17	14
	HPCM	23	19	0	45	36	4
2	HS	10	100	0	0	0	10
	CSF-α	38	33	0	28	39	13
	HPCM	50	12	0	44	44	6
3	HS	40	94	0	0	6	38
	SCM	38	98	0	0	2	37
	CSF-α	68	18	0	17	65	12
	HPCM	174	16	4	43	37	28
4	EDF	63	100	0	0	0	63
	SCM	67	100	0	0	0	67
	HPCM	220	20	28	22	30	44
5	EDF	12	100	0	0	0	12
	SCM	14	100	0	0	0	14
	CSF-α	78	22	7	17	54	17
	HPCM	108	22	25	19	33	24
6	EDF	6	100	0	0	0	6
	SCM	4	100	0	0	0	4
	HPCM	54	14	41	24	22	8
7	EDF	8	100	0	0	0	8
	SCM	25	81	0	0	19	20
	CSF-α	60	12	17	17	54	7
	HPCM	87	13	21	29	38	11
8	EDF	8	100	0	0	0	8
	SCM	8	100	0	0	0	8
	CSF-α	34	23	7	30	40	8
	HPCM	42	26	21	23	30	11

Normal human bone marrow cells were incubated for 14 days and stimulated as indicated. The mean absolute number of colonies was determined by scoring 4 replicate cultures. These cultures were then fixed and stained, and consecutive colonies were examined to determine the percentage of each colony type present. The number of cells cultured was 2-5×10^4 cells/ml and the results are expressed per 10^5 cells. Cultures were stimulated by CSF-α, HPCM, murine SCM, the hybridoma supernatant (HS) or EDF as indicated.

and these experiments confirmed the previous results (Table 3.3, experiments 4-8). Morphological examination of the day 14 colonies revealed that the hybridoma supernatant and the purified EDF almost exclusively stimulated colonies containing only eosinophils whereas CSF-α and HPCM also stimulated the formation of neutrophil, neutrophil-macrophage and macrophage colonies. In experiment 3, a few macrophage colonies were seen in cultures stimulated by the hybridoma supernatant but these were also seen in unstimulated cultures suggesting that these arose spontaneously in this particular bone marrow sample. In any one experiment, the total number of eosinophil colonies stimulated by EDF was similar to that stimulated by the hybridoma supernatant, SCM, CSF-α or HPCM suggesting that EDF was not stimulating only a subset of responsive eosinophil progenitors. This was also supported by the observation that the eosinophil colonies stimulated by EDF and HPCM were of similar size (Figure 3.4). Because of the higher frequency of clonogenic eosinophil precursors in human bone marrow compared with murine tissues, further studies on the actions of EDF were performed using human cells. To confirm that the stimulation of eosinophil colonies was a direct effect of EDF and not mediated via an accessory cell, two types of experiment were performed. First, human bone marrow cells were cultured at varying concentrations and it was shown that the relationship between cell concentration and number of eosinophil colonies stimulated by EDF was linear. The concentration of cells cultured ranged between 0.0625-2.0 $\times$ 10^5/ml and the numbers of colonies stimulated were between 0.5 $\pm$ 0.5 and 16.7 $\pm$ 1.2 (mean $\pm$ standard deviation).

The second type of experiment involved clone transfers. Bone marrow cells were initially stimulated by EDF for 7 days in primary culture, and developing clones (mean 15 cells per clone) were then
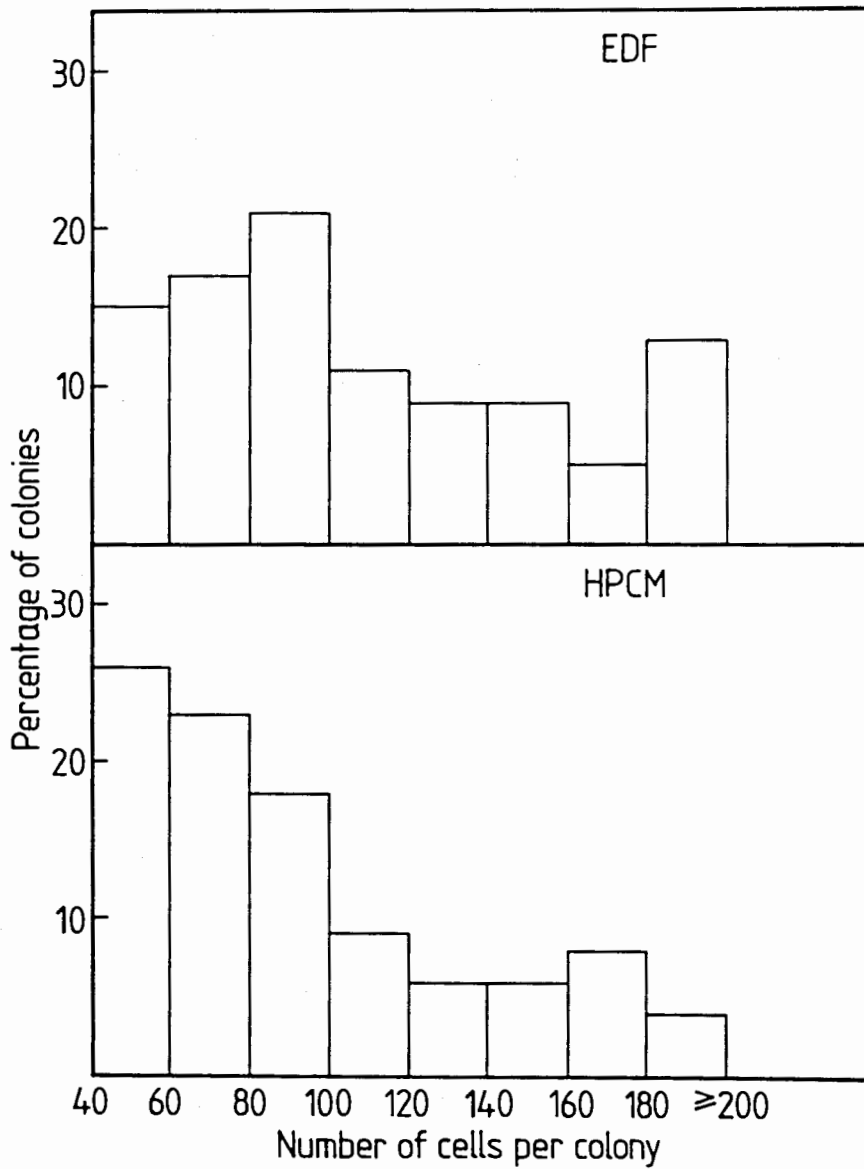
Fig. 3.4 Size distribution of human eosinophil colonies stimulated
by murine EDF and HPCM. 65 consecutive eosinophil colonies
were counted from experiments 4 and 5 shown in Table 3.3.

transferred to recipient culture dishes containing either EDF, CSF-α
or medium. This transfer results in a dilution of the initiating
CSF by a factor of 1:500 and allows an assessment of the direct
effect of CSF in cultures lacking other cell types (Metcalf and
Merchav, 1982). Following a further 9 days of incubation, a
significant proportion of the transplanted clones showed a progress-
ive increase in size in cultures containing EDF and CSF-α, whereas
clones decreased in size and died when transferred to cultures lack-
ing CSF (Figure 3.5). The proliferating clones were confirmed to
be eosinophilic in type.

3.2.3 Biological Activities of Recombinant Human GM-CSF

The biological activity of a human GM-CSF cDNA expressed in
monkey COS cells (Wong et al, 1985) was examined.

To minimize the risk of missing direct or indirect effects of
the recombinant human GM-CSF (rH GM-CSF), initial assays were
performed using serial dilutions of purified recombinant material
in unfractionated cultures of 50,000 normal human bone marrow cells.
Control cultures were stimulated by unfractionated human placental
conditioned medium or semi-purified fractions of CSF-α and β. The
recombinant material exhibited colony stimulating activity in agar
cultures of all 14 marrow samples tested and a typical example of
the results obtained is shown in Figure 3.6. From this titration
curve it was calculated that the colony stimulating activity of the
rH GM-CSF preparation was 4×10^7 units/mg. In the example shown
in Figure 3.6 the recombinant material stimulated plateau numbers
of both day 7 and day 14 colonies at a concentration of 5 ng/ml and
with progressive reduction of this concentration, a typical sigmoid
dose-response curve was obtained, the lowest concentration stimulat-
ing detectable colony formation being approximately 300 pg/ml. With
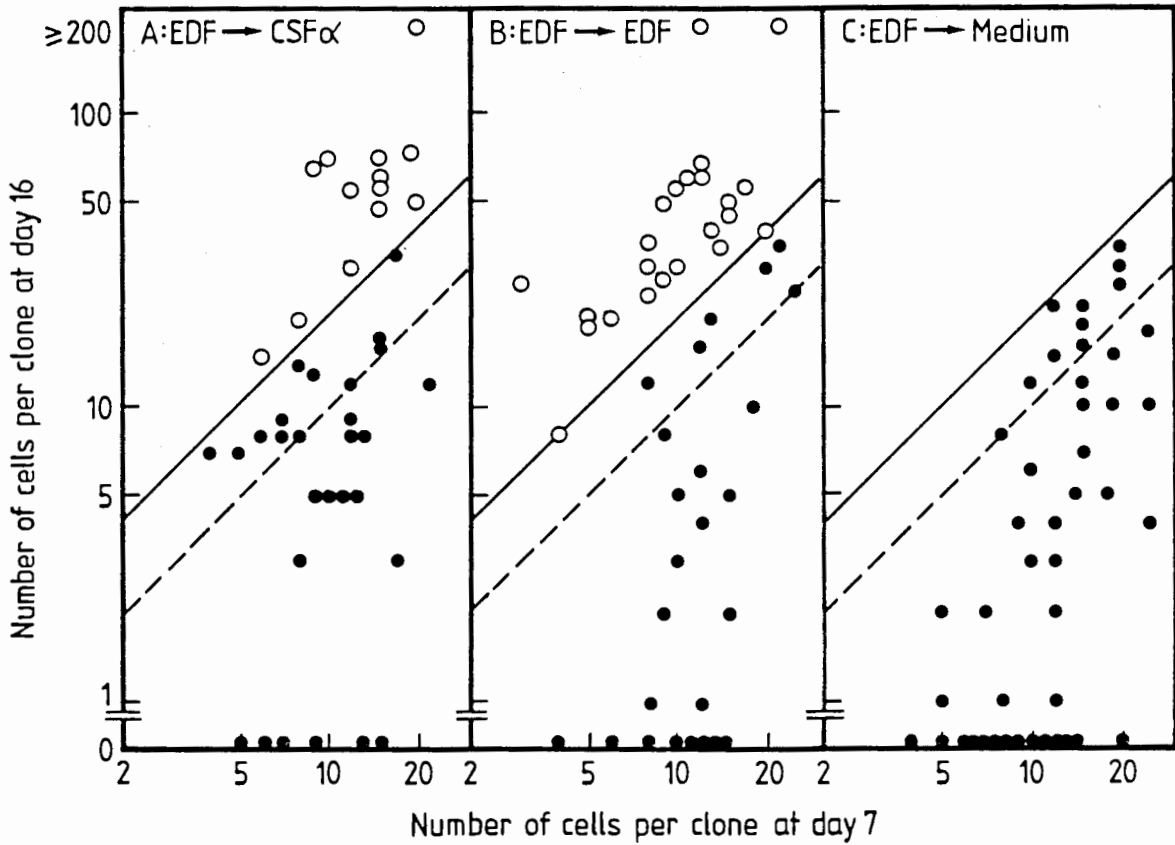
Fig. 3.5 Proliferation of eosinophil clones 9 days after transfer of
EDF-initiated clones to cultures containing, CSF-α, EDF or
medium.   The broken and solid lines indicate maintenance
or doubling of initial cell number respectively.   Clones
which had at least doubled in size were typed and confirmed
to be eosinophilic in type (open circles).   Several clones
that were neutrophil and macrophage in type were observed
following transfer from EDF to CSF-α.   It was not possible
however to attribute the initiation of these clones to
EDF as the primary agar culture was performed using 10^5
cells per ml.

the 14 marrow samples analyzed in detail, individual marrows varied up to 4-fold in their responsiveness to stimulation by the recombinant material and with some marrow samples maximal colony formation was observed with a concentration of 1 ng/ml, giving an estimate of specific activity of 1.6×10^8 units/mg.

For the 14 marrow samples analyzed, the mean colony counts with the various stimuli were: 7 day; rH GM-CSF 41 ± 30, HPCM 140 ± 110, CSF-α 27 ± 34, CSF-β 138 ± 77 and 14 days; rH GM-CSF 85 ± 55, HPCM 103 ± 96, CSF-α 54 ± 40, CSF-β 37 ± 35 (± standard deviations). It will be seen that, using rH GM-CSF, colony counts rose between 7 and 14 days (a characteristic of CSF-α but not CSF-β) and individual examples of these trends are shown in Figure 3.6 and Table 3.4. A characteristic feature of the cultures stimulated by rH GM-CSF was the relatively slow onset of development of colonies, a feature also seen in cultures stimulated by CSF-α-containing preparations and contrasting with the early onset and early disappearance of colony formation in cultures stimulated by CSF-β (Nicola et al, 1979; Morstyn et al, 1981).

As shown in the example in Figure 3.6, the number of colonies stimulated by rH GM-CSF was usually lower than that stimulated by the HPCM control preparation at the day 7 timepoint. HPCM contains both CSF-α and β and there is evidence that neither CSF-α nor β, acting alone, is able to stimulate all available progenitor cells (Morstyn et al, 1981). The colony counts in cultures stimulated by rH GM-CSF therefore suggested that the recombinant material was not able to stimulate the proliferation of all available granulocyte-macrophage progenitors.

Morphological analysis of day 7 colonies stimulated by rH GM-CSF showed that these were granulocytic, granulocyte-macrophage and mono-
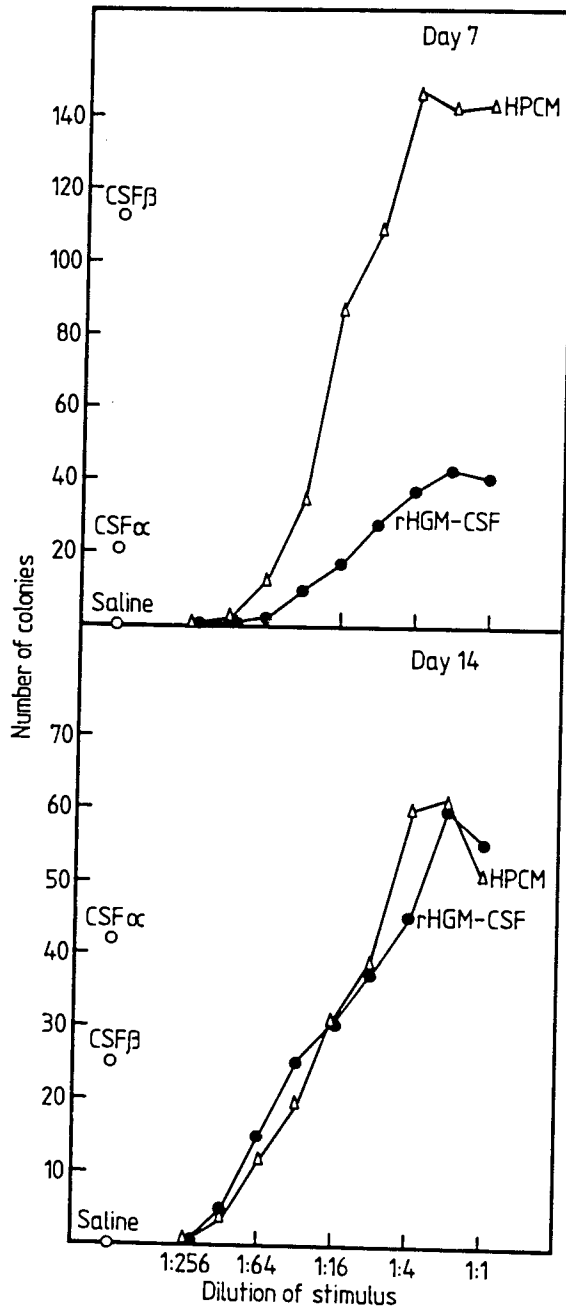
Fig. 3.6 Colony formation at days 7 and 14 in cultures of 50,000 human marrow cells stimulated by 0.1 ml of serial dilutions of a preparation containing 200 ng/ml purified recombinant human GM-CSF (rH GM-CSF). Control cultures contained 0.1 ml of serial dilutions of Stage II human placental conditioned medium (HPCM) or semi-purified preparations of CSF-α and CSF-β. Note rise with time in the number of colonies in cultures stimulated by rH GM-CSF.

TABLE 3.4. Morphological Types of Colonies in Human Marrow Cultures After 7 and 14 Days

of Stimulation by Human Recombinant GM-CSF

Incubation Time	Marrow Sample No.	Stimulus	Mean Number of Colonies	Percent Colonies			
				G	GM	M	Eosin.
7 days	1	Saline	0	-	-	-	-
		HPCM	143	62	34	4	0
		rH GM-CSF	41	10	65	25	0
		CSF-α	21	13	56	31	0
		CSF-β	113	45	44	11	0
	2	Saline	4	0	29	71	0
		HPCM	127	35	48	17	0
		rH GM-CSF	115	7	42	46	5
14 days	1	Saline	2	0	0	100	0
		HPCM	57	11	34	43	12
		rH GM-CSF	54	0	41	40	19
		CSF-α	42	2	45	38	15
		CSF-β	26	11	56	33	0
	2	Saline	4	0	0	100	0
		HPCM	33	34	26	34	6
		rH GM-CSF	99	4	31	44	21

5×10^4 human marrow cells cultured for 7 or 14 days. The various stimuli (human placental conditioned medium (HPCM), rH GM-CSF and CSF-α and β fractionated from U/5637 conditioned medium) were all used at a concentration 4-fold in excess of that stimulating maximal colony numbers to develop. Mean colony counts from duplicate cultures. After colony counts were performed, cultures were fixed and stained with Luxol-Fast-Blue/hematoxylin and differential colony counts performed on the entire colonies in each culture. G = neutrophilic granulocyte, GM = neutrophilic granulocyte-monocyte, M = monocyte-macrophage, Eosin. = eosinophil.

cyte-macrophage colonies together with a few small eosinophil colonies
(Table 3.4).   In the cultures stimulated by rH GM-CSF, the percentage
of monocyte-macrophage colonies was higher than in control cultures
stimulated by human placental conditioned medium.   This pattern
persisted at 14 days with the most frequent colony type again being
monocyte-macrophage.   It should be noted that day 14 cultures stimul-
ated by rH GM-CSF always contained prominent eosinophil colonies.
In control cultures stimulated by CSF-α, a similar pattern of colony
formation was observed to that in cultures stimulated by rH GM-CSF,
whereas in cultures stimulated by CSF-β, eosinophil colonies never
developed.

   To determine whether the colony stimulating effects of rH GM-CSF
were the result of direct action on responding clonogenic cells,
cultures were initiated using 50,000 marrow cells and 400 units rH
GM-CSF.   At 4 days, individual clones containing 4-10 cells (mean
size 6 cells) were removed using a fine pipette and transferred
intact with minimal surrounding agar to the surface of recipient
agar cultures containing 400 units rH GM-CSF or 400 units HPCM or
0.1 ml saline but no cells.   Analysis of the fate of these trans-
planted clones after a further 10 days of incubation (Figure 3.7)
showed that clone size increased progressively in recipient cultures
containing both rH GM-CSF and HPCM, whereas clones died when trans-
ferred to cultures lacking CSF. Clones exhibiting progressive pro-
liferation with continued stimulation by rH GM-CSF included both
granulocyte-macrophage and eosinophil colonies, indicating that
rH GM-CSF was able to act directly on responding clones of both
types.
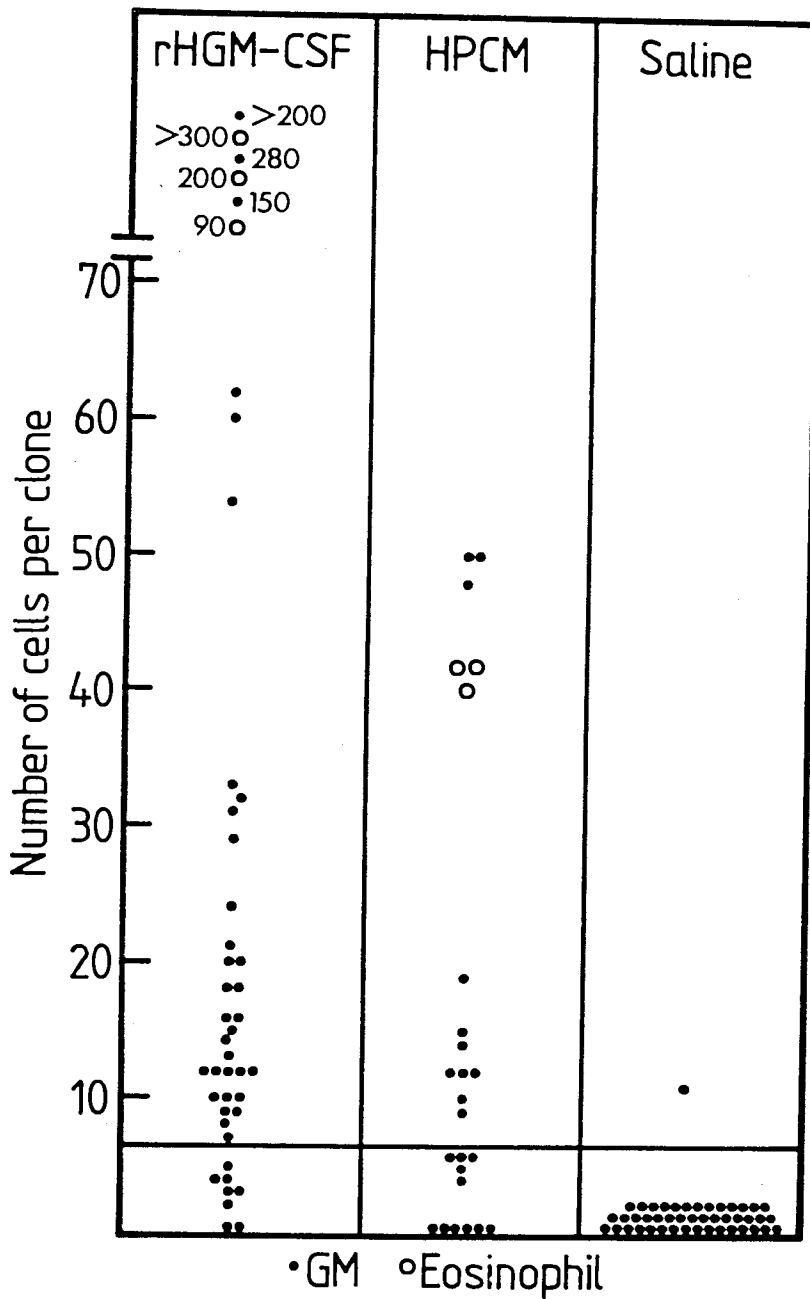
Fig. 3.7 Behaviour of 4 day clones initiated by rH GM-CSF after

transfer to recipient cultures containing rH GM-CSF,

human placental conditioned medium (HPCM) or saline.

The concentration of rH GM-CSF and HPCM in recipient

cultures was 400 units/ml. Line indicates mean size

of clones at transfer. Note progressive size increase

in clones after 10 further days of incubation in

cultures containing rH GM-CSF or HPCM.

3.2.3.1 Effects of rHGM-CSF on Erythroid and Multipotential

Colony Formation. No erythroid or multipotential

colonies developed in agar cultures of bone marrow or peripheral blood

cells stimulated by rHGM-CSF alone. However, addition of rH GM-CSF to

cultures of non-adherent peripheral blood cells containing 1.5 units

of erythropoietin consistently resulted in the formation of 2 to

3-fold higher numbers of burst erythroid colonies than in cultures

containing only erythropoietin and in the development of small

numbers of multipotential colonies containing erythroid cells. Two

examples of this effect are shown in Table 3.5 and in two other

experiments similar results were obtained. A similar pattern of

colony formation was observed in cultures stimulated by a mixture

of human placental conditioned medium and erythropoietin and in

cultures containing semi-purified CSF-α and erythropoietin. In

contrast, combination of semi-purified CSF-β with erythropoietin

did not lead to enhanced erythroid colony formation.

Titration experiments showed that enhanced erythroid colony

formation could be observed with dilutions of rH GM-CSF over the

same range as those able to stimulate granulocyte-macrophage colony

formation by direct action. However, in contrast to the action of

rH GM-CSF on granulocyte-macrophage and eosinophil progenitor cells,

150 sequentially sampled clones initiated by 200 units rH GM-CSF

for 5, 6 or 8 days (clone size 3-60 cells) generated only a single

erythroid, one mixed erythroid colony and one mixed neutrophil-

eosinophil colony when transferred to cultures containing either

erythropoietin or rH GM-CSF plus erythropoietin (Table 3.6). These

results suggested that most of the enhanced erythroid colony forma-

tion seen when rH GM-CSF was combined with erythropoietin was not

ascribable to an ability of rH GM-CSF, acting alone, to initiate

the proliferation of some BFU-E by direct action.

TABLE 3.5. Enhancement of Erythroid and Multipotential Colony Formation by Recombinant Human GM-CSF

Experiment No.	Stimulus	Erythropoietin	Mean Number of Colonies		
			Erythroid	Multipotential	Non-Erythroid
I	Saline	−	0	0	0
		+	13 ± 2	0	1 ± 1
	HPCM	−	0	0	68 ± 8
		+	55 ± 6	2 ± 1	52 ± 12
	CSF-α	−	0	0	36 ± 6
		+	36 ± 3	2 ± 2	39 ± 7
	CSF-β	−	0	0	32 ± 3
		+	8 ± 5	0	19 ± 5
	rH GM-CSF	−	0	0	38 ± 7
		+	36 ± 6	2 ± 2	40 ± 7
II	Saline	−	0	0	3 ± 1
		+	11 ± 1	0	2 ± 2
	HPCM	−	0	0	133 ± 9
		+	29 ± 3	1 ± 1	148 ± 9
	CSF-α	−	0	0	51 ± 9
		+	17 ± 8	1 ± 1	46 ± 10
	CSF-β	−	0	0	109 ± 14
		+	12 ± 3	0	94 ± 9
	rH GM-CSF	−	0	0	35 ± 10
		+	18 ± 3	1 ± 1	41 ± 9

Footnote to Table 3.5.

2×10^5 non-adherent peripheral blood cells were cultured in 1 ml agar cultures containing 400 units of test stimulus with or without 1.5 units semi-purified human urinary erythropoietin. Cultures scored after 14 days of incubation. Mean colony counts ± standard deviations of 4 replicate cultures.

TABLE 3.6. Development of Erythroid and Non-Erythroid Colonies from Transferred Clones

Initiated in Peripheral Blood Cultures Using rH GM-CSF

Day of Clone Transfer	Stimulus in Recipient Cultures	Number of Colonies					
		G	GM	M	Eosin.	E	Mixed
5	Saline	2	0	1	2	0	0
	rH GM-CSF	0	0	0	5	0	0
	rH GM-CSF + Epo	2	0	0	7	0	0
6	Saline	1	0	0	1	0	0
	rH GM-CSF	1	0	0	7	0	1
	rH GM-CSF + Epo	0	0	0	6	0	1
8	Saline	0	1	0	0	0	0
	Epo	1	0	0	0	1	0
	rH GM-CSF + Epo	0	0	0	5	0	0

Clones were initiated for 5, 6 or 8 days in cultures of 2×10^5 non-adherent peripheral blood cells using 200 units of rH GM-CSF. Twenty-five sequential clones of size 3-60 cells were transferred to each type of recipient culture. Recipient agar cultures contained 0.1 ml saline, 200 units rH GM-CSF and/or 1.5 units erythropoietin (Epo). Cultures were scored after a total incubation period of 14 days. G = neutrophilic granulocyte, GM = neutrophilic granulocyte plus monocyte, M = monocyte/macrophage, Eosin. = eosinophil, one colony of neutrophils, eosinophils and erythroid cells.

3.2.3.2. Inactivity of rH GM-CSF on Murine Cells. The
rH GM-CSF failed to stimulate any type of colony or cluster formation
in cultures of 75,000 C57BL bone marrow cells or 20,000 12 day CBA
foetal liver cells. In cultures initiated using rH GM-CSF to which
400 units of murine Multi-CSF were added after 2 days of incubation,
again no colonies or clusters were seen after a total incubation
period of 7 days. As shown in Table 3.7, these negative results
were similar to those obtained with CSF-α and were in contrast to
the ability of CSF-β-containing preparations to stimulate granulo-
cyte colony formation in cultures of adult mouse bone marrow or
foetal liver cells and the weak but reproducible ability of CSF-β
to initiate erythroid and multipotenital colony formation when
stimulation of colony formation was completed by the delayed addition
of murine Multi-CSF.

In microwell assays using the 32D murine haemopoietic cell line
(responding to murine Multi-CSF) and the murine FDC-P1 cell line
(responding to both murine Multi-CSF and murine GM-CSF), (Kelso and
Metcalf, 1985), rHGM-CSF failed to support the survival or stimulate
the proliferation of cells of either line.

In agar cultures of 300 WEHI-3B D$^+$ myelomonocytic leukemic cells,
rH GM-CSF and CSF-α failed to induce differentiation in the leukemic
colonies whereas, as shown in Figure 3.2, CSF-β fractions exhibited
high differentiation-inducing activity.

3.2.3.3. Biological Activities of Bacterially Synthesized

Human GM-CSF. The cDNA for human GM-CSF was isolated
from a cDNA library by Biogen, Geneva. The cDNA library was
constructed using mRNA from the human bladder carcinoma cell line
U/5637 by screening candidate clones with mixed oligonucleotides
prepared on the basis of the nucleotide sequence of murine GM-CSF.

TABLE 3.7.   Effect of Recombinant Human GM-CSF in Cultures of Mouse Foetal Liver Cells

Stimulus	Number of Colonies	
	Non-Erythroid	Pure or Mixed-Erythroid
Saline	0	0
MLCM	34 ± 8	0
SCM	74 ± 25	15 ± 13
HPCM	47 ± 4	0
CSF-α	2 ± 2	0
CSF-β	14 ± 1	0
rH GM-CSF	0	0
Delayed Addition of Murine Multi-CSF Day 2		
Saline	7 ± 2	0.3 ± 0.3
MCLM	69 ± 6	10 ± 2
HPCM	85 ± 4	6 ± 4
CSF-α	15 ± 9	0.5 ± 0.5
CSF-β	57 ± 18	3 ± 2
rH GM-CSF	6 ± 2	0

Footnotes to Table 3.7

20,000 12 day CBA foetal liver cells were cultured in 1 ml agar cultures containing 20% human plasma.
Cultures were scored after 7 days of incubation. Data are colony counts ± standard deviations from
four replicate cultures. MLCM = mouse lung conditioned medium, SCM = pokeweed mitogen-stimulated
mouse spleen conditioned medium, HPCM = human placental conditioned medium. CSF-α and β-containing
preparations were from U/5637 bladder cancer conditioned medium.

A full length cDNA for GM-CSF was cloned into an inducible expression vector so that authentic human GM-CSF would be produced. After induction of the GM-CSF synthesis, the bacteria were harvested and the GM-CSF extracted as described previously for murine GM-CSF (DeLamarter et al, 1985). The protein was purified with final purification achieved using gradient elution from a reversed-phase support on an ODS-Hypersil column (Burgess et al, 1986). SDS-poly-acrylamide gel analysis indicated that the protein associated with the biological activity had a single molecular weight (14,000 daltons). Amino acid sequence analysis of the purified rH GM-CSF yielded a single amino acid at each cycle, the first residue detected was methionine and the subsequent cycles yielded the sequence expected from the nucleotide sequence.

The purified rH GM-CSF was titrated and assayed on eight separate bone marrow aspirates (Figure 3.8). Both the 7 day and 14 day cultures indicated that maximal stimulation of colony formation was achieved at concentrations of at least 3.8 ng/ml. Half maximal stimulation occurred between 0.5-0.3 ng/ml and the minimum detectable rH GM-CSF concentration using the semi-solid agar assay was approximately 230 pg/ml for both time points.

The direct action of bacterially synthesized rH GM-CSF on human colony forming cells was examined by transfer of day 5 clones to plates containing the bacterially synthesized human GM-CSF, human placental conditioned medium or saline (Figure 3.9). The bacterially synthesized rH GM-CSF continued to stimulate colony growth.

The types of colonies stimulated by the rH GM-CSF were examined and two separate experiments are shown in Table 3.8 compared with the colony types obtained with unfractionated HPCM, CSF-α and CSF-β.
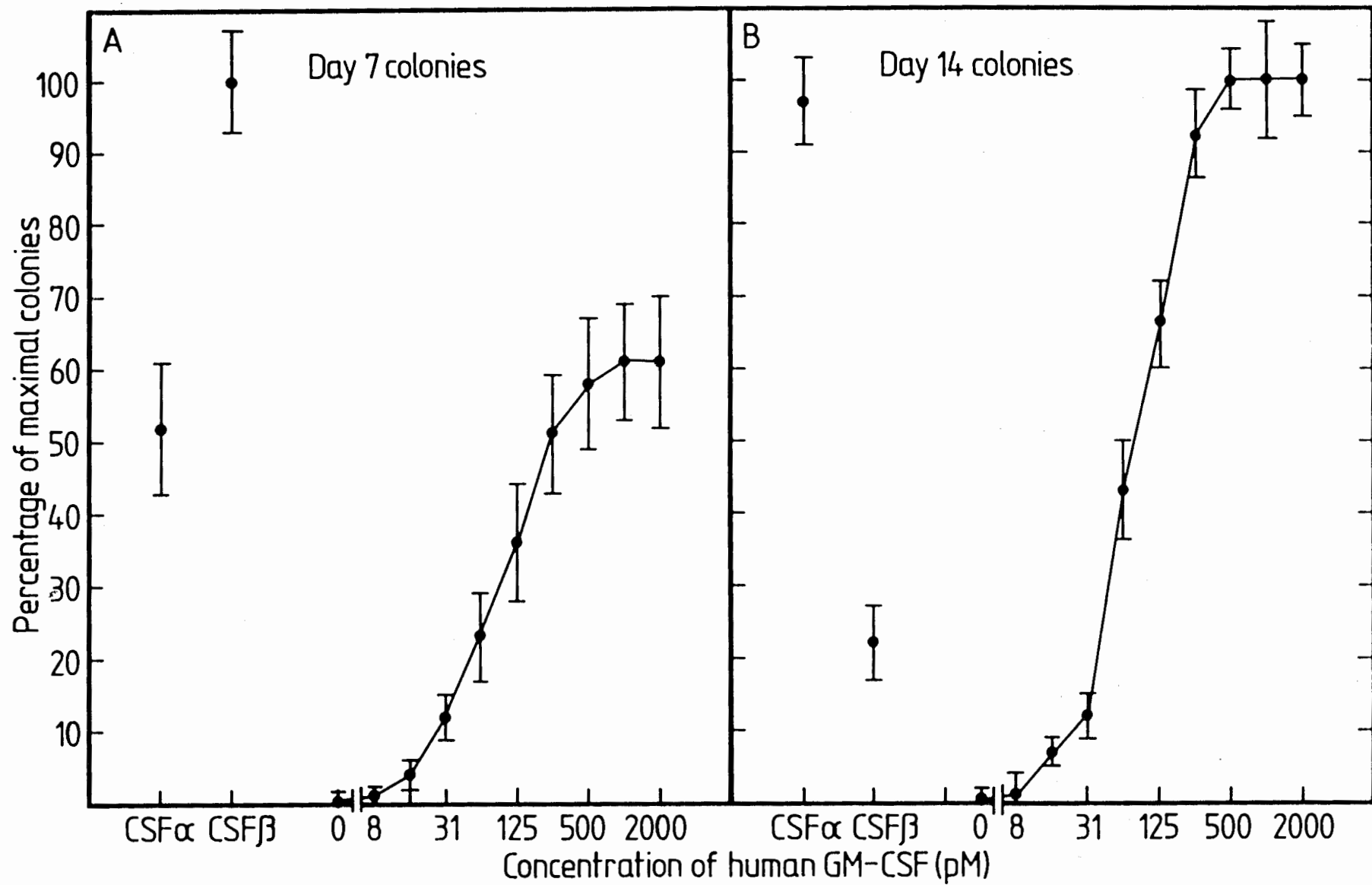
Fig. 3.8 Colony formation at days 7 and 14 in cultures of human
marrow cells stimulated by serial dilutions of bacterially-
synthesized rH GM-CSF. Control cultures contained HPCM or
semipurified CSF-α or CSF-β. Results are expressed as
percentage of maximum colonies stimulated by HPCM. Between
$3\text{-}5 \times 10^4$ cells were cultured per ml and replicate cultures
scored at day 7 and 14 of culture. Results are mean ±
standard deviation for 8 normal marrow samples.

Fig. 3.9 Behaviour of 5 day clones initiated by bacterially-
synthesized rH GM-CSF after transfer to recipient cultures
containing rH GM-CSF, HPCM or no CSF. The concentration
of rH GM-CSF and HPCM was 400 units/ml. The mean size of
clones at transfer was 17 cells per clone. There was
a progressive increase in clones after a further 11 days
of incubation in cultures containing rH GM-CSF and HPCM.
These clones were both neutrophil-macrophage (closed
circles) and eosinophil (open circles) in type.

TABLE 3.8. Morphology of Colonies Stimulated by Bacterially Synthesized Human GM-CSF

Incubation Time	Experiment Number	Stimulus	Mean Number of Colonies	Per Cent Colonies			
				G	GM	M	Eosin.
7 days	1	Saline	0	-	-	-	-
		HPCM	275	84	0	16	0
		rH GM-CSF	230	83	0	11	6
		CSF-α	232	83	0	17	0
		CSF-β	278	82	0	18	0
	2	Saline	0	-	-	-	-
		HPCM	146	64	2	34	0
		rH GM-CSF	98	58	3	36	3
		CSF-α	98	56	8	32	4
		CSF-β	149	64	5	31	0
14 days	1	Saline	0	-	-	-	-
		HPCM	98	42	6	39	13
		rH GM-CSF	95	40	3	42	15
		CSF-α	93	52	1	38	9
		CSF-β	31	72	0	28	0
	2	Saline	3	0	0	100	0
		HPCM	87	31	13	50	6
		rH GM-CSF	89	23	12	52	13
		CSF-α	85	24	13	49	14
		CSF-β	30	67	12	21	0

3×10^4 light density human bone marrow cells were cultured per ml and cultures examined after 7 and 14 days of incubation. Colony morphology was determined by sequential examination of colonies from glutaraldehyde-fixed cultures stained with Luxol-Fast-Blue and Haematoxylin. G = neutrophil granulocyte, M = macrophage, GM = neutrophil granylocyte-macrophage, Eosin. = Eosinophil.

As predicted from experiments using COS-cell rHGM-CSF, bacterially synthesized rH GM-CSF stimulated neutrophil, macrophage and eosinophil colonies. At day 7 all preparations of CSF stimulated mainly granulo-cytic colonies, however, the bacterially synthesized rH GM-CSF and CSF-α also stimulated a small proportion of eosinophil colonies. Thus, the bacterially synthesized rH GM-CSF stimulated a similar range of colonies to the COS-cell rH GM-CSF and CSF-α.

The bacterial rH GM-CSF was unable to stimulate haemopoietic progenitor cells in murine bone marrow. Even at a concentration of 300 ng/ml no colonies (or even clusters) were observed after 7 days in culture. Similarly, there was no effect of this rH GM-CSF on several murine cell lines which were known to respond to murine haemopoietic growth factors: WEHI-3B D$^+$ (G-CSF responsive), FDC-P1 (GM-CSF and Multi-CSF responsive) and 32D cells (Multi-CSF responsive). These results were completely consistent with the inability of the COS-cell rH GM-CSF to stimulate murine cells (Table 3.6).

3.3 DISCUSSION

Fractionation of U/5637-CM demonstrated the presence of two human-active granulocyte-macrophage CSF's with the biochemical and biological properties previously described for CSF-α and CSF-β obtained from HPCM (Nicola et al, 1979; Morstyn et al, 1981) and GCT-conditioned medium (Abboud et al, 1981). The major differences in the biological actions of these two molecules are the preferential stimulation of different subsets of granulocyte-macrophage progenitors and the stimulation of eosinophil progenitors by CSF-α but not CSF-β. The stimulation of eosinophil progenitors by CSF-α but not CSF-β is also reflected in stimulation of the functional activity of mature eosinophils by CSF-α but not CSF-β (Vadas et al, 1983).

It was thus of interest to determine the biological activities of the rH GM-CSF and its relation to CSF-α and CSF-β. In agreement with the original biological monitoring experiments undertaken during the cloning of the cDNA for human GM-CSF (Wong et al, 1985), the present experiments have indicated that rH GM-CSF, as synthesized by monkey COS-cells and bacteria, is an effective proliferative stimulus for human granulocyte-macrophage progenitor cells leading to typical colony formation in semisolid cultures. In addition however, the present experiments have shown that rH GM-CSF is also a highly effective proliferative stimulus for human eosinophil colony formation. Plateau levels of granulocyte-macrophage and eosinophil colonies were stimulated by a concentration of 1-5 ng/ml of COS-cell rH GM-CSF and minimum colony formation was observable with 300 pg/ml. The overall level of colony stimulating activity in the purified material was calculated as at least 4×10^7 units/mg, a figure in agreement with the previous estimate (Wong et al, 1985) and reasonably similar to the specific activity of the four purified murine granulocyte-macrophage CSF's in bone marrow cultures which vary from 10^8 to 10^9 units/mg (Metcalf, 1984). Results with bacterially-synthesized rH GM-CSF were very similar. The rH GM-CSF exhibited somewhat stronger stimulating activity for monocyte-macrophage colony formation than for granulocyte colony formation. This, coupled with the fact that plateau numbers of colonies at day 7 were always lower than with HPCM suggests that rH GM-CSF is not able to stimulate the proliferation of all granulocyte-macrophage progenitors and probably stimulates the same subset as stimulated by CSF-α.

A prominent feature of the action of rH GM-CSF was the stimulation of eosinophil colony formation. While eosinophil colony formation was observed in cultures stimulated by semi-purified CSF-α

preparations, a possible explanation for these observations was that the eosinophil colony formation was due to the presence in the preparations of a contaminating eosinophil-CSF. The present results with purified GM-CSF of recombinant origin clearly indicate that this molecule possess the capacity to stimulate eosinophil colony formation and suggests that the observed activity of CSF-α-containing preparations was not due to a contaminating eosinophil-CSF. Analysis using clone transfers also indicated that the rH GM-CSF acted directly on granulocyte-macrophage and eosinophil progenitor cells.

The rH GM-CSF enhanced the formation of erythroid and multi-potential colonies, however, experiments using clones initiated by rH GM-CSF showed that most of the enhanced erythroid colony formation in cultures containing rH GM-CSF and erythropoietin seemed not to be ascribable to a direct action of rH GM-CSF in initiating proliferation of BFU-E. Thus the situation with rH GM-CSF differs from that documented previously for murine GM-CSF where it was possible to demonstrate a direct action of GM-CSF on a major fraction of murine BFU-E (Metcalf et al, 1980). The basis for the observed erythroid enhancement requires further investigation. Two poss-ibilities needing investigation are (a) that rH GM-CSF and erythro-poietin need to act simultaneously on BFU-E or (b) that the action of rH GM-CSF may be indirect and be mediated by stimulation of some other cell type in the culture to produce an erythroid-enhancing factor.

The rH GM-CSF had no capacity to stimulate the proliferation of murine haemopoietic cells from either marrow or foetal liver or of cells of two murine continuous cell lines responding to murine Multi-CSF or GM-CSF. Furthermore, no differentiation-inducing activity was noted when rH GM-CSF was added to cultures of the murine myelomonocytic cell line WEHI-3B.

A summary of the observed biological activity in parallel assays of rH GM-CSF compared with those of semi-purified human CSF-α and β is presented in Table 3.9. It is clear that the activities of rH GM-CSF differ completely from those of CSF-β-containing material. The activities of rH GM-CSF are identical to those of CSF-α-containing preparations and it can be concluded provisionally that rH GM-CSF and CSF-α represent the same molecule. Verification of this conclusion should be possible since GM-CSF with the same N-terminal sequence has been purified from Mo cell conditioned medium (Wong et al, 1985) and the properties of this purified native molecule need to be determined using a range of assay systems. Similarly CSF-α could be purified and the amino acid sequence determined for comparison.

The purification has been reported of a human pluripoietin from medium conditioned by the bladder cancer cell line, U/5637 (Welte et al, 1985). From its reported ability to stimulate erythroid, multipotential and day 7 granulocyte-macrophage colonies and to induce differentiation in murine myeloid leukemia cells, this pluripoietin does not resemble CSF-α, CSF-β or rH GM-CSF. Furthermore, if it has any homology with murine Multi-CSF at the amino acid sequence level it would be unlikely to have homology with rH GM-CSF since there is no homology between murine GM-CSF and Multi-CSF (Gough et al, 1984). Therefore, despite the ability of rH GM-CSF to promote erythroid colonies, it is unlikely that rH GM-CSF is a candidate human analogue of murine Multi-CSF. In this context murine GM-CSF also potentiates erythroid and mixed colony formation by being able to initiate (but not sustain) the proliferation of erythroid and multipotential precursors (Metcalf et al, 1980). Previous studies have shown that murine GM-CSF is also able to stimulate eosinophil colony formation by adult and particularly foetal progenitor cells (Johnson and Metcalf, 1980). Thus, the ability of rH GM-CSF to

TABLE 3.9. Comparison of Range of Biological Activities of rH GM-CSF, Human CSF-α and Human CSF-β

Biological Activity	Type of Human CSF		
	rH GM-CSF	CSF-α	CSF-β
Stimulation of 7D GM colonies	+	+	++
14D GM colonies	++	++	+
eosinophil colonies	++	++	0
Potentiation of erythroid and multipotential colonies	+	+	0
Stimulation of murine GM colonies	0	0	++
Initiation of murine erythroid and mixed colonies	0	0	+
Induction of differentiation in murine leukaemic cells	0	0	++

stimulate the proliferation of human eosinophil precursors does not make it different in principle from its murine analogue.

Essentially the same results were observed with both the COS-cell and the bacterially-synthesized rH GM-CSF. Thus neither the addition of the extra N-terminal methionine residue nor the absence of carbohydrate appear to effect the in vitro activity of rH GM-CSF. (The activity of this molecule on human leukaemic cells is discussed in Chapter 8).

The activity of murine G-CSF on human granulocyte-macrophage progenitor cells was of interest and was examined in greater detail using fractionated populations of human cells (Chapter 6) and human leukaemic cells (Chapters 7 and 8).

The experiments using murine EDF documented this molecule as a genuine CSF with specificity for the eosinophil lineage for both murine and human cells. These results were not surprising given that EDF is a potent stimulus for proliferation of murine eosinophil precursors in liquid culture (Sanderson et al, 1985) and that a human-active Eo-CSF is present in murine SCM (Metcalf et al, 1983). The apparent inability of murine EDF and SCM-derived human Eo-CSF to stimulate murine eosinophil colonies in the earlier studies could be attributed to both the low frequency of eosinophil progenitors in mouse marrow and possibly suboptimal culture conditions. However, this study clearly demonstrated that murine EDF was an Eo-CSF and stimulated a small but significant number of eosinophil colonies in cultures of both murine and human cells.

The consistent occurrence of granulocyte and/or macrophage clusters and occasional colonies in EDF-stimulated cultures suggested that EDF may also have some action on other haempoietic cell types in keeping with the action of other CSF's at high concentrations (Metcalf, 1984). Unfortunately, the low frequency of clonogenic

eosinophil precursors in this assay using normal murine bone marrow precluded the clone transfer experiments which are necessary to establish unequivocally that EDF is a direct-acting CSF.

The colony-stimulating activity of EDF on human bone marrow was demonstrated after 14 days in culture. These colonies contained only eosinophils and were similar in number to those eosinophil colonies resulting from stimulation with HPCM or CSF-α, although these stimuli also stimulated the growth of neutrophil, neutrophil-macrophage and macrophage colonies. Since the size distributions of the eosinophil colonies generated by EDF and HPCM were also comparable, it is likely that they act on the same responsive cells. That this action was due to the direct effect of EDF on the progenitor cells was demonstrated by the clone transfer experiments, in which continuing clonal proliferation was propagated by EDF in the absence of accessory cells, and by the linear relationship between colony number and number of progenitor cells plated. These results also predict the existence of a corresponding human Eo-CSF (and specific receptor) that is closely related to murine EDF (Eo-CSF).

CHAPTER 4

FRACTIONATION OF HUMAN BONE MARROW CELLS

4.1 INTRODUCTION

Human marrow is a complex mixture of haemopoietic cells of different lineages at different stages of maturation. One approach to understanding the differentiation of progenitor cells to mature cells is to purify cells of each stage of maturity. The progenitor cells are present in normal marrow at a very low frequency (0.1-1%) and are recognised by their ability to respond in functional assays, for example by dividing and generating mature cells in vitro (Pike and Robinson, 1970). The objective of cell separation techniques for these cells must therefore be to separate and purify them under conditions in which they will retain their functional properties. Ideally such a separative procedure will also remove "accessory" cells (e.g. lymphocytes, monocytes) to allow study of the direct effect of regulatory molecules without possible indirect effects that may be mediated via such cell types.

Partial purification of progenitor cells has been achieved using a variety of physical separative procedures (Moore et al, 1972; Dicke et al, 1973; Dicke et al, 1973a; Burghouts et al, 1978) and fluorescence activated cell sorting using a variety of lectin (Morstyn et al, 1980; Nicola et al, 1980; Nicola et al, 1980a) and monoclonal probes (Goldschneider et al, 1980; Beverley et al, 1980; Janossy et al, 1978; Young and Hwang-Chen, 1981; Ball et al, 1982; Griffin et al, 1982; Bodger et al, 1983; Griffin et al, 1983; Andrews et al, 1983). Using these techniques progenitor cells have been identified as undifferentiated blast cells (Morstyn et al, 1980; Nicola et al, 1981; Beverley et al, 1980; Griffin et al, 1982) and some separation of the various lineage-specific progenitor cells has been achieved (Morstyn et al, 1980; Griffin et al, 1982; Andrews et al, 1983; Strife et al, 1983; Papayannopoulou et al, 1984).

It is intriguing that while numerous monoclonal antibodies have been raised against human granulocytes, two classes of immunoglobulin predominate. Several groups have described murine IgM monoclonal antibodies that bind to immature human myeloid cells and monocytes (Ball et al, 1982; Strauss et al, 1983; Civin et al, 1981; Perussia et al, 1982; Strauss et al, 1984; Majdic et al, 1981; Bernstein et al, 1982; Ferrero et al, 1983; Lopez and Vadas, 1985) and IgG mono-clonal antibodies that bind to more mature myeloid cells, from pro-myelocytes-myelocytes onwards (Griffin et al, 1981; Ball and Fanger, 1983; Perussia et al, 1982; Ball et al, 1982; Strauss et al, 1984; Ferrero et al, 1983; Lopez et al, 1985). The similarity between antibodies developed in different laboratories also includes similar molecular weights for the glycoprotein antigen(s) recognised by the IgM class antibodies (Skubitz et al, 1983; Tetteroo et al, 1984; Geurts van Kessel et al, 1983; Ferrero et al, 1983) and the pre-dominance of these two classes of antibodies implies two highly antigenic determinants on the surface of human myeloid cells. Furthermore, one study has demonstrated that the gene encoding an antigen recognised by 20 of the IgM class monoclonal antibodies is on chromosome 11 (Geurts van Kessel et al, 1984).

Limited studies have been performed to examine the expression of these antigens on the surface of progenitor cells. Studies have generally examined the formation of colonies after 14 days of incuba-tion (Ball and Fanger, 1983; Strauss et al, 1983; Strauss et al, 1984; Griffin et al, 1981) and occasionally after 7 days of culture (Skubitz et al, 1983). Few workers have examined cultures at both time-points (Young and Hwang-Chen, 1981; Griffin et al, 1982; Andrews et al, 1983) and no sequential studies have been performed.

Failure to recognise the two different subsets of granulocyte-macrophage progenitors has led to apparent confusion (Skubitz et al, 1983; Strauss et al, 1984), although these two subsets (day 7 CFC and day 14 CFC) have been clearly identified based on differences in sedimentation velocity (Johnson et al, 1977; Inoue and Ottenbreit, 1978; Jacobsen et al, 1978; Miller et al, 1978) and lectin binding characteristics (Morstyn et al, 1980; Nicola et al, 1980).

Several studies have been performed using populations of pro-myelocytes-myelocytes that were obtained from normal human bone marrow cells using the IgG class of monoclonal antibody (Griffin et al, 1981; Ball and Fanger, 1983; Griffin et al, 1983; Strauss et al, 1984; Andrews et al, 1983). These studies demonstrated an apparent inability of promyelocytes and myelocytes to exhibit clonal proliferation in vitro. This is in conflict with in vivo labelling studies using tritiated thymidine (Warner and Athens, 1964; Golde, 1983) demonstrating that such cells are clearly capable of proliferation. One likely explanation for these discrepant results was that promyelocytes and myelocytes might proliferate early during the incubation period but form small clones that disappear before the usual time of scoring such cultures.

The availability of the monoclonal antibodies WEM-G1 (Lopez and Vadas, 1985) a murine IgM raised against human neutrophils, and antibody WEM-G11 (Lopez et al, 1985) a murine IgG2a against human neutrophils, provided useful reagents with which to address these questions.

Two other monoclonal antibodies were studied to determine their binding characteristics to normal bone marrow cells. The monoclonal antibody HTF-1, was developed against human brain tissue factor and was initially thought to neutralise tissue factor-related procoagulant

activity. It has been shown, however to bind to lipopolysaccharide-stimulated monocytes but not to unstimulated monocytes, suggesting that expression of the antigen recognised by HTF-1 may be a marker for monocyte activation (Ewan et al, observations unpublished). Monocyte generation of tissue factor is a potent stimulus for activation of the extrinsic pathway of blood coagulation (reviewed, Edwards and Rickles, 1980) and appears to be controlled by immune (via T-lymphocytes) and non-immune (via for example endotoxin) regulatory pathways (Edwards et al, 1979; Niemetz and Morrison, 1977; Levy and Edgington, 1980). The surface expression and cytoplasmic expression of the antigen recognised by HTF-1 was examined on normal bone marrow cells.

Monoclonal antibody 25E11 was raised against natural killer cells and inhibits the lysis of K562 target cells by both monocytes and natural killer cells. Furthermore, this antibody binds to an epitope of the IIb-IIIa glycoprotein complex of platelets and inhibits platelet aggregation stimulated by ADP, adrenaline, collagen, arachidonic acid and thrombin but not ristocetin (Burns et al, unpublished observations). Because of the conflicting reports regarding the presence of the IIb-IIIa complex on cells other than platelets, (Burckhardt et al, 1982; Godstad et al, 1983; Bai et al, 1984; Coller et al, 1983; Levine and Rabellino, 1984; Clemetson et al, 1985) the binding of this monoclonal antibody to bone marrow cells was examined. An additional aim of these experiments was to examine the role, if any, natural killer cells play in modulating haemopoiesis. These cells have been reported to inhibit myeloid (Hansson et al, 1982; Barr and Stevens, 1982; Morris et al, 1980) and erythroid colony-formation (Mangan et al, 1984; Degliantoni et al, 1985), while others have reported enhanced growth of blood and bone marrow BFU-E

(Meytes et al, 1979; Mangan et al, 1982; Mangan et al, 1984;
Pistoia et al, 1985; Linch et al, 1985). An interaction between
natural killer cells and monocytes has been proposed (Pistoia et
al, 1985). While the number of natural killer cells in the bone
marrow is small (Abo and Baulch, 1981) and their activity low
(Lotzova et al, 1979; Targan et al, 1980) all studies have used
natural killer cells and blood/bone marrow cells in the ratio of at
least 0.5:1.

Monoclonal antibody 25E11 was used to separate populations of
natural killer cells and monocytes and the effect on colony formation
was examined.

4.2 RESULTS

4.2.1 Fractionation of Marrow Cells Using Monoclonal Antibody HTF-1

Light-density bone marrow cells were labelled with HTF-1 anti-
body (raised against human tissue factor) washed and incubated with
FITC-conjugated F(ab')$_2$ sheep anti-mouse immunoglobulin. Cells were
then analysed and sorted using the FACS. An analysis of the fluore-
scence of fixed bone marrow cells is shown in Figure 4.1 and the
fluorescence-profile for FITC-conjugated sheep anti-mouse immuno-
globulin (dotted) is shown for comparison. Approximately 30% of
all bone marrow cells were labelled with HTF-1. Cells were sorted
on the basis of fluorescence into negative-, intermediate- and high-
fluorescence groups. The cellular composition of these groups is
shown in Table 4.1 for both fixed and unfixed bone marrow cells.
All erythroid and almost all lymphoid cells were non-reactive or
weakly reactive with HTF-1. The majority of cells in the inter-
mediate- and high-fluorescence populations were myeloid cells and
monocytes. The percentage of basophils in the high-fluorescence

Fig. 4.1 FACS profile of normal human bone marrow cells labelled

with monoclonal antibody HTF-1 (solid line). The vertical

axes show cell numbers vs low angle light scatter

(horizontal axis, upper panel), high angle light scatter

(middle panel) and fluorescence (bottom panel). The

fluorescence profile of cells labelled with FITC-

conjugated, anti-mouse antibody alone is also shown

(dotted line).

TABLE 4.1.   COMPOSITION OF HUMAN BONE MARROW POPULATIONS FRACTIONATED USING THE MONOCLONAL ANTIBODY HTF-1

CELL STATUS	HTF-1 STATUS	PERCENTAGE OF CELLS						
		Blasts	Promyelocytes and myelocytes	Metamyelocytes and polymorphs	Lymphocytes	Monocytes	Basophils	Nucleated red blood cells
Fixed	-	2.0 ± 1.4	14.0 ± 2.8	49.0 ± 5.7	17.1 ± 0.7	7.5 ± 3.5	0.4 ± 0.4	10.0 ± 2.8
	Negative	2.0 ± 0.0	6.3 ± 3.2	30.9 ± 2.1	29.5 ± 0.7	5.8 ± 4.6	0.0 ± 0.0	25.5 ± 0.7
	Intermediate	1.0 ± 1.4	27.0 ± 18.3	58.0 ± 18.4	1.5 ± 2.1	6.0 ± 4.2	6.5 ± 4.9	0.0 ± 0.0
	Positive	0.0 ± 0.0	9.5 ± 1.4	49.0 ± 11.3	2.5 ± 0.7	18.0 ± 2.8	21.0 ± 13.5	0.0 ± 0.0
Unfixed	-	3.5 ± 0.7	13.5 ± 2.1	58.0 ± 14.1	12.5 ± 10.6	5.7 ± 1.4	0.3 ± 0.3	6.5 ± 2.1
	Negative	6.5 ± 4.9	12.0 ± 1.4	21.5 ± 6.4	32.5 ± 2.5	3.0 ± 0.5	0.0 ± 0.0	24.5 ± 13.4
	Intermediate	4.0 ± 1.4	15.5 ± 12.0	61.5 ± 12.0	2.5 ± 2.1	11.0 ± 8.5	1.5 ± 2.1	4.0 ± 2.8
	Positive	2.5 ± 0.7	4.5 ± 3.5	42.0 ± 11.3	7.0 ± 9.8	32.5 ± 6.4	7.5 ± 7.5	4.0 ± 0.0

Mean ± Standard deviation of percentage frequency of cells for 2 fixed and 2 unfixed bone marrow sample fractionated by fluorescence-activated cell sorting using the monoclonal antibody HTF-1. For the 4 bone marrow samples, the percentage of total cells in the fluorescence-negative fraction was 59.3 ± 10.6% (mean ± SD), in the intermediate-fluorescence fraction 35.8 ± 10.8% and positive-fluorescence fraction 4.9 ± 0.9%.

population was striking, but was even more evident when more highly fluorescent cells were collected (constituting approximately 1% of bone marrow cells). In these populations between 45-67% of cells were basophils and the remainder of the cells were myeloid cells and monocytes (although this represents only approximately 1% of the total myeloid cells and monocytes).

The cell populations were examined for their ability to generate colonies of haemopoietic cells in vitro. One result is shown in Table 4.2. The cells generating day 7 and day 14 clones were almost entirely confined to the non-or weakly-fluorescent populations, and thus this antibody did not serve to fractionate populations of progenitor cells.

4.2.2 Fractionation of Marrow Cells Using Monoclonal Antibody 25E11

The distribution of normal bone marrow cells according to cell size (low angle or 0 degree light scatter), high angle (90 degree) light scatter (a function of nuclear segmentation and cytoplasmic granulation) and fluorescence with 25E11 is shown in Figure 4.2. Fluorescent cells showed the low angle light scatter characteristics of granulocytes (Morstyn et al, 1980) and the majority of non-fluorescent cells showed the size distribution of small lymphocytes. When cells were analysed according to high angle light scatter characteristics and fluorescence (Figure 4.2, panel B) three populations of cells were evident. The most highly fluorescent cells were in the region of high angle light scatter expected for monocytes, the intermediate-fluorescence population in the region of granulocytes and the negative-fluorescence population showed almost no high angle light scatter (lymphocytes and blast cells). The low level of fluorescence observed for myeloid cells was a feature of all 6 marrow

TABLE 4.2.   FREQUENCY OF CLONOGENIC CELLS IN HUMAN MARROW POPULATIONS

FRACTIONATED USING THE MONOCLONAL ANTIBODY HTF-1

HTF-1 STATUS	FREQUENCY PER 10^4 CULTURED CELLS				
	DAY 7		DAY 14		
	Total Colonies	Total Clusters	Total Nonerythroid Colonies	Total Clusters	Total Erythroid Colonies
-	47	69	20	52	17
Negative	48	60	27	88	22
Intermediate	65	36	5	65	0
Positive	0	55	0	40	0

Results are mean number of clones from 4 replicate cultures. Day 7 results were obtained from cultures stimulated by HPCM alone and day 14 results from cultures stimulated by HPCM and erythropoietin. Colonies contained >40 cells and clusters <40 cells. For unfractionated marrow 3×10^4 cells were culture per millilitre, for the negative- and intermediate-fluorescence populations 4×10^3 cells were cultured per millilitre and for the positive-fluorescence cells 10^4 cells per millilitre.
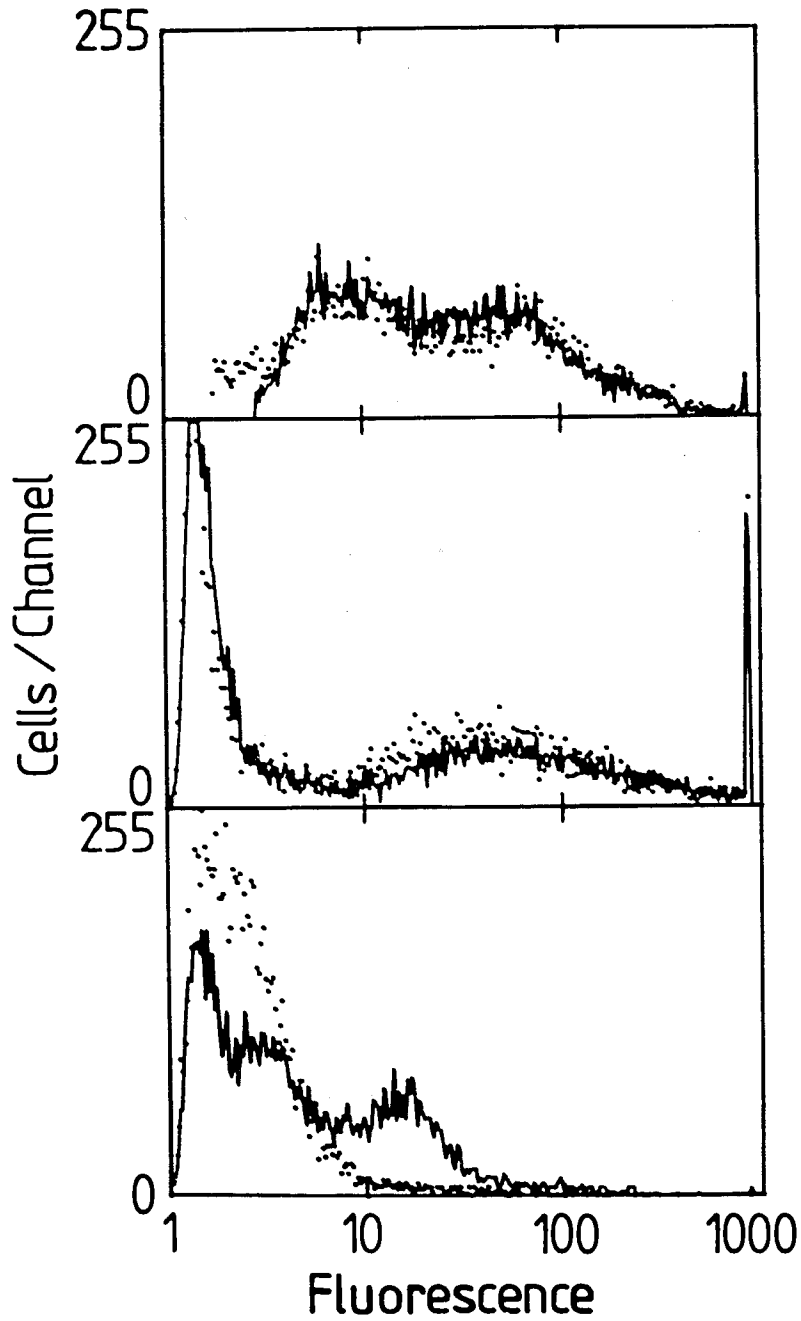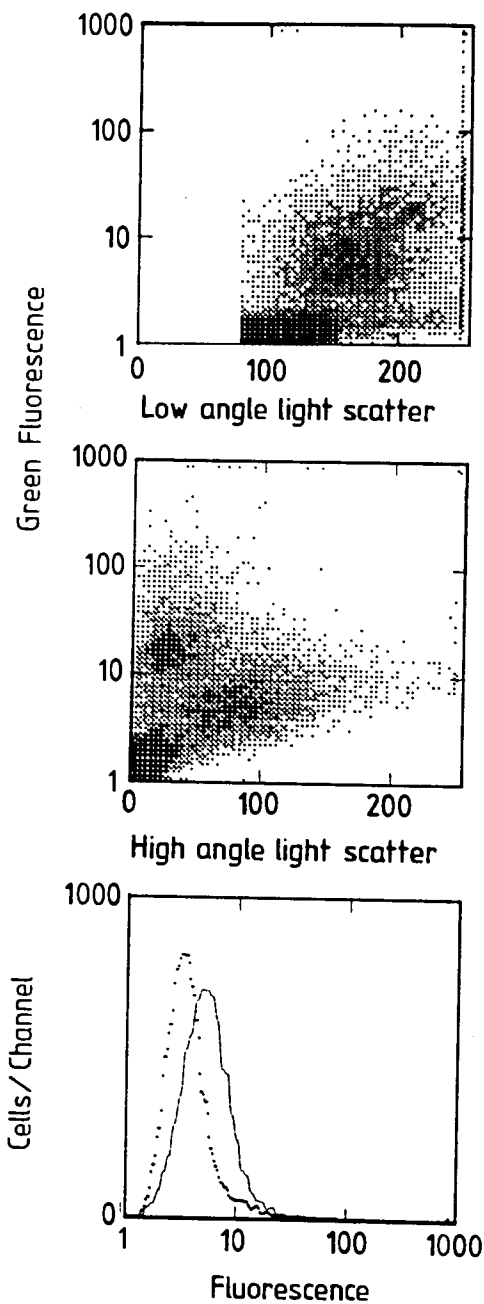
<u>Fig. 4.2</u> FACS profile of normal human bone marrow cells labelled
with monoclonal antibody 25E11. The vertical axes show
fluorescence vs low angle light scatter (horizontal axis,
upper panel) and high angle light scatter (horizontal
axis, middle panel). The bottom panel shows cell number
(vertical axis) vs fluorescence (horizontal axis) for
the granulated myeloid cells and compares the fluorescence
distribution of granulated myeloid cells labelled with
25E11 (solid line) with an irrelevant anti-platelet
antibody (dotted line).

samples examined and was consistently greater than the negative-fluorescence observed for myeloid cells with a platelet specific monoclonal antibody (Thurlow et al, 1985) (Figure 4.2, panel C).

The fluorescence negative cells consisted of primarily blast cells, small lymphocytes and nucleated red blood cells (Table 4.3). Intermediate-fluorescence cells were, as expected from the FACS analysis, primarily myeloid cells while the high-fluorescence cells consisted of monocytes and large lymphocytes (both granular and agranular). The small number of myeloid cells in this fraction probably represented cross-contamination as sorting windows were contiguous.

4.2.2.1 Clonal Proliferation by 25E11 Fractionated Cells. The clonal proliferation of cells derived from bone marrow and fractionated using 25E11 is shown in Table 4.4. The day 14 colony-forming cells and the majority of day 7 colony-forming cells were restricted to the negative-fluorescence population, although a small number of colony-forming cells were obtained from the intermediate- and high-fluorescence populations. This probably also represented cross-contamination from the negative-fluorescence population. The calculated distribution for the recovery of clonogenic cells is shown in Table 4.5. The recovery of clonogenic cells was consistently about 200%, for all marrow samples studied, and suggested that inhibitory cell(s) had been removed during the fractionation procedure. Despite this, mixing experiments failed to demonstrate any consistent inhibition of colony formation when cells from the high-fluorescence population (large lymphocytes; probable natural killer cells, and monocytes) were mixed in appropriate amounts with cells from the negative-fluorescence population (colony-forming cells) (Table 4.6).

TABLE 4.3.   COMPOSITION OF HUMAN MARROW POPULATIONS FRACTIONATED USING THE MONOCLONAL ANTIBODY 25E11

25E11 STATUS	PERCENTAGE OF CELLS						
	Blasts	Promyelocytes and Myelocytes	Metamyelocytes and Polymorphs	Lymphocytes	Monocytes	Eosinophils	Nucleated Red Blood cells
-	7.2 ± 2.5	17.6 ± 1.3	37.2 ± 9.7	16.8 ± 4.7	7.7 ± 3.3	0.5 ± 0.5	13.0 ± 7.6
Negative	26.8 ± 5.3	5.0 ± 4.1	0.5 ± 0.5	41.5 ± 12.1	1.5 ± 1.5	0.0 ± 0.0	24.7 ± 12.0
Intermediate	1.3 ± 1.3	33.7 ± 3.8	57.8 ± 2.0	2.2 ± 2.9	0.8 ± 1.4	0.0 ± 0.0	4.2 ± 2.8
Positive	8.8 ± 3.2	3.8 ± 3.8	1.0 ± 1.7	22.7 ± 4.3	52.7 ± 12.5	5.3 ± 0.6	5.7 ± 3.5

Mean ± Standard deviation of percentage frequency of various cells in four normal bone marrow samples fractionated by fluorescence-activated cell sorting using the monoclonal antibody 25E11. The percentage of the total cells present in each fraction was 44.3 ± 11.4% (mean ± standard deviation for 4 marrow samples) in the negative fraction, 41.8 ± 16.7% in the intermediate- and 14.0 ± 16.7 in the positive-fluorescence fraction. Occasional megakaryocytes were noted in the fluorescence-positive fraction.

TABLE 4.4.   FREQUENCY OF CLONOGENIC CELLS IN HUMAN MARROW POPULATIONS FRACTIONATED

USING THE MONOCLONAL ANTIBODY 25E11

25E11 STATUS	FREQUENCY PER 10^4 CULTURED CELLS					
	DAY 7			DAY 14		
	Total Colonies	Total Clusters		Total Nonerythroid Colonies	Total Clusters	Total Erythroid Colonies
—	66	69		16	18	5
Negative	273	157		110	93	35
Intermediate	45	110		0	40	0
Positive	40	53		13	21	0

Results are mean clone numbers from 4 replicate cultures.   Day 7 results were obtained from cultures stimulated by HPCM alone and day 14 results from cultures stimulated by HPCM and erythropoietin.   Colonies contained >40 cells and clusters <40 cells. For unfractionated marrow, 3×10^4 cells were cultured per millilitre.   For fractionated populations, 2×10^3 cells were cultured per millilitre.

TABLE 4.5.   CALCULATED YIELDS OF CLONOGENIC CELLS IN HUMAN MARROW POPULATIONS FRACTIONATED

USING THE MONOCLONAL ANTIBODY 25E11

| 25E11 STATUS | CALCULATED PERCENTAGE RECOVERY | | | | | |
| | DAY 7 | | | DAY 14 | | |
	Total Colonies	Total Clusters		Total Nonerythroid Colonies	Total Clusters	Total Erythroid Colonies
Negative	150	82		250	186	252
Intermediate	40	93		0	129	0
Positive	4	7		5	7	0
Total	194	182		255	322	252

The percentage of total clonogenic cells recovered gives the distribution of the clonogenic cells in each fraction.

These results were obtained by multiplying the frequency of clones generated within one cell fraction (see Table 4.4)

by the percentage of total cells present in that fraction (computed by the FACS computer) and dividing by the

frequency of clones generated by the unfractionated marrow cells.

TABLE 4.6.   FREQUENCY OF CLONOGENIC CELLS IN HUMAN MARROW POPULATIONS FRACTIONATED BY

25E11 AND IN ADMIXED POPULATIONS

EXPERIMENT NUMBER	25E11 STATUS		FREQUENCY PER 10^4 CULTURED CELLS				
			DAY 7		DAY 14		
			Total Colonies	Total Clusters	Total Nonerythroid Colonies	Total Clusters	Total Erythroid Colonies
1	Negative		273	157	110	93	35
	Positive		40	53	13	21	0
	Negative	Observed	225	140	113	62	23
	+ Positive	Expected	293	184	116	105	35
2	Negative		120	70	53	25	42
	Positive		76	99	70	55	8
	Negative	Observed	188	180	71	82	47
	+ Positive	Expected	158	120	88	48	46

Results are means from 4 replicate cultures.   Cells were fractionated using 25E11 and fluorescence negative and fluorescence positive populations cultured alone and admixed.   Day 7 results were obtained from cultures stimulated by HPCM alone and day 14 results from cultures stimulated by HPCM and erythropoietin.   The negative and positive populations were cultured alone using 2 x 10^3 cells per millilitre.   The mixed populations contained 2 x 10^3 negative- and 10^3 positive-fluorescence cells per millilitre.   Colonies contained >40 cells and clusters <40 cells.

This suggested that interactions between these cell types did not simply result in inhibition of colony growth, and as suggested by others (Bjornson et al, 1984) perhaps implicated mature granulocytes as potential inhibitors of in vitro colony formation.

Further evidence of a possible role for natural killer cells and monocytes in inhibitory colony formation in vitro was obtained in experiments in which monoclonal antibody 9.1C3 was added, without complement, to unfractionated bone marrow cells. This antibody blocks both natural killer cell and monocyte killing of K562 cells and is directed against a component of the T200 molecule (which is present on T cells and haemopoietic cells and their progenitors) (Burns et al, 1984; Newman et al, 1983). In experiments 1 and 2 (Table 4.7) the addition of 9.1C3 to cells 15 min. prior to culture resulted in enhanced numbers of day 7 colonies as well as day 14 non-erythroid and erythroid colonies. In other experiments (experiment 3) there was no change in colony numbers while the most consistent result observed was an increase in the number of day 14 erythroid colonies (experiment 4). All cultures were stimulated with supramaximal, non-inhibitory concentrations of HPCM and erythropoietin and the effect of 9.1C3 addition occurred without the addition of complement.

4.2.3 Fractionation of Marrow Cells Using Monoclonal Antibody WEM-G1

Cells from the light-density fraction of human bone marrow were labelled with FITC-conjugated WEM-G1 then analysed and sorted using a modified FACS II instrument (as described in Chapter 2). Figure 4.3 shows a typical analysis of cell distribution on the basis of low angle (0 degree) light scatter, high angle (90 degree) light scatter and fluorescence. Fluorescent cells were distributed in the region of 0 degree light scatter previously shown to contain a high proportion of granulocytes (Morstyn et al, 1980). There were

TABLE 4.7.   FREQUENCY OF CLONOGENIC CELLS IN HUMAN MARROW SAMPLES FOLLOWING THE ADDITION
OF MONOCLONAL ANTIBODY 9.1C3 WITHOUT COMPLEMENT

		FREQUENCY PER 10^5 CULTURED CELLS				
EXPERIMENT NUMBER	9.1C3 ADDITION	DAY 7			DAY 14	
		Total Colonies	Total Clusters	Total Nonerythroid Colonies	Total Clusters	Total Erythroid Colonies
1	-	155	395	60	210	124
	+	266	701	89	411	223
2	-	555	945	170	330	209
	+	745	1555	198	352	308
3	-	79	276	60	110	35
	+	88	237	62	88	34
4	-	54	156	41	69	54
	+	55	155	43	92	86

Results are mean frequencies of 4 replicate cultures. Day 7 results were obtained using cultures maximally stimulated by HPCM alone and day 14 cultures were stimulated by HPCM and erythropoietin. Colonies contained >40 cells and clusters <40 cells. The number of cells cultured was between 2-5 x 10^4 per millilitre.
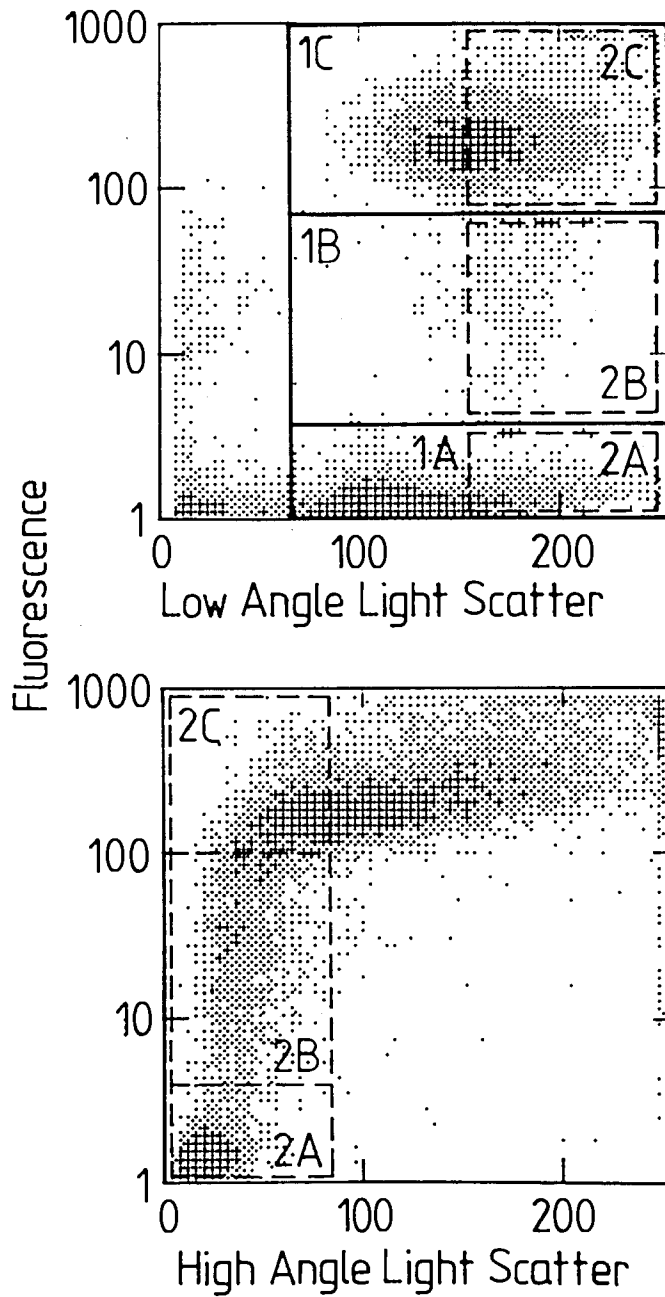
Fig. 4.3   FACS profile of normal human bone marrow cells labelled
           with monoclonal antibody WEM-G1. The vertical axis show
           fluorescence vs low angle (0°) light scatter (horizontal
           axis, upper panel) and high angle (90°) light scatter
           (horizontal axis, lower panel). Fractions labelled 1
           were separated on the basis of fluorescence only (following
           exclusion of dead cells) into negative-(A), intermediate-(B)
           and positive-fluorescence-(C) fractions. Fractions labelled
           2 were cells of high 0° light scatter and low 90° light
           scatter separated on the basis of fluorescence into
           negative-(A), intermediate-(B) and positive-(C) fractions.

a small number of cells of intermediate fluorescence that displayed
0 degree properties somewhat greater than the highly fluorescent
cells, reflecting cells of larger size. The majority of non-fluore-
scent cells showed 0 degree light scatter characteristics consistent
with lymphocytes, but a small number of larger cells were also non-
fluorescent.

The analysis of 90 degree light scatter and fluorescence revealed
three populations of cells. The most highly fluorescent cells showed
90 degree light scatter characteristics of granulocytes, and was in
contrast to the pattern observed with antibody 25E11. Cells of inter-
mediate fluorescence showed lower 90 degree light scatter properties
while non-fluorescent cells had very low 90 degree light scatter
characteristics.

To examine the composition of each of these populations, 6
fractions of cells were analysed and sorted. After exclusion of
dead cells (of very low 0 degree light scatter) fractions labelled
"1" were sorted solely on the basis of fluorescence into groups of
negative-(1A), intermediate-(1B), and highly-fluorescent (1C) cells.
Because cells of high 0 degree light scatter and low 90 degree light
scatter have previously been shown to be enriched for progenitor
cells (Morstyn et al, 1980; Beverley et al, 1980), fractions labelled
"2" were sorted using three parameters. Selecting only those cells
of high 0 degree light scatter and low 90 degree light scatter, cells
were then fractionated on the basis of fluorescence into negative-(2A),
intermediate-(2B) and highly-fluorescent (2C) fractions.

The cellular composition of the various sorted populations from
normal human bone marrow cells is shown in Table 4.8. Cell popula-
tions sorted on the basis of fluorescence alone consisted of blast
cells, lymphocytes and erythroblasts in the fluorescence-negative

TABLE 4.8.   COMPOSITION OF HUMAN MARROW POPULATIONS FRACTIONATED USING THE MONOCLONAL ANTIBODY WEM-G1

SORTING PROCEDURE	WEM-G1 STATUS	PERCENTAGE OF CELLS					
		Blasts	Promyelocytes and Myelocytes	Metamyelocytes and Polymorphs	Lymphocytes	Monocytes	Nucleated Red Blood Cells
-		6.0 ± 1.6	24.0 ± 4.0	35.5 ± 4.0	12.5 ± 1.0	14.0 ± 1.6	8.0 ± 1.5
1 (A)	Negative	18.0 ± 3.9	1.3 ± 0.6	0.0 ± 0.0	64.6 ± 7.0	5.6 ± 1.9	10.5 ± 2.6
1 (B)	Intermediate	11.4 ± 2.2	26.8 ± 8.9	4.0 ± 2.4	3.1 ± 1.4	33.7 ± 6.2	21.0 ± 6.6
1 (C)	Positive	2.1 ± 0.1	26.3 ± 6.7	45.4 ± 11.5	0.1 ± 0.1	24.0 ± 5.2	2.1 ± 1.3
2 (A)	Negative	56.4 ± 5.1	2.1 ± 1.0	0.0 ± 0.0	14.5 ± 6.7	11.4 ± 2.8*	15.6 ± 2.1
2 (B)	Intermediate	16.6 ± 4.6	24.5 ± 5.9	0.6 ± 0.6	4.8 ± 1.5	35.4 ± 7.5*	18.1 ± 6.9
2 (C)	Positive	4.4 ± 1.9	37.9 ± 2.4	25.1 ± 3.0	0.0 ± 0.0	30.0 ± 2.1*	2.6 ± 1.7

Mean ± standard deviation of percentage frequency of various cells in four normal bone marrow samples fractionated by fluorescence -activated cell sorting using the monoclonal antibody WEM-G1. In procedure 1, only dead cells were excluded by cell sorting. In procedure 2, cells were sorted after exclusion of cells with low 0 degree light scatter and high 90 degree light scatter.

* Cells included in this category were of undifferentiated "monocytoid" appearance.

fraction. Cells of intermediate fluorescence were blast cells, promyelocytes and myelocytes and monocytes. The nucleated red blood cells in this fraction were primarily normoblasts. The most highly fluorescent cells were primarily promyelocytes, myelocytes, meta-myelocytes and monocytes.

The exclusion of cells of low 0 degree light scatter and high 90 degree light scatter resulted in a relative depletion of small lymphocytes and metamyelocytes from the sorted populations. Therefore the fluorescence-negative population was more highly enriched for blast cells while the most highly fluorescent population was enriched for promyelocytes and myelocytes with 30% monocytes and 4% blast cells.

4.2.3.1 <u>Clonal Proliferation by WEM-G1 Fractionated Cells.</u>
The clonal proliferation of the various cell populations was examined for 4 normal bone marrow samples. A typical result is shown in Table 4.9. For the fluorescence-negative populations, the total number of clones remained relatively constant between days 7 and 14 of culture. These fractions generated all the day 14 erythroid colonies and the majority of the day 14 non-erythroid colonies. The number of day 7 colonies generated by these fractions was small and the maintenance of clone numbers throughout the incubation period suggested that the day 7 colonies formed by these fractions might persist and be scored as colonies again at day 14. In contrast, the fractions of inter-mediate and high-fluorescence generated the majority of day 7 colonies and clusters. Few of these clones persisted for 14 days, although the majority of day 14 clusters were generated by these fractions. As expected, sorting procedure 2 resulted in an increased enrichment for all clonogenic cells when compared with sorting procedure 1.

TABLE 4.9. FREQUENCY OF CLONOGENIC CELLS IN HUMAN MARROW POPULATIONS FRACTIONATED

USING THE MONOCLONAL ANTIBODY WEM-G1

SORTING PROCEDURE AND WEM-G1 STATUS	FREQUENCY PER 10^4 CULTURED CELLS				
	DAY 7		DAY 14		
	Total Colonies	Total Clusters	Total Nonerythroid Colonies	Total Clusters	Total Erythroid Colonies
NIL	57	80	16	10	6
1 (A) Negative	34	26	32	4	20
1 (B) Intermediate	485	255	37	23	0
1 (C) Positive	187	93	2	26	0
2 (A) Negative	55	100	100	0	75
2 (B) Intermediate	730	165	80	185	0
2 (C) Positive	600	520	5	60	0

In sorting procedure 1, only dead cells were excluded. In sorting procedure 2, cells of low 0 degree light scatter and high 90 degree light scatter were excluded. Day 7 culture results were obtained with HPCM alone as stimulus and day 14 results with HPCM and erythropoietin. For unfractionated marrow, 3×10^4 cells were culture per millilitre. For sorting procedure 1, 5,000 cells were cultured per millilitre. For sorting procedure 2, 1,000 cells were cultured per millilitre. Colonies contained 40 or more cells and clusters less than 40 cells but greater than 2 cells. Each result is the mean of 4 replicate cultures.

The percentage of clonogenic cells for the fluorescence-negative population sorted by procedure 2 was 1.7% and in other experiments, the maximum percentage obtained was 14.3%. The percentage of clonogenic cells for the intermediate-fluorescence population (procedure 2) was 9.0% (maximum observed 14.0%) and the high-fluorescence population was 11.2% (maximum observed 14.5%). This did not allow any definite conclusions about the morphology of the clonogenic cells in these fractions.

The calculated percentage recovery for clonogenic cells within each sorted population is shown in Table 4.10. This gives the distribution of the recovered clonogenic cells within each of the sorted populations. It is evident that the major percentage of day 14 colonies was generated by the fluorescence-negative cell population. Although the intermediate-fluorescence populations generated the greatest frequency of day 7 colones (Table 4.9), the high-fluorescence populations contained the greatest proportion of the day 7 colony-forming cells. For all types of clonogenic cell, the recovery was at least 98% using sorting procedure 1. The recovery of 150-250% for some clone types suggested the removal of an inhibitory cell. Sorting procedure 2 resulted in the recovery of the majority of clonogenic cells and confirmed that such a separation procedure could be used to obtain a high enrichment of clonogenic cells but without just selecting out a small subset of these cells.

While it was evident that the erythroid colonies were generated by the fluorescence-negative cells, it was not clear what cell types contributed to the day 14 non-erythroid colonies. As WEM-G1 binds to mature eosinophils (Lopez and Vadas, 1985) it was of interest to determine whether the antibody also identified eosinophil progenitor cells. The morphology of colonies was examined and is presented in

TABLE 4.10. CALCULATED YIELDS OF CLONOGENIC CELLS IN HUMAN MARROW POPULATIONS

FRACTIONATED USING THE MONOCLONAL ANTIBODY WEM-G1

SORTING PROCEDURE AND WEM-G1 STATUS	DAY 7			DAY 14		
	Total Colonies	Total Clusters		Total Nonerythroid Colonies	Total Clusters	Total Erythroid Colonies
1 (A) Negative	27	15		90	18	150
1 (B) Intermediate	80	30		22	22	0
1 (C) Positive	149	53		6	118	0
TOTAL	256	98		118	158	150
2 (A) Negative	8	10		51	0	102
2 (B) Intermediate	78	13		31	113	0
2 (C) Positive	111	68		3	63	0
TOTAL	197	91		85	176	102

CALCULATED PERCENTAGE RECOVERY

The percentage of total clonogenic cells recovered gives the distribution of the clonogenic cells. These results were

calculated by multiplying the frequency of clones generated (see Table 3.2) by the percentage of total cells present in

that sorted populated (computed by the FACS computer) and dividing by the frequency of clones generated by the

unfractionated marrow cells.

Table 4.11. The small number of erythroid colonies generated by the intermediate-fluorescence population from some bone marrow samples probably represented cross-contamination of cells from the fluorescence-negative populations because sorting windows were contiguous. Eosinophil colonies were equally distributed between the negative-fluorescence and intermediate-fluorescence populations, there were however no eosinophil clones in the high-fluorescence population. The small number of day 14 colonies observed in the high-fluorescence population did not allow accurate assessment of particular colony types. The day 14 clusters generated by the high-fluorescence population were 66 ± 4% (mean ± SD) neutrophil, 24 ± 5% macrophage and 10 ± 4% neutrophil-macrophage.

4.2.4 Fractionation of Marrow Cells Using Monoclonal Antibody WEM-G11

FITC-conjugated $F(ab')_2$ fragments of WEM-G11 were used to label light density bone marrow cells. Figure 4.4 shows a typical FACS analysis of these cells. Two populations of cells were evident; non-fluorescent and fluorescent cells with few cells of intermediate fluorescence. This profile was identical to that previously described for another IgG mouse anti-human monoclonal antibody (Griffin et al, 1981).

The composition of labelled and unlabelled populations was determined by sorting four fractions of cells. After exclusion of dead cells, fractions labelled "1" were sorted solely on the basis of fluorescence into positive and negative fractions. Fractions labelled "2" were sorted using three parameters: cells of high 0 degree light scatter (upper panel) and low high angle (90 degree) light scatter (lower panel), were fractionated on the basis of fluorescence into positive and negative fractions. Table 4.12 shows the mean

TABLE 4.11. MORPHOLOGY OF DAY 14 COLONIES PRODUCED BY WEM-G1-FRACTIONATED CELLS

WHEN CULTURED IN AGAR

Expt. Sorting Procedure No. and WEM-G1 Status	PERCENTAGE OF DAY 14 COLONIES				
	Neutrophil	Neutrophil-Macrophage	Macrophge	Eosinophil	Erythroid
1 NIL	13	29	13	13	32
1 (A) Negative	10	38	8	15	29
1 (B) Intermediate	13	40	20	27	0
2 (A) Negative	13	31	13	17	26
2 (B) Intermediate	13	55	13	19	0
2 NIL	7	23	13	12	45
1 (A) Negative	4	23	16	10	47
1 (B) Intermediate	21	40	13	13	13
2 (A) Negative	7	23	7	14	49
2 (B) Intermediate	50	25	24	0	1

Results are percentages of sequentially sampled clones from cultures of cells fractionated by WEM-G1. The small number of day 14 colonies present in the most fluorescent populations did not allow accurate assessment of colony types.
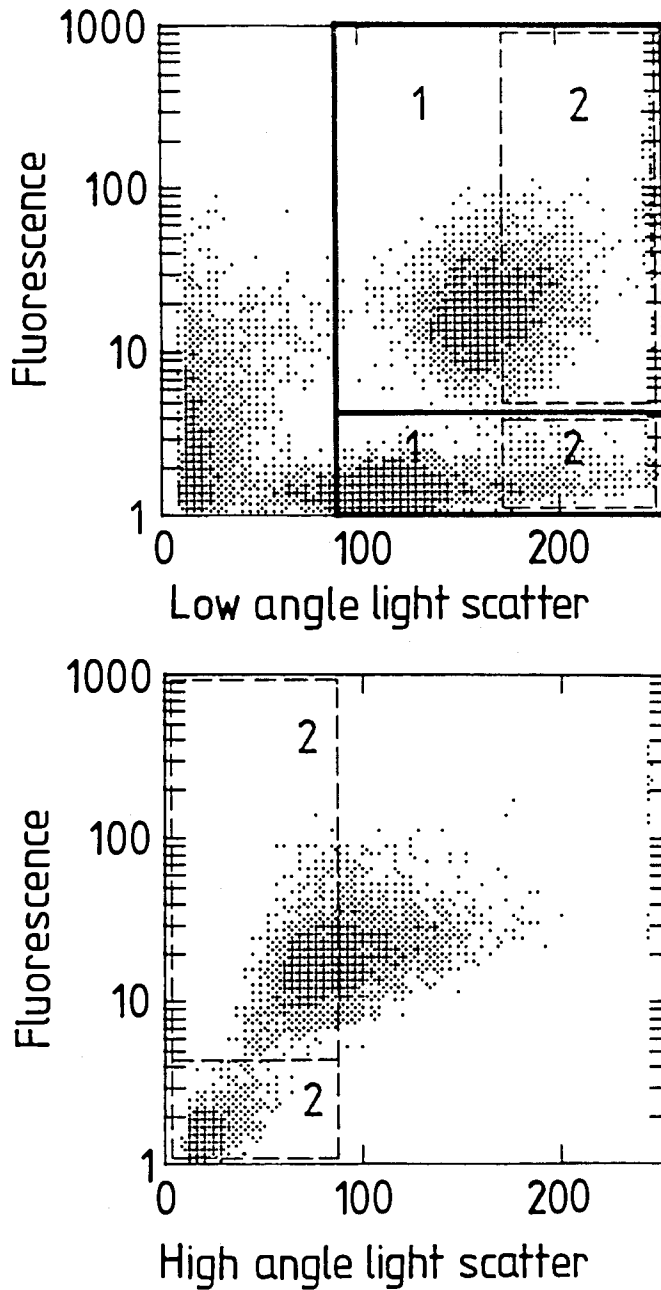
Fig. 4.4 FACS profile of normal human bone marrow cells labelled
with (Fab')2 fragments of the monoclonal antibody WEM-G11.
Upper panel shows low angle (0°) light scatter (horizontal
axis) and fluorescence intensity (vertical axis).
Fractions labelled 1 were separated on the basis of
fluorescence only (following exclusion of dead cells).
Fractions labelled 2 were cels of high 0° light scatter.
Lower panel shows high angle (90°) light scatter
(horizontal axis) and fluorescence intensity (vertical
axis). Fractions labelled 2 were cells of high 0°
light scatter and low 90° light scatter separated on
the basis of fluorescence.

TABLE 4.12. COMPOSITION OF HUMAN MARROW POPULATIONS FRACTIONATED USING THE MONOCLONAL ANTIBODY WEM-G11

Sorting Procedure	WEM-G11 Status	Percentage of Cells							
		Blasts	Promyelo- cytes	Myelo- cytes	Metamyelo- cytes and Polymorphs	Lympho- cytes	Mono- cytes	Erythro- blasts	Normo- blasts
Nil		6.3 ± 5.7	5.6 ± 1.7	12.1 ± 2.8	32.1 ± 9.6	22.2 ± 9.1	11.1 ± 10.5	0.9 ± 0.9	9.7 ± 2.8
1	Neg.	14.2 ± 6.5	5.2 ± 4.5	0.3 ± 0.3	0.0 ± 0.0	43.9 ± 17.3	21.8 ± 14.0	1.7 ± 1.7	12.9 ± 9.0
1	Pos.	0.1 ± 0.1	8.0 ± 2.0	18.6 ± 7.0	71.4 ± 7.0	0.5 ± 0.5	0.5 ± 0.5	0.0 ± 0.0	0.9 ± 0.8
2	Neg.	29.8 ± 8.8	4.8 ± 4.5	0.6 ± 0.6	0.0 ± 0.0	15.1 ± 7.1	32.4 ± 8.2*	5.4 ± 3.7	11.9 ± 7.4
2	Pos.	0.4 ± 0.4	12.5 ± 5.4	25.4 ± 7.7	57.8 ± 12.2	0.8 ± 0.6	1.4 ± 1.4	0.0 ± 0.0	1.7 ± 1.1

Mean ± standard deviations of the percent frequency of various cells in eight separate bone marrow samples fractionated by fluorescence-activated cell sorting using the monoclonal antibody WEM-G11. In procedure 1, only dead cells were excluded by cell sorting. In procedure 2 cells were sorted after exclusion of cells with low 0° light scatter and high 90° light scatter.

* Cells included in this category were difficult to identify accurately as monocytes because of their undifferentiated appearance. They would be more accurately described as "monocytoid".

data on the cellular composition of the various sorted populations from 8 normal bone marrow specimens. For populations sorted only on the basis of fluorescence, it was evident that non-fluorescent cells included blast cells, lymphocytes, monocytes and nucleated red blood cells. Some early promyelocytes were present in this fraction but these cells differed from the blast cells only by their content of a small number of primary granules. The fluorescence-positive fraction contained more mature promyelocytes, myelocytes, metamyelocytes and mature granulocytes. This population was depleted of blast cells, lymphocytes and monocytes, and contained almost exclusively cells of the granulocytic lineage. While 70% of fluorescent cells were post-mitotic metamyelocytes and polymorphs, more than 25% were promyelocytes and myelocytes. Thus, from the viewpoint of cells potentially able to proliferate following stimulation by GM-CSF, the WEM-G11-positive population could be regarded as a population of promyelocytes and myelocytes essentially free of blast cells (candidate-committed progenitor or colony-forming cells).

The separation of blast cells from promyelocytes and myelocytes achieved using WEM-G11 was accentuated by exclusion of cells with low 0 degree light scatter and high 90 degree light scatter (Fractions 2). After such sorting, the fluorescence-negative fraction contained 30% blast cells while the fluorescence-positive fraction contained up to 35-40% of promyelocytes and myelocytes and less than 0.5% of blast cells.

4.2.4.1 Clonal Proliferation by WEM-G11 Fractionated Cells. Since progenitor (colony-forming) cells have the morphology of blast cells (Beverley et al, 1980; Nicola et al, 1981; Griffin et al, 1982), it was anticipated that fluorescence-negative fractions would generate increased numbers of granulocyte-macrophage colonies in GM-CSF stimulated cultures compared with unfractionated marrow.

As shown in a typical result in Table 4.13 this expectation was confirmed when the frequencies of day 14 colonies formed by cells from the various fractions were compared. Furthermore, fluorescence-positive, blast cell-depleted, fractions formed relatively low numbers of day 14 colonies. However a different situation was observed in cultures scored at day 7. At this earlier timepoint, the promyelocyte-myelocyte fraction generated a relatively large number of clones of mainly sub-colony size (clusters). Those colonies that did develop were of small size and tended to disappear on continued incubation to 14 days. The day 7 colonies formed by the promyelocyte-myelocyte fractions were usually of a dispersed nature when compared with the larger, more compact day 7 colonies formed by the blast cell-enriched fractions.

The calculated yields for cells cultured following sorting procedure 1 (Figure 4.4) were 150% total recovery for day 7 colonies, 88% total recovery for day 7 clusters and 89-125% total recovery for day 14 clones (Table 4.14). This suggested inhibitory cells might have been removed from the population forming day 7 colonies during the fractionation procedure. As 30% of clusters in the unfractionated marrow at day 7 are macrophage (see Table 4.15) the calculated enrichment for granulocytic clusters in the WEM-G11-positive population (procedure 1) was 110-190% (mean 125%).

The total yields for cells cultured following sorting procedure 2 (Figure 4.4) varied significantly with different marrow specimens. For the experiment shown in Table 4.13 the calculated yields were 40% for day 7 colonies, 10% for day 7 clusters and 23-45% for day 14 clones. In other experiments, the calculated recovery of day 7 clusters from the promyelocyte-myelocyte-enriched population (Fraction 2) varied between 5% (Table 4.14) and 84% (mean 30%) and

TABLE 4.13.   Frequency of Clonogenic Cells in Human Marrow Populations Fractionated Using the Monoclonal Antibody WEM-G11

Sorting Procedure and WEM-G11 Status	Cell composition	Frequency per 10^4 Cultured Cells				
		Day 7			Day 14	
		Total Colonies	Total Clusters	Total Non-erythroid Colonies	Total Clusters	Total Erythroid Colonies
Nil		54	126	55	32	22
1 Negative		196	134	120	74	49
1 Positive		5	95	3	17	0
2 Negative	Blast cell-enriched	240	72	204	160	66
2 Positive	Promyelocyte-myelocyte-enriched	88	176	6	66	0

In sorting procedure 1 only dead cells were excluded. In procedure 2 cells were sorted after exclusion of cells with low 0° light scatter and high 90° light scatter.

Results were obtained using human placental conditioned medium alone as the stimulus for day 7 culture results. Human placental conditioned medium and erythropoietin were used for day 14 results. For unfractionated marrow 3 x 10^4 cells were cultured per ml. For sorting procedure 1, 5000 cells were cultured per ml. For sorting procedure 2, 1000 cells were cultured per ml. Colonies contained 40 or more cells and clusters less than 40 cells, but greater than 2 cells.

TABLE 4.14. Calculated Yields of Clonogenic Cells in Human Marrow Populations Fractionated Using the Monoclonal Antibody WEM-G11.

Sorting Procedure and WEM-G11 Status	Calculated Percentage Recovery				
	Day 7		Day 14		
	Total Colonies	Total Clusters	Total Nonerythroid Colonies	Total Clusters	Total erythroid Colonies
1 Negative	144	42	86	93	1 90
1 Positive	6	46	3	32	0
TOTAL	150	88	89	125	90
2 Negative	34	5	28	38	23
2 Positive	6	5	1	7	0
TOTAL	40	10	29	45	23

The percentage of total clonogenic cells recovered gives the distribution of clonogenic cells in each fraction. Results were calculated by multiplying the frequency of clones generated by the percentage of total cells present in that fraction (computed by the FACS computer) and dividing by the frequency of clones generated by the unfractionated marrow cells.

TABLE 4.15. Morphology of Clones Produced by WEM-G11 Fractionated Cells When Cultured in Agar

| | Day 7 | | | | | | Day 14 | | | | | |
| | Neutrophils | | Macrophages | | Eosinophils | | Neutrophils | | Macrophages | | Eosinophils | |
	Colony	Clusters	Colony	Cluster	Colony	Cluster	Colony	Clusters	Colony	Clusters	Colony	Cluster
Unfractionated Marrow	27±9	46±9	0	23±14	0	4±2	23±3	5±2	3±2	56±10	5±4	8±6
Blast cell fraction	36±18	14±5	5±4	41±22	0	4±4	28±14	3±3	7±4	51±13	8±4	3±3
Promyelocyte-myelocyte fraction	8±8	84±11	0	5±4	0	3±2						

Results are the means ± standard deviations as percentages, of sequentially sampled clones from cultures of cells fractionated from 4 normal human bone marrows cultured with human placental conditioned medium. For the unfractionated marrow, 3×10^4 cells were cultured per ml. For both fractionated populations 1000 cells were cultured per ml.

the calculated recovery of promyelocytes-myelocytes paralleled these percentages. These lower yields were the consequence of deliberate selection of a sorted population giving a higher degree of enrichment of promyelocytes and myelocytes (between 2.6 and 4.7-fold for different experiments).

The percentage of clonogenic cells for 9 unfractionated marrow samples ranged between 2.3-2.5% (mean 1.9%) at day 7 of culture. For the blast cell-enriched fraction (Fraction 2) the percentage of clonogenic cells present at day 7 was 1.3-7.3% (mean 4.5%) and for the promyelocyte-myelocyte-enriched fraction (Fraction 2) 1.6-8.5% (mean 4.8%).

The morphology of clones produced by each cell fraction was examined and is presented in Table 4.15. At day 7, clones formed by the blast cell-enriched fraction consisted of neutrophilic colonies and clusters of macrophages. In contrast, the promyelocyte-myelocyte-enriched fraction generated primarily neutrophilic clusters and colonies with few macrophage and eosinophil clusters. The small number of macrophage and eosinophil clusters may represent contamination of the promyelocyte-myelocyte population by cells from the fluorescent-negative blast population since the fluorescence sorting windows used were contiguous (Figure 4). However, since WEM-G11 binds to mature eosinophils (Lopez et al, 1985) the eosinophil clusters may have been formed by eosinophilic promyelocytes-myelocytes, although no cells of this type were seen in the necessarily restricted sample of cells analyzed for morphology.

4.3 UNDERLINE_DISCUSSION

Although monoclonal antibody HTF-1 did not serve to separate out subpopulations of progenitor cells, the morphological analysis of the cell types identified by this antibody were of interest, particularly those cells that were clearly of positive fluorescence. These cells constitute only approximately 5% of the total marrow cells and included primarily myeloid cells, monocytes and basophils. Of all myeloid cells and monocytes, however only 5% and 12% respectively were present in the fluorescence-positive population. This may in part explain the apparent discrepancy between this result and reports that the antigen recognised by HTF-1 is not present on unstimulated monocytes (Ewan et al, unpublished observations) nor is there detectable tissue factor procoagulant activity in human neutrophils (de Moerloose et al, unpublished observations). Other possible explanations include the exposure of cells to small levels of endotoxin during preparation for cell sorting (Edwards and Rickles, 1980).

The unequivocal binding of HTF-1 to marrow basophils was unexpected. Because of the very low frequency of these cells in unfractionated bone marrow, in some experiments the calculated recovery of basophils was greater than 300%, and in all experiments was at least 100%. This antibody could thus be used as a single step technique in combination with FACS sorting to prepare enriched populations of basophils. Whether the binding of HTF-1 to basophils represents expression of "tissue factor related antigen" on these cells or merely the expression of an antigenically-related but functionally-unrelated molecule (Lane and Kaprowski, 1982) remains to be determined. It is of interest however that an antibody has been described that binds to both murine inflammatory macrophages and mast cells (Katz et al, 1981). These points could be resolved

by determining the procoagulant activity of the HTF-1 positive

basophils and by determining the biochemical characteristics of the

antigen present on different cell types. HTF-1 did not identify

basophil colony-forming cells, although the culture conditions used

would have supported their growth (Denburg et al, 1983).

Monoclonal antibody 25E11 was raised against natural killer

cells and recognises the IIb-IIIa complex of platelets. The most

highly fluorescent cells were lymphocytes (granular and agranular),

monocytes and unexpectedly eosinophils. Myeloid cells, from pro-

myelocytes onwards were weakly but consistently positive in all six

marrow samples examined and binding was confirmed to be specific by

using a platelet-specific monoclonal antibody that did not bind to

granulocytes and by using $F(ab')_2$ fragments of 25E11. This provided

initial evidence for the presence of the IIb-IIIa complex on cells

other than platelets. This was subsequently confirmed using immuno-

precipitates of platelet and neutrophil surface antigens (Burns et

al, 1985). Preliminary experiments were also performed to analyse

the action of natural killer cells and monocytes on colony formation.

The inconsistent results obtained were in keeping with the conflicting

results reported by different groups (and even conflicting results

from within one group). Thus reports include inhibition of myeloid

(Hansson et al, 1982; Barr and Stevens, 1982; Morris et al, 1980)

and erythroid colonies (Mangan et al, 1984; Degliantoni et al, 1985),

and stimulation of erythroid colonies (Meytes et al, 1979; Mangan

et al, 1982; Mangan et al, 1984; Pistoia et al, 1985; Linch et al,

1985). All such studies have used natural killer cells and blood/

bone marrow cells in the ratio of at least 0.5:1. The relevance of

all these studies to the microenvironment of progenitor cells in

vivo, at concentrations much greater than 10^4-10^5 cells per ml is

dubious, particularly as the effect of one cell type (monocytes) clearly changes with changes in cell concentrations (To et al, 1983).

The experiments using monoclonal antibody 9.1C3 may have provided some additional evidence for an inhibitory effect of natural killer cells and monocytes on colony formation. This antibody blocks the killing function of both cell types and in some marrow samples resulted in enhanced colony formation up to 170%. This effect was however inconstant and although 9.1C3 does block natural killer cells and monocytes (Burns et al, 1984) the antibody is directed against the T200 molecule which is present on all T-lymphocytes and haemo-poietic progenitor cells. The addition of one reagent to a crude population of cells provides very limited information about the direct effects of that reagent, particularly when it is known to react with many cell types. It was however of interest that in no experiments did the results suggest a stimulatory role for natural killer cells and monocytes (although this may only reflect an unintentional sampling error). The enhanced colony growth with 9.1C3 occurred in cultures stimulated by supramaximal, non-inhibitory concentrations of CSF. Such cultures may therefore significantly underestimate the absolute number of clonogenic cells present in some marrow samples.

In all these experiments the difficulty remains that considerable individual heterogeneity exists, and the relevance of results obtained using even purified populations of cells is of doubtful significance when attempting to understand the in vivo interactions. For these reasons, despite the availability of monoclonal antibodies to dissect out subpopulations of lymphocytes and monocytes, no further experi-ments were performed to analyse these interactions.

Monoclonal antibody WEM-G1 served to identify a population of myeloblasts, granulated myeloid cells and monocytes. The more

immature cells were of lower fluorescence intensity and generated the highest frequency of day 7 colonies but very few day 14 colonies. These results were consistent with other reports of murine IgM monoclonal antibodies against human granulocytes that apparently did not identify human GM-CFC when cultures were scored after 14 days (Ball and Fanger, 1983; Strauss et al, 1983; Strauss et al, 1984; Griffin et al, 1981). Cultures had either not been examined after 7 days or if they were, it was not recognised that two subsets of human GM-CFC could be identified (Skubitz et al, 1983; Strauss et al, 1984). From an analysis of the distribution of the day 7 CFC it was evident that although the intermediate-fluorescence population generated the highest frequency of day 7 colonies, the high-fluorescence population contained the majority of day 7 CFC's. The antibody thus separated the day 7 and day 14 CFC's. These cell populations have been previously separated on the basis of differences in sedimentation velocity characteristics (Johnson et al, 1977; Inoue and Ottenbreit, 1978; Jacobsen et al, 1978; Miller et al, 1978) and lectin binding characteristics in combination with the FACS (Morstyn et al, 1980; Nicola et al, 1980). It was therefore not surprising that these cells might also display different surface antigens as detected by monoclonal antibodies.

The binding of the IgM-class murine monoclonal antibodies to human eosinophil progenitors had not previously been examined. These cells appeared to be equally distributed between the negative- and intermediate-fluorescence populations. This may therefore represent "spurious" fluorescence and reflect cross-contamination of cell populations. However as mature eosinophils express this antigen (Lopez and Vadas, 1985) it is possible that the antigen recognised by WEM-G1 is acquired by a subset of eosinophil progenitors. Experi-

ments were not performed to examine the use of WEM-G1 in combination with lectin binding to obtain more highly purified populations of CFC's, but such a procedure might also result in an increased frequency of eosinophil progenitors as these cells are also identified by lectin binding (Morstyn et al, 1980). It would also be possible to combine labelling with WEM-G1 and WEM-G11. This would be expected to identify a population of blast cells (WEMG-1 positive but WEM-G11 negative) that generate only day 7 colonies.

WEM-G11, as with other murine IgG anti-granulocyte monoclonal antibodies (Griffin et al, 1981; Ball and Fanger, 1983; Griffin et al, 1983; Strauss et al, 1984; Andrews et al, 1983) identified a population of promyelocytes, myelocytes and metamyelocytes and polymorphs. These cells generated primarily neutrophil clusters and a small number of neutrophil colonies. Additional studies were performed to examine the nature of the clonal proliferation of these cells in normal and leukaemic states and results of these studies are presented in subsequent chapters.

CHAPTER 5

THE BIOLOGY OF NORMAL HUMAN PROMYELOCYTES AND MYELOCYTES

5.1 INTRODUCTION

Despite the suggestion that immature granulocytes possess proliferative potential in vitro (Morstyn et al, 1980; Francis et al, 1981; Francis et al, 1983) and the clear evidence that such cells are capable of proliferation in vivo (Warner and Athens, 1964; Golde, 1983) studies using purified populations of these cells failed to demonstrate clonal proliferation in vitro (Griffin et al, 1981; Ball and Fanger, 1983).

The monoclonal antibody WEM-G11 was used in combination with the FACS to obtain fractions of normal bone marrow cells that were highly enriched for promyelocytes and myelocytes. Sequential analysis of semisolid cultures of such cells documented the ability of these cells to proliferate clonally and characterised the composition and fate of these clones in conventional CSF-stimulated cultures. The proliferation of these cells was also examined in microwell suspension cultures.

Microwell suspension cultures of haemopoietic cells have been used to monitor the proliferative effects of the CSF's and permit convenient and rapid assays. Several microassay systems have been described that are dependent on proliferation of target cells to detect CSF. These assays are rapid, permit the use of small volumes of CSF and the murine assays display a heightened sensitivity to CSF (Schrader et al, 1981; Ihle et al, 1982; Tushinski et al, 1982; Burgess et al, 1982; Griffin et al, 1984).

5.2 RESULTS

5.2.1 Clonal Proliferation of Normal Promyelocytes and Myelocytes

As described in Chapter 4, WEM-G11 was used to obtain a population of promyelocytes and myelocytes (depleted of blast cells, mono-

cytes and lymphocytes) and a population of blast cells, monocytes
and lymphocytes. The promyelocyte-myelocyte population generated
day 7 clusters while the blast cell population generated day 7 and
14 colonies.

The difference in clonal proliferation exhibited by the blast
cell versus promyelocyte-myelocyte fractions was emphasized by a
sequential analysis of cultures of cells in which total clone numbers
per culture were monitored from day 1-14 of culture. Figure 5.1
shows the results of such an analysis for cells sorted by procedure
1 (i.e. cells sorted on the basis of fluorescence alone with exclusion
of dead cells (see Figure 4.4). The composition of the fluorescence-
negative population when sorted using procedure 1 was 14.2 ± 6.5%
(mean ± S.D.) blast cells, 5.2 ± 4.5% promyelocytes, 43.9 ± 17.3%
lymphocytes, 21.8 ± 14.0% monocytes and erythroid cells. The fluore-
scence-positive population was 8.0 ± 2.0% promyelocytes, 18.6 ± 7.0%
myelocytes, 71.4 ± 7.0 metamyelocytes and less than 1% blasts, lympho-
cytes and monocytes.

The blast cell fractions (procedure 1) generated maximum numbers
of clones after 7 days of incubation, with total clone numbers being
maintained throughout the 14 day culture period. Cultures of the
promyelocyte-myelocyte fractions (procedure 1) generated maximum
numbers of clones between days 3 and 5 of culture with clone numbers
declining rapidly thereafter (Figure 5.1). The frequency of clones
generated by the promyelocyte-myelocyte fraction at day 3 represented
between 2-7% of all cells cultured. As the only cells capable of
proliferation in this fraction were promyelocytes and myelocytes
this suggested that up to 25% of these cells were capable of prolifera-
tion in vitro.

Fig. 5.1. Total clone numbers followed over 14 days in cultures of cells from 4 normal human bone marrows fractionated using the monoclonal antibody WEM-G11. The results are mean values ± standard deviations expressed as per 10^4 plated cells. For both populations, replicate plates were examined using 3×10^3 cells cultured per ml and stimulated by HPCM alone.

Similar experiments were performed with cells sorted by procedure 2, however as this fractionated procedure resulted in a higher enrichment of promyelocytes and myelocytes, a higher frequency of clonogenic cells was expected. The fluorescence-negative fraction (blast cell-enriched) consisted of 29.8 ± 8.8% blasts, 4.8 ± 4.5% promyelocytes, 15.1 ± 7.1% lymphocytes, 32.4 ± 8.2% "monocytoid" cells and nucleated red blood cells. The fluorescence-positive fraction (promyelocyte-myelocyte-enriched) consisted of 12.5 ± 5.4% promyelocytes, 25.4 ± 7.7% myelocytes, 57.8 ± 12.2% metamyelocytes and approximately 1% blasts, lymphocytes and monocytes.

The pattern of proliferation for cells sorted by procedure 2 paralleled that of cells sorted by procedure 1. As shown in Figure 5.2, the blast cell-enriched fractions (procedure 2) generated peak numbers of clones by day 5 of incubation with total clone numbers remaining relatively constant for the remainder of the observation period and some clones enlarging progressively to form the day 14 colonies. In contrast, in cultures of fractions enriched for promyelocytes and myelocytes (procedure 2) peak clone numbers occurred between 3 and 5 days and after this time clone numbers declined rapidly to between 30-50% of peak values by day 7 and to almost zero by day 14.

At day 3, the frequency of clones generated by promyelocyte-myelocytes represented 9-18% of all cells cultured. Since essentially the only cells capable of proliferation in such fractions were promyelocytes and myelocytes (38% of total cells), this indicates a likely cloning efficiency for such cells of 25-50%. With fractions from some marrows, the frequency of day 3 clones exceeded the percentage of promyelocytes, suggesting that some myelocytes in these fractions must have been capable of proliferation.
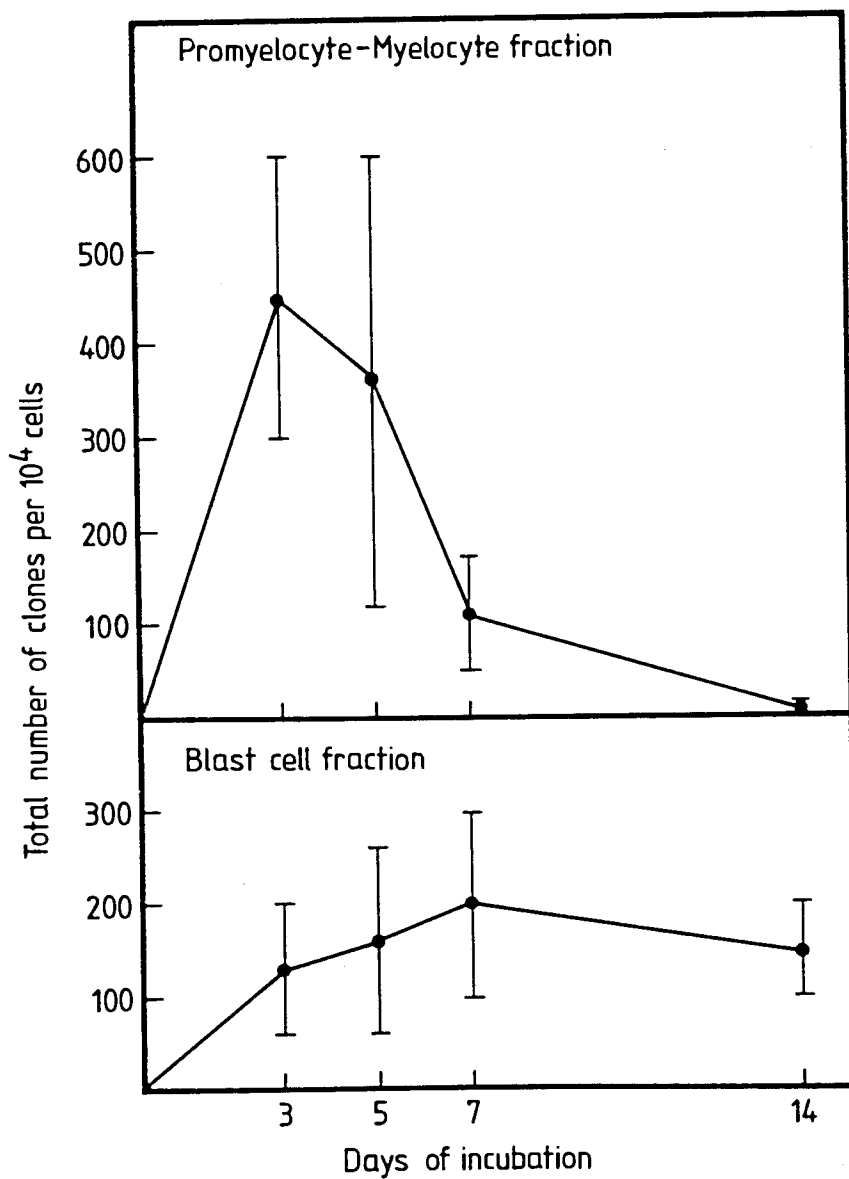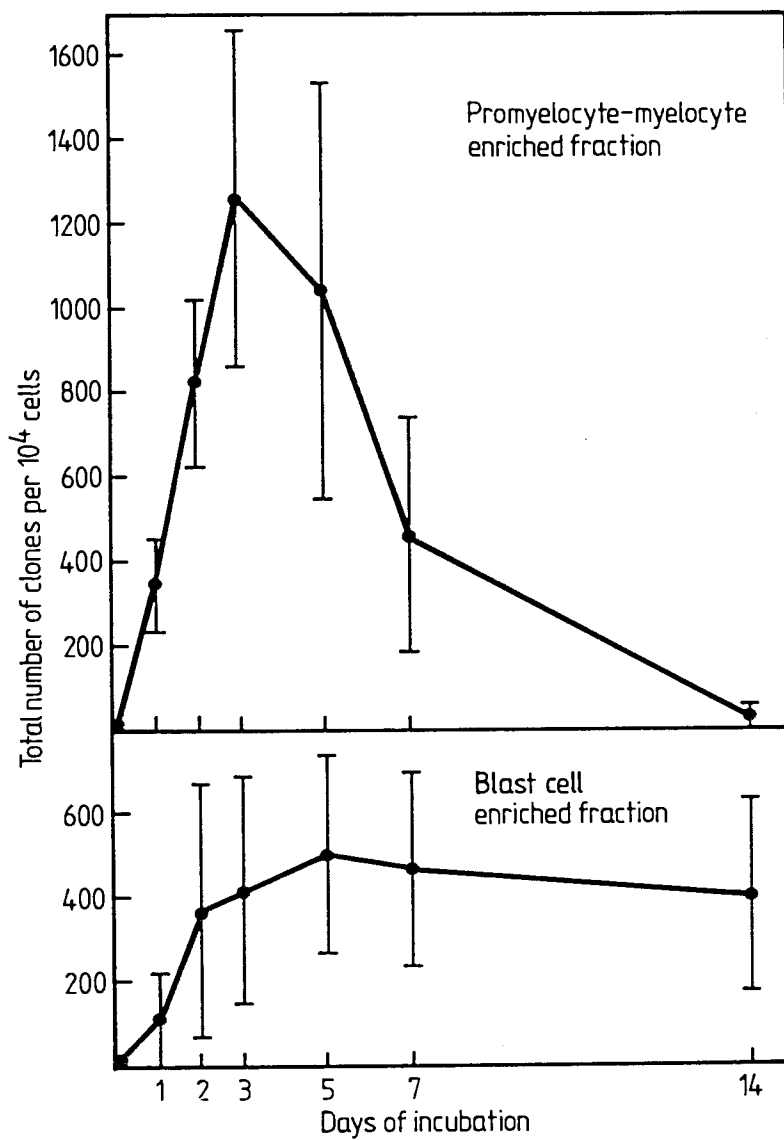
Fig. 5.2 Total clone numbers followed over 14 days in cultures of
cells from 9 normal human bone marrows fractionated using
the monoclonal antibody WEM-G11. The results are mean
values ± standard deviations expressed as per 10^4 plated
cells. For both populations, replicate plates were examined
using 10^3 cells cultured per ml and stimulated by HPCM alone.

Because of the greater frequency of clonogenic cells in popula-
tions sorted by procedure 2 (i.e. cells with high 0 degree light
scatter characteristics and low 90 degree light scatter character-
istics, separated on the basis of fluorescence) this procedure was
used in subsequent experiments to examine the clonal proliferation
of these cell populations in greater detail.

To establish the time at which individual cells initiated clonal
proliferation and the subsequent fate of such clones, a series of
culture mapping experiments were performed. A typical result is
presented in Table 5.1. Cells in the blast cell-enriched fractions
were slow to initiate proliferation and the majority of clones present
at day 5 persisted for 14 days. In contrast, cells of the promyelo-
cyte-myelocyte-enriched fractions exhibited an earlier onset of
proliferation but most clones subsequently disappeared, some as early
as day 3-5 of incubation. Few clones survived for 14 days. With
the use of cultures containing only 1000 cells, it was clear that for
some of the clones, cell death was the cause of clonal disappearance
but in the majority of cases dispersion of clone cells into interclonal
areas probably occurred. Similar results were obtained in the four
other marrow samples examined and for cells sorted both by procedure 1
and procedure 2.

5.2.2 Size and Composition of Clones

The size distribution of clones produced by the blast cell-
enriched and promyelocyte-myelocyte-enriched populations was compared
by counting the number of cells in consecutive clones present at
day 7. A typical result is shown in Figure 5.3. The majority of
clones present in cultures of the blast cell-enriched population
contained more than 40 cells. In cultures of the promyelocyte-
myelocyte-enriched fractions most clones contained fewer than 40

Table 5.1. Onset of Proliferation and Fate of Individual Clones in Mapped Areas in Cultures of Cells Fractionated Using the Monoclonal Antibody WEM-G11

Blast Cell-Enriched Fraction							Promyelocyte-Myelocytes-Enriched Fraction						
Day of Culture						Number of Examples (as Percentage)	Day of Culture						Number of Examples (as percentage)
1	2	3	5	7	14		1	2	3	5	7	14	
							C	C	C	†			4
							C	C	C	C	†		7
							C	C	C	C	C	†	5
	C	C	C	C	†	3		C	C	†			11
C	C	C	C	C		22		C	C	C	†		16
		C	C	†		2		C	C	C	C	†	11
		C	C	C	†	5		C	C	C	C	C	<1
		C	C	C	C	35			C	†			6
			C	†		<1			C	C	†		17
			C	C	†	1			C	C	C	†	14
			C	C	C	26			C	C	C	C	<1
				C	†	<1				C	†		3
				C	C	6				C	C	†	6
										C	C	C	<1
											C	†	<1

Footnote to Table 5.1.

Time of initiation and fate of individual clones in mapped areas of cultures of cells from a human marrow fractionated using the monoclonal antibody WEM-G11.

For both populations, 1000 cells were cultured per ml with human placental conditioned medium as the source of CSF.

Clones of 2 or more cells were recorded during 14 days of culture. For the blast cell fraction 130 clones were followed.

For promyelocyte-myelocyte fraction 355 clones were followed.

C = clone present.

† = lysis or disappearance of clone.
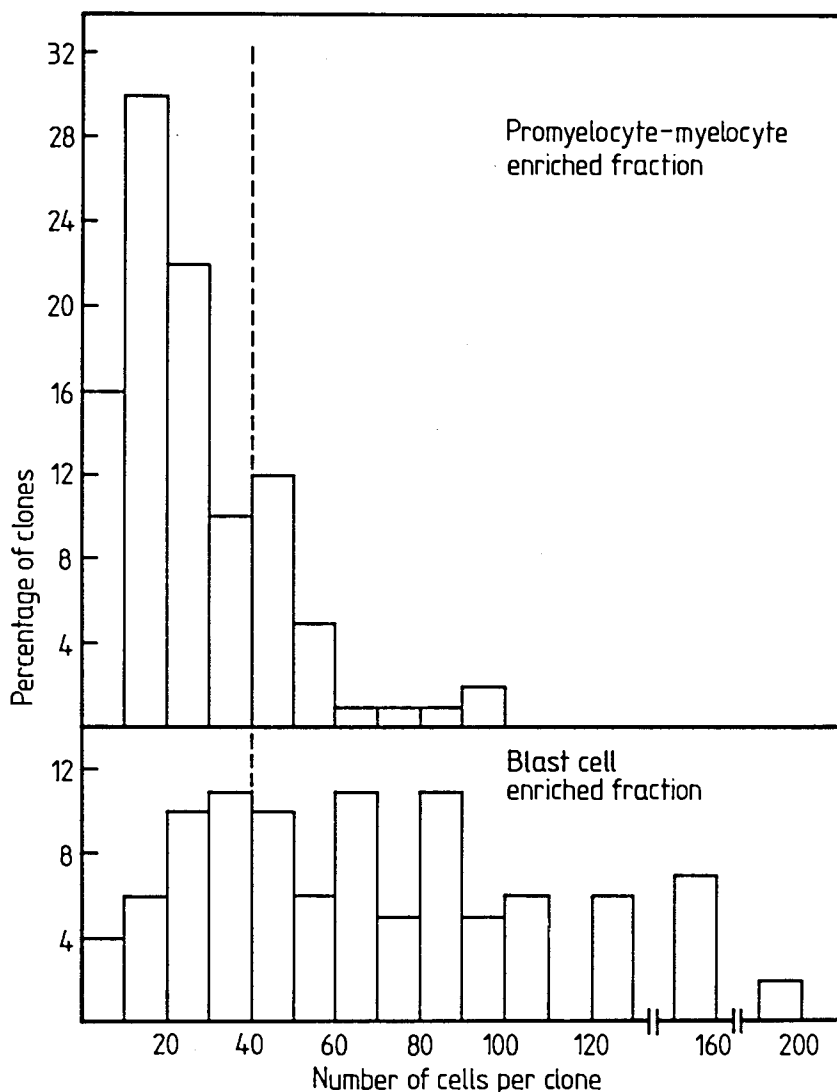
Fig. 5.3 Size distribution of sequentially-sampled 7 day clones
formed by human marrow cells fractionated using the
monoclonal antibody WEM-G11. Both fractions were cultured
using 1000 cells per ml with human placental conditioned
medium as the source of CSF. The dashed line indicates
clones of greater than 40 cells that were scored as colonies.
For the blast cell-enriched fraction of this marrow a total
of 103 clones were counted and for the promyelocyte-myelo-
cyte-enriched fraction 111 clones were counted. The mean
number of cells per clone ± standard deviation was 65 ± 43
for the blast cell-enriched fraction and 26 ± 18 for the
promyelocyte-myelocyte-enriched fraction.

cells. Many of the clones in cultures of this latter population had disappeared by day 7 (Figure 5.2) and only the larger clones remained. The small number of colonies present may be attributable to the blast cells contaminating this population however their dispersed nature when compared with the compact day 7 colonies of the blast cell-enriched population may indicate that they originate from promyelocytes.

Both the blast cell-enriched and promyelocyte-myelocyte-enriched populations were demonstrated to be CSF-dependent (Figure 5.4) with cells from the promyelocyte-myelocyte fraction being slightly more responsive than cells from the blast cell-enriched fraction. Both populations demonstrated a minor degree of proliferation in the absence of exogenous CSF. These clones were of 2 cells in size with up to 10 clones developing per 1000 cultured cells in both populations. For the blast cell-enriched population this proliferation may be due to the presence of lymphocytes and monocytes. Proliferation in unstimulated cultures was less in the promyelocyte-myelocyte-enriched fraction, and although fractions from most marrows were almost free of lymphocytes and monocytes, this fraction from one marrow did contain 34% monocytes.

The relationship between the number of cells cultured and the number of clones generated by both cell populations is presented in Table 5.2. The relationship was linear for both the promyelocyte-myelocyte and blast cell fractions, suggesting that CSF was acting directly on the target cells within both populations, despite the presence of candidate accessory cells.

The morphology of clones produced by WEM-G11 fractionated cell populations when cultured in agar was shown in Table 4.15. At day 7, clones formed by the blast cell-enriched population were neutrophil
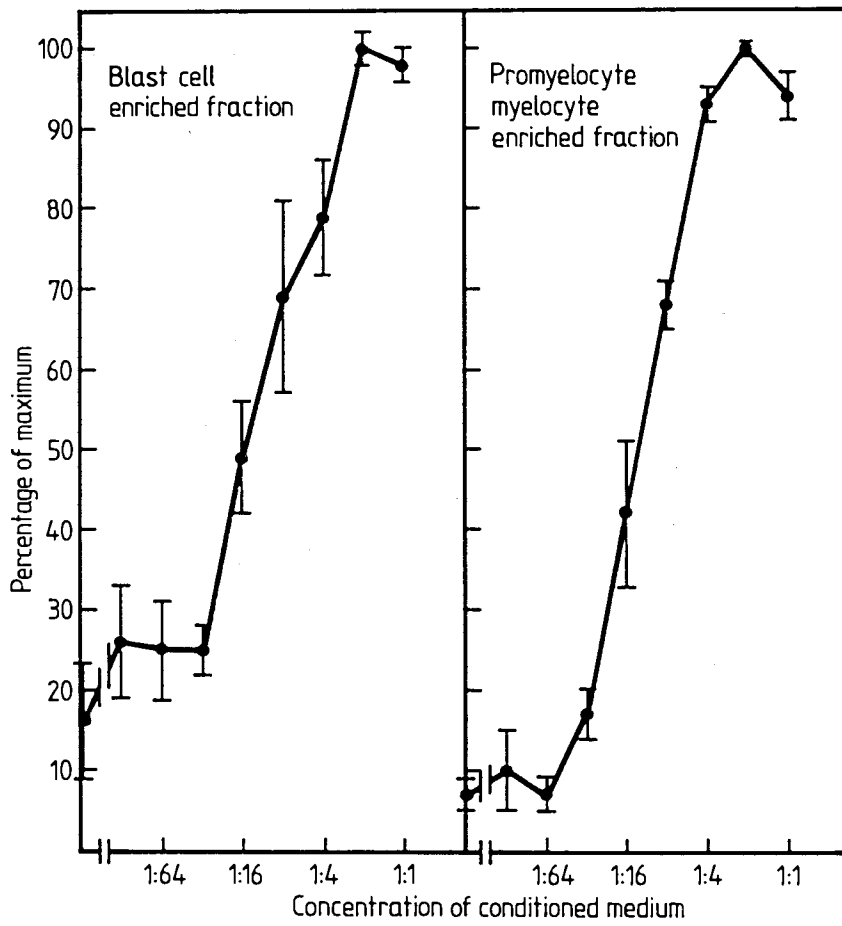
Fig. 5.4 Dose-response curves to stimulation by CSF of blast cell-
enriched and promyelocyte-myelocyte-enriched fractions
from normal human marrow separated using the monoclonal
antibody WEM-G11. For both populations 1000 cells were
cultured per ml with serial dilutions of human placental
conditioned medium. The results are the mean values ±
standard errors for 4 normal human marrows. Duplicate
cultures were scored at day 5 for the promyelocyte-
myelocyte-enriched fraction and at day 7 for the blast
cell-enriched fraction.

TABLE 5.2. Linear Relationship Between Number of Cells Cultured and Number of Clones Generated in Cultures of Marrow Cells Fractionated Using Monoclonal Antibody WEM-G11

Cell Population	Number of Cells Cultured				
	4,000	2,000	1,000	500	250
Blast Cell Fraction	98 ± 4	48 ± 3	22 ± 2	12 ± 3	6 ± 2
Promyelocyte-Myelocyte Fraction	204 ± 11	92 ± 4	45 ± 5	25 ± 4	10 ± 4

Results are mean (± standard deviation) number of clones generated in cultures of normal marrow cells fractionated using monoclonal antibody WEM-G11. Varying numbers of cells were cultured (as indicated) and replicate cultures were scored at day 5 (promyelocyte-myelocyte fraction) and day 7 (blast cell fraction). All cultures were stimulated by HPCM.

colonies and macrophage clusters, and at day 14 there were both
colonies and clusters of neutrophils, macrophages and eosinophils.
The promyelocyte-myelocyte fractions generated clones that were 92%
neutrophil, 5% macrophage and 3% eosinophil.

5.2.3 Relationship Between Day 7 and Day 14 Clones Generated by the Blast Cell Fractions

Studies examining clones generated by the promyelocyte-myelocyte
fractions demonstrated that the vast majority of day 7 neutrophil
clusters were transient. Culture mapping experiments were performed
using clones generated by the blast cell fractions to examine the
relationship between day 14 clusters and colonies and the clones
that were present following 7 days of culture. Results are presented
in Table 5.3. The origin of day 14 clusters was determined to be
primarily from day 7 clusters (of which 75% were macrophage,
Table 4.15). These day 7 clusters generated 73% of day 14 clusters.
Day 7 colonies (primarily neutrophil) generated 20% of day 14
clusters and 7% of day 14 clusters developed from clones that were
not noted at day 7. The day 14 colonies were generated by day 7
clusters (56%) and day 7 colonies (44%).

5.2.4 The Proliferation of Fractionated Populations in Micro-well Cultures

Having examined the clonal proliferation of fractionated cell
populations in conventional agar cultures, studies were performed
to analyse the behaviour of these cells in microtitre liquid suspen-
sion cultures.

Figure 5.5 shows a typical example of the proliferative pattern
exhibited by 200 cells from human marrow fractions enriched for
promyelocytes-myelocytes or blast cells in wells containing unfraction-
ated bladder cancer cell conditioned medium. In wells lacking CSF,
the majority of the cells originally cultured were dead by day 2.

TABLE 5.3.   Relationship Between Day 14 Clones and Day 7 Clones in Mapped Areas of

Cultures of Blast Cell-Enriched Fractions

		Clone Status at Day 14		
		Colony	Cluster	Not Observed
Clone Status at Day 7	Colony	23	36	11
	Cluster	29	131	20
	Not Observed	0	12	0

Fate of day 7 colonies and clusters and origin of day 14 colonies and clusters in mapped areas of cultures of blast cell-enriched fractions obtained by fractionating normal marrow cells using WEM-G11.   HPCM was used as stimulus and 1,000 cells cultured per ml.   Results are number of examples of sequentially sampled clones from two bone marrow samples. Colonies contained >40 cells and clusters <40 cells.

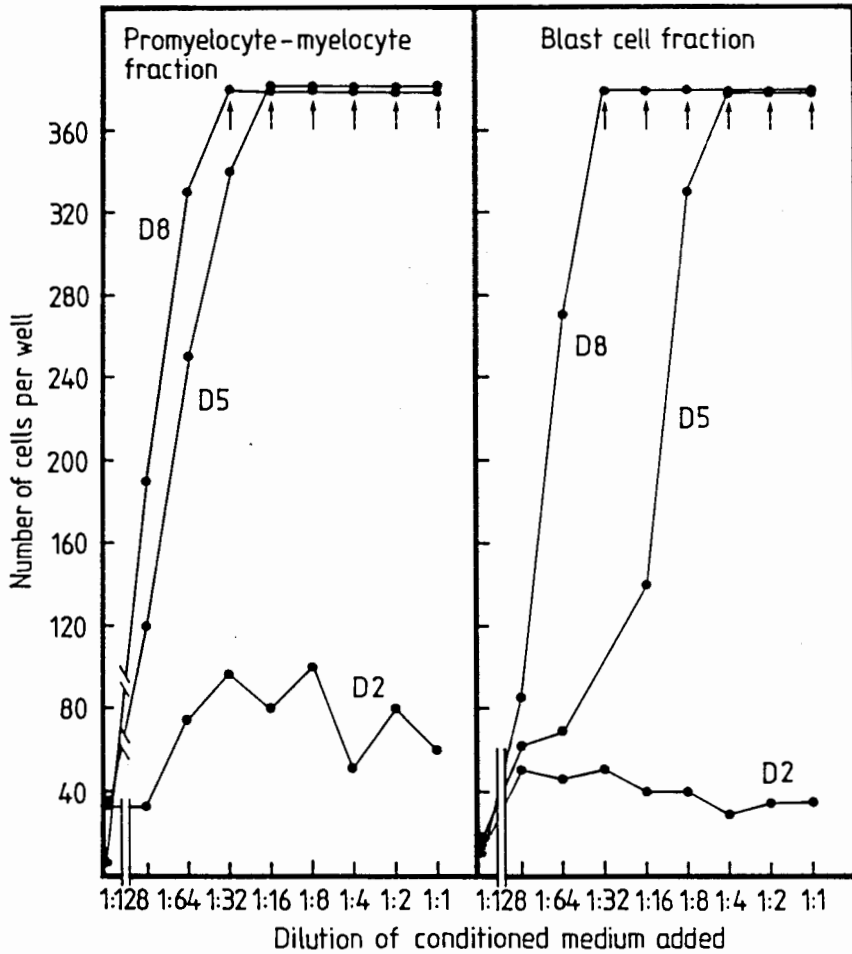Fig. 5.5 Cell proliferation induced by serial dilutions of unfractionated bladder cancer cell-conditioned medium in microwell cultures of 200 cells from promyelocyte-myelocyte-enriched or blast cell-enriched fractions of human marrow cells. Wells with the highest CSF concentration contained 12,500 units/ml of CSF. Each point is the mean cell count of two replicate wells at days 2, 5 and 8 of incubation.

At this time, wells containing increasing concentrations of CSF
contained 30-100 cells which represented probably CSF-induced survival
of the original cells and/or some stimulation of cell division. In
cultures of promyelocyte-myelocytes, concentration-dependent CSF
stimulation of proliferation was clearly evident by day 5 of incuba-
tion and this was more marked by day 8. After this time, wells
exhibited little further proliferation. In cultures of blast cell-
enriched fractions, initiation of proliferation was slower and more
variable than in cultures of promyelocytes-myelocytes, resulting in
a smaller increase in cell numbers by day 5. By day 8 however,
extensive concentration-dependent CSF stimulation of proliferation
was again evident.

The delayed onset of proliferation observed in microwell cultures
was consistent with the delayed onset of clonal proliferation observed
in agar cultures of the blast cell-enriched fraction (Table 5.1).
The apparent discrepancy between the time of maximum clone numbers
in agar cultures (between 3-5 days) and the time of maximum cell
numbers in liquid cultures (8 days) is probably explained by the
dispersion of clone-cells into interclonal areas in agar cultures.

It should be noted in the example in Figure 5.5 that while there
was some persistence of surviving cells in unstimulated cultures of
both cell fractions, there was usually no evidence of proliferation
by such cells, suggesting that the fractionation procedure had removed
most endogenous CSF-producing cells. This was also in keeping with
the observations in agar cultures, where up to 10 clones developed
per 10^3 cells in unstimulated cultures of blast cell and promyelocyte-
myelocyte fractions.

The data suggest that only about 20-30% of cells in the promyelo-cyte-myelocyte fraction were capable of CSF-stimulated proliferation, a finding also in agreement with data from the agar culture of such cells.

Similar kinetics of initiation and extensiveness of proliferation were observed in cultures stimulated by unfractionated HPCM and in cultures stimulated either by semipurified human CSF-α or β prepared by hydrophobic chromatography from bladder cancer cell conditioned medium (Figure 5.6). It is evident from Figure 5.6 that both cell fractions responded to stimulation by both forms of human-active CSF although again a relatively delayed and more restricted prolifer-ative response was observed with cells from the blast cell-enriched fractions. Although in Figure 5.6 CSF-β stimulated a greater proliferative response than equivalent concentrations of CSF-α, this was not a consistent finding with fractions from different marrow specimens. This patient-to-patient variability in responsiveness to CSF-α and β has also been observed in agar cultures of unfraction-ated marrow cells and may be based on the presence of varying propor-tions of subsets of granulocyte-macrophage precursors (Morstyn et al, 1981). Weak proliferative responses of blast cells to stimulation by CSF-α as exemplified in Figure 5.6 are also ascribable to the fact that the progenitors stimulated by CSF-α tend to exhibit their major proliferation in the second week of culture (Morstyn et al, 1981). In both the microwell cultures and agar cultures, the promyelocyte-myelocyte fractions were slightly more responsible to CSF than the blast cell fractions.
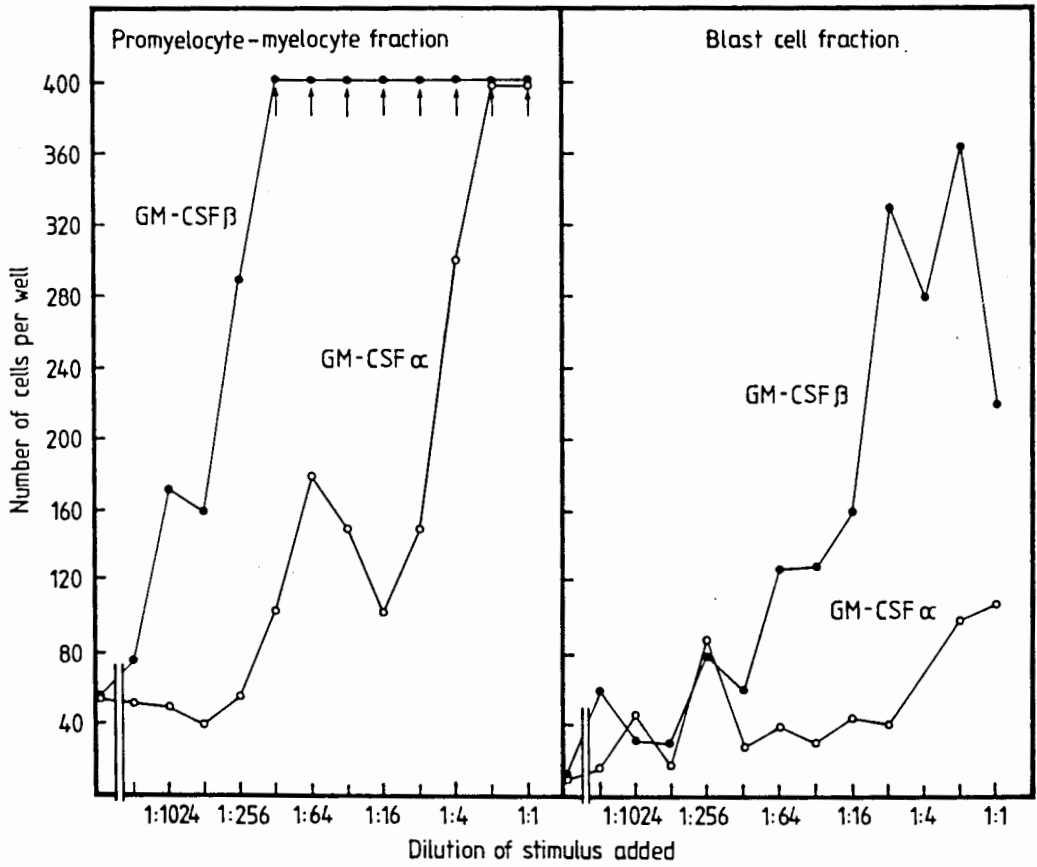
Fig. 5.6   Cell proliferation induced by CSF-α or CSF-β in microwell

cultures of 200 cells from promyelocyte-myelocyte-enriched

or blast cell-enriched fractions of human marrow cells.

CSF-α and β were semipurified preparations from bladder

cancer cell-conditioned medium and wells with the highest

CSF concentration contained 8000 units/ml of CSF.   Each

point represents the mean cell count of two replicate

wells at day 8 of incubation.

5.2.4.1 <u>Responsiveness to Stimulation by Purified Murine</u>

<u>CSF's</u>. Early experience using agar cultures of human marrow cells stimulated by unfractionated conditioned media suggested that murine CSF's were unable to stimulate the proliferation of human granulocyte-macrophage precursor cells since no obvious colony formation was observed after 7 or 14 days of incubation (Metcalf, 1977).

With the availability of purified murine G-CSF, GM-CSF, M-CSF and Multi-CSF, this question was re-explored using microwell cultures containing fractions enriched either for promyelocytes-myelocytes or blast cells. The results of one such experiment are shown in Figure 5.7. It was usual in cultures of blast cell fractions for none of the four murine CSF's to be able to stimulate any significant cell proliferation. However, in cultures of fractions enriched for promyelocytes and myelocytes, G-CSF was consistently able to stimulate cell proliferation. Analysis of the progeny cells produced following stimulation for 8 days by G-CSF (Table 5.4) showed that murine G-CSF stimulated mainly the formation of maturing granulocytes although small numbers of monocytes were also produced. The composition of this progeny population was essentially similar to that of populations generated after stimulation by human-active CSF's.

Murine Multi-CSF and M-CSF failed to stimulate cell proliferation in cultures of human promyelocytes and myelocytes. GM-CSF also usually failed to stimulate proliferation but with promyelocyte-myelocyte fractions from some marrow samples moderate cell proliferation was observed. In each of these latter instances the fraction contained 5-30% monocytes and the action of GM-CSF may have been indirect.

5.2.4.2 <u>Development of Adherent Spindle-Shaped Cells</u>.

In cultures from fractions enriched for promyelocytes and myelo-cytes, all cells remain spherical and non-adherent to the wells throughout the incubation period. However in cultures of blast cell-
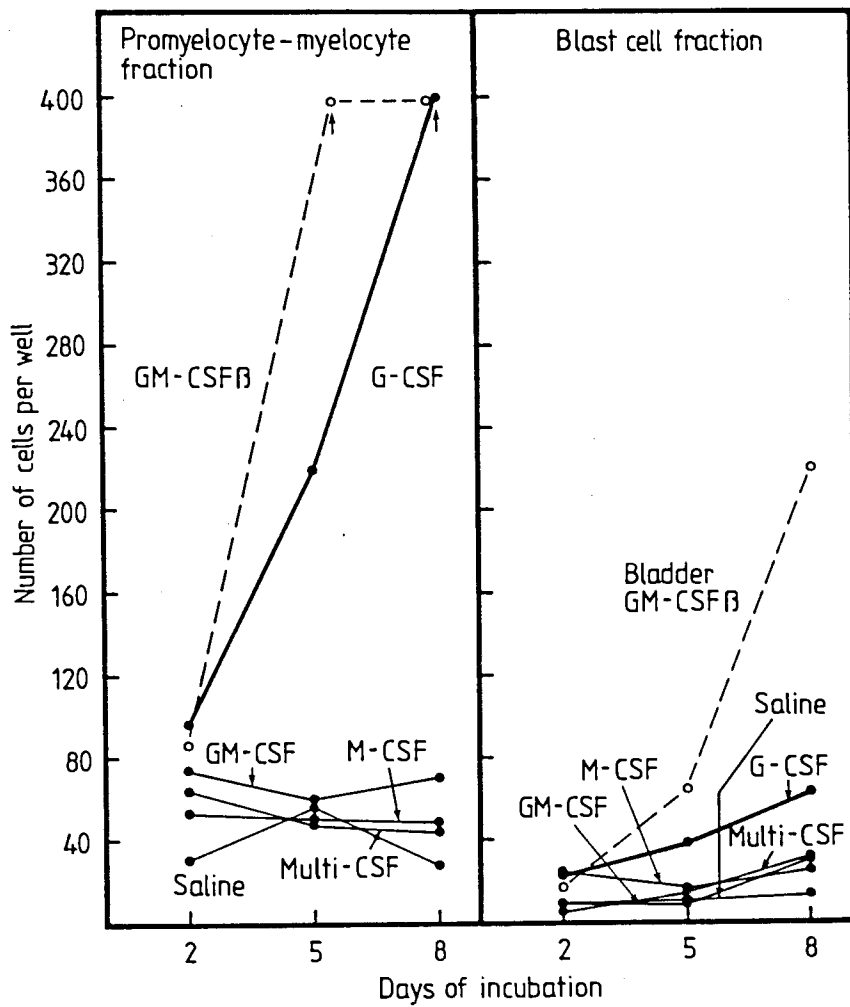
Fig. 5.7 Cell proliferation stimulated by 25 units/well of purified mouse G-CSF in cultures of 200 cells from promyelocyte-myelocyte-enriched or blast cell-enriched fractions of human marrow cells. Each point represents mean cell counts of two replicate wells. Note the inability of 25 units of purified murine GM-CSF, M-CSF or Multi-CSF to stimulate significant cell proliferation. Control wells were stimulated by 40 units of CSF-β from bladder cancer cell-conditioned medium.

TABLE 5.4. Morphology of Progeny Generated by Fractionated Human Marrow Cells After Stimulation by Mouse G-CSF

Cells Cultured	Stimulus	Percentage of Cells					
		Blasts	Promyelocytes	Myelocytes	Metamyelocytes and Polymorphs	Monocytes	Eosinophils
Promyelocyte-myelocyte fraction	HPCM	0	11	31	50	8	0
	G-CSF	0	26	37	35	2	0
Blast cell fraction	HPCM	0	13	39	34	10	4
	G-CSF	2	20	38	25	15	0

200 cells from promyelocyte-myelocyte-enriched fractions were cultured per well in 15 µl medium containing 50 units CSF in partially purified human placental conditioned medium (HPCM) or 40 units of purified murine G-CSF.

Cells were harvested from microculture wells after 8 days of incubation and stained with May-Grumwald Giemsa.

No erythroid or lymphoid cells were detected.

enriched fractions, after day 5, the progressive development of adherent,
often spindle-shaped, cells was observed.   No such cells developed
in cultures lacking added CSF but as shown in Table 5.5, small numbers
of the adherent spindle-shaped cells developed in cultures containing
human or murine CSF's.   The number of such cells was higher in cultures
containing higher numbers of progeny cells although the ratio of
adherent to non-adherent cells varied widely between different cultures.
It is of interest that even the murine CSF's unable to stimulate
significant proliferation were able to elicit the formation of some
adherent cells.

The nature and origin of these adherent cells have not been
further investigated although they resemble closely the adherent
macrophages noted previously in microwell cultures of murine
hemopoietic cells formed by purified progenitor cells following CSF
stimulation (Nicola and Metcalf, 1982).

5.2.4.3   Sensitivity of Microassay Compared with Colony

Assay.   The sensitivity of the microassay to CSF
was compared with the sensitivity of the agar assay and one result is
shown in Figure 5.8.   There was no difference in the CSF-responsive-
ness of the blast cell fraction when assayed either in agar cultures
or microwell cultures.   The promyelocyte-myelocyte cells were slightly
more CSF-responsive when studied in microcultures compared with agar
cultures. Figure 5.8 again illustrates the increased CSF-responsive-
ness of the promyelocyte-myelocyte fraction compared with the blast
cell fraction.

5.3   DISCUSSION

The studies described clearly documented the ability of normal
human promyelocytes and myelocytes to proliferate clonally in vitro.

TABLE 5.5.   Development of Spindle-Shaped Adherent Cells in Cultures

of Blast Cell Fractions

Stimulus	Total Cells per Well	Total Adherent Spindle Cells per Well
Unfractionated HPCM	350 ± 30	30 ± 4
Bladder CM -		
CSF-α	100 ± 10	17 ± 3
CSF-β	220 ± 25	16 ± 4
Pure G-CSF	63 ± 6	8 ± 2
Pure GM-CSF	30 ± 38	2 ± 3
Pure M-CSF	25 ± 14	12 ± 3
Pure Multi-CSF	23 ± 8	4 ± 2
Saline	13 ± 13	0 ± 0

Two hundred cells were cultured per well and scored after 8 days of
incubation.   Results are mean cell counts ± standard deviations from
four replicate wells.   Wells contained 50 units of human-derived CSF
or purified murine CSF's.
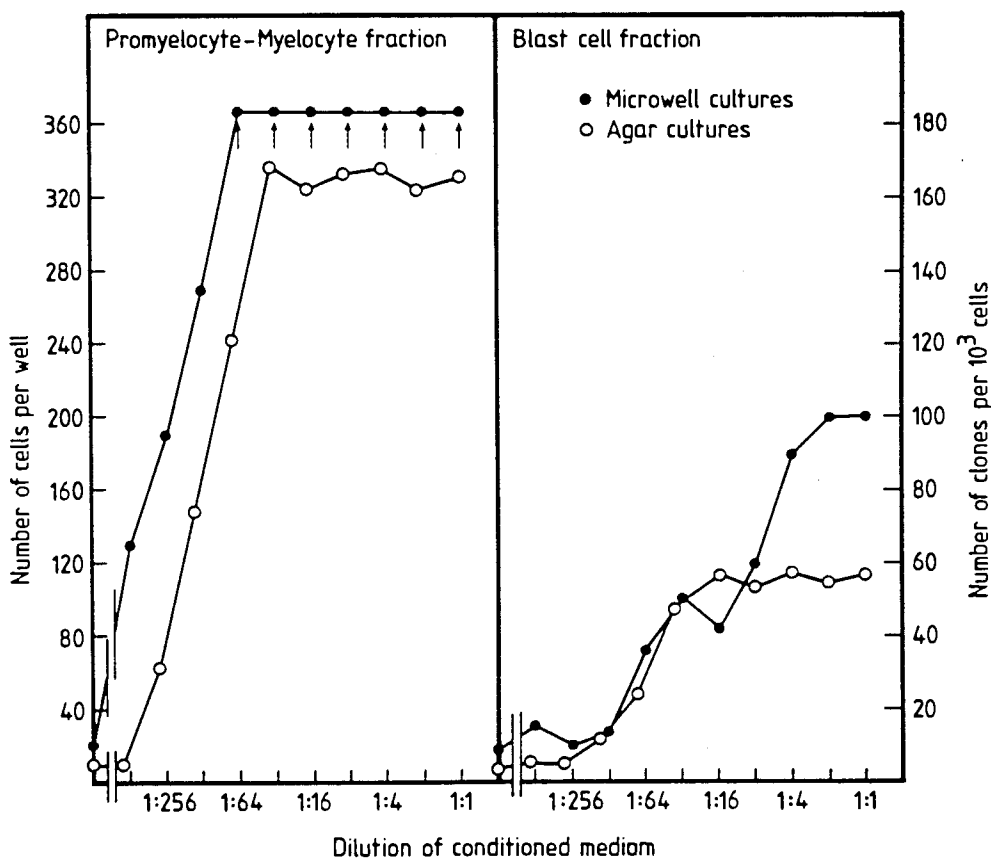
Fig. 5.8   Comparison of the dose-response curves to stimulation by

CSF of blast cell-enriched and promyelocyte-myelocyte

enriched fractions in agar cultures and microwell cultures.

In microwell cultures, 200 cells per well were stimulated

by serial dilutions of HPCM and replicate wells counted

at day 8 of culture. In agar cultures, 1000 cells per

1 ml culture were stimulated by serial dilutions of

HPCM and replicate cultures scored at day 5 for the

promyelocyte-myelocyte fraction and day 8 for the

blast cell fraction.

These cells generated maximum clone numbers between days 3 and 5 of culture and clones disappeared rapidly thereafter. These clones were primarily of subcolony size (less than 40 cells) at day 7 of culture although some colonies were generated by these cells. Up to 25-50% of promyelocyte-myelocytes were capable of in vitro proliferation and this proliferation was dependent on stimulation by CSF.

Both the blast cell and promyelocyte-myelocyte cell fractions proliferated in microwell cultures when stimulated by CSF. The frequency of responding cells and the pattern of proliferation observed was similar in both culture systems. In both agar cultures and microwell cultures the promyelocyte-myelocyte cells were more CSF-responsive than the blast cell fractions. The liquid culture system was not a more sensitive assay for CSF than agar cultures although this has been reported for other microwell culture systems (Schrader et al, 1981; Ihle et al, 1982; Tushinski et al, 1982; Burgess et al, 1982).

The transient proliferation observed for clones generated by promyelocytes and myelocytes explains the apparent failure of such cell populations to proliferate in vitro when scored at the conventional day 7 and day 14 timepoints (Griffin et al, 1981; Ball and Fanger, 1983; Griffin et al, 1983; Andrews et al, 1983; Strauss et al, 1983). In other studies the frequency of clonogenic cells was too low to detect proliferation of promyelocytes and myelocytes (Andrews et al, 1983; Strife et al, 1983). One study using fractionated human cells (Strife et al, 1983) did however demonstrate a tenfold fall in total clone numbers between days 3 and 7. These fractions contained enriched populations of promyelocytes and myelocytes and the pattern of proliferation is consistent with that described here.

The clones produced by the promyelocyte-myelocyte population had a mean of 26 cells per clone present at day 7. Thus the presence of a surface antigen detected by binding of monoclonal antibody allowed the separation of granulocytic cluster-forming cells (derived from promyelocytes-myelocytes) from the colony-forming cells (derived from the blast cell population). The observed size of the clones derived from the promyelocyte-myelocyte population at day 7 is greater than that predicted by mathematical models based on tritiated thymidine studies (Warner and Athens, 1964). However the majority of smaller clones have disappeared by this timepoint and the mean cell number per clone noted at day 7 in the present study probably overestimates the average size of clones formed by promyelocytes.

From the present data it is evident that promyelocyte-myelocyte fractions can generate characteristic dispersed granulocytic clones of transient nature and variable size. The largest and most persistent of these would be scorable as colonies at the conventional day 7 timepoint. It seems likely however that the occasional larger day 7 colonies and the day 14 colonies formed by this cell population originated from the small percentage of contaminating blast cells.

It should be noted that the promyelocytes-myelocytes recovered in procedure 2 comprised between 5-84% (mean 30%) of total promyelocytes-myelocytes in the starting cell suspensions and these cells were used for most of these studies. While it is possible that the remaining cells might exhibit different proliferative characteristics from the cells sorted by procedure 2, this is unlikely based on the results of experiments examining total clone numbers and the behaviour of individual clones generated by cell populations sorted by both procedure 1 and procedure 2. The increased enrichment obtained using procedure 2 made these populations of cells more suitable for study

but clearly may have resulted in the inadvertant selection of a sub-population of promyelocytes-myelocytes.

The high frequency of clonogenic cells and the early onset of clonal proliferation made the promyelocyte-myelocyte fractions an ideal population for study in a microwell assay system. Such culture systems are most effective in monitoring the proliferative effects of CSF's when uniformly CSF-responsive cell lines are used (Ihle et al, 1982; Schrader et al, 1981) or when cell populations are obtained from CSF-prestimulated cultures (Tushinski et al, 1982). The frequency of CSF-responsive cells is too low in human or murine bone marrow to allow the use of these cells in such systems without prior fractionation to produce an enriched population of target cells (Griffin et al, 1984; Burgess et al, 1982). While murine microassay systems show a heightened sensitivity to CSF this has not been shown for human microcultures (Griffin et al, 1984).

Using WEM-G11-fractionated populations of cells it was demonstrated that both target cell populations can be used to document CSF-induced cellular proliferation in microassay cultures. However, as neither enriched population was absolutely free of candidate accessory cells, the results cannot be used as formal evidence for the direct proliferative effects of CSF on human progenitor cells.

Both studies in agar cultures and microwell cultures suggested that the promyelocyte-myelocyte fractions were more sensitive to CSF than the blast cell fractions. While the difference between the populations was small, it is consistent with observations of others demonstrating that more differentiated cells are more CSF-responsive (Francis et al, 1981). The promyelocyte-myelocyte cells were slightly more CSF-responsive when studied in the microculture

assay compared with the agar assay, however this difference was not comparable to the heightened sensitivity of the murine microassays. This difference between the murine and human assays probably reflects a property of the cells being studied rather than being a consequence of liquid or agar cultures but also reflects the ability to detect CSF-induced survival of target cells in microwell cultures. The measurement of CSF-induced survival allows the detection of lower levels of CSF than are required to stimulate proliferation (Burgess et al, 1982).

Previous experiments had suggested that crude preparations containing murine CSF's were unable to stimulate clonal proliferation in cultures of human granulocyte-macrophage progenitors (Metcalf, 1977). However the present experiments using purified preparations of the four major murine CSF's have shown that G-CSF has a readily demonstrable capacity to stimulate proliferation of the human promyelocyte-myelocyte populations but little or no detectable capacity in this type of culture to stimulate fractions enriched for blast cells. Murine Multi-CSF and M-CSF exhibited no detectable proliferative effects on either human population and GM-CSF had variable effects that appeared to depend on the presence of monocytes in the target cell population. The previous failure to detect the activity of G-CSF in human cultures can be ascribed to the transient nature of clones formed in agar by the promyelocyte-myelocyte fractions.

As expected both from the action of murine G-CSF on normal mouse haemopoietic cells (Metcalf and Nicola, 1983) and from the morphology of clones generated by the promyelocyte-myelocyte fractions in agar cultures, the proliferation stimulated by murine G-CSF led to the formation almost exclusively of granulocytes.

Based on the results of these studied, the action of the human CSF's (α and β) and purified murine CSF's was examined in agar cultures of WEM-G11-fractionated cells.

CHAPTER 6

CLONAL PROLIFERATION OF NORMAL PROMYELOCYTES-MYELOCYTES STIMULATED BY

HUMAN AND MURINE COLONY STIMULATING FACTORS

6.1 INTRODUCTION

An intriguing aspect regarding the regulation of murine hemo-
poiesis is the evidence of overlapping actions of the CSF's. The
production of granulocyte colonies can be stimulated using GM-CSF
(Burgess and Metcalf, 1977), Multi-CSF (Ihle et al, 1983), or G-CSF
(Nicola et al, 1983; Metcalf and Nicola, 1983) while the production
of macrophage colonies can be stimulated by three molecules with
very different structures; GM-CSF, Multi-CSF and M-CSF (Burgess and
Metcalf, 1977; Ihle et al, 1983; Stanley et al, 1976). From clone
transfer studies it has been shown that both GM-CSF and Multi-CSF
can stimulate cells of the same clone (Metcalf et al, 1980). Similar
studies using human cells failed to confirm this redundancy and
suggested instead that the responsiveness to different CSF's was
differentiation-associated (Morstyn et al, 1981) with different sub-
sets of progenitor cells being responsive to one type of CSF.

In this respect, the results described in Chapter 5 were intrig-
uing. Normal human promyelocytes-myelocytes proliferated when stimul-
ated by both CSF-α and CSF-β. Although the small number of lympho-
cytes and monocytes in these cell populations did not allow conclusions
about direct effects of CSF, the results suggested that promyelocytes-
myelocytes were able to respond to two human CSF's or that subpopula-
tions of promyelocytes-myelocytes existed that were responsive to
only one type of CSF. Furthermore these cells also responded to murine
G-CSF. The experiments presented here were performed to further
analyse the response of normal human promyelocytes-myelocytes to
human CSF-α, CSF-β and murine G-CSF.

6.2 RESULTS

6.2.1 Clonal Proliferation Stimulated by Human CSF's

Table 6.1 shows the results of a typical culture experiment of cells sorted using the monoclonal antibody WEM-G11. Cells were stimulated by HPCM (a source of CSF-α and CSF-β), CSF-α alone or CSF-β alone. The day 14 colonies were generated by the blast cell-enriched fraction (and consisted of granulocyte, macrophage and eosinophil colonies as shown in Table 4.15). At days 7 and 14 of culture, the number of colonies stimulated by HPCM was greater than that stimulated by either CSF-α or CSF-β. CSF-α consistently stimulated day 14 rather than day 7 colony formation whilst CSF-β preferentially stimulated day 7 colony formation. These effects were consistent with previous descriptions of the activity of these molecules (Nicola et al, 1979; Morstyn et al, 1981). The promyelocyte-myelocyte-enriched fraction generated few day 7 colonies, and few clones (of 2 or more cells) persisted for 14 days regardless of the stimulus used.

The differences in proliferative responses following stimulation by CSF were again emphasized by a sequential analysis of 8 unselected normal human bone marrow samples. The results shown in Figure 6.1 are the mean ± standard deviation for total clone numbers generated by the blast cell-enriched and promyelocyte-myelocyte-enriched fractions when stimulated by CSF-α. The blast cell-enriched fractions generated maximum clone numbers at day 5 of incubation and the total clone numbers remained constant until day 14. In contrast, the promyelocyte-myelocyte-enriched fractions generated maximum clone numbers by day 5 of incubation with rapid disappearance of clones thereafter. The number of clones generated by 10^3 cells of this fraction at day 5 varied from 16-148 (mean 62) for the 8 marrow samples.

TABLE 6.1. Frequency of Clonogenic Cells in Subpopulations of Normal Human Bone Marrow Cells
Fractionated Using the Monoclonal Antibody WEM-G11

| Cell Population | Stimulus | Frequency per 10^4 Cultured Cells | | | |
| | | Day 7 | | Day 14 | |
		Colonies	Clusters	Colonies	Clusters
Light density marrow	HPCM	39	81	21	27
cells	CSF-α	10	105	18	37
	CSF-β	16	99	10	30
Blast cell-enriched	HPCM	153	314	200	267
	CSF-α	40	440	107	393
	CSF-β	94	376	74	257
Promyelocyte-myelocyte-	HPCM	85	320	0	30
enriched	CSF-α	0	530	0	35
	CSF-β	20	460	0	10

The blast cell-enriched population consisted of ~30% undifferentiated blasts. The promyelocyte-myelocyte-enriched
population consisted of ~40% of promyelocytes-myelocytes with the remainder being postmitotic metamyelocytes.
Results were obtained using supramaximal concentrations of the CSF preparations. For the light density
marrow cells 2 x 10^4 cells were cultured per ml. For the blast cell-enriched fraction 1.5 x 10^3 cells
cultured per ml and 10^3 cells per ml for the promyelocyte-myelocyte-enriched fraction. Colonies
contained 40 or more cells and clusters less than 40 cells.

Fig. 6.1 Total clone numbers followed over 14 days in cultures of cells from 8 normal human bone marrow samples fractionated using the monoclonal antibody WEM-G11. The results are mean values ± standard deviations expressed per 10^3 plated cells. For both populations replicate plates were examined and a maximum of 1500 cells cultured per ml. Cultures were stimulated by CSF-α alone.

Figure 6.2 shows the total number of clones generated following stimulation by CSF-β. The results are for the same 8 marrow samples shown in Figure 6.1. With stimulation by CSF-β, the blast cell-enriched fraction attained maximum clone numbers between days 5-7 of culture. Thereafter the total clone numbers declined, consistent with the reported "day 7" nature of this stimulus. With stimulation by CSF-β, 10^3 cells of the promyelocyte-myelocyte-enriched fraction generated maximum clone numbers at day 5 of incubation. The number of clones present at this time ranged from 35-167 (mean 84). Although not evident from the pooled data shown in Figures 6.1 and 6.2 the absolute numbers of clones generated by the promyelocyte-myelocyte-enriched fraction stimulated by CSF-α were only 36-94% (mean 74%) of the numbers stimulated by CSF-β. This difference was statistically significant when total clone numbers formed by individual marrow samples were compared ($p < 0.01$, Students method of paired differences test). There was no difference between the total number of clones stimulated by CSF-β and HPCM (a source of CSF-α and CSF-β) when respective marrow samples were compared (Table 6.2).

The total number of clones generated by the blast cell-enriched fraction and the promyelocyte-myelocyte-enriched fraction obtained from individual marrow samples was compared. For both Figure 6.1 (cultures stimulated by CSF-α) and Figure 6.2 (cultures stimulated by CSF-β), the differences in clone numbers obtained from the blast cell-enriched fraction when compared with the promyelocyte-myelocyte-enriched fraction were significant at the $p < 0.05$ level at the day 3, 5 and 14 timepoints (Students method of paired differences test).
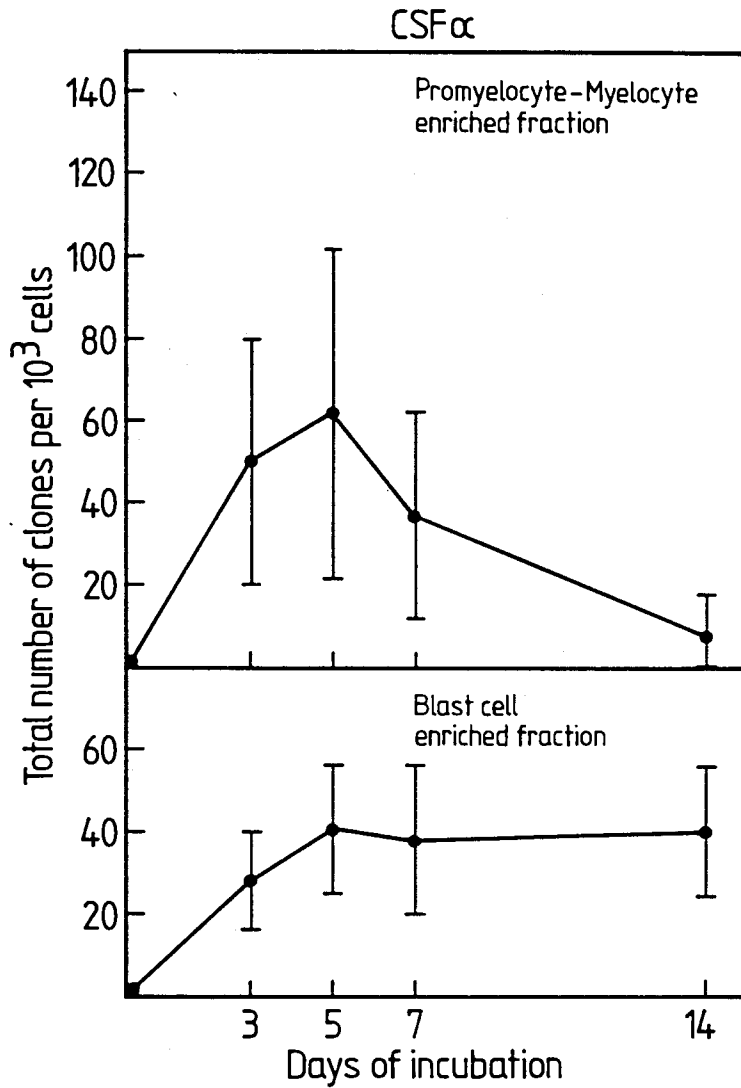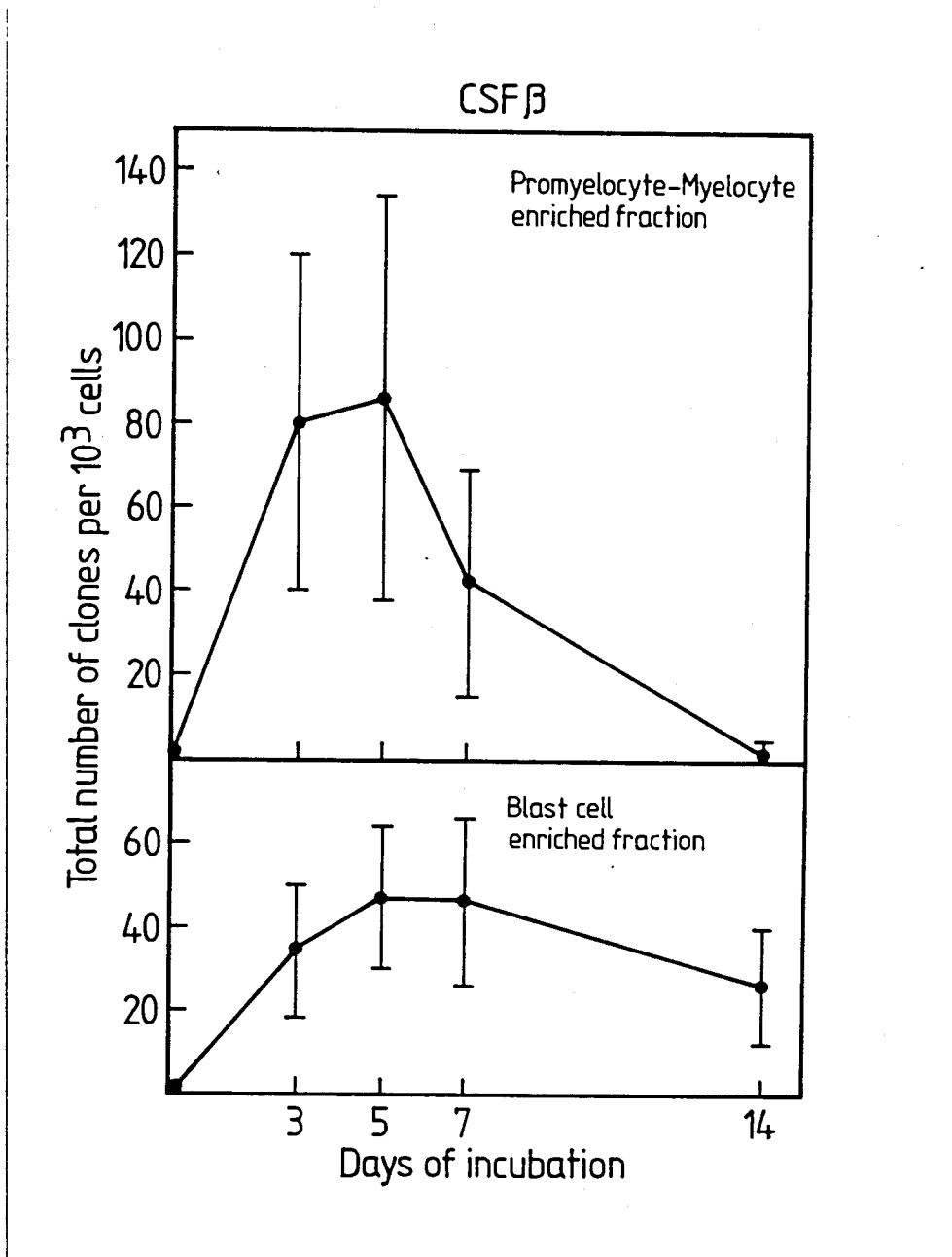
Fig. 6.2 Total clone numbers followed over 14 days in cultures of cells from 8 normal human bone marrow samples fractionated using the monoclonal antibody WEM-G11. The results are mean values ± standard deviations expressed per 10^3 plated cells. For both populations replicate plates were examined and a maximum of 1500 cells were cultured per ml. Cultures were stimulated by CSF-β alone.

TABLE 6.2. Frequency of Clonogenic Cells in Promyelocyte-Myelocyte Enriched Fractions

Experiment Number	Frequency Per 10^3 Cultured Cells								
	HPCM			CSF-α			CSF-β		
	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7
1	103	74	41	59	70	53	119	92	48
2	53	37	13	39	35	12	50	34	11
3	49	35	16	23	16	9	56	40	14
4	227	162	53	140	100	33	205	142	47

Results are mean number of clones generated by promyelocyte-myelocyte enriched fractions when stimulated by HPCM, CSF-α and CSF-β. 10^3 cells were cultured per ml and triplicate cultures scored at days 3, 5 and 7 of culture.

6.2.1.1 <u>Responsiveness of Both Populations to CSF</u>. The
responsiveness of both the blast cell-enriched and promyelocyte-
myelocyte-enriched populations to CSF-α and CSF-β is shown in
Figure 6.3. Cultures of the blast cell-enriched fraction were scored
on day 7 and of the promyelocyte-myelocyte-enriched fraction on day
5. Both populations appeared equally responsive to CSF-α, but the
promyelocyte-myelocyte population appeared more responsive to CSF-β
than the blast cell-enriched fraction. Both the blast cell-enriched
and the promyelocyte-myelocyte-enriched populations displayed some
proliferation in the absence of CSF. For both cell populations, these
clones were usually 2 cells in size with up to 10 clones developing
per 10^3 cultured cells.

6.2.1.2 <u>Clone Size of Promyelocyte-Myelocyte Fractions</u>.
Based on the titrations shown in Figure 6.3, supramaximal concentra-
tions of CSF-α and CSF-β were used in experiments to analyze the
size of clones generated by cells of the promyelocyte-myelocyte-
enriched fraction. A typical result is shown in Figure 6.4 where
the size of sequential clones was compared at days 5 and 7 of incuba-
tion. The clones stimulated by CSF-α were significantly smaller in
size at day 5 (p<0.01, λ^2 = 28.3) and at day 7 (p<0.05, λ^2 = 20.4)
than clones stimulated by CSF-β. Clones stimulated by CSF-α or CSF-β
were of smaller size than clones stimulated by HPCM (a source of
both CSF-α and CSF-β) (see Figure 5.3). The majority of clones formed
by cells of the promyelocyte-myelocyte fraction contained fewer than
40 cells at day 7 (clusters) regardless of the stimulus.

6.2.1.3 <u>Proliferation of Clones Following Transfer</u>. The
observed proliferation of promyelocyte-myelocyte populations following
stimulation by CSF-α and CSF-β was consistent with the possibility
either that two subsets of promyelocytes-myelocytes existed, each
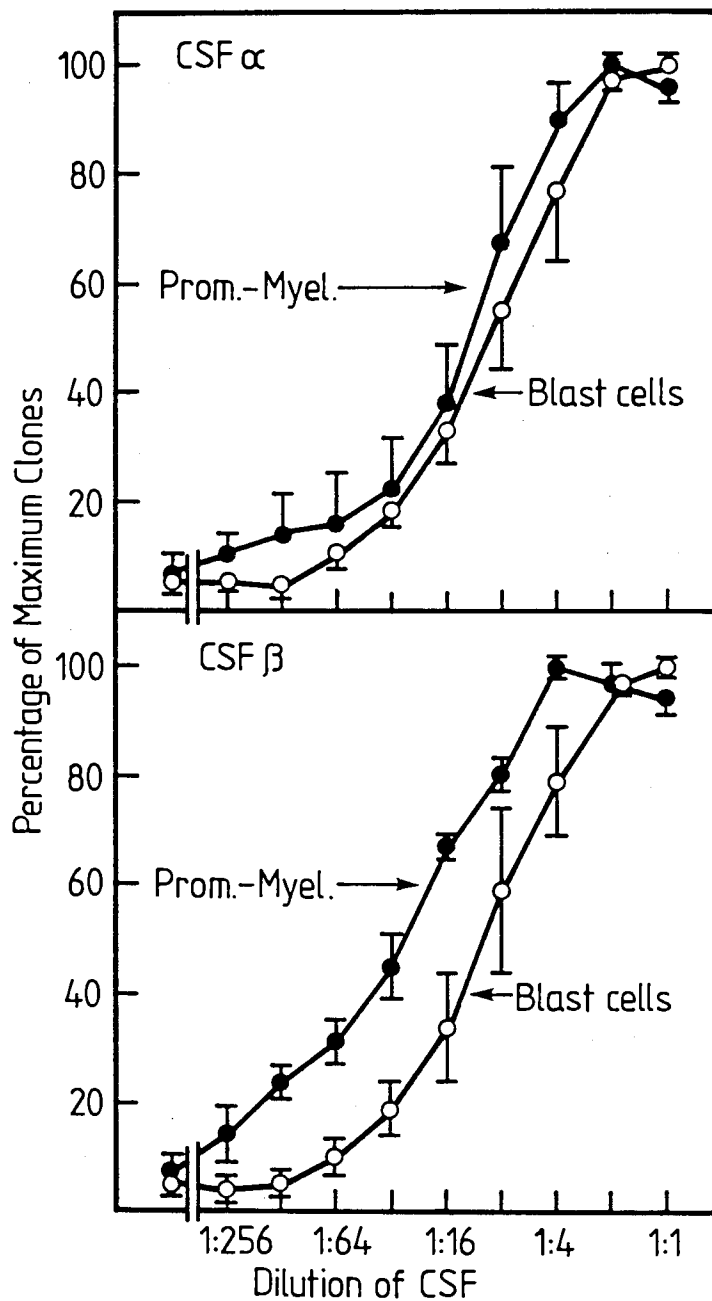
Fig. 6.3 CSF responsiveness of the blast cell-enriched population
(o———o) and the promyelocyte-myelocyte-enriched popula-
tion (●———●) when stimulated with CSF-α (Panel A) or
CSF-β (Panel B) following fractionation with the mono-
clonal antibody WEM-G11. Results are mean ± standard
errors for 3 normal marrow samples. Duplicate cultures
were scored on day 5 for the promyelocyte-myelocyte-
enriched fraction and day 7 for the blast cell-enriched
fraction. Cells were either cultured at 1500 cells
per ml (blast cell-enriched fraction) or 1000 cells per
ml (promyelocyte-myelocyte-enriched fraction) and
stimulated by serial dilutions of CSF.

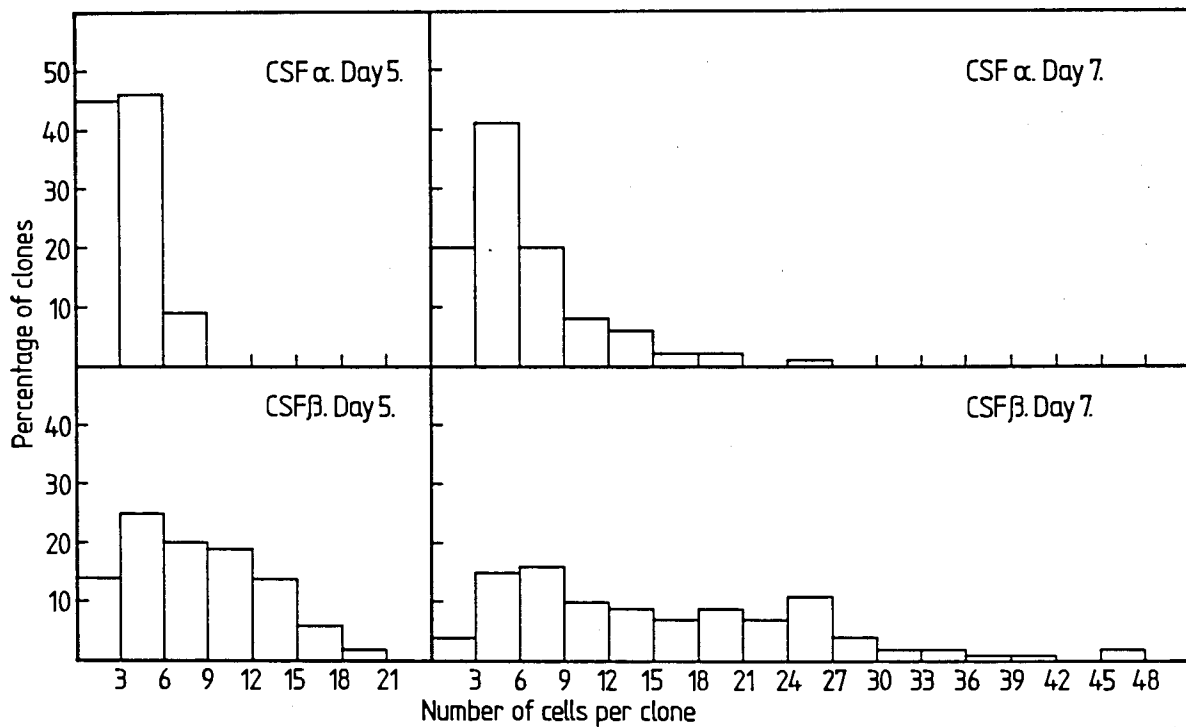Fig. 6.4 Size distribution of sequentially samples clones formed by

the promyelocyte-myelocyte-enriched fraction separated

from normal marrow cells using the monoclonal antibody

WEM-G11. Cells were cultured at 1000 cells per ml with

either CSF-α or CSF-β alone as the stimulus. Clones

were counted at day 5 and day 7 of culture. At least

96 sequential clones were examined at each timepoint.

responsive to only one form of CSF or that the majority of promyelo-cytes-myelocytes were responsive to both CSF-α and CSF-β. To test these possibilities, clone transfer experiments were performed. Following 2 days of incubation, clones that were initiated by either CSF-α or CSF-β were transferred to cultures containing CSF-α, CSF-β or no stimulus. The size of clones was recorded at the time of transfer and the majority were only 2 cells in size at this timepoint. Following a further three days of incubation, the size of clones was again recorded.

When clones initiated by CSF-α were transferred to CSF-β, 52% achieved a doubling in size (Figure 6.5A). This compared with 53% of clones that doubled in size upon transfer from CSF-α to CSF-α (Figure 6.5B) and 13% that doubled in size following transfer to no stimulus (Figure 6.5C). This indicated that both CSF-α and CSF-β were able to stimulate the progeny of promyelocytes-myelocytes initiated by CSF-α. In comparison, for clones initiated by CSF-β, 50% achieved a doubling in size upon transfer to CSF-α (Figure 6.5D) 65% doubled in size upon transfer to CSF-β (Figure 6.5E) and 15% doubled in size following transfer to no stimulus (Figure 6.5F) (see Table 6.3 for statistical analysis). The lower percentage of CSF-β-initiated clones doubling in size following transfer to CSF-α (Figure 6.5D) compared with CSF-β (Figure 6.5E), while not statistic-ally significant, suggested that CSF-α might be a less adequate stimulus for clones initiated by CSF-β. These results indicated how-ever that both CSF-α and CSF-β were able to stimulate the majority of progeny of promyelocytes-myelocytes initiated by CSF-β.

More clones showed a greater than three-fold size increase following transfer to CSF-β than to CSF-α regardless of the initiat-ing stimulus ($p < 0.05$, $\lambda^2 = 5.4$). This suggested that while promyelo-
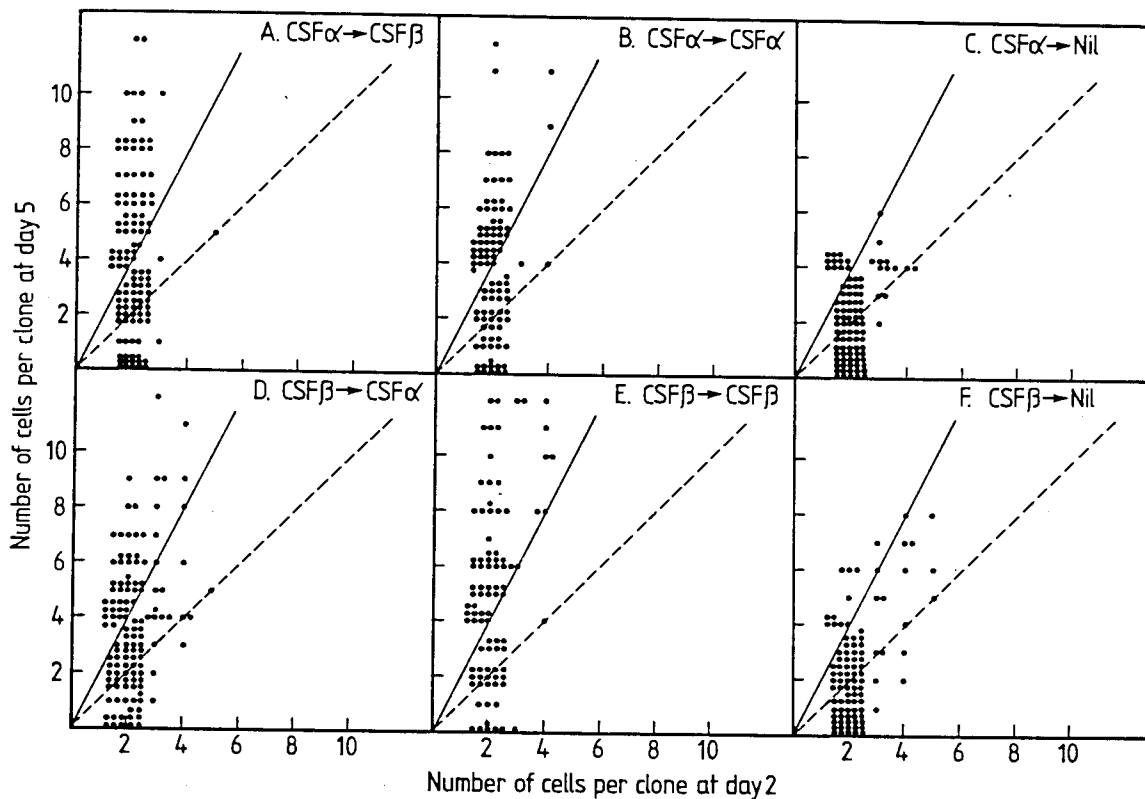
Fig. 6.5 Fate of clones generated from the promyelocyte-myelocyte-
enriched fraction obtained from normal human marrow
fractionated with the monoclonal antibody WEM-G11. Cells
were cultured in the presence of CSF-α or CSF-β for 2 days
then clones transferred to cultures containing CSF-β,
CSF-α or no stimulus. Size was compared following a
further 3 day incubation period. Each point represents
an individual clone. Similar results were obtained from
the two marrow samples shown. The solid line represents
the size attained had the clone doubled in size following
transfer. Broken line indicates clone size remaining
unchanged following transfer. See Table 6.3 for
statistical analysis.

TABLE 6.3. Chi Square Analysis of Significance of Data in Figure 6.5

Panel	λ^2	Total number of clones transferred	p value
A x B	0.005	211	NS
A x C	12.0	209	<0.01
B x C	12.4	192	<0.01
D x E	0.77	215	NS
D x F	9.7	211	<0.01
E x F	14.9	180	<0.01
C x F	0.09	183	NS

Chi square analysis of clones that doubled in size following transfer compared with those that did not.

cytes-myelocytes could respond to both CSF-α and CSF-β, CSF-β appeared to be a superior stimulus for these cells.

As expected, some clones disappeared during the three day culture period after transfer, even in cultures containing CSF. This is consistent with earlier observations that 20% of clones disappear by day 5 of culture (Table 5.1). Many clones initiated by either CSF-α or CSF-β exhibited limited proliferation following transfer to cultures with no stimulus, and some generated clones twice the size of the original clone (Figure 6.5C, 6.5F).

6.2.2 Clonal Proliferation Stimulated by Murine CSF's

The clonal proliferation of blast cell and promyelocyte-myelocyte fractions was examined when stimulated by murine CSF's. A typical result is shown in Table 6.4 for fractionated cells stimulated by murine G-CSF. Murine G-CSF stimulated a small number of clones from the blast cell fraction and in some marrow samples these clones formed colonies at day 7 and day 14. The action of murine G-CSF was more striking when the promyelocyte-myelocyte fractions were examined. In these fractions murine G-CSF stimulated many clones to develop with numbers of clones being comparable to the number stimulated by human CSF-β.

The responsiveness of the promyelocyte-myelocyte cells to murine G-CSF is shown in Figure 6.6. Murine GM-CSF, M-CSF and Multi-CSF were inactive on these cells (and on the blast cell fractions). The dose-response curves for proliferation of promyelocyte-myelocytes were almost identical in shape when cells were stimulated by CSF-α, CSF-β and murine G-CSF (Figure 6.7a). This was compared with the dose-response curves for inducing differentiation of WEHI-3B D$^+$ colonies where CSF-β and murine G-CSF also showed very similar dose-response curves while CSF-α was inactive (Figure 6.7b). Interestingly,

TABLE 6.4. Frequency of Clonogenic Cells in Subpopulations of Normal Human Bone Marrow Cells Fractionated Using Monoclonal Antibody WEM-G11 and Stimulated by Murine G-CSF

Cell Population	Stimulus	Frequency Per 10^3 Cultured Cell				
		Day 5	Day 7		Day 14	
		Clusters	Colonies	Clusters	Colonies	Clusters
Blast cell-enriched	Nil	6	0	3	0	1
	HPCM	56	14	42	18	36
	CSF-α	55	0	54	7	42
	CSF-β	52	10	44	3	27
	G-CSF	21	1	20	3	14
Promyelocyte-myelocyte-enriched	Nil	7	0	6	0	0
	HPCM	162	2	51	0	1
	CSF-α	100	0	33	0	0
	CSF-β	142	2	45	0	0
	G-CSF	150	0	51	0	0

Results are mean number of clones from replicate cultures stimulated by different CSF's (as shown) 10^3 cells were cultured per ml for both cell populations. Colonies consisted of 40 or more cells and clusters less than 40 cells (but greater than 2 cells).
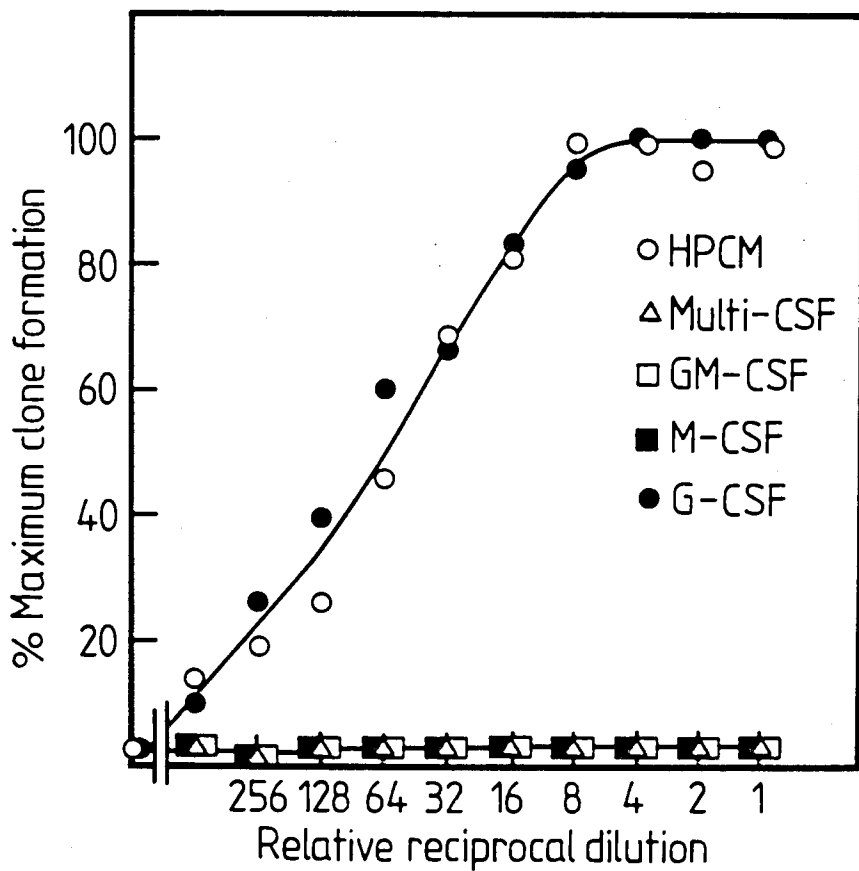
<u>Fig. 6.6</u> Ability of murine G-CSF but not murine Multi-CSF, GM-CSF

nor M-CSF to stimulate clonal proliferation of human

promyelocyte-myelocyte cells when compared with HPCM.

Each CSF was titrated in serial 2-fold dilutions.

Clones were scored at day 5 of culture. 10^3 cells were

cultured per ml.
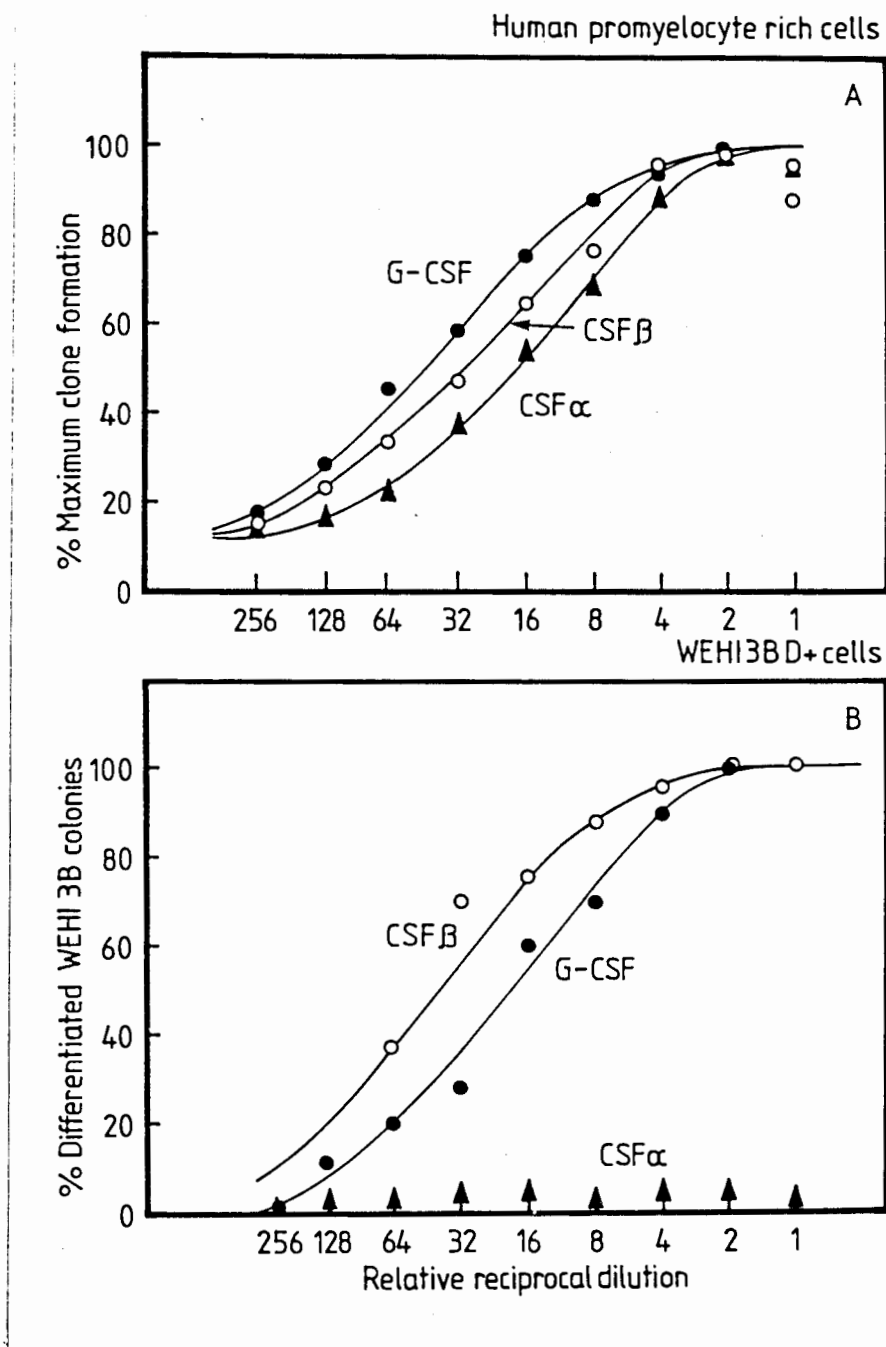
Fig. 6.7 Ability of human CSF's and murine G-CSF to stimulate clonal proliferation of human promyelocyte-myelocyte cells or induce differentiation in murine WEHI-3B D+ leukaemia cells. Each CSF was titrated in serial 2-fold dilutions. Clones from human promyelocytes-myelocytes were scored at day 5 of culture. 10^3 cells were cultured per ml. The same CSF's were assayed for their ability to induce differentiation in murine WEHI-3B D+ colonies after 7 days of culture. The 1:1 dilutions of samples in panel B were 8-fold diluted relative to the 1:1 dilution of samples in panel A.

the dose-response curves for inducing differentiation of WEHI-3B D$^+$ colonies were left-shifted by 8-fold for both CSF-β and murine G-CSF compared with their dose-response curves for human progenitor cells. The action of murine G-CSF on human promyelocytes-myelocytes was also very similar to the action of CSF-β on these cells both in terms of the total number of clones stimulated (Table 6.4) and the size of clones (Table 6.5). The number of cells per clone was 13.1 ± 7.6 (mean ± S.D.) for G-CSF, 15.9 ± 10.6 for CSF-β and 7.0 ± 4.7 for CSF-α after 7 days of culture. There was no significant difference between the size of clones stimulated by G-CSF and CSF-β (p<0.80, λ^2 = 9.7) while clones stimulated by CSF-α were significantly smaller (Figure 6.4).

6.3 DISCUSSION

These studies showed that normal human colony-forming cells and cluster-forming cells were responsive to both CSF-α and CSF-β. While the colony-forming cell (blast cell) population and the promyelocyte-myelocyte population appeared equally responsive to CSF-α, the promyelocyte-myelocyte population was more responsive to CSF-β than the colony-forming cell population.

Due to contaminating lymphocytes and monocytes, firm conclusions regarding the direct effects of CSF cannot be reached from observations of the blast cell population. However the promyelocyte-myelocyte population is more suitable for the study of the direct effects of CSF, being relatively free of cells capable of generating CSF. Clone transfer techniques were also employed to document the direct action of·both CSF-α and CSF-β upon the progeny of promyelocyte-myelocyte clones. The transfer of clones to secondary plates results in a 500-fold dilution of the initiating CSF and guarantees that

TABLE 6.5   Size Distribution of Clones Derived From Promyelocytes-myelocytes Fractionated
Using Monoclonal Antibody WEM-G11

Stimulus	Number of Cells Per Clone							
	2-6	7-12	13-18	19-24	25-30	31-36	37-42	43-49
CSF-β	19	26	16	16	15	4	2	2
G-CSF	13	40	24	15	4	2	2	

Results are number of clones as percentages.   Sequentially sampled clones were examined in cultures of 10^3 cells

per ml stimulated by CSF-β and murine G-CSF.   Clone size was examined after 7 days of culture and a minimum of

100 consecutive clones examined.   There was no significant difference between clones stimulated by G-CSF and

CSF-β (p<0.80, λ^2 = 9.7).   The results shown are for the same experiment shown in Figure 6.4.

these clones are free from cells capable of generating CSF (Metcalf and Merchav, 1982).

The observation that both CSF-α and CSF-β preparations stimulated the same clone could be explained by the incomplete separation of the two CSF's by the fractionation procedure. However, both fractions are widely separated from one another during the separation procedure, and moreover, CSF-α was able to stimulate eosinophil proliferation while CSF-β was not.

In cultures stimulated using a supramaximal concentration of CSF, the promyelocyte-myelocyte population generated fewer clones and clones of smaller size with CSF-α than with CSF-β. However, the transient nature and small size of clones formed by promyelocytes-myelocytes were independent of the type of CSF used, suggesting that this pattern of proliferative activity was an intrinsic property of the cells rather than a consequence of the type of CSF used.

There was considerable variation with different marrow samples in the comparative response of promyelocyte-myelocytes to CSF-α and CSF-β. The absolute number of clones generated by promyelocytes-myelocytes stimulated by CSF-α was between 34-94% of the number stimulated by CSF-β. In the titration curve shown in Figure 6.3 there was no evidence of high-dose inhibition with the CSF-α preparation and plateau levels of clone formation were apparently obtained. This would suggest that a small and variable subpopulation of promyelocytes-myelocytes were CSF-α-unresponsive. The CSF-α preparation was, however only partially purified and an alternative possibility was that this preparation contained non-specific inhibitory material to which cells from some individuals were more sensitive than others. This could have been discounted by performing a full titration curve on all samples studied or by using fully purified material.

One study (Morstyn et al, 1981) demonstrated that granulocyte-macrophage clones from cultures of unfractionated human marrow would proliferate following transfer from CSF-α to CSF-β but failed to demonstrate the converse. This failure may reflect a genuine differ-ence in the responsiveness of a subset of cells within the blast cell population or may have been artifactual because of the hetero-geneous population of clonogenic cells used, the lower frequency of genuinely clonogenic cells sampled or the later timepoints used for the study. The present results indicating that both human CSF's are active upon both normal colony and cluster-forming cell popula-tions is consistent with reports of both molecules stimulating the functional activity of mature granulocytes (Vadas et al, 1983; Vadas et al, 1983a) although it remains possible that these mature cells contain subpopulations responding better to one type of CSF than the other.

The present data on the dual responsiveness of individual human GM precursor cells to stimulation by both CSF-α and CSF-β are in agreement with the data from murine GM precursor cells that indicate that individual cells are able to bind and respond to stimulation by more than one type of CSF (Metcalf, 1985, 1985a).

Most murine haemopoietic growth factors were inactive on human cells but the species cross-reactivity of murine G-CSF demonstrated here appeared quite unambiguous. Both CSFβ and G-CSF are hydrophobic molecules of similar size (30,000 and 25,000 respectively) (Nicola et al, 1979; Nicola et al, 1983) and both were able to stimulate granulocyte colony formation by cells from both species with similar dose-response relationships and similar numbers of target cells responsive to either stimulus. The similarities in biological properties of CSF-β and G-CSF suggested that these two molecules might be closely related. This was of particular importance given

the action of G-CSF in inducing differentiation in murine WEHI-3B D$^+$ leukaemia cells and the possible potential implications this may have for human leukaemias. For these reasons, further studies were performed to examine the biological properties of human leukaemic promyelocytes and the role G-CSF might play with respect to these cell populations.

CHAPTER 7

COMPARATIVE STUDY OF NORMAL AND LEUKAEMIC

PROMYELOCYTES-MYELOCYTES

7.1 INTRODUCTION

These studies were performed to examine the clonal proliferation in vitro of human leukaemic promyelocytes-myelocytes. Numerous monoclonal antibodies have been described that identify surface antigens present on both normal and leukaemic cells (Griffin et al, 1981; Young and Hwang-Chen, 1981; Morstyn et al, 1981; Bernstein et al, 1982; Strauss et al, 1984) and thus monoclonal antibody WEM-G11 was predicted to identify a population of leukaemic promyelocytes-myelocytes equivalent to the normal cells identified by this antibody. Furthermore, studies on CML progenitor cells have demonstrated that the day 7 and day 14 subsets of GM progenitors are preserved in this disease (Broxmeyer et al, 1978) and sedimentation velocity studies have shown that the most rapidly sedimenting CML cells parallel the transient proliferative behaviour of normal cells in vitro, although the cells responsible for this transient proliferation were not identified (Strife et al, 1983). In contrast, normal in vitro growth characteristics are not preserved in acute myeloid leukaemias and there does not appear to be any correlation between the morphological subtype and the in vitro growth pattern (Moore et al, 1974; Spitzer et al, 1976; Elias and Greenberg, 1977).

7.2 RESULTS

7.2.1 Clonal Proliferation of AML (M3) Promyelocytes

Leukaemic promyelocytes were obtained from patients with acute promyelocytic leukaemia (AML M3). The clonal proliferation of these cells was studied using cultures containing 10^3 cells. The behaviour of the leukaemic-derived clones throughout the incubation period is shown for 6 patients in Figure 7.1. In contrast to normal promyelocyte-myelocyte clones, clones generated by these cells showed no uniform pattern of behaviour. In two examples, clones were transient
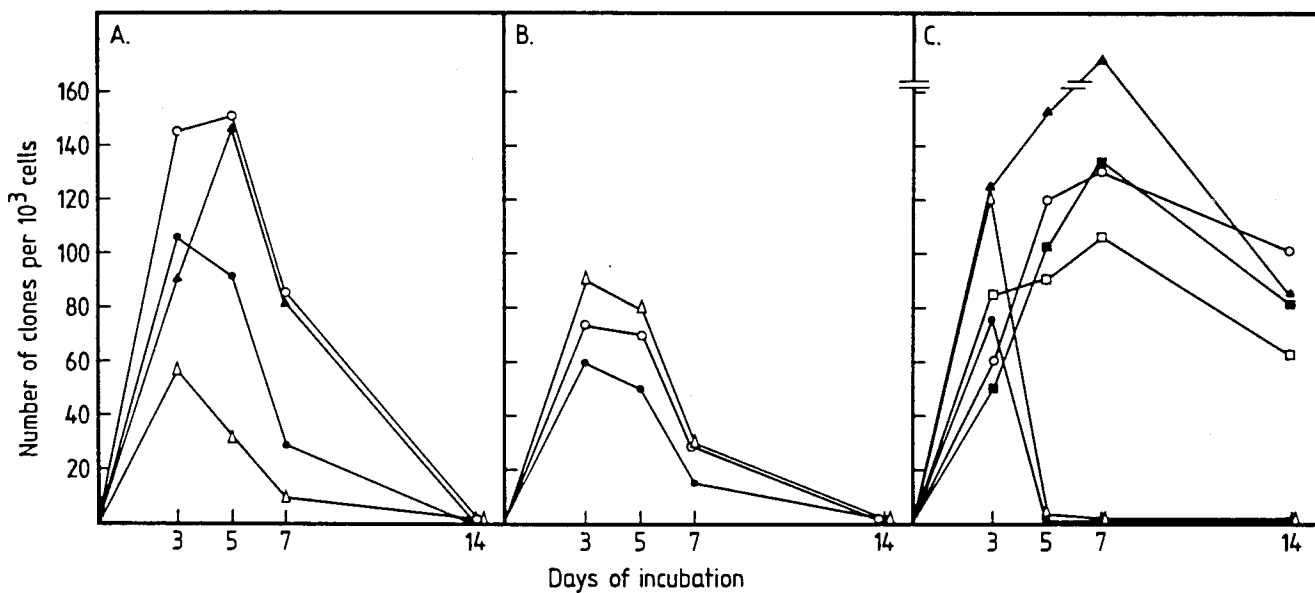
Fig. 7.1 Clonal proliferation in vitro of normal promyelocytes-

myelocytes (panel A), CML blood promyelocytes-myelocytes

(panel B) obtained using monoclonal antibody WEM-G11 and

the FACS, and acute leukaemic promyelocytes from patients

with AML M3 (panel C). 10^3 cells were cultured per ml and

the total number of clones examined throughout the incuba-

tion period. Results are means of replicate cultures.

All cultures were stimulated by HPCM. A maximum of 290

clones was generated by one leukaemic sample (panel C).

with no clones present at the day 7 or 14 timepoints. This transient
proliferation, was not observed in the other examples and in 3 cases
the total clone numbers remained relatively constant between days 7
and 14. There was no difference between cells obtained from blood
or bone marrow of these patients.

The responsiveness of these leukaemic promyelocytes to different
CSF's was examined for 3 patients, and the titration curves for HPCM,
CSF-α, CSF-β and murine G-CSF are shown in Figure 7.2. The responsive-
ness of the leukaemic cells was very similar for each of the CSF
preparations examined and in each case showed no significant differ-
ence to the responsiveness of populations of normal human promyelo-
cytes-myelocytes. All samples failed to display any proliferation
in the absence of CSF, even when cells were cultured at 3-4 x 10^4
cells/ml.

The behaviour of clones following stimulation by different CSF's
was examined both in terms of total clone numbers and behaviour of
individual clones. Figure 7.3 shows one example of total clone numbers
followed throughout the incubation period when cells were stimulated
by different CSF's. The absolute number of clones stimulated was
the same for HPCM, CSF-β and murine G-CSF. Fewer clones were stimul-
ated by CSF-α (between 70-75% in different experiments). Based on
the titration curve shown in Figure 7.2 this suggested that a subgroup
of leukaemic promyelocytes were CSF-α-unresponsive. There was, however
no significant difference between the size of clones stimulated by
CSF-α compared with those stimulated by CSF-β, or murine G-CSF
(Table 7.1).

To examine the onset of clonal proliferation and the subsequent
fate of clones, a series of plate mapping experiments were performed
comparing the behaviour of leukaemic clones stimblated by different
CSF's. A typical result is shown in Table 7.2 and refers to the
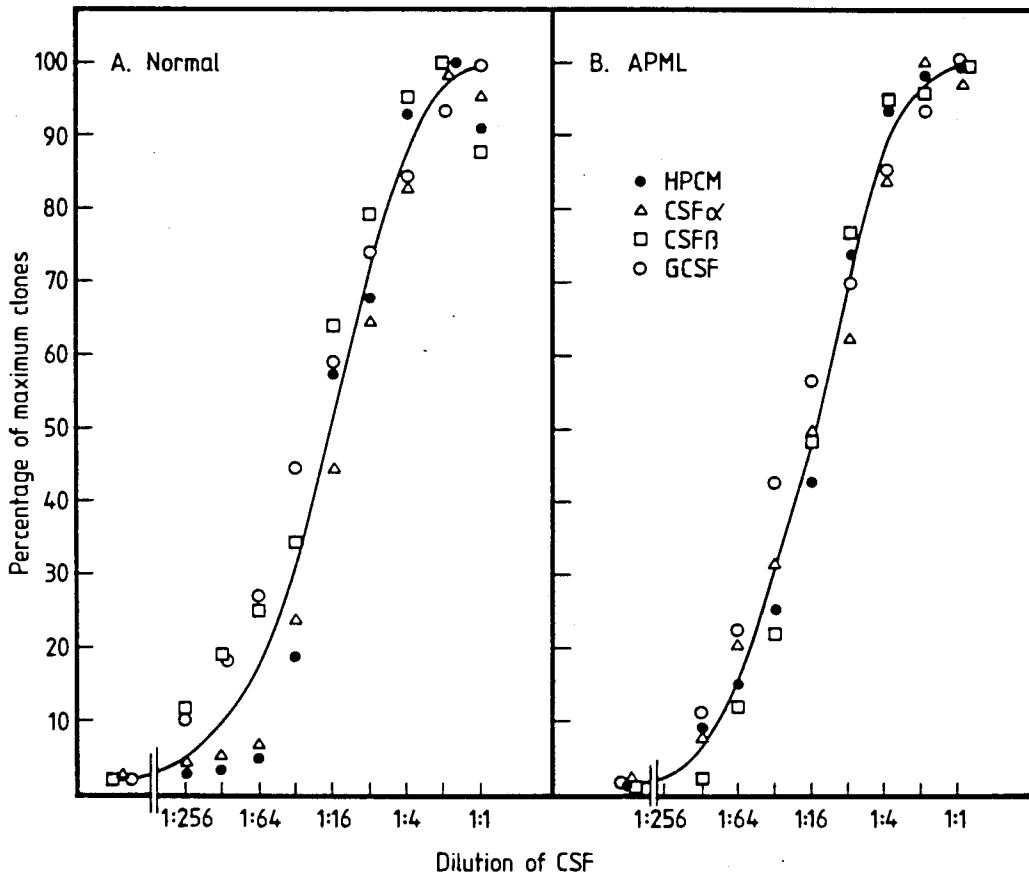
Fig. 7.2   Responsiveness of acute promyelocytic leukaemia cells to

CSF.   Cells were cultured in dilutions of human placental

conditioned medium, CSF-α, CSF-β and murine G-CSF.   The

number of clones were counted in replicate cultures

containing 10^3 cells per ml.   The responsiveness of normal

promyelocyte-myelocyte cells obtained using the FACS and

monoclonal antibody WEM-G11 is shown for comparison.   The

same CSF preparations were used to stimulate cultures

containing 10^3 cells per ml.
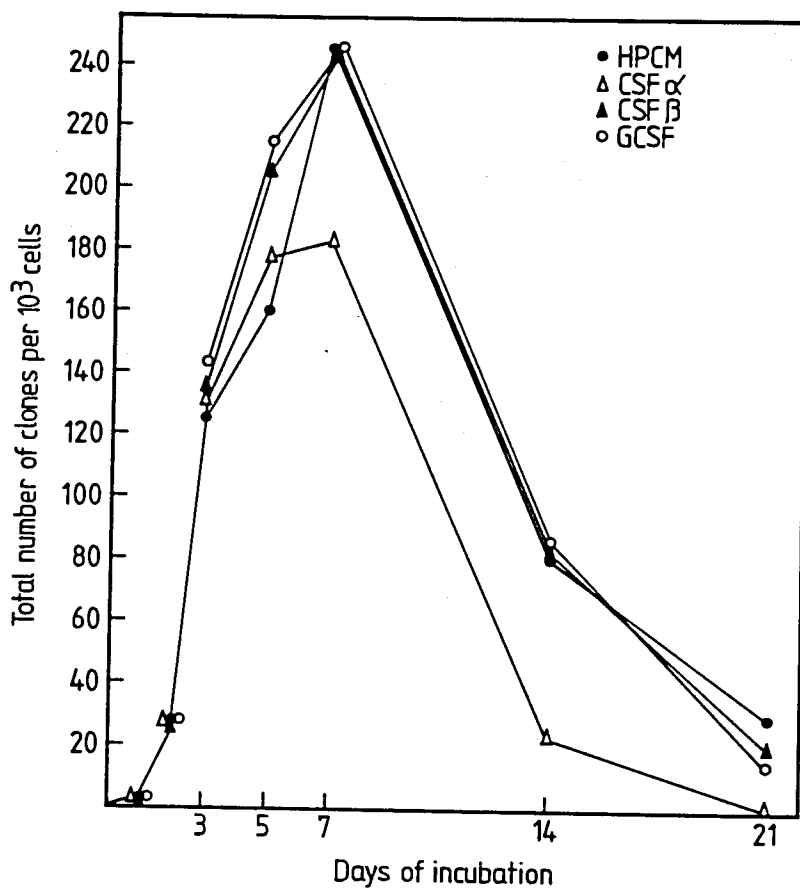
Fig. 7.3 Clonal proliferation in vitro of promyelocytes from one individual with acute promyelocytic leukaemia. Cells were stimulated by human placental conditioned medium, CSF-α, CSF-β and murine G-CSF in cultures containing 10^3 cells per ml. The number of clones was counted at varying timepoints and each point represents the mean of replicate cultures.

TABLE 7.1.   Size Distribution of Clones Generated by Acute Promyelocytic Leukaemic Cells

Stimulus	Number of Cells Per Clone					
	2	3-4	5-6	7-8	9-10	11-12
CSF-α	68	30	2			
CSF-β	32	43	16	4	3	2
G-CSF	55	32	12	1		

Results are size distribution of clones obtained from AML (M3) cells and expressed as percentages.   Clones were examined after 7 days of culture when stimulated by CSF-α, CSF-β or murine G-CSF. 10^3 cells were cultured per ml and a minimum of 100 sequential clones examined.

TABLE 7.2. Onset of Proliferation and Fate of Individual Clones in
Mapped Areas of Cultures of Leukaemic Promyelocytes (AML M3)

| Day of Culture | | | | | | Percentage of Examples Stimulus | | | |
2	3	5	7	10	14	HPCM	CSF-α	CSF-β	Murine G-CSF
C	C	†				3	1	1	1
C	C	C	†			1	3	2	1
C	C	C	C	†		3	4	2	3
C	C	C	C	C	†	3	5	4	4
C	C	C	C	C	C	7	2	4	5
	C	†				7	3	3	3
	C	C	†			8	8	8	9
	C	C	C	†		14	15	12	15
	C	C	C	†		8	18	13	9
	C	C	C	C		18	3	14	14
		C	†			3	8	5	8
		C	C	†		5	11	9	11
		C	C	C	†	5	16	9	6
		C	C	C	C	9	1	11	9
			C	C	†	1	1	<1	<1
			C	C	C	5	1	3	2
Total Number of Clones Followed						393	370	423	440

Leukaemic promyelocytes were obtained from a patient with acute promyelocytic leukaemia. Cells were cultured (10^3 cells per ml) with a source of CSF as indicated. Clones of 2 or more cells recorded and observed during 14 days of culture. C = clone present; † = lysis or disappearance of clone.

experiment shown in Figure 7.3. These results showed that the mainten-
ance of clone numbers was due to persistence of clones throughout
the 14 day culture period. The behaviour of clones was indistinguish-
able regardless of whether cells were stimulated by HPCM, CSF-α,
CSF-β or murine G-CSF. No clones developed in cultures unstimulated
by CSF.

Attempts were made to examine whether the stimulation of leukaemic
cells by CSF was associated with differentiation. Cells were placed
in liquid cultures and analyzed at various timepoints. Morphological
differentiation occurred in cultures stimulated by HPCM, CSF-α, CSF-β
and murine G-CSF but was also observed in cultures lacking any exogen-
ous CSF (Table 7.3). As well as displaying morphological evidence
of differentiation, these cells did not proliferate when subsequently
placed in agar, although all examples proliferated in primary agar
cultures. The morphologically differentiated cells did not arise
solely because of selective cell death (with persistence of mature
cell) as in one sample (experiment 1, Table 7.3) proliferation was
observed in liquid cultures. In the other two samples, the prolifera-
tion observed in agar cultures (Figure 7.1) was probably masked in
liquid cultures by concomitant cell death, however in both cases
the final cell numbers suggested that the majority of differentiation
cells had arisen from immature precursors during the culture.

Cells from 4 patients with acute promyelocyte leukaemia were
examined with monoclonal antibody WEM-G11. Cells from three of these
patients reacted positively with this antibody and cells from one
patient did not. There was no change in antibody-status with cells
following liquid culture, although in all cases there were changes
in the low angle and high angle light scatter characteristics
consistent with the results shown in Table 7.3.

TABLE 7.3. Morphological Differentiation in Liquid Cultures of Leukaemic Promyelocytes (AML M3)

Experiment Number	Cell Population and Stimulating CSF	Percentage of Cells					
		Blasts	Promyelocytes	Myelocytes	Metamyelocytes	Lymphocytes	Monocytes
1	Starting cells	1.0	67.5	17.0	10.0	4.5	0.0
	CSF-α	0.0	39.0	42.5	8.5	6.0	4.0
	CSF-β	0.0	35.0	46.0	12.5	4.0	2.5
	G-CSF	0.0	44.0	42.0	8.0	5.0	1.0
	Unstimulated	0.0	43.5	38.0	12.5	5.0	1.0
2	Starting Cells	44.0	31.0	3.0	2.0	18.0	2.0
	CSF-α	6.0	22.5	8.0	12.5	5.5	40.5
	CSF-β	2.0	17.0	13.0	10.0	9.0	49.0
	G-CSF	2.0	15.0	19.0	12.5	6.0	45.5
	Unstimulated	3.5	17.5	18.0	8.5	12.0	40.0
3	Starting cells	79.5	16.5	0.0	0.0	2.5	1.5
	CSF-α	1.5	10.5	49.5	30.0	5.0	3.5
	CSF-β	0.0	7.0	48.5	38.5	3.5	2.5
	G-CSF	0.0	3.0	57.5	34.5	2.0	3.0
	Unstimulated	1.0	8.0	61.5	21.5	5.0	3.0

Footnote to Table 7.3

Light density cells were obtained from blood and bone marrow samples from 3 patients with untreated acute promyelocytic leukaemia. Cells were cultured for 6 days with 10^6 cells per ml. Cells were harvested from liquid cultures and a minimum of 200 cells were examined. Cell counts were performed on all samples and the ratio of final to initial cell counts were approximately 1.2, 0.5 and 0.5 for experiments 1, 2 and 3 respectively. Samples 1 and 2 were both positive when examined with monoclonal antibody WEM-G11 and there was no change following culture. Sample 3 was negative with WEM-G11 and there was no change following culture.

7.2.2 Fractionation Procedure for Promyelocytes-Myelocytes
from Patients with CML

Light density bone marrow and peripheral blood cells were obtained from patients with chronic myeloid leukaemia (CML) and incubated with the FITC-conjugated monoclonal antibody WEM-G11. These cells were then analyzed using the FACS. The profile obtained was very similar to that obtained with normal marrow cells. Fluorescence-positive cells with high 0 degree light scatter and low 90 degree light scatter characteristics were collected (as described in Chapter 4). The composition of the fluorescence-positive populations is shown in Table 7.4. Approximately 45% of the cells obtained from CML bone marrow were promyelocytes and myelocytes, with the majority of the cells being post-mitotic metamyelocytes and polymorphs. These cells were relatively free from lymphocytes and monocytes. A similar population was obtained from CML peripheral blood samples. The procedure resulted in an enrichment of promyelocytes-myelocytes of approximately 2-fold and also served to separate these cells from other cells capable of proliferation.

7.2.2.1 Clonal Proliferation of Promyelocytes-Myelocytes

In Vitro. The clonal proliferation of fractionated peripheral blood promyelocytes-myelocytes obtained from patients with CML is shown in Figure 7.1. Maximum clone numbers were obtained prior to 7 days of incubation and few clones were present after 14 days. This transient pattern of proliferation was very similar to that described for normal promyelocytes-myelocytes in vitro (Chapters 5 and 6). Similar results were obtained for CML marrow promyelocytes-myelocytes and also for fractionated promyelocytes-myelocytes from 2 patients with chronic myelomonocytic leukaemia and myelofibrosis (Figure 7.4).

TABLE 7.4   COMPOSITION OF PROMYELOCYTE-MYELOCYTE ENRICHED POPULATIONS OBTAINED FROM CHRONIC MYELOID LEUKAEMIA

CELLS FRACTIONATED USING THE MONOCLONAL ANTIBODY WEM-G11

Cell Source	Sorting Procedure	Percentage of Cells							
		Myelo-blasts	Promyelo-cytes	Myelo-cytes	Metamyelo-cytes + Polymorphs	Lymphocytes	Monocytes	Basophils+ Eosinophils	Nucleated Red Blood Cells
CML Bone Marrow	Nil	2.0±0.7	6.5±2.8	14.0±1.4	59.0±8.5	4.5±0.7	4.5±3.2	5.0±1.4	4.5±4.6
	WEM-G11 Positive Cells	0.0±0.0	19.8±5.3	26.9±5.3	53.0±4.2	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.3
CML Blood	Nil	1.3±0.6	3.5±0.5	11.0±4.9	52.9±6.8	16.3±9.2	7.0±2.8	3.8±2.8	4.2±0.8
	WEM-G11 Positive Cells	0.0±0.0	9.7±2.1	19.8±6.0	68.8±6.1	0.0±0.0	1.2±1.0	0.5±0.5	0.0±0.0

Mean ± SD of the percentage frequency of various cell types from three individuals with untreated chronic myeloid leukaemia.

Promyelocyte-myelocyte enriched populations were obtained from peripheral blood and bone marrow cells fractionated by

fluorescence-activated cell sorting using the monoclonal antibody WEM-G11.
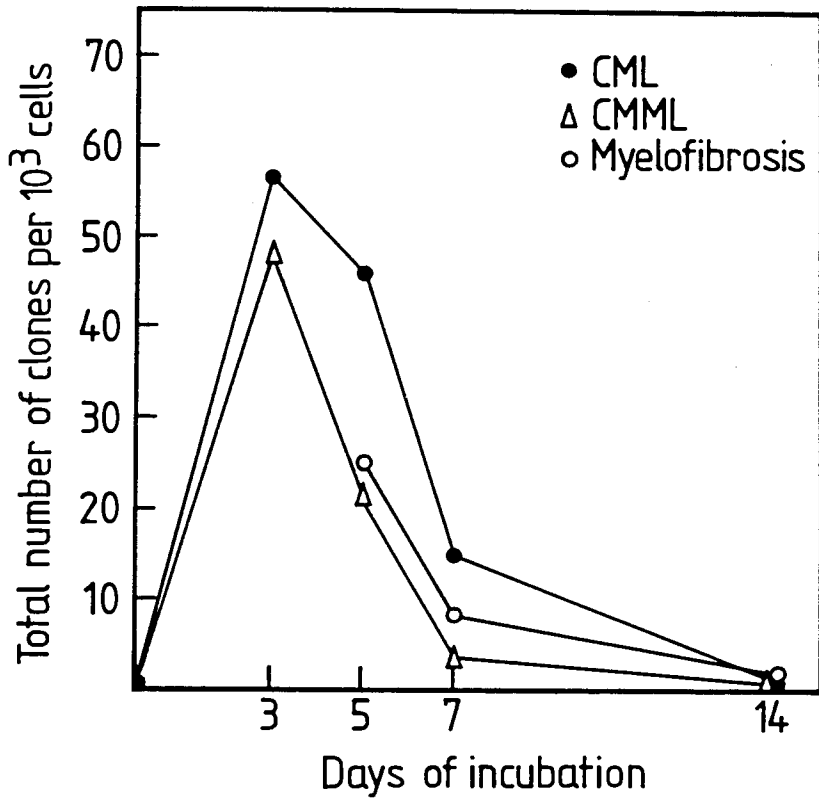
Fig. 7.4 Clonal proliferation of promyelocyte-myelocyte cells

obtained from patients with untreated chronic myelomono-

cytic leukaemia and myelofibrosis. Peripheral blood cells

were fractionated using the FACS and monoclonal antibody

WEM-G11. The fractionated populations consisted of

promyelocytes-myelocytes (18.5% and 19.5% for CMML and

myelofibrosis respectively), metamyelocytes (71.0% and

79.5%), lymphocytes (0.0% and 1.0%) and monocytes

(0.5% and 0.0%). 10^3 cells were cultured per ml and

stimulated by HPCM.

The frequency of clonogenic cells following 3 days of incubation was between 6-8% of the total number of cells cultured. This suggested that approximately 25% of CML blood-derived promyelocytes-myelocytes were capable of in vitro proliferation. The number of CML bone marrow-derived promyelocytes-myelocytes that demonstrated clonal proliferation in vitro was between 60-75%. The frequency of clonogenic cells was greater than the percentage of promyelocytes, suggesting that some CML-myelocytes were capable of proliferation in vitro.

7.2.2.2 Proliferative Response to Different CSF's. The proliferative response of CML promyelocytes-myelocytes was examined when stimulated by different CSF's. A typical result is shown in Figure 7.5.   CSF-α stimulated fewer clones to develop than CSF-β, murine G-CSF or HPCM (a source of both CSF-α and CSF-β). This difference was most striking with promyelocytes-myelocytes of bone marrow origin. The transient nature of clones was, however independent of the stimulating CSF as evidenced by changes in total numbers of clones (Figure 7.5) and behaviour of individual clones when studied in culture mapping experiments (Table 7.5). These results suggested the transient proliferation observed with promyelo-cytes-myelocyte clones reflected a property of the cells themselves rather than a consequence of the stimulating CSF. The morphology of clones was >95% neutrophils for all stimulating CSF's and clones consisted of morphologically differentiated cells.

All CML promyelocytes-myelocyte cell samples displayed some proliferation in unstimulated cultures. This was most evident with blood-promyelocytes-myelocytes where the number of clones that developed was between 60-100% of the number stimulated by CSF and was also observed in unstimulated cultures of CMML promyelocytes-
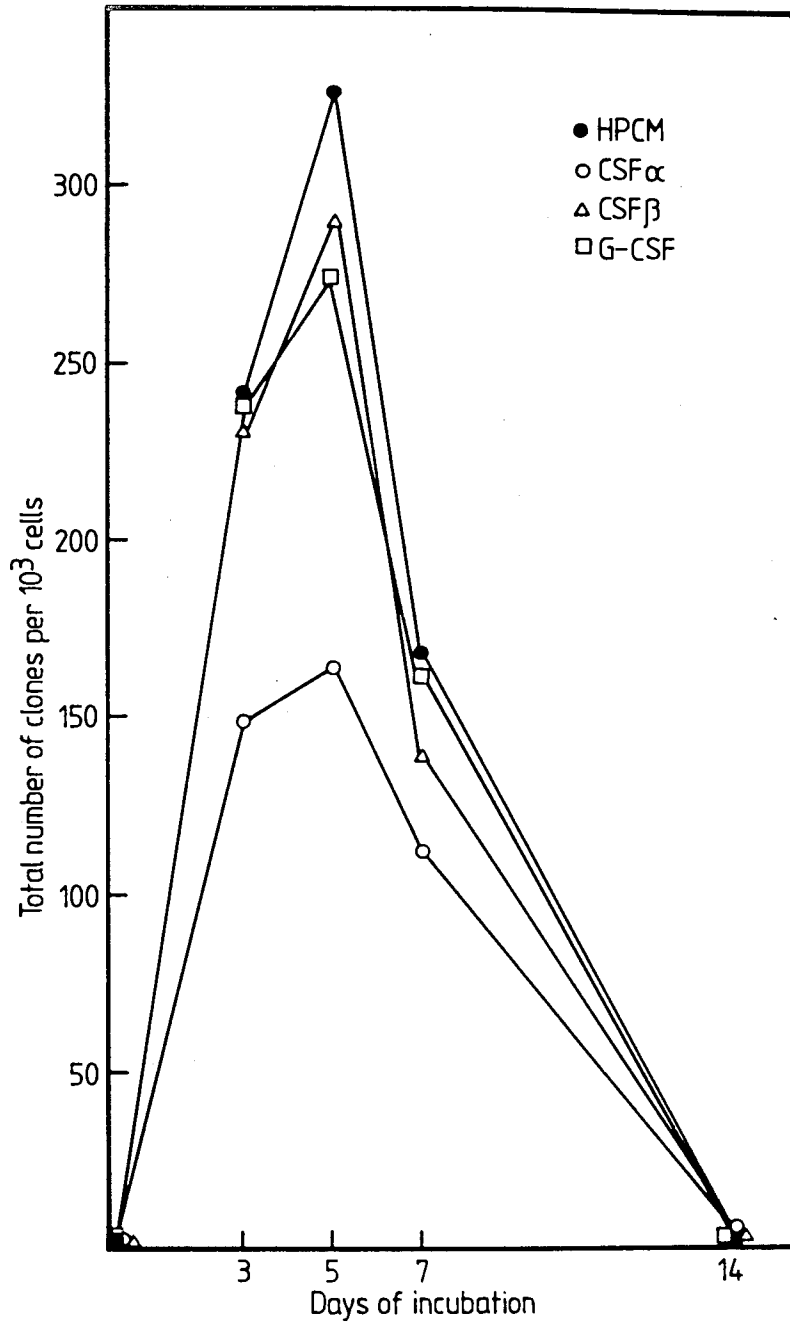
Fig. 7.5 The clonal proliferation in vitro of promyelocyte-myelocyte enriched cells obtained from CML bone marrow cells. The total number of clones was examined in cultures containing 10^3 cells per ml and stimulated by human placental conditioned medium, CSF-α, CSF-β and murine G-CSF. Each point is mean of replicate cultures.

TABLE 7.5. ONSET OF PROLIFERATION AND FATE OF INDIVIDUAL CLONES IN MAPPED
AREAS OF CULTURES OF CHRONIC MYELOID LEUKAEMIA-DERIVED
PROMYELOCYTES AND MYELOCYTES

Day of Culture						Percentage of Examples			
							Stimulus		
2	3	5	7	14		HPCM	CSF-α	CSF-β	Murine G-CSF
C	C	†				19	9	19	15
C	C	C	†			31	22	30	26
C	C	C	C	†		9	13	4	17
C	C	C	C	C		0	0	1	0
	C	†				10	4	8	8
	C	C	†			15	14	18	14
	C	C	C	†		3	9	1	10
	C	C	C	C		1	0	0	1
		C	†			7	20	17	6
		C	C	†		4	8	2	3
			C	†		1	1	0	0
Total Number of Clones Followed						118	127	139	155

Cells enriched for promyelocytes-myelocytes were obtained by fractionation
of CML blood cells using the fluorescence activated cell sorter and
monoclonal antibody WEM-G11. 10^3 cells were cultured per ml with a source
of CSF as indicated. Clones of 2 or more cells were recorded and observed
during 14 days of culture.

myelocytes.   In unstimulated cultures, marrow derived CML promyelo-
cytes-myelocytes generated 20-25% of the clones stimulated by CSF.
All these clones, whether derived from blood or marrow promyelocytes-
myelocytes were only 2 cells (minimum 90%) or 3 cells (maximum 10%)
in size.   This contrasted with the size of clones stimulated by CSF
(Table 7.6) where clones were significantly larger. There was no
significant difference between the size of clones stimulated by
different CSF's.

7.2.3 Proliferation of Normal Promyelocytes-Myelocytes
Following Pulsing with CSF

The observed clonal proliferation of CML promyelocytes-myelo-
cytes in unstimulated cultures was intriguing.   Limited prolifera-
tion of normal promyelocytes-myelocytes was consistently observed in
unstimulated cultures of these cells, although the number of clones
generated was only about 10% of the total number of clones obtained
when cells were maximally stimulated by CSF. One possible explanation
for this phenomenon was that promyelocytes-myelocytes were able to
demonstrate limited proliferation following prior exposure to CSF.
To test this possibility, promyelocytes-myelocytes were obtained
from normal marrow samples and "pulse" stimulated by CSF for 45
minutes.   Cells were then washed and cultured either stimulated by
CSF or without CSF.   The results of pulse stimulation by CSF-α are
shown in Figure 7.6.   The total number of clones generated by normal
promyelocytes-myelocytes was the same whether cells were "pulsed"
by CSF and then cultured without CSF, or whether cells were stimul-
ated continuously by CSF. Two control populations of cells were
those not pulsed by CSF and then either stimulated by CSF or cultured
without CSF.   There was no significant difference between the total
number of clones generated by the three groups of cells stimulated by

TABLE 7.6.   SIZE DISTRIBUTION OF CLONES GENERATED BY CML PROMYELOCYTES-
MYELOCYTES FRACTIONATED USING MONOCLONAL ANTIBODY WEM-G11

Stimulus	Number of Cells Per Clone					
	2-5	6-9	10-14	15-19	20-24	25-29
HPCM	34	28	12	15	6	5
CSF-α	54	22	10	5	6	3
CSF-β	47	30	12	8	2	1
G-CSF	34	39	18	7	2	
NIL	100					

Size distribution of CML promyelocyte-myelocyte clones expressed as
percentages and obtained by stimulating cells with HPCM CSF-α, CSF-β,
murine G-CSF and no CSF.   Clones were examined after 7 days of culture
and the number of cells per clone was counted for a minimum of 100
consecutive clones.

Fig. 7.6 Total clone numbers followed during 14 days of culture

for normal promyelocytes-myelocytes. 10^3 cells were

cultured per ml and replicate cultures examined at each

timepoint. Cells were pulse stimulated by CSF-α for

45 minutes and then cultured with or without CSF-α.

Control cells were cultured either in CSF-α stimulated

or unstimulated cultures.

CSF, regardless of whether this stimulation was transient or contin-
uous. Similar results were also obtained when cells were pulsed
stimulated by CSF-β (Figure 7.7).

Plate mapping experiments were performed to examine the onset
of clonal proliferation and fate of clones generated by normal
promyelocytes-myelocytes when pulsed by CSF. The results shown in
Table 7.7 are for cells pulsed with CSF-β. There was no difference
between the behaviour of promyelocytes-myelocytes pulsed by CSF and
cultured without CSF when compared with cells that were continuously
stimulated by CSF. In both situations, approximately 50% of clones
did not initiate proliferation until the third day of culture. The
behaviour of both groups of cells showed no difference to control
cells that were not pulsed by CSF. In particular, the cells that
were pulsed by CSF did not demonstrate an earlier onset of clonal
proliferation when compared with unpulsed cells. The behaviour of
clones derived from cells that were not exposed to CSF in vitro is
also shown in Table 7.7. The absolute number of clones generated
by these cells was consistently approximately 10% of the total number
of clones generated when cells were maximally stimulated by CSF.

7.2.3.1 Size of Clones Following Pulsing with CSF. The
size of clones generated by the normal promyelocytes-myelocytes when
pulsed by CSF is shown in Figure 7.8. Sequential clones were examined
and their size recorded throughout the incubation period. Panel A
shows the size distribution of clones generated from promyelocytes-
myelocytes pulsed by CSF and then cultured without CSF. The majority
of these clones were 2 cells in size. Similarly, the clones generated
by cells that had not been exposed to CSF in vitro were of 2 cells in
size (panel C) and the total number of clones generated were signific-
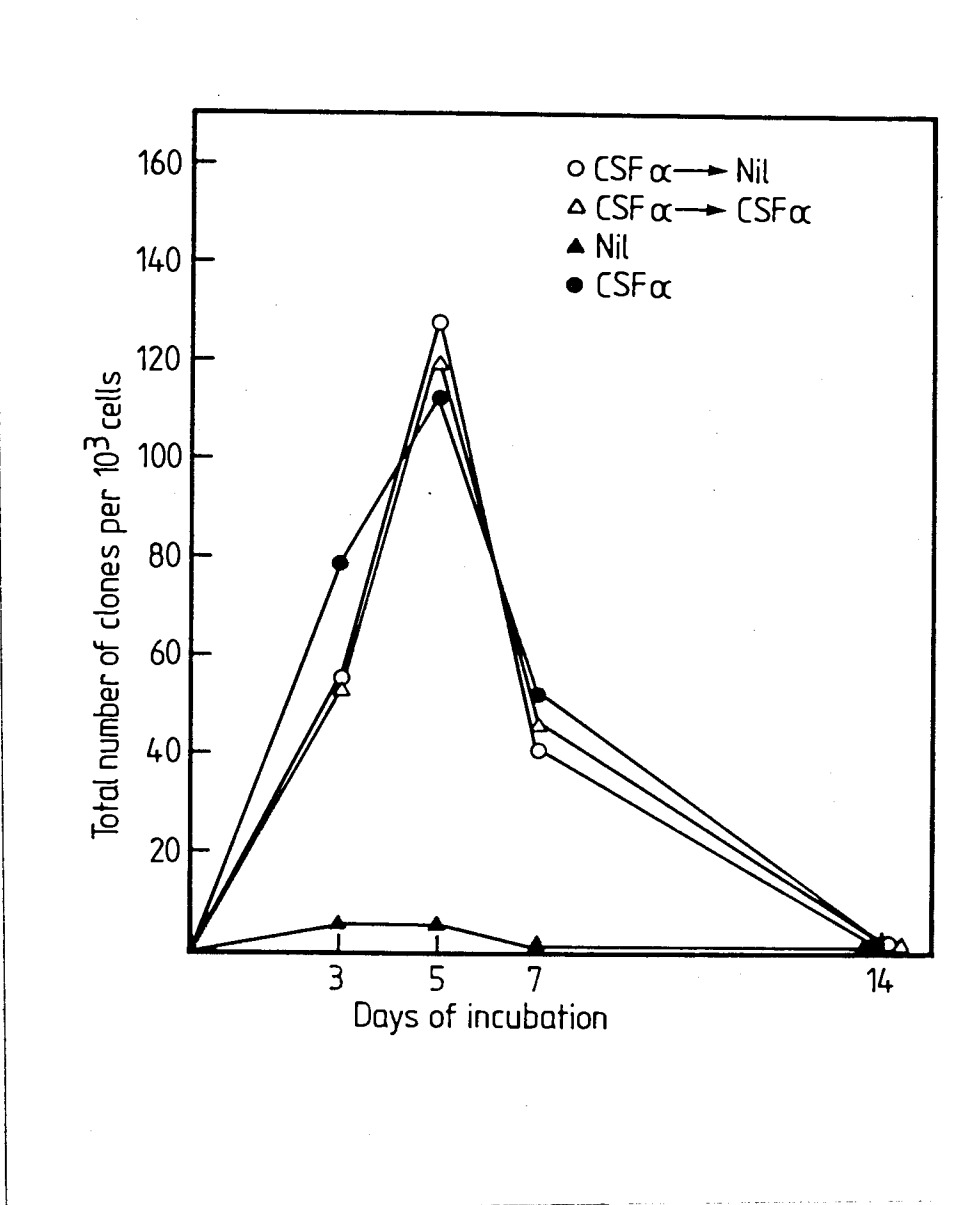antly less. Panels B and D show the size distribution of clones when
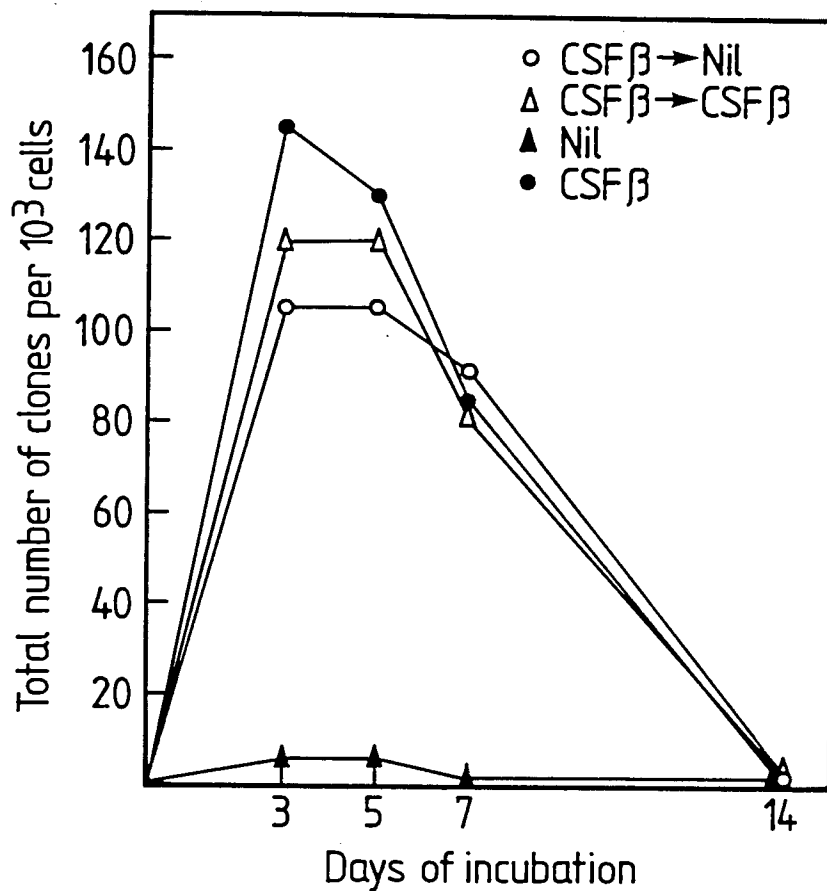
Fig. 7.7 Total clone numbers followed during 14 days of culture

for normal promyelocytes-myelocytes. 10^3 cells were

cultured per ml and replicate cultures examined at each

time point. Cells were pulse stimulated by CSF-β for

45 minutes and then cultured either with or without

CSF-β. Control groups of cells were not pulse stimulated

by CSF-β but were cultured either with or without CSF-β.

TABLE 7.7. Onset of Clonal Proliferation and Fate of Individual Clones in Cultures of Normal Promyelocytes-Myelocytes Pulse Stimulated by CSF

Day of Culture						Number of Examples (%)		
						CSF-β Pulsed		Nil
1	2	3	5	7	14	CSF-β	Nil	Nil
C	C	C	†			8	13	4
C	C	C	C	†		7	7	18
C	C	C	C	C	†	9	10	4
	C	†				0	0	4
	C	C	†			7	7	14
	C	C	C	†		3	8	20
	C	C	C	C	†	14	8	7
		C	†			9	8	17
		C	C	†		10	12	4
		C	C	C	†	18	22	4
			C	†		4	1	4
			C	C	†	10	4	0
				C	†	1	0	0
Absolute Number of Clones in Monitored Areas						151	156	28

Time of initiation and fate of individual clones in mapped ares of cultures of normal promyelocyte-myelocyte cells. Promyelocyte-myelocyte enriched populations were obtained using the fluorescence activated cell sorter and the monoclonal antibody WEM-G11. 10^3 cells were cultured per ml and clones of 2 or more cells were observed during the 14 day culture period. Cells were pulse stimulated by CSF-β for 30 minutes and were then placed in cultures either stimulated by CSF-β or lacking CSF. For comparison cells that were not exposed to CSF in vitro were also studied. The absolute number of clones generated in the monitored areas is shown for the three groups of cells.

    C = clone present
    † = lysis or disappearance of clone.
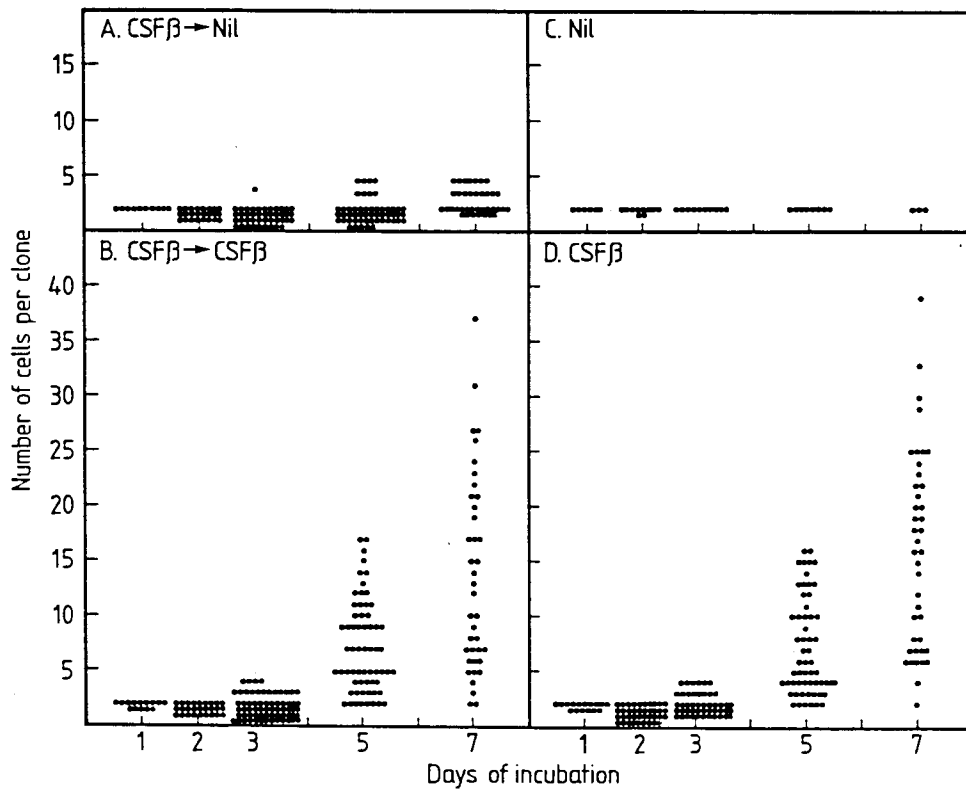
Fig. 7.8 Size distribution of clones generated by normal marrow

promyelocytes-myelocytes fractionated using monoclonal

antibody WEM-G11. 10^3 cells were cultured per ml and

the size of clones recorded throughout the culture period.

In panel A, normal promyelocyte-myelocytes were pulse

stimulated by CSF-β for 30 min and then placed in cultures

lacking CSF. In panel B, cells were pulse stimulated by

CSF-β for 30 min. and then placed in cultures stimulated

by CSF-β. In panel C, cells were not exposed to CSF

in vitro. In panel D cells were not pulsed with CSF but

were placed in cultures stimulated by CSF-β. Similar

results were obtained for promyelocytes-myelocytes when

pulse stimulated by CSF-α.

promyelocytes-myelocytes were continuously stimulated by CSF. These

clones were primarily of 2 cells in size up until 3 days of culture.

Thereafter there was a marked increase in the size of clones. The

behaviour of the normal promyelocytes-myelocytes following pulsing

by CSF (Figure 7.8A) was reminiscent of the proliferation of CML

promyelocytes-myelocytes observed in the unstimulated cultures.

7.2.3.2 Pulsing of Normal Blast Cell Populations by CSF.

Blast cell enriched populations were obtained from normal bone marrow

samples and pulsed stimulated by either CSF-α or CSF-β for 45 minutes.

A typical result is shown in Figure 7.9. Cells were obtained from

the same bone marrow samples as the normal promyelocytes-myelocytes,

but in contrast, there was no increase in the number of clones

generated by the pulsed cells compared with the number of clones

present in unstimulated cultures.

7.3 DISCUSSION

In this study the proliferation of leukaemic promyelocytes-

myelocytes was examined. CML-promyelocytes-myelocytes were obtained

using the monoclonal antibody WEM-G11 and the fluorescence activated

cell sorter. As with other monoclonal antibodies that recognise

both normal and leukaemic cells (Griffin et al, 1981; Young and

Hwang-Chen, 1981; Morstyn et al, 1981a; Bernstein et al, 1982;

Strauss et al, 1984), the cells from patients with CML that were

identified by WEM-G11 were very similar to those obtained from normal

bone marrow cells. The behaviour of the normal and CML-promyelocytes-

myelocytes in vitro was very similar. Clones derived from CML-

promyelocytes-myelocytes showed transient proliferation in vitro,

generated clones of subcolony size (less than 40 cells) and responded

to three CSF's, CSF-α, CSF-β and murine G-CSF. As with normal
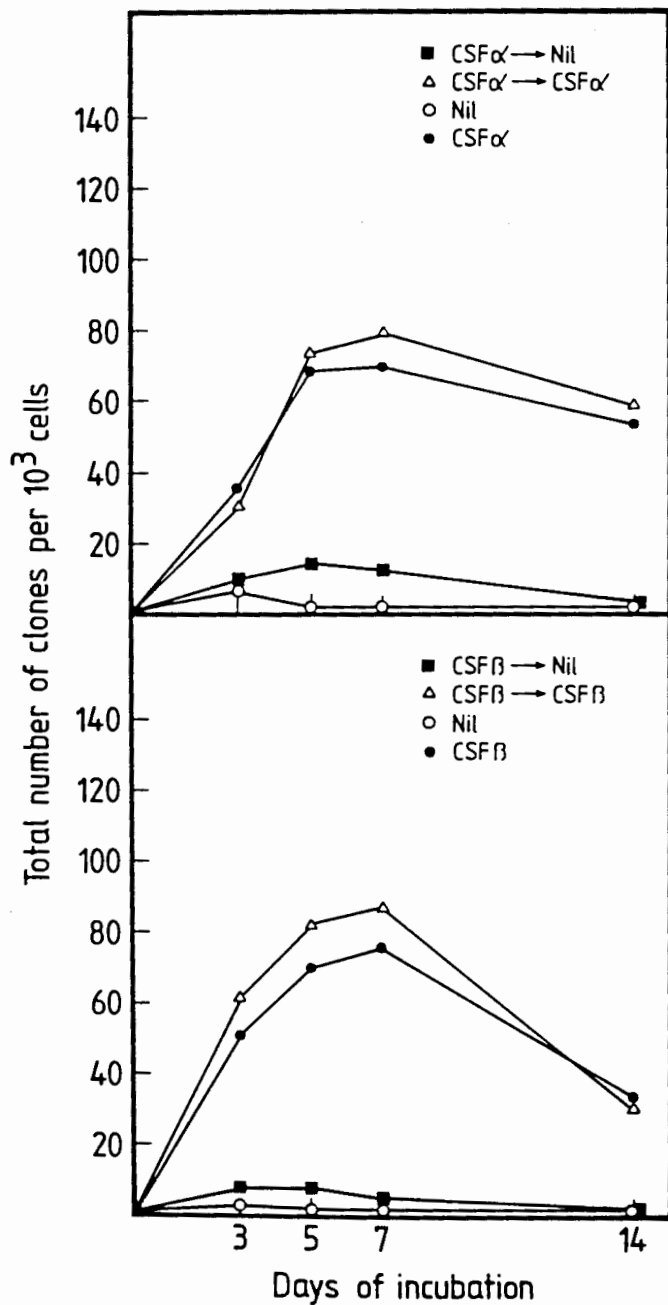
Fig. 7.9 Total number of clones followed during 14 days of incubation. 10^3 cells were cultured per ml and replicate cultures examined at each time point. Cells were fractionated from normal marrow using monoclonal antibody WEM-Gll and the blast cell enriched population obtained. Cells were pulse stimulated by CSF-α (upper panel) and CSF-β (lower panel) and cultured either with or without CSF. Control cells were not pulsed but were cultured either in CSF stimulated or unstimulated cultures. The results shown for this blast cell fraction were obtained from the same marrow sample shown in Figures 7.6 and 7.7.

promyelocytes-myelocytes these characteristics appeared to be intrinsic to the cells themselves. The frequency of clonogenic cells was between 25-75%.

The preservation of normal proliferative characteristics of CML-promyelocytes-myelocytes is consistent both with observations that day 7 and day 14 subsets of granulocyte-macrophage colony forming cells are also preserved in CML (Broxmeyer et al, 1978), and with sedimentation velocity studies that have shown the most rapidly sedimenting CML cells parallel the transient proliferative behaviour of normal cells in vitro, although the cells responsible for this proliferation were not identified (Strife et al, 1983).

In contrast, promyelocytes from patients with acute promyelocytic leukaemia showed no consistent pattern of behaviour. Previous studies have also failed to demonstrate a correlation between growth patterns and morphological subtypes (Moore et al, 1973, 1974). Some promyelocyte clones were transient in culture. These samples would have been regarded as not displaying clonal proliferation had they been scored at the day 7 timepoint alone. This transient proliferation may partly explain the inability of approximately 20-30% of acute myeloid leukaemia cell samples to proliferate in agar cultures when scored following 7 days of culture (Moore et al, 1973, 1974; Spitzer et al, 1976). The promyelocytic leukaemia samples showed a subset of leukaemic promyelocytes that were unresponsive to CSF-α. Selective stimulation of different subsets of leukaemic cells has been previously reported with different crude sources of CSF (Swart et al, 1982; Francis et al, 1980). Maximum numbers of clones were stimulated however by both human CSF-β and murine G-CSF and clone mapping experiments showed no difference in the behaviour of clones stimulated by these CSF's. There was no proliferation in unstimulated cultures.

The striking difference between CML promyelocytes-myelocytes
and normal promyelocytes-myelocytes was the proliferation, albiet
limited of the leukaemic cells in the absence of exogenous CSF.
Possible explanations for this limited proliferation include for
example the generation of CSF by lymphocytes or monocytes in the
cultures, but sufficient for only one cell division. However, the
phenomenon was mimicked by normal promyelocytes-myelocytes when pulse
stimulated by CSF for only 30 minutes. This indicated that these
normal cells were able to "remember" a CSF signal for prolonged
periods after CSF withdrawal, and is the probable explanation for
the observed limited proliferation of these cells previously recorded
in the absence of CSF or following transfer of clones to cultures
lacking CSF (Figures 6.3, 6.5). The dilution factor involved in
the pulsing experiments ensured that the carry-over of CSF could
not account for these results, even assuming the washing procedure
was inadequate.

It has been shown previously that fewer than 5% of murine marrow
granulocyte-macrophage colony forming cells are able to survive for
more than 24 hours in the absence of GM-CSF (Metcalf, 1970; Lin and
Stewart, 1974). Transfer of colonies initiated by GM-CSF to cultures
lacking GM-CSF led to cessation of colony growth and death of colony
cells (Metcalf and Foster, 1967; Paran and Sachs, 1968). Studies
on paired daughter cells indicated that few cells could complete a
cell cycle in progress at the time of CSF withdrawal (Metcalf, 1980).
These observations have led to the conclusion that GM-CSF is required
continuously for proliferation of mouse granulocyte-macrophage progen-
itor cells. However, continued proliferation has been documented
for murine cells following withdrawal of CSF (Metcalf and Burgess,
1982) with up to 30% of cells proliferating following transfer to
cultures lacking CSF (Metcalf and Merchav, 1982). Studies using

human marrow cells have also documented the requirement for CSF for
proliferation, but again 30% of clones continued to proliferate
following transfer to cultures lacking CSF (Morstyn et al, 1981).
One trivial explanation for these results might be the carry-over
of CSF in the agar-droplet during clone transfer experiments.
Alternatively these results might be explained in part by the ability
of some cells to respond by proliferating following CSF withdrawal
as shown here for normal promyelocytes-myelocytes. The observed
limited proliferation of CML promyelocytes-myelocytes described here
in unstimulated cultures may also be attributable to exposure of
these cells to CSF in vivo, particularly as patients with CML are
known to have elevated levels of CSF (Metcalf, 1977).

The absence of this phenomenon in the normal blast cell popula-
tions may relate to the reduced sensitivity of these cells to CSF
(Figures 5.4, 6.3). It may also be that these cells require repeated
pulsing by CSF for proliferation to occur.

The observation of normal promyelocyte-myelocyte proliferation
following pulse stimulation by CSF may have relevance for future in
vivo studies of the action of CSF as the serum half life of these
molecules may bear little relationship to the biological half-life
at their target-cell level.

CHAPTER 8

COLONY STIMULATING FACTORS AND HUMAN LEUKAEMIC CELLS:

RESPONSIVENESS AND DIFFERENTIATION-INDUCTION

8.1 FRESH HUMAN LEUKAEMIC CELLS

8.1.1 Introduction

A possible alternative to the explanation presented in Chapter 7
for the limited proliferation observed in unstimulated cultures of
CML promyelocytes-myelocytes was the elaboration of a growth factor
by the leukaemic cells themselves. Such a mechanism has been
suggested as playing a causative role in the genesis of leukaemia
(Schrader, 1984) although human leukaemic cells appear to be absol-
utely dependent on CSF for proliferation (Moore et al, 1973; Metcalf,
1984). Based on the observations with CML promyelocytes-myelocytes
the question of leukaemic cell dependence on CSF was re-examined.

Murine G-CSF stimulated the proliferation of normal human pro-
myelocytes-myelocytes (Chapter 6), CML promyelocytes-myelocytes and
acute promyelocytic leukaemic cells (Chapter 7). Further experiments
were performed to examine the response of other human leukaemias to
murine G-CSF and the human CSF's, and the nature of the receptor
for murine G-CSF on human cells. The aim of these studies was to
determine whether the action of murine G-CSF on human leukaemic cells
was in any way analogous to its action on murine WEHI-3BD$^+$ cells in
inducing differentiation commitment.

8.1.2 Results

8.1.2.1 Clonal Proliferation of Leukaemic Cells In Vitro.
Fresh human leukaemic cells were cultured using 10^3 cells per ml
and developing clones observed at days 3, 5, 7 and 14 of culture.
Results are shown in Table 8.1 for 20 representative, consecutive
examples. Several samples displayed only transient proliferation
and would have been regarded as not displaying proliferation in vitro
had they been scored at the day 7 timepoint alone (patients 1, 4, 6,
10, 15, 19). There was no correlation between morphological features

TABLE 8.1   THE CLONAL PROLIFERATION OF ACUTE MYELOID LEUKAEMIC CELLS
IN CSF-STIMULATED AND UNSTIMULATED CULTURES

Patient No.	FAB Classification	Stimulus		Day of maximum clone number
		Nil	HPCM	
1	M2	43	170	3
2	M1	0	19	5
3	M1	7	800	7
4	M2	0	27	3
5	M4	20	185	7
6	M3	2	75	3
7	M3	4	115	7
8	M3	6	129	7
9	M4	101	100	7
10	M1	0	13	3
11	M5	89	85	3
12	M1	92	320	7
13	M3	3	243	7
14	M5	50	205	3
15	M1	0	137	3
16	M4	128	130	7
17	M5	41	165	7
18	M2	0	14	7
19	M3	0	125	3
20	M1	5	178	3

Light density cells were obtained from individuals with acute
leukaemia and 10^3 cells were cultured per ml. The behaviour of clones
was examined in both unstimulated cultures and cultures stimulated by
400 U/ml of human placental conditioned medium. Cultures were examined
at day 3,5,7 and 14 of incubation. The results shown are the maximum
number of clones generated by each sample.

and transient proliferation. The behaviour of clones in unstimulated
cultures was also examined. The early timepoints and clone size of
2 cells or more were deliberately chosen for examining unstimulated
cultures to increase the likelihood of detecting small amounts of
CSF that might be generated by the leukaemic cells. The number of
artefactural "clones" present immediately following plating of cells
varied from experiment to experiment, but was not never more than
10 "clones" per culture. For the patients shown in Table 8.1, 14
were classified as lacking morphological or cytochemical evidence
of monocytic differentiation (FAB classification M1, M2, M3). Of
these patients only two displayed any significant proliferation in
the unstimulated cultures (Patients 1 and 12). These clones were
mainly 2 cells in size (100% for patient 1; 90% for patient 12).
Both patients had monocytes present in these samples (8% for patient
1, 4% for patient 12). Both patients showed a decline in the total
number of clones in the unstimulated cultures while the number of
clones increased in stimulated cultures. This limited proliferation
may have been due either to the elaboration of CSF by monocytes in
the culture or to exposure of the leukaemic cells to CSF in vivo
(as postulated for CML promyelocytes in Chapter 7 and based on the
proliferation observed with normal cells when pulse stimulated by
CSF). The majority of human leukaemic cells lacking evidence of mono-
cytic differentiation however showed no significant proliferation
in unstimulated cultures.

In contrast, 6 samples showed evidence of monocytic different-
iation (FAB M4, M5) and in all cases, some clones were generated in
unstimulated cultures. Furthermore, in some cases there was no
difference between the behaviour of clones in stimulated or unstimul-
ated cultures either in terms of the total number of clones generated

(Figure 8.1) or the size of clones (Table 8.2). Bone marrow cells from patients with acute and chronic lymphoblastic leukaemia were examined under the same conditions but failed to proliferate either in CSF-stimulated or unstimulated cultures (see Table 8.5).

8.1.2.2 <u>Murine G-CSF and Human Leukaemic Cells</u>. The action of murine G-CSF and human CSF-α and CSF-β on human leukaemic cells was examined. Table 8.3 shows the ability of these three molecules to stimulate the proliferation of acute leukaemic cell populations and Table 8.4 shows proliferation of other leukaemic cell populations. Results are shown for day 7 and 14 timepoints although some samples demonstrated only transient proliferation with no clones present at these timepoints. Murine G-CSF stimulated the proliferation of leukaemic cells in all examples studied and the total number of clones stimulated was very similar to the number stimulated by CSF-β. In some cases however there was significant variation between the number of clones stimulated by different CSF preparations. CSF-α and CSF-β did not always stimulate equal numbers of clones nor did they stimul- ate the number of clones stimulated by HPCM yet all cultures contained 400 units per ml of CSF. This again suggested (as described for promyelocytic leukaemic cells in Chapter 7) that subsets of leukaemic cells existed that were not responsive to different CSF's.

Studies were also performed to examine the nature of the binding of murine G-CSF to human leukaemic cells. ^{125}I-labelled G-CSF demon- strates specific, saturable binding to WEHI-3BD^{+} cells and normal murine bone marrow cells (Nicola and Metcalf, 1984). ^{125}I G-CSF also bound specifically to normal human bone marrow and blood cells as well as a variety of fresh human myeloid leukaemic cells (Tables 8.3, 8.4). The binding of ^{125}I G-CSF to human leukaemias paralleled its specificity of action on normal target cells. Lymphocytic leukaemia cells showed relatively little binding (Table 8.5) but
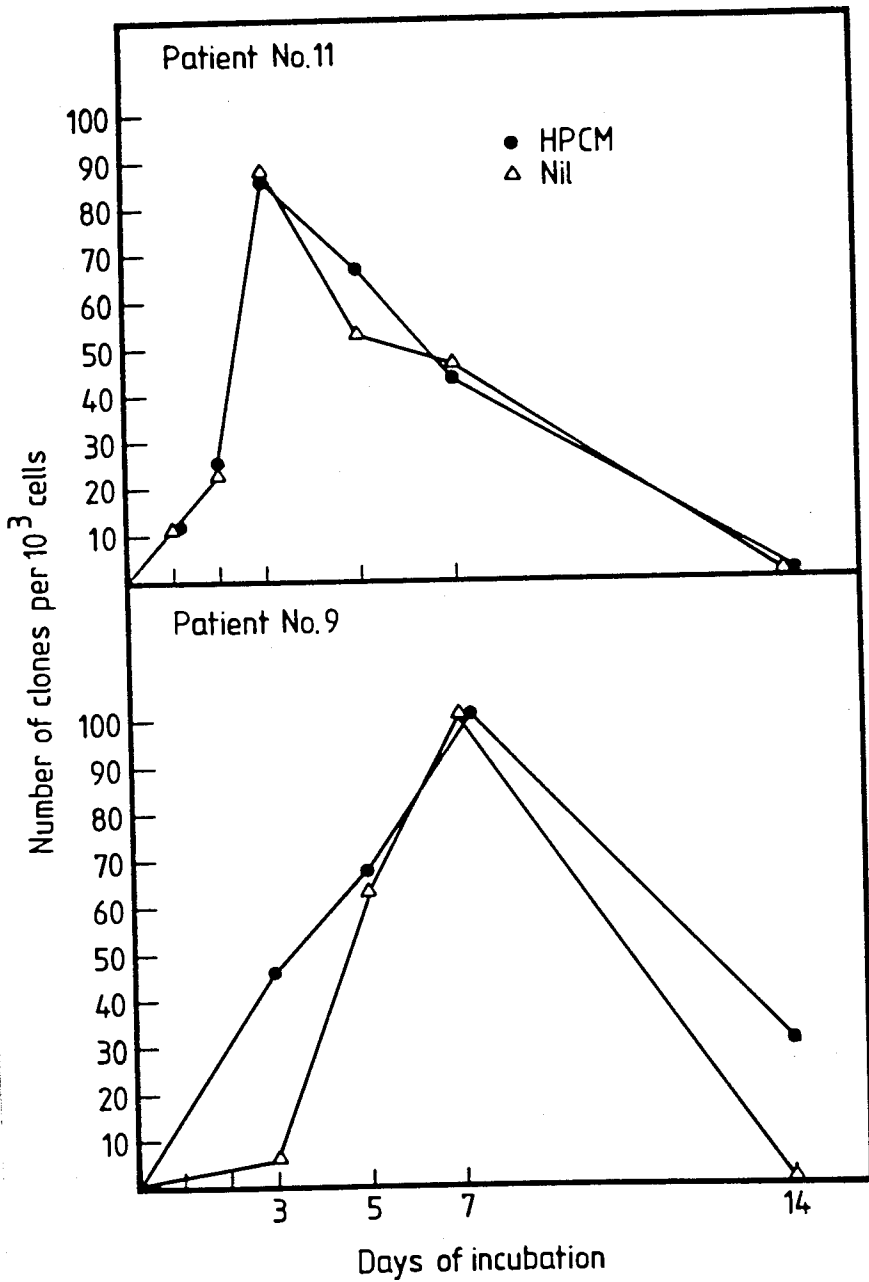
<u>Fig. 8.1</u> The total number of clones generated in CSF-stimulated

and unstimulated cultures. Cells were obtained from two

patients with acute myeloid leukaemia (M5, upper panel

and M4, lower panel) and 10^3 cells cultured per ml.

Cultures were stimulated by HPCM or no stimulating CSF

and replicate cultures examined at different timepoints.

TABLE 8.2 Size Distribution of Clones Derived from Acute

Monoblastic Leukaemic Cells

Stimulus	Number Cells Per Clone		
	2	3-4	5-6
Nil	87	11	2
HPCM	82	15	3

Size of clones generated in CSF-stimulated or unstimulated cultures

of cells obtained from patient with AML (M5). 10^3 cells were

cultured per ml and a minimum of 91 sequential clones were counted.

Results are expressed as percentages.

TABLE 8.3 PROLIFERATION AND BINDING CHARACTERISTICS OF HUMAN ACUTE MYELOID LEUKAEMIC CELLS

| Source of AML Cells | Total Clusters (Colonies) per 20,000 Cells | | | | | | | | Specific Binding of ^{125}I-G-CSF (% of WEHI-3B D$^+$) | |
| | HPCM | | CSF-α | | CSF-β | | G-CSF | | | |
	D7	D14	D7	D14	D7	D14	D7	D14	Marrow	Blood
M1	6,400 (0)	0 (0)	3,000 (0)	0 (0)	7,200 (0)	0 (0)	6,600 (0)	0 (0)	32	24
M1	50 (0)	146 (12)	-	-	45 (0)	6 (0)	25 (0)	5 (0)	36	13
M1	140 (0)	125 (2)	30 (0)	48 (0)	140 (0)	41 (0)	143 (0)	41 (0)	98	38
M1	220 (0)	275 (0)	230 (0)	286 (0)	12 (0)	5 (0)	12 (0)	4 (0)	140	49
M1	750 (0)	175 (0)	700 (0)	200 (0)	150 (0)	40 (0)	165 (0)	40 (0)	18	11
M1	40 (0)	0 (0)	35 (0)	0 (0)	40 (0)	0 (0)	39 (0)	0 (0)	36	10
M1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	68	-
M1	130 (0)	10 (0)	145 (0)	10 (0)	40 (0)	0 (0)	39 (0)	0 (0)	62	19
M3	2,580 (0)	1,980 (0)	2,180 (0)	1,590 (0)	2,600 (0)	1,100 (0)	2,500 (0)	740 (0)	24	10
M3	2,800 (0)	1,600 (154)	-	-	-	-	2,800 (0)	1,600 (140)	163	-
M3	4,860 (0)	1,590 (0)	3,640 (0)	540 (0)	4,500 (0)	1,400 (0)	4,840 (0)	1,640 (0)	82	20

M3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	14	7
M4	2,000 (0)	620 (0)	1,760 (0)	360 (0)	2,000 (0)	360 (0)	2,200 (0)	740 (0)	24	10
M4	300 (0)	20 (0)	-	-	-	-	200 (0)	5 (0)	16	11
M4	1,500 (0)	0 (0)	950 (0)	0 (0)	1,500 (0)	0 (0)	1,250 (0)	0 (0)	7	5
M4	25 (0)	10 (0)	66 (0)	8 (0)	55 (2)	28 (0)	28 (0)	9 (0)	49	-
M4	10 (0)	0 (0)	10 (0)	0 (0)	9 (0)	0 (0)	10 (0)	0 (0)	77	2
M4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5	15
M5	860 (0)	0 (0)	860 (0)	0 (0)	640 (0)	0 (0)	720 (0)	0 (0)	83	43
M5	70 (0)	0 (0)	50 (0)	0 (0)	80 (0)	0 (0)	40 (0)	0 (0)	-	30
M5	3,200 (18)	2,800 (18)	3,300 (16)	3,200 (16)	3,200 (17)	3,300 (16)	3,000 (18)	3,200 (16)	25	27

Cells were obtained from patients with acute myeloid leukaemia. All samples were at least 70% leukaemic blast cells.
Cells were stimulated by HPCM, CSF-α, CSF-β and murine G-CSF and replicate cultures examined at day 7 (D7) and day 14 (D14)
of culture. 10^3 and 2×10^4 cells were cultured per ml. Colonies were greater than 40 cells and clusters less than 40 cells.
Specific binding of ^{125}I-labelled murine G-CSF is expressed as percentage of binding to the same numbers of WEHI-3B D$^+$ cells
performed on the same day.

TABLE 8.4 PROLIFERATION AND BINDING CHARACTERISTICS OF HUMAN MYELOID LEUKAEMIC CELLS

Source of Cells	Total Clusters (Colonies per 20,000 Cells								Specific Binding of ^{125}I-G-CSF (% of WEHI-3B D$^+$)	
	HPCM		CSF-α		CSF-β		G-CSF			
	D7	D14	D7	D14	D7	D14	D7	D14	Marrow	Blood
CMML	1,400 (400)	500 (150)	1,700 (500)	320 (280)	1,740 (440)	240 (160)	2,160 (80)	300 (160)	72	58
CML	350 (255)	300 (150)	340 (220)	300 (160)	360 (245)	300 (80)	350 (200)	300 (100)	10	15
CML	600 (372)	210 (175)	580 (163)	160 (65)	575 (352)	200 (188)	575 (345)	200 (100)	89	50
CML	100 (34)	55 (9)	80 (3)	40 (2)	80 (13)	10 (0)	110 (4)	11 (1)	100	62
CML/AML	288 (0)	2 (0)	320 (0)	6 (0)	-	-	190 (0)	3 (0)	110	79
CML/AML	60 (2)	35 (2)	60 (1)	15 (0)	50 (0)	20 (0)	20 (0)	18 (0)	22	73
CML/AML	80 (2)	30 (2)	55 (2)	5 (0)	-	-	90 (2)	5 (0)	81	20
CML/AML	135 (10)	170 (0)	135 (4)	180 (0)	124 (4)	50 (0)	120 (7)	49 (0)	37	36
RAEB	90 (0)	4 (0)	90 (0)	3 (0)	-	-	84 (0)	2 (0)	62	-
RAEB	760 (0)	0 (0)	130 (0)	0 (0)	435 (0)	0 (0)	440 (0)	0 (0)	81	-
RAEB	250 (5)	250 (2)	265 (0)	245 (0)	324 (2)	70 (0)	300 (0)	80 (0)	70	10
RAEB	100 (20)	30 (7)	40 (0)	26 (0)	60 (10)	20 (0)	50 (0)	20 (0)	11	-

Footnote to Table 8.4

Bone marrow and blood cells were obtained from untreated patients with chronic myeloid leukaemia (CML), chronic myelomono-cytic leukaemia (CMML) and preleukaemia or refractory anaemia with excess blasts (RAEB). Cells were also obtained from patients with CML during acute myeloblastic transformation (CML/AML). 10^3 and 2×10^4 cells were cultured per ml and stimulated by HPCM, CSF-α, CSF-β and murine G-CSF. Replicate cultures were scored at day 7 (D7) and day 14 (D14). Colonies were greater than 40 cells and clusters less than 40 cells. Specific binding of ^{125}I-labelled murine G-CSF is expressed as percentage of binding to the same number of WEHI-3B D$^+$ cells performed on the same day.

TABLE 8.5 PROLIFERATION AND BINDING CHARACTERISTICS OF HUMAN LYMPHOID LEUKAEMIC CELLS

Source of Cells	Total Clusters per 10^5 Cells								Specific Binding of ^{125}I-G-CSF (% of WEHI-3B D$^+$)	
	HPCM		CSF-α		CSF-β		G-CSF			
	D7	D14	D7	D14	D7	D14	D7	D14	Marrow	Blood
ALL	0	0	0	0	0	0	0	0	0	1
ALL	0	0	0	0	0	0	0	0	2	0
ALL	0	0	0	0	0	0	0	0	0	0
ALL	44	7	60	17	56	14	80	6	11	-
ALL	20	0	15	0	19	0	20	0	4	0
ALL	0	0	0	0	0	0	0	0	0	0
ALL	0	0	0	0	0	0	0	0	0	0
CLL	0	0	0	0	0	0	0	0	-	0
CLL	0	0	0	0	0	0	0	0	0	0
CLL	0	0	0	0	0	0	0	0	0	0
CLL	0	0	0	0	0	0	0	0	1	0

Footnote to Table 8.5

Bone marrow cells were obtained from 7 patients with untreated acute lymphoblastic leukaemia (ALL) and 4 patients with chronic lymphatic leukaemia (CLL). All samples consisted of >70% leukaemic cells. 10^5 cells were cultured per ml and the number of clones counted at day 7 (D7) and day 14 (D14) of culture. All clones were <40 cells. Cultures were stimulated by HPCM, CSF-α, CSF-β and murine G-CSF. Specific binding of ^{125}I-labelled murine G-CSF is expressed as percentage of binding to the same number of WEHI-3B D$^+$ cells performed on the same day.

cells from all acute myeloblastic leukaemias, chronic myeloid
leukaemias, acute promyelocytic leukaemias and pre-leukaemias showed
higher levels of binding than the range seen for lymphocytic
leukaemias. Acute myelomonocytic leukaemias showed relatively little
binding of ^{125}I G-CSF compared to the granulocytic leukaemias. This
granulocytic specificity of the binding exhibited by ^{125}I-G-CSF was
confirmed by autoradiography of human bone marrow cells (Figure 8.2)
which showed that cells of the granulocytic cell lineages were
specifically labelled (promyelocytes more intensely labelled than
polymorphs) while lymphoid and erythroid cells were not. For
comparison, specific labelling of acute promyelocytic leukaemic
blasts is also shown in Figure 8.2.

The binding of ^{125}I G-CSF to WEHI-3B D$^+$ cells is inhibited in
a dose-dependent manner by unlabelled G-CSF but not by M-CSF, GM-CSF,
Multi-CSF or any of a number of unrelated growth factors (Nicola
and Metcalf, 1984). Figure 8.3c shows that human CSF-β inhibited
^{125}I G-CSF binding to WEHI-3B D$^+$ cells to the same extent as
unlabelled G-CSF and that the dose-response relationship was
indistinguishable from that for G-CSF when the data were standard-
ized to the number of units added, based on the human promyelocyte -
assay described in Chapter 6. In contrast, human CSF-α and murine
GM-CSF showed no inhibition of binding over the same dose range.
Light density marrow cells from patients with untreated chronic
myeloid leukaemia or untreated acute myeloid leukaemia (with greater
than 90% leukaemic blast cells) gave almost identical binding data
to those seen for murine WEHI-3B D$^+$ cells (Figure 8.3a,b). Moreover,
the dose-response relationships for inhibition of ^{125}I G-CSF binding
by unlabelled G-CSF or CSF-β were identical and occurred over the
same concentration range as the inhibition of binding to WEHI-3B D$^+$
cells while again, CSF-α and GM-CSF were unable to inhibit binding.
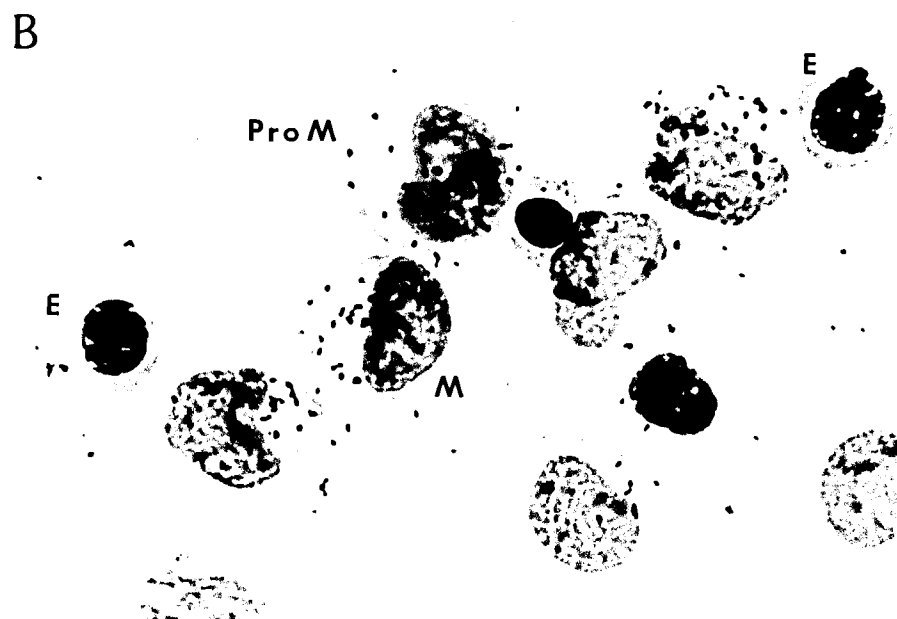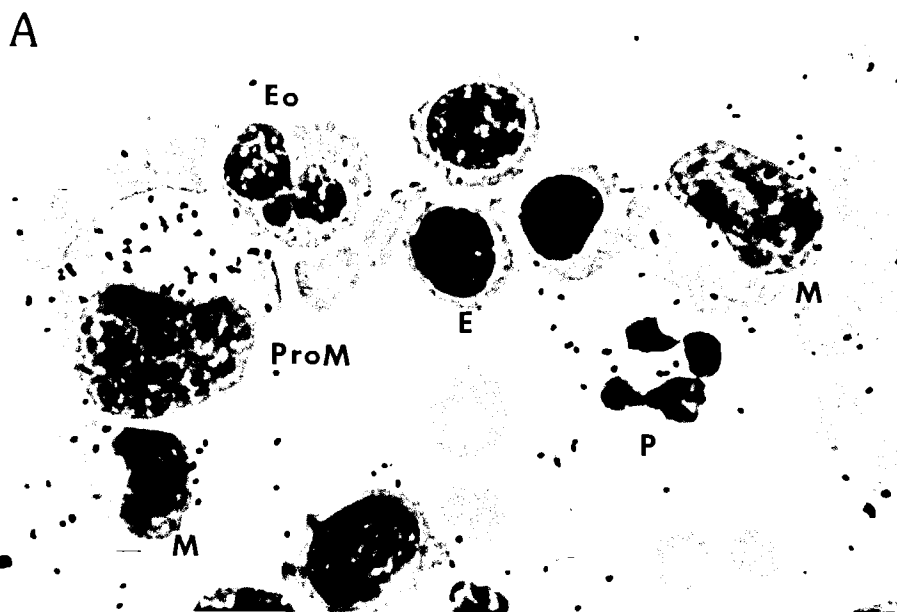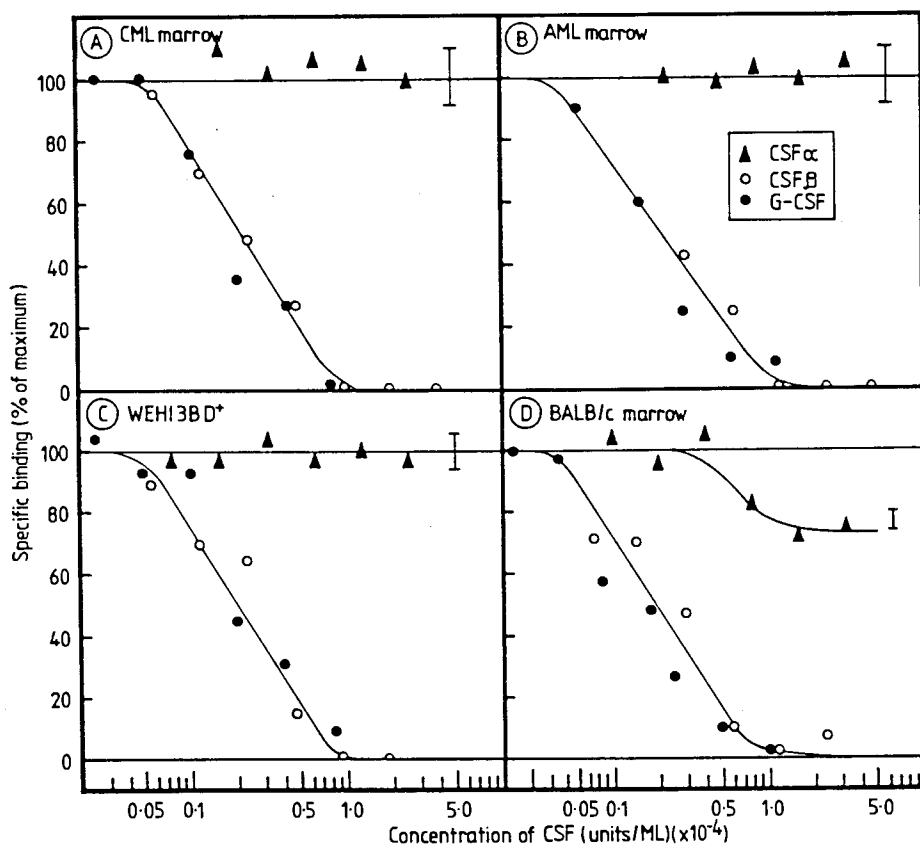
Fig. 8.2 Autoradiographs of the binding of ^{125}I-G-CSF to (A) normal human marrow. Note heavily labelled promyelocyte (ProM) and labelled myelocytes (M) and polymorph (P) but unlabelled eosinophil (Eo) and erythroid (E) cells. (B) Acute promyelocytic leukaemic marrow. Note labelled promyelocytes (ProM) and myelocytes (M) but unlabelled erythroid cell (E). Exposure time 1 month.

Fig. 8.3 Ability of human and murine colony-stimulating factors to compete for the binding of murine granulocyte colony-stimulating factor to human and murine cells. Human CSF-α and CSF-β pools and murine G-CSF Units were standardized in the assay on human promyelocyte-enriched cells described in Chapter 6 (50 Units being that amount giving 50% of a maximal clone response). In all panels open circles are unlabelled CSF-β, closed circles unlabelled G-CSF and closed triangles unlabelled CSF-α. Iodinated G-CSF and increasing amounts of unlabelled G-CSF, CSF-α or CSF-β as shown were added to 5 x 10^6 human chronic myeloid leukaemic bone marrow cells (panel A), 5 x 10^6 human acute myeloid leukaemic bone marrow cells (panel B), 2 x 10^6 WEHI-3B D^+ cells (panel C) or 5 x 10^6 BALB/c murine bone marrow cells (panel D). Maximum (100%) specific binding was that in the absence of competitors (mean of duplicate tubes) and 0% specific binding was that in the presence of a 50-fold excess of unlabelled G-CSF (mean of duplicate tubes). The bar on the right side of each panel represents the % specific binding seen in the presence of 100,000 Units of murine GM-CSF (range for triplicate or more tubes).

Figure 8.3d shows that the binding of ^{125}I G-CSF to BALB/c bone marrow cells was equally competed for by unlabelled G-CSF or CSF-β and that CSF-α like GM-CSF, could inhibit only 30% of the specific binding sites. This latter effect appears to result from down-regulation of G-CSF receptors rather than receptor cross-reactivity (Walker et al, 1985).

Saturation binding and Scatchard analyses were performed for the binding of ^{125}I G-CSF to human acute myeloid leukaemic bone marrow cells (>95% leukaemic blast cells) and murine leukaemic WEHI-3B D$^+$ cells (Figure 8.4). Because the Scatchard analysis of binding data for WEHI-3B D$^+$ cells becomes non-linear at low levels of bound ^{125}I G-CSF (Nicola and Metcalf, 1984) the dissociation constants were determined from the limiting slopes of the Scatchard curves. These gave a dissociation constant of 102 pM for binding to WEHI-3B D$^+$ cells and 98 pM for binding to the acute myeloid leukaemic blast cells. The number of binding sites per cell estimated from the intercepts on the abscissa were 300 and 480 for WEHI-3B D$^+$ and human acute myeloid leukaemic cells, respectively.

8.1.2.3 Recombinant Human GM-CSF and Human Leukaemic Cells. Based on the studies described in Chapter 3 showing a similar spectrum of biological activities for rH GM-CSF and CSF-α, and the results shown in Table 8.3 it was predicted that rH GM-CSF would stimulate the proliferation of leukaemic cells. This prediction was confirmed and results of stimulation of leukaemic cells by rH GM-CSF are shown in Table 8.6. The total number of clones stimulated by HPCM and rH GM-CSF was very similar, although in some cases HPCM was a superior stimulus for leukaemic cells. As the number of units of HPCM and rH GM-CSF was the same in all cultures (400 U/ml) this again suggested that a subset of some leukaemic cell populations were unresponsive to rH GM-CSF.
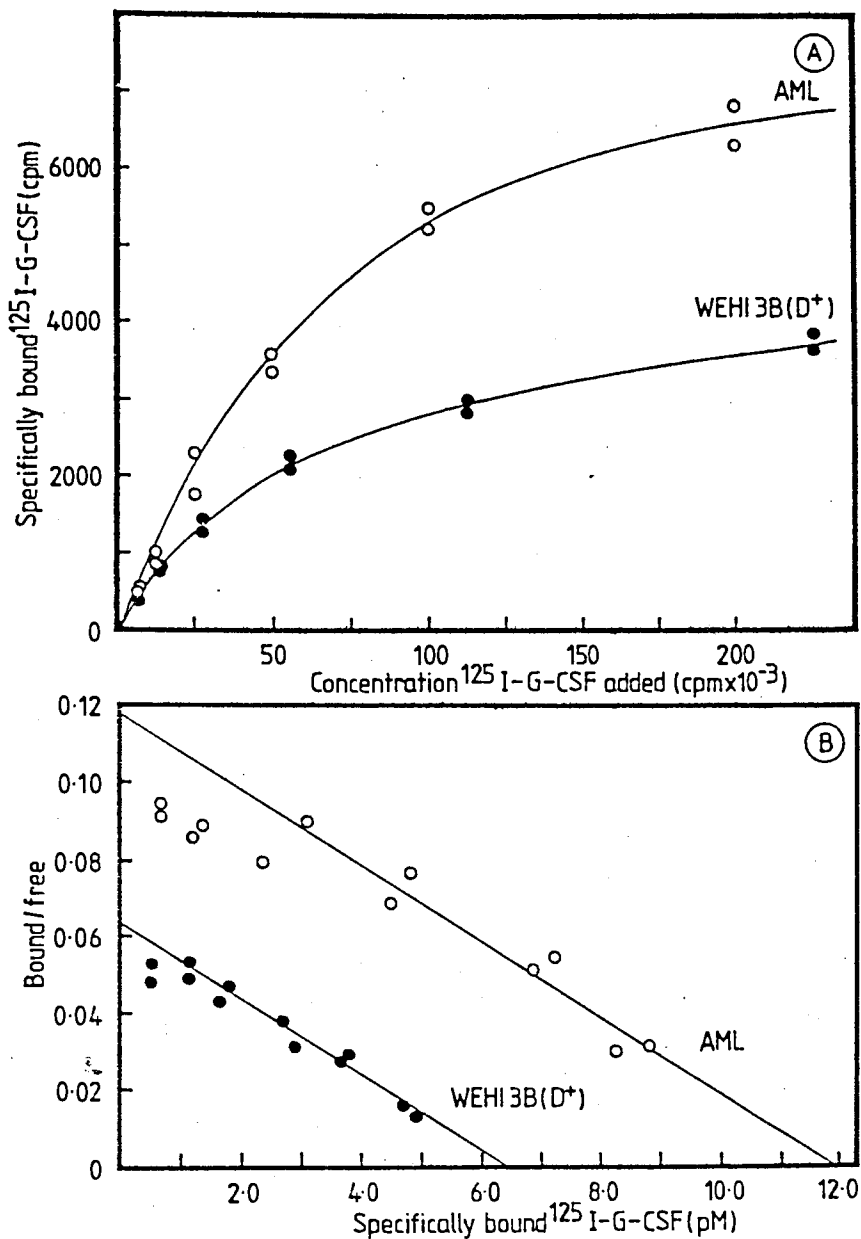
Fig. 8.4   Saturation analysis of binding of [125]I G-CSF to human acute
myeloid leukaemic cells and murine myelomonocytic leukaemic
cell line WEHI-3B D+.   AML cells (2.5 x 10^{6}) or WEHI-3B
D+ cells (2.0 x 10^{6}) were incubated with increasing concentra-
tions of [125]I G-CSF as shown, with or without an excess
of unlabelled G-CSF (250 ng/ml) at 37°C for 1 hr.   Tubes
were then placed on ice, cells resuspended and layered
over 200 µl cold FCS in 0.5 ml plastic centrifuge tubes
and centrifuged at 300 g for 5 min.   The cell pellet and
supernatants were counted separately in a gamma counter.
Non-specific binding (that in the presence of excess
unlabelled G-CSF) was linear with respect to the amount
of [125]I G-CSF added and was maximally 15% of the specific
binding in these experiments.   Specific binding was deter-
mined as total binding - non-specific binding and the
results of duplicate determinations are shown in Panel A.
Panel B shows the conversion of this data to Scatchard
plots (specific cpm bound/cpm in the supernatant versus
the concentration of [125]I G-CSF specifically bound to cells).

TABLE 8.6   CLONAL PROLIFERATION OF HUMAN ACUTE LEUKAEMIC CELLS

Source of AML Cells	rHGM CSF-responsiveness	Total Clusters (Colonies) Per 20,000 Cells					
		HPCM			rH GM-CSF		
		D3	D7	D14	D3	D7	D14
M1	Hypo-	300	136 (0)	12 (0)	288	140 (0)	12 (0)
M2	Normo-	3,700	540 (0)	640 (40)	3,840	600 (0)	640 (104)
M2	Normo-	-	4,900 (0)	3,300 (36)	-	3,100 (0)	1,900 (16)
M2	-	120	8 (0)	0 (0)	120	8 (0)	0 (0)
M2	Hyper-	244	140 (0)	28 (0)	240	140 (0)	36 (0)
M4	Hyper-	1,940	2,080 (0)	1,400 (156)	2,000	2,200 (0)	1,500 (160)
M4	Hyper-	4,600	12,000 (0)	4,000 (8)	4,500	12,000 (0)	4,000 (12)
M4	Normo-	336	872 (12)	332 (48)	316	880 (0)	332 (36)
M4	Normo-	404	12 (0)	0 (0)	432	8 (0)	0 (0)
M5	Normo-	-	20,000 (80)	8,000 (22)	-	20,000 (80)	20,000 (60)
M5	Normo-	460	296 (4)	0 (0)	460	260 (4)	0 (0)
M5	Hyper-	-	180 (12)	4,000 (200)	-	260 (20)	4,000 (200)
M5	* -	-	500 (0)	250 (0)	-	360 (0)	90 (0)
CML/AML	Hypo-	396	128 (0)	0 (0)	392	120 (0)	0 (0)

Footnote to Table 8.6

Cells were obtained from patients with untreated acute myeloid leukaemia and one patient with chronic myeloid leukaemia during acute transformation (CML/AML). All samples were at least 70% leukaemic blast cells. Cells were stimulated by HPCM and recombinant human GM-CSF (rH GM-CSF) and clones from replicate cultures counted at days 3 (D3), 7 (D7) and 14 (D14) of culture. Colonies were of greater than 40 cells and clusters of less than 40 cells. CSF-responsiveness was determined using serial dilutions of rH GM-CSF. Hypo-responsiveness samples did not reach a plateau phase of the titration curve when stimulated by 400 Units (20 ng/ml) of rH GM-CSF. Hyper-responsive leukaemic samples were at least 4-fold more responsive to rH GM-CSF than the most CSF-responsive normal cells and were thus outside 2 standard deviations of the normal CSF-response curve (see Chapter 3). In one patient (*) CSF-responsiveness could not be determined because proliferation occurred in unstimulated cultures and generated 360 clones present at day 7 and 90 clones at day 14.

The titration curves for different leukaemic samples were compared and two contrasting examples are shown in Figure 8.5. There was considerable variation in the responsiveness of different leukaemic cell populations, with some leukaemic samples being "hypo-responsive", some being "hyper-responsive" and other showing a responsiveness very similar to that of normal cells (Figure 7.2). The example of the "hypo-responsive" leukaemia shown in Figure 8.5 raised the possibility that the transient proliferation in vitro observed in some leukaemic cell populations may have been a reflection of CSF hypo-responsiveness of the cells. However, in other examples it appeared that transient proliferation was not related to CSF hypo-responsiveness. Transient proliferation was also observed in cell samples that were normo-responsive and hyper-responsive to CSF (Table 8.6). Furthermore stimulation of hypo-responsive leukaemic cells by 750 ng/ml of rH GM-CSF (approximately 15,000 Units) increased the number of clones but did not alter the size nor the transient behaviour of these clones. The significance of hypo-responsiveness or hyper-responsiveness at the receptor level is unknown but could reflect either altered receptor numbers or altered affinity of receptors for CSF.

8.1.2.4   Differentiation Induction in Human Leukaemic Cells

In an attempt to examine whether CSF induced differentiation in myeloid leukaemic cells, experiments were performed similar to those described for acute promyelocytic leukaemic cells in Chapter 7. Results for two examples at one timepoint are shown in Table 8.7. As with the acute promyelocytic leukaemic cells, differentiation was observed in CSF-stimulated and unstimulated cultures. The cell counts during the incubation period suggested that the differentiated cells had arisen from undifferentiated blast cells and that prolifera-
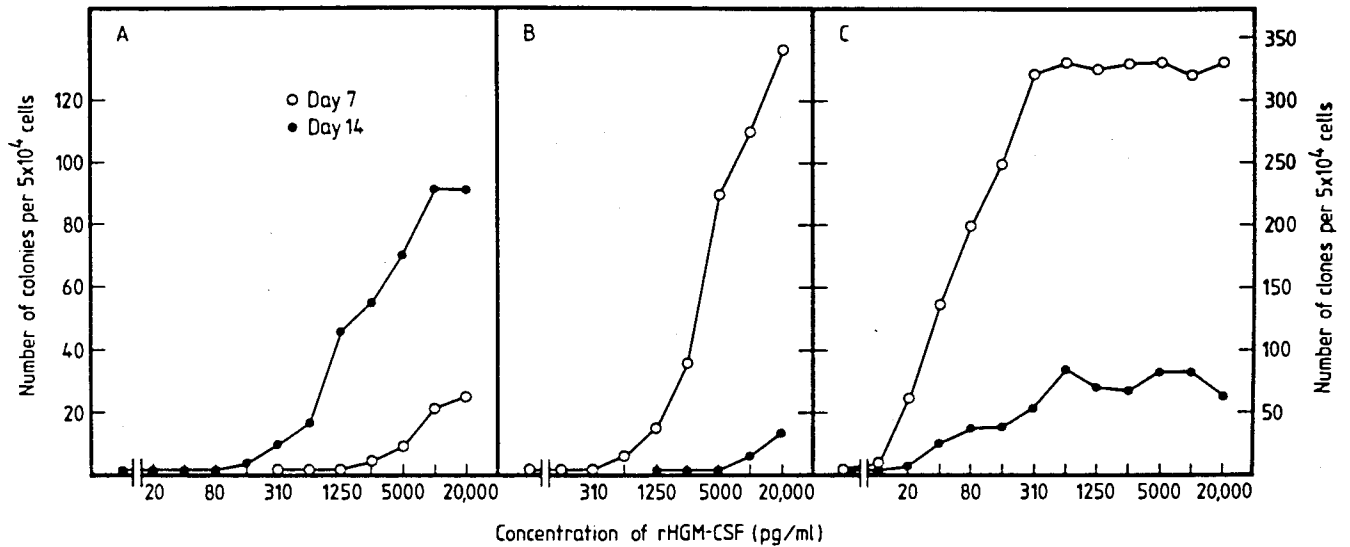
Fig. 8.5   Dose-response curves for recombinant human GM-CSF

(rH GM-CSF) stimulated proliferation of clonogenic cells.

Panel A shows a typical result for normal human bone marrow

cells.   Panel B shows the result for one fresh human

leukaemia (FAB M1) and Panel C for another fresh human

leukaemia (FAB M2).   For all samples replicate cultures

were examined after 7 and 14 days of incubation in cultures

stimulated by varying concentrations of rH GM-CSF.   The

number of colonies (of greater than 40 cells) and clones

(of greater than 3 cells) was recorded.   The number of

cells cultured per ml was 3×10^4, 2×10^4 and 5×10^4

for panels A, B and C respectively.

TABLE 8.7   MORPHOLOGY OF HUMAN LEUKAEMIC CELLS IN LIQUID CULTURES

FAB Classific- ation	Stimulating CSF	Percentage of Cells						Cell Count FINAL INITIAL
		Blasts	Promyelocytes	Myelocytes	Metamyelocytes	Lymphocytes	Monocytes	
M2	Starting Marrow	93.0	0.0	0.0	0.0	3.0	4.0	-
	No CSF	0.0	0.0	27.0	55.0	1.0	17.0	0.8
	CSF-α	1.5	2.5	33.5	40.5	3.5	18.5	0.8
	CSF-β	0.0	4.0	36.0	35.0	4.5	20.5	0.8
	G-CSF	0.0	2.0	31.0	36.5	4.5	26.0	0.8
M1	Starting Marrow	94.5	1.5	0.0	0.0	3.5	0.5	-
	No CSF	42.0	53.0	0.0	0.0	3.0	2.0	1.0
	CSF-α	46.0	47.0	0.0	0.0	4.0	3.0	1.0
	CSF-β	40.0	56.0	0.0	0.0	4.0	0.0	1.0
	rH GM-CSF	41.0	55.0	0.0	0.0	3.0	1.0	1.0

Light density bone marrow cells were cultured at 10^6 cells per ml for 9 days (first experiment) and 3 days (second experiment) in CSF-stimulated and unstimulated cultures. Cells were harvested, viable cell counts performed and cells stained with May-Grünwald and 4% Giemsa for morphological examination. A minimum of 200 cells was examined.

tion was masked by concomitant cell death. Consistent with the differentiated phenotype, these cells did not proliferate when subsequently placed in agar cultures, regardless of the source of CSF, although all samples proliferated in primary agar cultures. The observation of "spontaneous" differentiation induction is similar to the experience of others (Breitman et al, 1981; Golde and Cline, 1973).

In addressing the question of differentiation induction in leukaemic cell populations, the crucial issue is suppression of leukaemic-cell self-renewal. Although an assay system has been well-described that allows the study leukaemic stem cells with self-renewal properties (Buick et al, 1979) numerous attempts to establish this assay system were unsuccessful. Because of the importance of a possible factor present in PHA-LCM that was not present in HPCM or U/5637-CM, batches of PHA-LCM were prepared but these were also unable to support the growth of leukaemic blast cell colonies. For this reason, further studies on differentiation-induction were performed using the HL60 human leukaemic cell line.

8.2 HL60 HUMAN LEUKAEMIC CELLS: A MODEL FOR DIFFERENTIATION-COMMITMENT BY CSF

8.2.1 Introduction

Because of the difficulty in demonstrating CSF-stimulated differentiation-commitment in fresh leukaemic cells, experiments were performed using the human leukaemic cell line, HL60 (Collins et al, 1977). This cell line consists predominantly of promyelocytes but can be induced to terminally differentiate to functionally mature granulocytes or monocytes by a wide variety of agents. These include dimethyl sulphoxide and related compounds (Collins et al, 1978), retinoic acid (Breitman et al, 1980), phorbol diesters

(Rovera et al, 1979), inhibitors of DNA synthesis (Griffin et al, 1982a), butyric acid (Collins et al, 1978), γ-interferon (Ball et al, 1984) and proteolytic enzymes (Fibach et al, 1985). Several studies have also been performed to examine the action of crude CSF-containing supernatants on these cells. One study (Ruscetti et al, 1981) demonstrated that the number and size of HL60 colonies growing in semi-solid medium were increased by medium conditioned by several types of human cells, although these materials were reported not to induce differentiation nor to suppress self-generation of clonogenic cells. The stimulation of proliferation of HL60 cells was evident with early passages of these cells while later passages displayed little CSF-dependent proliferation and in contrast many of these cells proliferated in the absence of CSF-preparations. There was however no detectable HL60-stimulating activity nor CSF-activity present in medium conditioned by HL60 cells.

Medium conditioned by mitogen-stimulated human peripheral blood cells could induce differentiation in HL60 cells (Elias et al, 1980; Olsson et al, 1981) and this activity at least in part can be attributed to γ-interferon (Ball et al, 1984). One study reported that HL60 cells themselves were able to condition medium to stimulate the proliferation of HL60 colony cells but the active factor appeared unable to stimulate the proliferation of normal progenitor cells (Brennan et al, 1981). HL60-stimulatory activity has however, not been observed by others (Ruscetti et al, 1981; Metcalf, 1983).

Metcalf (1983) examined the action of HPCM, CSF-α and CSF-β on HL60 cells. These preparations stimulated proliferation of HL60 cells as evidenced by an increase in the total number of cells and an increase in the number of clonogenic cells per colony after 14 days of culture. As evidence of differentiation, the percentage of

colonies displaying a peripheral halo of loosely dispersed cells was also increased, however the most important single parameter of differentiation in leukaemic populations, the level of clonogenic (stem cells) within the population was increased. Murine CSF's including G-CSF were reported to be inactive on these cells.

8.2.2 Results

8.2.2.1 Clonal Growth of HL60 Cells in Primary Agar

Cultures. The optimal growth of HL60 cells is greatly dependent on the batch of FCS that is used (Boyd and Metcalf, 1984). Twenty-five batches of FCS were therefore screened until one was selected that supported optimal growth of HL60 cells in unstimulated cultures. This batch was used in all subsequent experiments and the frequency of clonogenic cells was between 53-92% (mean 80%) of the number of HL60 cells cultured.

The result of a typical primary agar culture is shown in Table 8.8. Similar results were observed in 6 other experiments. There was no increase in the size of colonies stimulated by CSF compared with unstimulated cultures although this was previously reported (Metcalf, 1983) and this difference is probably due to the batch of FCS used in these experiments. During the screening of different batches of FCS it also became evident that the peripheral halo of cells around "differentiated" colonies of HL60 cells was also very dependent on the batch of FCS used. With some batches of FCS, 20% of colonies in unstimulated cultures appeared "differentiated" and thus this criterion of differentiation was not used in these studies.

The results of primary agar culture experiments suggested that CSF might suppress the clonogenicity of HL60 cells. In cultures stimulated by CSF-α, the total number of clonogenic HL60 cells was between 58-88% (mean 77%) of the number in unstimulated cultures.

TABLE 8.8   CLONAL PROLIFERATION OF HL60 CELLS IN AGAR CULTURES
STIMULATED BY COLONY STIMULATING FACTOR

STIMULUS	7 Days		14 Days	
	Total Clones	Colonies	Total Clones	Colonies
Nil	158	140	139	133
CSF-α	126	62	120	89
CSF-β	120	74	132	92
rH GM-CSF	114	91	135	101
G-CSF	120	71	132	100

HL60 cells were stimulated by CSF-α, CSF-β, recombinant human GM-CSF
(rH GM-CSF) and murine G-CSF.   200 cells were cultured per ml and total
clone numbers (of >2 cells) and total colonies (of >40 cells) examined
in replicate cultures after 7 and 14 days of culture.

Similarly with CSF-β the number of clonogenic cells was between 46-100% (mean 76%) of unstimulated cultures. With both rH GM-CSF and murine G-CSF similar results were obtained 47-86% (mean 73%) and 69-86% (mean 79%) respectively.

8.2.2.2 CSF-Stimulated Differentiation of HL60 Cells

in Liquid Cultures. To further examine the

action of CSF on HL60 cells, liquid cultures were employed. This allowed more accurate assessment of cellular morphology and analysis of changes in surface antigen expression. Preliminary experiments indicated that a culture period of up to five days resulted in no detectable morphological change in HL60 cells nor was there any significant change in surface antigen expression. Furthermore, subsequent culture of these cells in agar showed no difference compared with primary agar cultures of HL60 cells stimulated by CSF.

Figure 8.6 shows the results of one experiment in which changes in surface antigen expression were examined when HL60 cells were stimulated by CSF for 1 week. The results shown are for cells stimulated by rH GM-CSF (upper panel) and murine G-CSF (lower panel) and examined using three monoclonal antibodies. Similar results were obtained when cells were stimulated by CSF-α and CSF-β. Although the change in fluorescence after 1 week was small, a similar change was observed in all experiments and was more striking when HL60 cells were stimulated by CSF for 2 weeks (Figure 8.7). Table 8.9 summarizes the results of 10 experiments. The Table shows both the change in median fluorescence of the cells (compared with CSF-unstimulated HL60 cells) and the percentage change in fluorescence that this represents for the whole population. For comparison, in 4 experiments HL60 cells were cultured with 1.2% dimethylsulphoxide which induces HL60 cells to differentiate into neutrophils (Collins et al,

Fig. 8.6. Change in surface antigen expression of HL60 cells after 1 week in cultures stimulted by recombinant human GM-CSF (upper panel, solid lines) and murine G-CSF (lower panel, solid lines). The left hand panels show results obtained with monoclonal antibody 25E11; middle panels, NIMP-R10; right hand panels, WEM-G11. The results for unstimulated cells are also shown (dotted line). In all experiments dead cells were excluded from analysis by propidium-iodide staining.
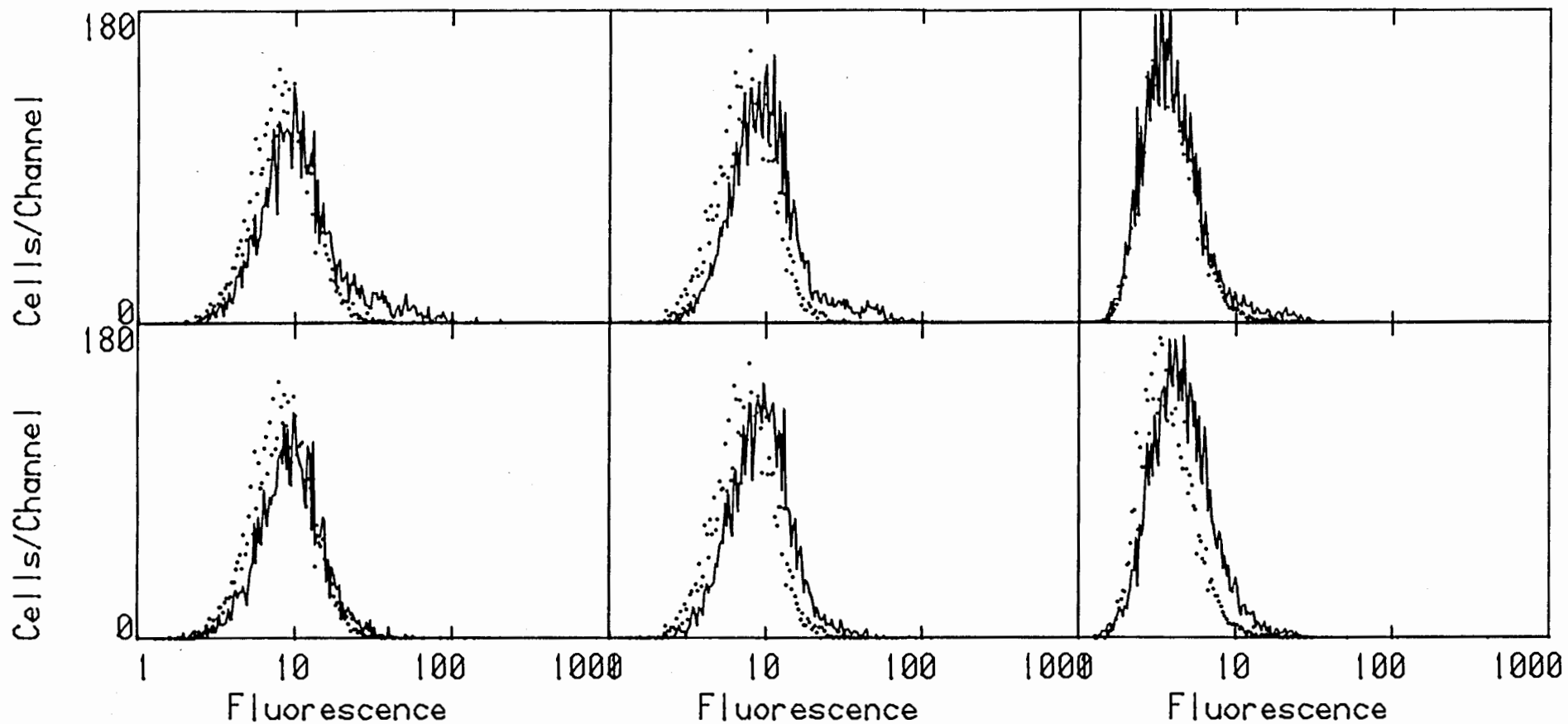
Fig. 8.7. Change in surface antigen expression of HL60 cells after 2 weeks in cultures stimulated by recombinant human GM-CSF (upper panel, solid lines) and murine G-CSF (lower panel, solid lines). The left hand panels show results obtained with monoclonal antibody 25E11; middle panels, NIMP-R10; right hand panels, WEM-G11. The results for unstimulated HL60 cells are also shown (dotted lines). In all experiments dead cells were excluded by propidium-iodide staining.
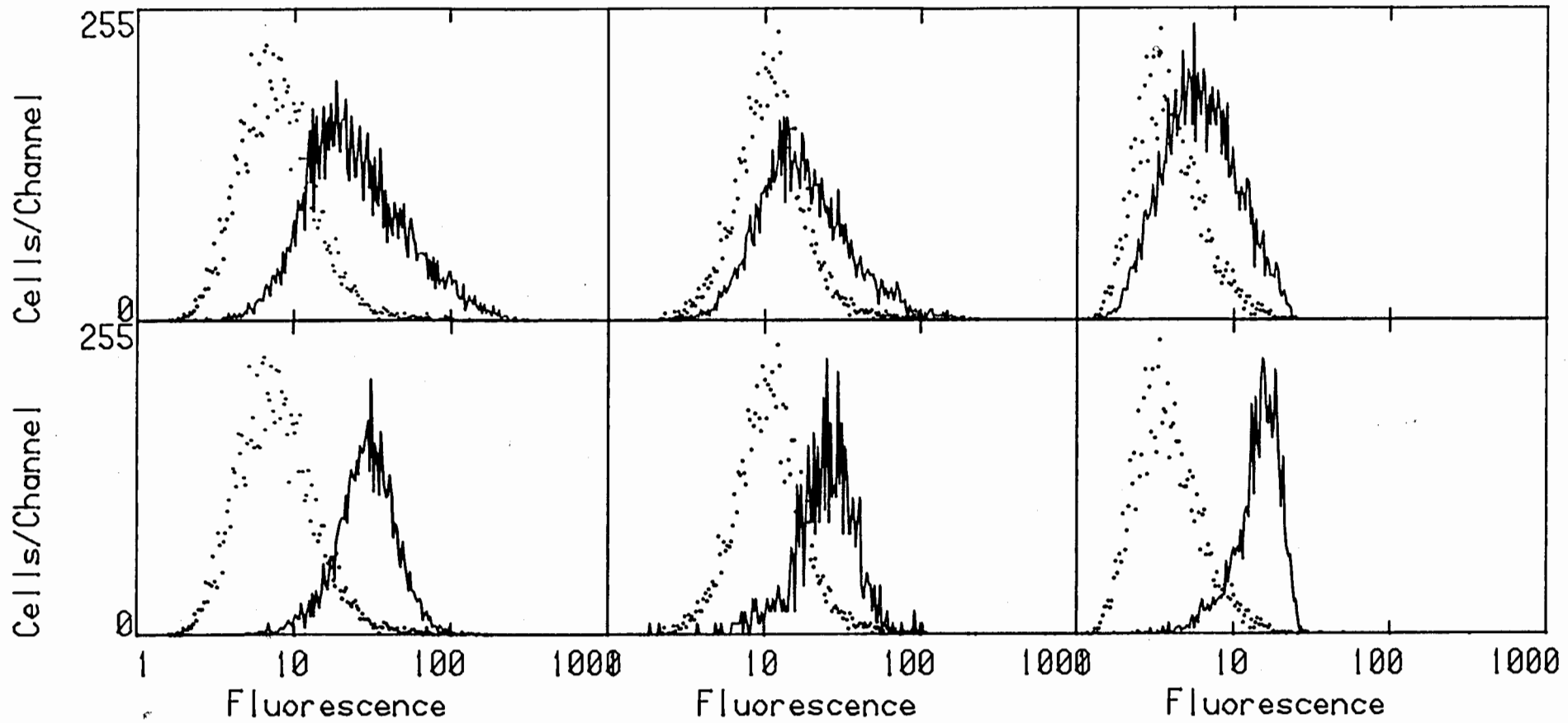
1978; Tarella et al, 1982a). After 1 week of culture, the change in
median fluorescence was determined using three monoclonal antibodies
(Table 8.9) and was 7.8 ± 8.7 (mean ± SD) channels for antibody 25E11,
9.3 ± 6.5 channels for antibody NIMP-R10 and 3.8 ± 4.5 channels for
antibody WEM-G11.

The morphology of HL60 cells following stimulation by CSF was
also examined. As shown in Table 8.10, there was no detectable change
in the morphology of these cells, despite the changes in surface
antigen expression. Cells were also stained with chloroacetate-
and non-specific esterases but no change was detected with CSF-
stimulated compared with unstimulated HL60 cells. Similarly, cells
induced to differentiate with dimethyl sulphoxide showed no morphol-
ogical evidence of differentiation although such cells were noticably
smaller in size compared with untreated HL60 cells. The aquisition
of cell surface markers in the absence of morphological change has
been previously reported for HL60 cells (Graziano et al, 1983) and
suggests that in these cells or some sublines of these cells the
two processes are separable. These results also caution against
the use of morphology alone as an index of differentiation.

8.2.2.3 Suppression of Clonogenic HL60 Cells by CSF.
Although there was evidence of differentiation commitment by CSF
acting on HL60 cells (demonstrated by changes in the expression of
surface antigens), the question of most importance related to the
possible ability of CSF to suppress clonogenicity of HL60 cells in
primary agar cultures (Table 8.8). Cells were therefore taken after
1 and 2 weeks of liquid culture and the number of clonogenic HL60
cells determined. One result is shown in Table 8.11 for cells
stimulated by 400 Units of each CSF for 1 week and then cultured in
agar for a further 14 days. Proliferation occurred in CSF-stimulated

TABLE 8.9   SURFACE ANTIGEN EXPRESSION OF HL60 CELLS STIMULATED BY COLONY STIMULATING FACTOR

Time (Weeks)	Stimulus	25E11		NIMP-R10		WEM-G11	
		Change in Channel No.	Relative Fluorescence (%)	Change in Channel No.	Relative Fluorescence (%)	Change in Channel No.	Relative Fluorescence (%)
1	CSF-α	9.1 ± 6.7	134 ± 24	6.7 ± 5.1	121 ± 17	3.3 ± 3.6	111 ± 10
	CSF-β	6.1 ± 4.9	127 ± 31	6.0 ± 4.4	118 ± 18	3.4 ± 2.6	110 ± 8
	rH GM-CSF	9.7 ± 6.2	134 ± 24	6.3 ± 5.5	120 ± 18	2.4 ± 2.5	107 ± 7
	G-CSF	11.6 ± 12.8	145 ± 63	8.0 ± 5.4	124 ± 17	2.6 ± 2.8	108 ± 10
2	CSF-α	20.1 ± 9.8	178 ± 53	18.3 ± 11.9	170 ± 55	18.8 ± 12.3	173 ± 63
	CSF-β	16.3 ± 12.1	164 ± 63	15.4 ± 12.2	160 ± 54	22.8 ± 15.2	197 ± 84
	rH GM-CSF	26.9 ± 12.5	214 ± 79	16.3 ± 10.2	154 ± 44	15.5 ± 5.8	153 ± 22
	G-CSF	15.1 ± 14.7	160 ± 89	16.1 ± 13.7	165 ± 62	23.3 ± 19.3	210 ± 126

HL60 cells were incubated for 1 and 2 weeks in cultures stimulated by CSF (as indicated) and containing 25% foetal calf serum. 2×10^4 cells were cultured per ml. Cells were analysed for surface antigen expression as detected by monoclonal antibodies 25E11, NIMP-R10 and WEM-G11 and compared to unstimulated cells. Results are mean (± SD) for 10 experiments and show the change in channel number of median fluorescence measured by the FACS (logarithmic scale). The increase in fluorescence that this represents relative to unstimulated cells (100% fluorescence) is shown. rH GM-CSF = recombinant human GM-CSF synthesized by bacteria. G-CSF = murine G-CSF.

TABLE 8.10   MORPHOLOGY OF HL60 CELLS STIMULATED BY COLONY STIMULATING FACTOR

Duration of Culture	Stimulus	CELL TYPE (%)				
		Blast cells	Promyelocytes	Myelocytes	Metamyelocytes	Monocytes
7 Days	Nil	2.5	94.0	3.0	0.0	0.5
	CSF-α	4.0	93.5	1.5	0.0	1.0
	CSF-β	2.0	96.5	1.0	0.0	0.5
	rH GM-CSF	0.5	95.5	4.0	0.0	0.0
	G-CSF	1.5	96.5	1.0	0.0	1.0
14 Days	Nil	2.0	91.5	5.0	0.0	1.5
	CSF-α	1.5	93.5	4.0	0.0	1.0
	CSF-β	1.0	94.0	4.0	0.0	1.0
	rH GM-CSF	1.5	94.5	3.5	0.0	0.5
	G-CSF	2.0	92.5	5.5	0.0	0.0

HL60 cells were incubated for 7 and 14 days in cultures stimulated by CSF-α, CSF-β, recombinant human GM-CSF (rH GM-CSF) and murine G-CSF. The morphology of cells was examined using cytocentrifuge smears stained with May-Grünwald and 4% Giemsa.

TABLE 8.11   SUPPRESSION OF CLONOGENIC HL60 CELLS BY COLONY STIMULATING FACTOR

| Liquid Culture (7 Days) | | AGAR CULTURE | | | | | Total |
| Stimulus | Cell Count per ml $(x10^{-5})$ | Stimulus | 7 Days | | 14 Days | | Clonogenic Cells per ml $(x10^{-5})$ |
			Total Clones	Colonies	Total Clones	Colonies	
Nil	1.8	Nil	313	295	327	327	2.2
CSF-α	1.0	CSF-α	15	15	13	13	0.10
		Nil	19	19	21	21	0.14
CSF-β	1.6	CSF-β	92	68	81	64	0.54
		Nil	93	88	95	95	0.64
rH GM-CSF	1.8	rH GM-CSF	30	17	32	25	0.21
		Nil	44	36	47	47	0.32
G-CSF	1.7	G-CSF	73	45	92	69	0.61
		Nil	97	91	98	88	0.66

HL60 cells were incubated for 7 days in liquid cultures stimulated by CSF-α, CSF-β, recombinant human GM-CSF (rH GM-CSF) and murine G-CSF. All cultures were performed using 25% foetal calf serum and the initial cell concentration was 25,000 cells per ml. An equal volume of cells was taken from all cultures and cultured in agar. Agar cultures were scored after 7 and 14 days of incubation and the total number of clones (of greater than 2 cells) and total colonies (of greater than 40 cells) counted from replicate cultures. The total number of clonogenic cells was calculated (per ml of liquid culture) and compares with an initial value of 0.25×10^5 per ml for all cultures.

and unstimulated liquid cultures and to minimize possible errors involved in cell counts, an equal aliquot was taken from all liquid cultures for the secondary agar cultures. All CSF preparations suppressed the number of clonogenic HL60 cells and this suppression was more pronounced when cells were further stimulated by CSF in agar cultures. Although in the example shown in Table 8.11, CSF-α reduced the total number of clonogenic cells to below the initial number of clonogenic cells, this was not always the case in other experiments. In cultures stimulated by CSF-α for 1 week, the number of clonogenic HL60 cells was 32.6 ± 25.3% (mean ± SD of 15 experiments) of the total number of clonogenic cells in unstimulated cultures. For CSF-β-stimulated cells, the number of clonogenic HL60 cells was 47.1 ± 25.3%, for rH GM-CSF 31.1 ± 19.5% and for murine G-CSF 46.2 ± 26.0% of the total number of clonogenic HL60 cells in control, unstimulated cultures. For comparison, in 4 experiments using 1.2% dimethylsulphoxide, the number of clonogenic cells after 1 week of liquid culture was 30.5 ± 30.9% of the number in control cultures.

Table 8.12 shows the result of a typical experiment in which the number of clonogenic HL60 cells was determined after 2 weeks liquid culture. Proliferation occurred in all cultures. The suppression of HL60 cells by CSF was more striking after 2 weeks liquid culture and the number of clonogenic cells in CSF stimulated cultures was approximately 2% of the number present in unstimulated cultures (Table 8.12). In other experiments, the number of clonogenic cells was 11.5 ± 13.2% (mean ± SD of 15 experiments) for CSF-α, 13.7 ± 13.4% for CSF-β, 10.8 ± 11.1% for rH GM-CSF and 10.9 ± 9.3% for murine G-CSF. In all experiments there was marked suppression of the total number of clonogenic cells and in 3 experiments total clonogenic cells were reduced to zero. For comparison, in 4 experi-

TABLE 8.12   SUPPRESSION OF CLONOGENIC HL60 CELLS BY COLONY STIMULATING FACTOR

| Liquid Culture (14 Days) | | AGAR CULTURE | | | | | Total Clonogenic |
| Stimulus | Cell Count per ml $(x10^{-5})$ | Stimulus | 7 Days | | 14 Days | | Cells per ml $(x10^{-5})$ |
			Total Clones	Colonies	Total Clones	Colonies	
Nil	2.5	Nil	281	281	300	300	25.0
CSF-α	0.6	CSF-α	9	6	11	11	0.90
		Nil	11	9	24	24	2.0
CSF-β	0.3	CSF-β	8	5	6	6	0.50
		Nil	7	6	9	9	0.75
rH GM-CSF	1.6	rH GM-CSF	1	0	2	1	0.17
		Nil	1	0	2	2	0.17
G-CSF	0.3	G-CSF	2	1	2	1	0.17
		Nil	2	2	2	2	0.17

HL60 cells were incubated in liquid cultures stimulated by CSF-α, CSF-β, recombinant human GM-CSF (rH GM-CSF) and murine G-CSF. All cultures were performed using 25% foetal calf serum and the initial cell concentration was 25,000 cells per ml. After 7 days of liquid culture (Table 8.11) 100 µl of cells was taken and fresh medium added. After a further 7 days of culture, an equal volume of cells was taken from all cultures and cultured in agar. Agar cultures were scored after 7 and 14 days of incubation and the total number of clones (of greater than 2 cells) and total colonies (of greater than 40 cells) counted from replicate cultures. The total number of clonogenic cells was calculated (per ml of liquid culture) and compares with an initial value of 0.25×10^{5} per ml for all cultures.

ments using 1.2% dimethylsulphoxide, the number of clonogenic cells after 2 weeks of liquid culture was 14.5 ± 16.9% of the number in untreated cultures.

     8.2.2.4 <u>CSF-Stimulated Proliferation of HL60 Cells</u>. CSF containing preparations have been reported to stimulate the proliferation of HL60 cells (Metcalf, 1983) although this is more evident with early rather than later passages of these cells where autonomous growth predominates (Ruscetti et al, 1981). Similarly, studies with murine leukaemic WEHI-3B D$^+$ cells have demonstrated that murine G-CSF, a powerful inducer of differentiation also stimulates proliferation of these cells (Metcalf, 1982).

     In 2 of 17 experiments in which HL60 cells were cultured for 1 week in liquid and subsequently in agar cultures, there was a suggestion of CSF-stimulated proliferation. The number of clonogenic cells in CSF-stimulated cultures compared with unstimulated cultures was 128 ± 33% (mean ±SD) for CSF-α, 128 ± 26% for CSF-β, 123 ± 33% for rH GM-CSF and 147 ± 38% for murine G-CSF. However, to study differentiation-induction by CSF, culture conditions had been deliberately selected to support the optimal autonomous growth of HL60 cells. It seemed possible therefore that CSF-stimulated proliferation had been masked in other experiments because maximal or near maximal proliferation had occurred in unstimulated cultures. An attempt was therefore made to manipulate the culture system to allow CSF-stimulated proliferation to be evidenced and experiments were performed using lower concentrations of foetal calf serum (FCS).

     Of 12 experiments performed using 2% FCS, 6 showed evidence of CSF-stimulated proliferation while 6 experiments demonstrated only CSF-suppression of clonogenic HL60 cells. The degree of suppression observed was very similar to that seen in cultures using 25% FCS.

For example, after 1 week in liquid cultures the number of clonogenic cells in cultures stimulated by rH GM-CSF compared with unstimulated cultures was 33.6 ± 19.2% (mean ± SD of 6 experiments) in 2% FCS compared with 31.1 ± 19.5% (mean ± SD of 15 experiments) in 25% FCS.

One example of CSF-stimulated proliferation of HL60 cells is shown in Table 8.13. Proliferation occurred in both CSF-stimulated and unstimulated cultures but the total number of cells was always greater in the CSF-stimulated cultures. Similarly, there was an increase in the number of clonogenic cells in CSF-stimulated liquid cultures. In the example shown in Table 8.13 the increase in total number of cells and number of clonogenic cells occurred in parallel for all CSF preparations. However, this was not always constant, so that in some cases, for example rH GM-CSF stimulated an increase in the total number of total cells but the proportion of clonogenic cells was decreased. This variability was taken to reflect a balance between the opposing actions of CSF in stimulating both proliferation and suppression of clonogenic HL60 cells. Some variability is also evidence in Table 8.13. After 7 days in agar culture, the number of clonogenic cells in rH GM-CSF and G-CSF stimulated cultures was decreased compared with the number of clonogenic cells in the paired-unstimulated agar cultures. In contrast the total number of clones was increased in CSF-β-stimulated versus the paired-unstimulated agar cultures.

An increase in number of total HL60 cells after 7 days liquid culture was observed with all CSF preparations in 6 experiments and an increase in clonogenic cells was observed in 3 experiments with CSF-α and rH GM-CSF, 4 experiments with CSF-β and 5 experiments with murine G-CSF. The mean increase in clonogenic cells was 188 ± 22% (mean ± SD) for CSF-α, 164 ± 46% for CSF-β, 181 ± 33% for rH GM-CSF and 163 ± 32% for murine G-CSF.

TABLE 8.13   CSF-STIMULATED PROLIFERATION OF CLONOGENIC HL60 CELLS CULTURED IN 2% FOETAL CALF SERUM

| Liquid Culture (7 Days) | | AGAR CULTURE | | | | | Total |
| Stimulus | Cell Count per ml $(x10^{-5})$ | Stimulus | 7 Days | | 14 Days | | Clonogenic |
			Total Clones	Colonies	Total Clones	Colonies	Cells per ml $(x10^{-5})$
Nil	10.2	Nil	630	550	650	650	13.0
CSF-α	15.8	CSF-α	716	560	954	805	19.1
		Nil	733	590	985	985	19.7
CSF-β	12.2	CSF-β	1005	550	815	630	16.3
		Nil	845	695	1130	1130	22.6
rH GM-CSF	15.3	rH GM-CSF	658	535	632	482	12.6
		Nil	920	805	1095	1095	21.9
G-CSF	11.5	G-CSF	460	370	651	420	13.0
		Nil	700	593	850	850	17.0

HL60 cells were incubated in liquid cultures stimulated by CSF-α, CSF-β, recombinant human GM-CSF (rH GM-CSF) and murine G-CSF. All cultures were performed using 2% foetal calf serum and the initial cell concentration was 20,000 cells per ml. After 7 days of culture cells were counted and an equal volume from each culture was cultured in agar. Agar cultures were scored after 7 and 14 days and total clones (of greater than 2 cells) and total colonies (of greater than 40 cells) scored from replicate cultures.

As expected from the results shown in Table 8.13 there was evidence of suppression of clonogenic cells following incubation for a second week in CSF-stimulated cultures (Table 8.14). The number of clonogenic cells in CSF-stimulated compared with unstimulated cultures was $5.5 \pm 3.1\%$ (mean $\pm$ SD of 4 experiments) for CSF-α $24.0 \pm 15.3\%$ for CSF-β, $8.4 \pm 5.6\%$ for rH GM-CSF and $10.6 \pm 4.8\%$ for murine G-CSF.

While in most experiments the number of clonogenic cells continued to decline when cells were stimulated by CSF for longer periods of time, occasionally this was not the case. An example is shown for CSF-β in Table 8.15 and similar results were obtained with all the CSF preparations and in cultures containing 25% FCS. This probably reflected the emergence of HL60 cells that were resistant to CSF-suppression (analogous to CSF-unresponsive mouse leukaemia cells) (Metcalf and Nicola, 1982). Alternatively this could have represented a protracted CSF-stimulated proliferative phase prior to CSF-suppression of cells and in some experiments this seemed to be the case.

8.2.2.5 FCS-Poor Culture Conditions Induce Surface Antigen Changes in HL60 Cells.

The expression of surface antigens by HL60 cells cultured in 2% FCS was examined. An unexpected result was obtained when cells were cultured in decreasing concentrations of FCS in unstimulated cultures (Table 8.16). For both antibodies examined there was increased surface antigen expression in unstimulated cultures containing decreasing concentrations of FCS. Similar results were obtained in 5 experiments. In 2% FCS cultures stimulated by CSF there was no increase in surface antigen expression above that which was evident in 2% FCS unstimulated cultures. This was despite the CSF-stimulated proliferation and suppression of these cells. There was no detectable morphological change in the cells.

TABLE 8.14 CSF-STIMULATED SUPPRESSION OF CLONOGENIC HL60 CELLS CULTURED IN 2% FOETAL CALF SERUM

| Liquid Culture (14 Days) | | AGAR CULTURE | | | | | Total |
| Stimulus | Cell Count per ml $(x10^{-5})$ | | 7 Days | | 14 Days | | Clonogenic |
		Stimulus	Total Clones	Colonies	Total Clones	Colonies	Cells per ml $(x10^{-5})$
Nil	7.2	Nil	658	597	674	629	56.2
CSF-α	5.1	CSF-α	32	18	22	20	1.8
		Nil	36	15	27	20	2.3
CSF-β	3.7	CSF-β	160	96	161	112	13.4
		Nil	255	140	249	205	20.8
rH GM-CSF	2.6	rH GM-CSF	72	40	47	37	3.9
		Nil	79	42	50	39	4.2
G-CSF	4.2	G-CSF	83	46	46	36	3.8
		Nil	79	49	65	47	5.4

HL60 cells were incubated in liquid cultures stimulated by CSF-α, CSF-β, recombinant human GM-CSF (rH GM-CSF), and murine G-CSF. All cultures were performed using 2% foetal calf serum and the initial cell concentration was 20,000 per ml. After 7 days of culture (Table 8.13) 200 μl of cells were taken and fresh medium added. After a further 7 days of culture an equal volume of cells was taken from all cultures and cultured in agar. Replicate cultures were scored and the total number of clones (of greater than 2 cells) and colonies (of greater than 40 cells) counted.

TABLE 8.15   CLONAL PROLIFERATION OF HL60 CELLS STIMULATED BY COLONY STIMULATING FACTOR

| Liquid Culture (21 Days) | | AGAR CULTURE | | | | | Total Clonogenic |
Stimulus	Cell Count per ml $(x10^{-5})$	Stimulus	7 Days Total Clones	Colonies	14 Days Total Clones	Colonies	Cells per ml $(x10^{-5})$
Nil	3.7	Nil	245	50	260	260	520.0
CSF-α	1.5	CSF-α	4	2	2	2	4.0
		Nil	9	5	9	9	18.0
CSF-β	8.6	CSF-β	400	180	410	400	820.0
		Nil	450	200	445	420	890.0
rH GM-CSF	1.3	rH GM-CSF	0	0	0	0	0.0
		Nil	2	1	2	2	4.0
G-CSF	1.6	G-CSF	3	1	2	2	4.0
		Nil	10	7	11	9	22.0

HL60 cells were incubated in liquid cultures stimulated by CSF (as indicated). All cultures were performed using 2% foetal calf serum and the initial cell concentration was 20,000 cells per ml. After 7 and 14 days of culture 200 µl of cells were taken and fresh medium added. After a further 7 days of culture the number of cells was counted and an equal volume from all cultures was cultured in agar. Replicate cultures were scored after 7 and 14 days and the total number of clones (of greater than 2 cells) and colonies (of greater than 40 cells) recorded. The total number of clonogenic cells was calculated and compares with an initial value of 0.2×10^{5} cells per ml.

TABLE 8.16   CHANGE IN SURFACE ANTIGEN EXPRESSION OF UNSTIMULATED HL60 CELLS CULTURED IN VARYING
CONCENTRATIONS OF FOETAL CALF SERUM

Concentration	25E11		NIMP-R10	
of F.C.S.	Change in Channel No.	Relative Fluorescence (%)	Change in Channel No.	Relative Fluorescence (%)
25%	0	100	0	100
10%	30	202	18	162
5%	46	311	22	181
2%	49	337	37	271

20,000 HL60 cells per ml were incubated in varying concentrations of foetal calf serum (FCS) for

7 days.   Cultures were not stimulated by CSF.   Cells were then stained with monoclonal antibodies

25E11 and NIMP-R10.   Results change in channel number of median fluorescence (as measured by the

FACS on a logarithmic scale) and the percent increase in fluorescence that this represents

(relative to cells cultured in 25% FCS).   Similar results were obtained in 4 other experiments

and the change in channel number was 36 ± 10 (mean $\pm$ SD) channels ($276 \pm 70\%$) for cells

cultured in 25% F.C.S compared with cells cultured in 2% F.C.S.

Although there was increased surface antigen expression in unstimulated cells cultured in FCS-poor conditions, there was no apparent change in the frequency of clonogenic cells. This was in contrast to CSF-stimulated cultures where suppression (with or without prior proliferation) of clonogenic cells was evident. These results suggested that the acquisition of surface markers alone may occur in the absence of any change in clonogenic (self-renewal) properties and furthermore these changes may be the result of relatively trivial alterations in culture conditions. It is therefore potentially hazardous to attribute such changes alone to a specific effect of a particular agent. However, the concomitant changes in clonogenic cells argue for a specific action of CSF in inducing differentiation in HL60 cells.

8.3 DISCUSSION

The observation that human leukaemic cells are dependent on stimulation by CSF for clonal proliferation is not new, having been first reported by Moore et al in 1973. More recently however, it has been suggested that these cells elaborate their own growth factors as a mechanism for leukaemogenesis (Schrader, 1984). The results presented here are not consistent with this hypothesis except perhaps for some leukaemic cells displaying monocytic differentiation where CSF synthesis can be attributed to monocytes of leukaemic origin (Golde et al, 1974; Goldman et al, 1976; Greenberg et al, 1978). Therefore, while it is clear that leukaemic cells may be generated in vitro in association with the acquisition of CSF independence (Schrader and Crapper, 1983) or following insertion of the GM-CSF cDNA into factor-dependent cell lines (Lang et al, 1985) this does not appear to be a common mechanism in the development of human leukaemia.

The action of murine G-CSF on normal human promyelocytes and leukaemic promyelocytes was extended in these studies to other human myeloid leukaemic cells. Most murine CSF's are inactive on human cells, but the species cross-reactivity of murine G-CSF and human CSF-β is striking. Both molecules stimulated granulocyte colony formation by cells from both species with similar dose-response relationships and similar numbers of responsive target cells (Chapter 6). Both molecules induced differentiation in murine WEHI-3B D$^+$ cells. Both are hydrophobic molecules of similar size (30,000 for CSF-β and 25,000 for G-CSF). That these molecules are directly cross-reactive was indicated by the demonstration of specific binding sites for ^{125}I G-CSF on human and murine granulo-cytic cells and the equal abilities of G-CSF and CSF-β to inhibit this binding.

The frequency of binding sites on leukaemic cells was similar to that on normal cells and the apparent binding affinity for ^{125}I G-CSF appeared to be approximately the same on all the cell types studied. Moreover, most human leukaemic cells were analogous to murine WEHI-3B D$^+$ cells in that they expressed receptors for murine G-CSF (and by implication, CSF-β). These were functional receptors and the preservation of a functional receptor on human leukaemic cells for murine G-CSF, a profound inducer of different-iation in murine leukaemic cells, suggests possible potential clinical applications for this molecule.

The variation in responsiveness to rHGM-CSF observed with human leukaemic cells is consistant with observations made by Francis (1979) who described hypo-responsiveness in leukaemic cells and the report of one hyper-responsive leukaemia (Brennan et al, 1979). These reports were in conflict with the observations of Metcalf (1977)

showing normal responsiveness of leukaemic cells (although the
standard deviation reported was considerable). The discrepancy may
be explained in part by the degree of individual variation noted in
this study. Some leukaemic samples were clearly hypo-responsive,
some were hyper-responsive and some displayed responsiveness similar
to normal cell populations. This degree of variability is greater
than the individual variation seen with normal cells (Chapter 3)
and does not appear to explain the transient nature of some leukaemic
clones in vitro. The significance of these observations at a receptor
level remains to be determined, but probably reflects either altered
receptor numbers or altered receptor-affinity for CSF molecules.

Attempts to examine differentiation-commitment by CSF using
fresh leukaemic cells were unsuccessful and therefore studies were
performed using the human leukaemic cell line HL60. These studies
demonstrated CSF-stimulated surface antigen changes in these cells
associated with significantly reduced numbers of clonogenic cells
after 2 weeks in liquid and 2 weeks in agar cultures. Consistent
with other reports (Graziano et al, 1983) there was no detectable
morphological change in these cells. These results are very similar
to those obtained with murine WEHI-3B D^+ cells where suppression of
clonogenic cells by murine G-CSF can be demonstrated (Metcalf and
Nicola, 1982) but usually requires between 14-28 days (Metcalf, 1980)
because of the asynchronous nature of this event. In several experi-
ments with HL60 cells, clonogenic cells were completely suppressed.
Several other reports have failed to demonstrate suppression of clono-
genicity of HL60 cells by CSF (Ruscetti et al, 1981; Metcalf, 1983)
although there was some evidence of differentiation-induction as
measured by motility through agar (Metcalf, 1983). The differences
are probably due to the different culture systems that were employed

as in this study primary agar cultures alone also failed to demonstrate any clear CSF-suppression of clonogenic cells. The action of murine G-CSF to stimulate differentiation in HL60 cells reported here is also in conflict with published results (Metcalf, 1983) but the experiments presented here used concentrations of G-CSF 10-fold higher than those used previously. This higher concentration was used because of the reduced sensitivity of human cells compared with murine cells for murine G-CSF (Chapter 6).

The proliferation of HL60 cells has previously been shown to be stimulated by CSF (Ruscetti et al, 1981; Metcalf, 1983) although in these experiments this effect was apparently masked in cultures containing 25% FCS. In FCS-poor cultures, CSF-stimulated proliferation became evident in some experiments. Using such culture conditions however, such a result may also have been seen with the addition of, for example, insulin or transferrin (although cellular proliferation did occur in control FCS-poor cultures suggesting the presence of adequate levels of such compounds). A CSF-specific stimulated proliferation of HL60 cells seems likely, however for several reasons. A proliferative effect of CSF is seen when murine WEHI-3B D$^+$ cells are stimulated to differentiate by murine G-CSF (Metcalf, 1982) and is also evident with fresh human leukaemic cells stimulated by CSF. Furthermore, even in cultures in which proliferation was observed, the most striking result was the ultimate suppression of clonogenic cells.

The increased surface antigen expression of cells in unstimulated but FCS-poor cultures was intriguing. This was not however associated with an apparent reduction in the frequency of clonogenic cells as observed in CSF-stimulated cultures. These results suggested that surface marker changes alone (as evidence of "differentiation") may occur in the absence of changes in self-renewal potential. Experi-

ments were not performed to further examine this question but these results suggest two separate mechanisms by which changes in surface antigens may be achieved.

Based on the suppression of clonogenic HL60 cells by CSF-α and rHGM-CSF, murine G-CSF and the human equivalent CSF-β, it is tempting to speculate regarding a possible role for such molecules in the suppression of self-renewal potential in fresh human leukaemias. This remains to be determined. However the possible action of these molecules in stimulating proliferation of HL60 cells and their unequivocal action stimulating fresh human leukaemic cells would necessitate the careful use of such agents in vivo (perhaps following cytotoxic therapy). Furthermore, the existence of CSF-unresponsive WEHI-3B cells (Metcalf and Nicola, 1982) and the probable emergence of CSF-unresponsive HL60 cells in these experiments may potentially limit the clinical usefulness of such agents.

CHAPTER 9

PURIFIED COLONY STIMULATING FACTORS ENHANCE THE
SURVIVAL OF MATURE HUMAN NEUTROPHILS AND EOSINOPHILS

9.1 INTRODUCTION

One important action of the CSF's is the stimulation of the functional activities of mature cells. M-CSF stimulates the ability of macrophages to kill tumour cells (Wing et al, 1982) as well as stimulating production and release of several important molecules (Moore et al, 1980; Kurland et al, 1979; Fleit and Rabinovitch, 1981). Several of these functions can also be stimulated by GM-CSF (Handman and Burgess, 1979) and Multi-CSF (Crapper et al, 1985). In mature granulocytes, GM-CSF stimulates RNA and protein synthesis (Burgess and Metcalf, 1977; Stanley and Burgess, 1983) and ability of neutrophils to exhibit antibody-dependent cytotoxicity of tumour cells (Lopez et al, 1983). Human neutrophils and eosinophils are stimulated by CSF-α, but CSF-β is unable to stimulate eosinophils (Vadas et al, 1983; 1983a). Two studies have suggested that CSF's may have a role in enhancing the survival of mature cells (Stanley and Burgess, 1983; Vadas et al, 1983) similar to the action on progenitor cells (Morstyn et al, 1981; Metcalf and Merchav, 1982).

An intriguing aspect regarding the action of CSF's that has emerged from the studies described in this thesis is that the "lineage-specific" murine molecules (granulocyte CSF (G-CSF); eosinophil differentiation factor/eosinophil CSF (EDF/EoCSF)) are completely active and retain their "lineage-specificity" on normal human cells. These observations are consistent with previous reports that murine G-CSF is able to stimulate mature human neutrophils but not eosinophils (Lopez et al, 1983) and reports that murine M-CSF and human urinary M-CSF display antigenic and biochemical reactivities across species (Shadduck and Metcalf, 1975; Das et al, 1980). In contrast, and despite the high degree of homology at the molecular

level (Wong et al, 1985) neither the rH GM-CSF nor the purified
murine GM-CSF displayed cross-species reactivities (Chapter 3;
Lopez et al, 1983).

The experiments described here were performed to examine whether
the human-active CSF's (of murine and recombinant origin) were active
on mature human neutrophils and eosinophils. As the action of CSF
on numerous activities of mature cells had previously been studied,
it was decided to examine the question of mature cell survival where
it might be predicted that CSF could have an effect and where previous
studies were lacking.

9.2 RESULTS

9.2.1 Neutrophil Survival In Vitro

Figure 9.1 shows the results of a typical experiment in which
the survival of purified peripheral blood neutrophils was examined.
For the first 15 hours of the culture period there was no difference
between the survival of neutrophils in control cultures compared
with cultures containing approximately 10 units/ml (as determined
by colony formation of normal human marrow cells) of rH GM-CSF, CSF-α
or murine G-CSF. In Figure 9.1, the first timepoint at which dead
cells were noted in control cultures was at 20 hours. The time at
which cell death was first evident varied between 14-20 hours for
neutrophils obtained from different individuals. On continued
incubation, cell numbers continued to decline and after approximately
35 hours fewer than 10% of cells remained in control cultures of
neutrophils from 8 individuals examined.

Neutrophils in cultures containing CSF showed a consistent
enhanced survival compared with cells in control cultures. In the
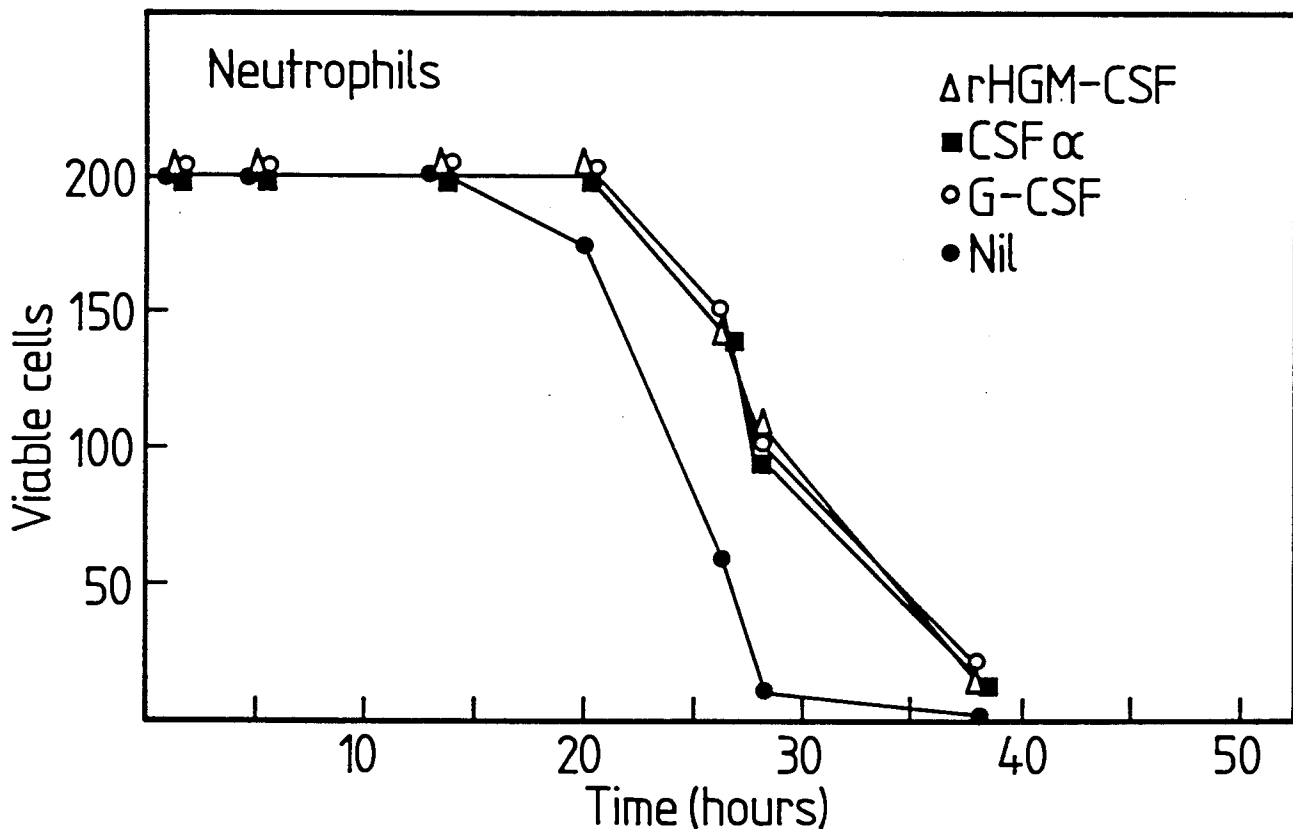example in Figure 9.1, this difference was most striking at the 28

Fig. 9.1 The survival of human blood neutrophils (>98% pure) in vitro in control cultures and cultures containing CSF. Cells were placed in microtiter trays (200 cells per well) and the number of viable cells was counted in 4 replicate wells at various timepoints thereafter. Cell survival was enhanced in cultures containing recombinant human GM-CSF (rH GM-CSF), semi-purified human CSF-α, and murine G-CSF. The concentration of CSF was approximately 10 units/ml as determined by normal human bone marrow colony formation.

hour timepoint, when the mean number of cells in control cultures was 10 compared with 100 cells in wells containing CSF. There was no apparent difference between rH GM-CSF, CSF-α and murine G-CSF in terms of their ability to enhance neutrophil survival.

9.2.2 Eosinophil Survival In Vitro

The survival of purified human eosinophils in vitro was also enhanced when cells were cultured in the presence of CSF (Figure 9.2). At early timepoints there was no difference between control cultures and cultures containing CSF. Dead cells were first observed in control cultures at 26 hours (range 18-28 hours for different individuals). Cell death was progressive in control cultures and fewer than 10% of cells remained after approximately 40 hours for all individuals examined. The survival of eosinophils was enhanced in cultures containing CSF. The difference between control and CSF-containing cultures was most evident at the 40 hour timepoint (Figure 9.2) when the mean number of cells was 20 in control cultures compared with 108 in CSF-containing cultures. At equivalent concentrations there was no apparent difference between rH GM-CSF, CSF-α and murine EDF (Eo-CSF) in terms of their ability to enhance eosinophil survival in vitro.

Eosinophils consistently showed a longer survival in vitro than neutrophils obtained from the same individual. The mean time taken for 50% of cells to die in unstimulated cultures was 20 hours (mean of 4 experiments) for neutrophils and 30 hours for eosinophils.

To confirm that cell viability was being accurately assessed by direct visual examination, viable cell counts were performed in the presence and absence of eosin. The results are shown in Figure 9.3. For neutrophils, cell viability could be accurately assessed
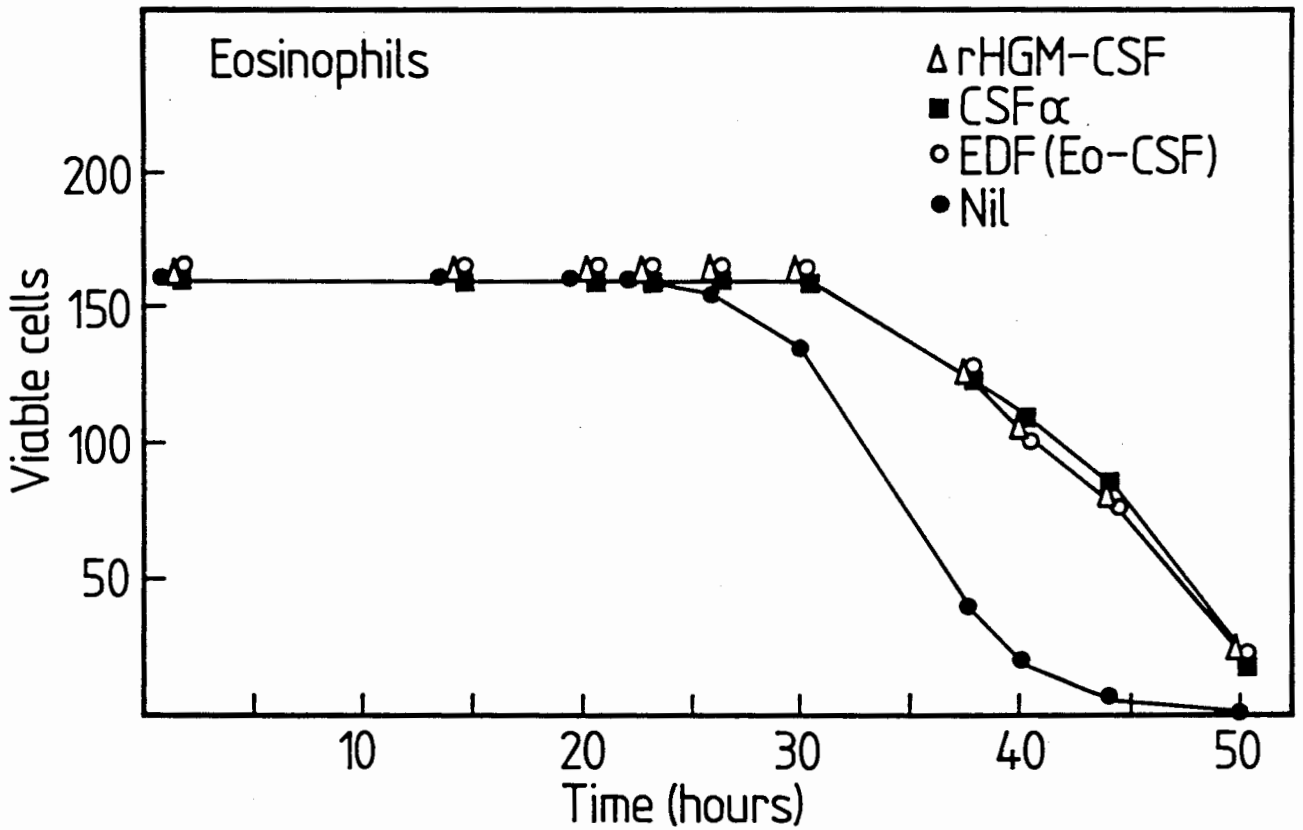
Fig. 9.2 The survival of human blood eosinophils (>95% pure)
in vitro in control cultures and cultures containing
CSF. Cells were placed in microtiter trays (160 cells
per well) and the number of viable cells was counted
at varying times thereafter. Results shown are means
of 4 replicate wells. Cell survival was enhanced in
cultures containing recombinant human GM-CSF (rH GM-CSF),
semi-purified human CSF-α and murine eosinophil differ-
entiation factor (EDF: eosinophil-CSF (Eo-CSF)). The
concentration of CSF was approximately 10 units/ml as
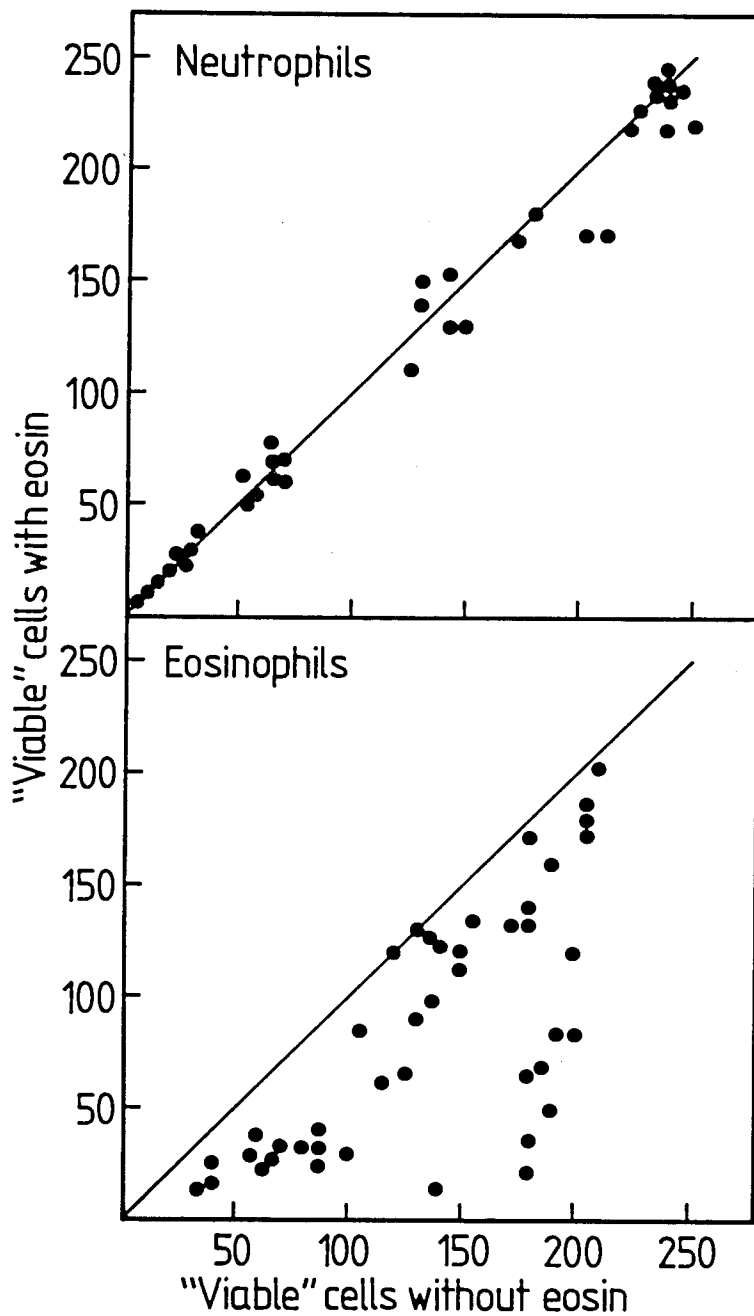determined by normal human bone marrow colony formation.

Fig. 9.3 Cell viability was examined either in cultures without
eosin or in cultures with eosin added. 200 cells
(either neutrophils or eosinophils) were placed in
microtiter wells and examined at various timepoints
thereafter. There was no difference in cell survival
of neutrophils as assessed by direct inspection or the
use of eosin (upper panel). Following the onset of
cell death in cultures of eosinophils, eosin was required
to accurately assess numbers of remaining viable cells
(lower panel).

from the morphology of the cells in the absence of eosin (Figure 9.3, upper panel). In contrast, eosinophils maintained their apparent integrity for many hours after they had ceased being able to exclude eosin (Figure 9.3, lower panel). For this reason all eosinophil cell counts were performed using eosin.

9.2.3 Titration of Survival-Enhancement Induced by rH GM-CSF

The activity of varying concentrations of purified recombinant human GM-CSF (rH GM-CSF) in enhancing cell survival was examined and a typical result is shown in Figure 9.4. Similar results were also obtained for equivalent concentrations of the partially-purified native molecule CSF-α (which shares all the biological activities of the rH GM-CSF described in Chapter 3).

The upper panel (Figure 9.4) shows a titration of the rH GM-CSF at the 28 hour timepoint when purified neutrophils were used as the target cell population. This molecule was also effective in enhancing eosinophil survival (Figure 9.4, lower panel) and the responsiveness of the two cell types to CSF was very similar. These results were consistent with previous reports of this molecule being active in stimulating the functional activity of mature cell populations (Vadas et al, 1983). The initial concentration of rH GM-CSF shown in Figure 9.4 was 670 pg/ml. The lowest concentration of rH GM-CSF detected as enhancing survival was approximately 1.3 pg/ml.

9.2.4 "Lineage-Specific" Enhancement of Survival of Mature Human cells by Murine G-CSF and EDF (Eo-CSF)

Murine G-CSF and murine EDF (Eo-CSF) are able to stimulate the proliferation of human progenitor cells in a "lineage-restricted" manner (Chapters 3,6) and therefore the action of these purified murine molecules was examined and is shown in Figure 9.4.
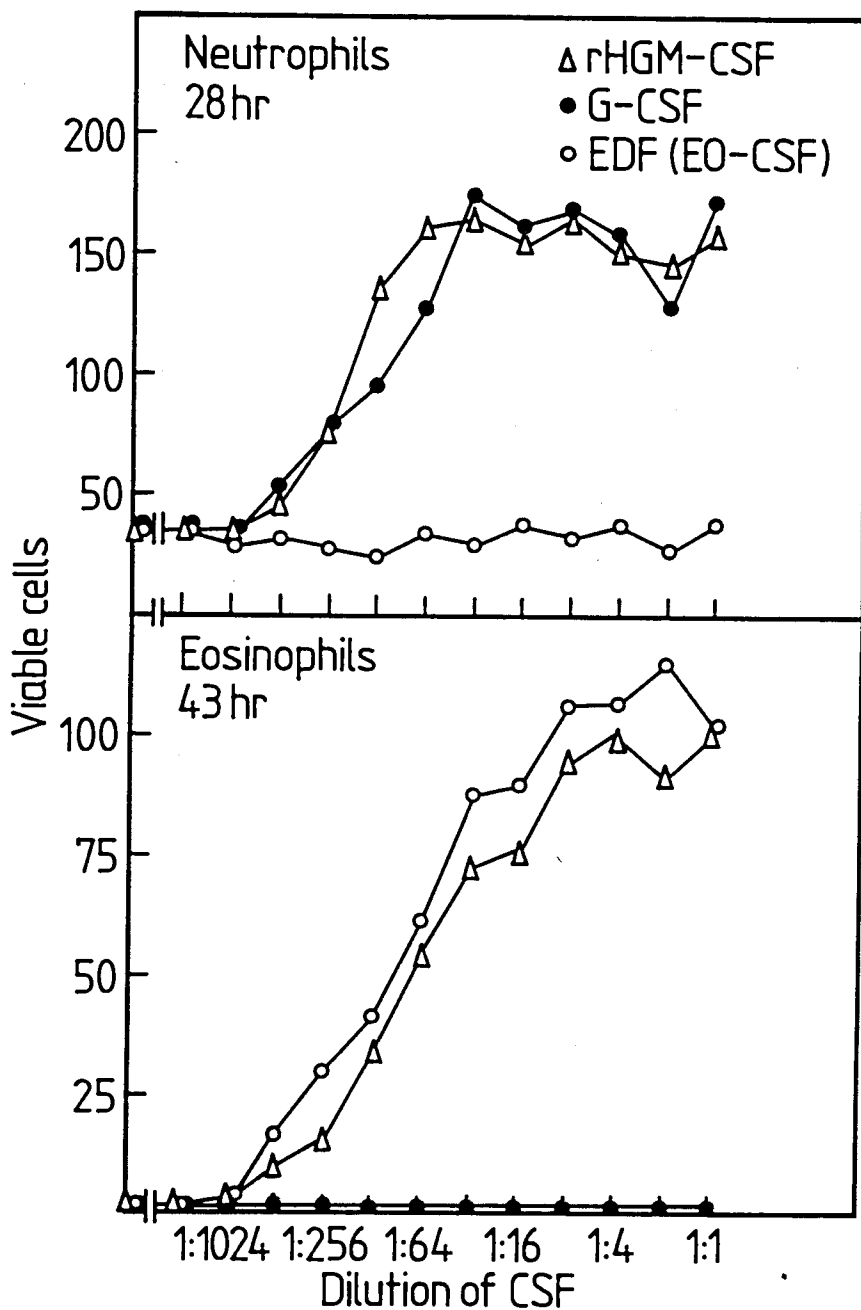
<u>Fig. 9.4</u> Dose-response relationship for purified blood neutrophils
(upper panel) and eosinophils (lower panel), when cultured
with recombinant human GM-CSF (rH GM-CSF), murine granulo-
cyte-CSF (G-CSF) and murine eosinophil differentiation
factor (EDF: Eosinophil-CSF (Eo-CSF)). 200 cells were
placed in microtiter trays and the numbers of viable
neutrophils was counted after 28 hours. Viable eosino-
phils were counted after 43 hours. Results are means
of replicate wells stimulated by two-fold dilutions of
each CSF preparation. The initial concentration of
rH GM-CSF was 670 pg/ml.

Neutrophil survival was enhanced when cells were cultured in the presence of murine G-CSF. Similar results were obtained when cells were cultured with human CSF-β, the human analogue of murine G-CSF (as shown in Chapters 6,8). Murine EDF (Eo-CSF) was inactive (Figure 9.4, upper panel).

Eosinophil survival was enhanced by murine EDF (Eo-CSF) while murine G-CSF (and human CSF-β) were inactive upon these cells (Figure 9.4, lower panel). These results confirmed and extended the previous descriptions of the activities of these purified murine molecules upon specific populations of human cells.

9.2.5 Morphological Changes Induced by CSF

At high concentrations of CSF both neutrophils and eosinophils underwent a striking morphological change similar to that previously described for neutrophils for example with N-formylmethionine-leucine-phenylalanine or influenza virus (Abramson et al, 1984). Cells ceased to be spherical and assumed irregular shapes, becoming elongated, "tear drop" and convoluted in appearance. These morphological changes were maximal within ½-1 hour and reversed over several hours. The viability of these cells subsequently was compromised compared with cells held at lower concentrations of CSF where no such change was observed but where survival was prolonged. The rH GM-CSF and CSF-α induced this morphological change in both neutrophils and eosinophils. Murine G-CSF and human CSF-β induced similar acute morphological changes in neutrophils but not eosinophils and the murine EDF (Eo-CSF) induced changes in eosinophils but not neutrophils, indicating that this was a specific result of stimulation by CSF. The concentration of rH GM-CSF above which this morphological change was observed in greater than 50% of cells at 1 hour was approximately 50 pg/ml. Similarly, higher concentrations were required for the other CSF's

than the concentrations at which survival was enhanced. Less than 5% of cells in unstimulated cultures demonstrated this change.

9.2.6 Higher Sensitivity of Survival Assay Compared with Colony Assay

Figure 9.5 shows the stimulation by purified rH GM-CSF of colony formation by normal human bone marrow cells. This factor preferentially stimulated the day 14 subset of human colony-forming cells which includes neutrophil, macrophage and eosinophil colonies (Chapter 3). The starting concentration was 18.2 ng/ml of rH GM-CSF. A titration of the rH GM-CSF on neutrophil and eosinophil survival is shown for comparison. The starting concentration was 0.67 ng/ml in the microwell assay. The lowest concentration of rH GM-CSF detected in the colony-forming assay was approximately 280 pg/ml, compared with approximately 0.7 pg/ml in the microassay.

The sensitivity of the microassay was greater than that of the colony assay by 5×10^2-fold. In other experiments the greater sensitivity of the microassay ranged between 10^2-10^3-fold for the rH GM-CSF. This heightened degree of sensitivity was also seen with CSF-α, CSF-β and murine G-CSF.

9.3 DISCUSSION

This study demonstrated that purified CSF's induce enhanced survival of purified populations of mature human neutrophils and eosinophils in vitro. Previous experiments have shown that survival of progenitor cells is dependent upon CSF (Morstyn et al, 1981; Metcalf and Merchav, 1982) and these observations have now been extended by the demonstration of a similar type of "lineage-specific" action for CSF upon mature cells. Furthermore, the "lineage specificity" of murine G-CSF and murine EDF (Eo-CSF) for murine cells (Nicola et al, 1983; Sanderson et al, 1985) is retained in their
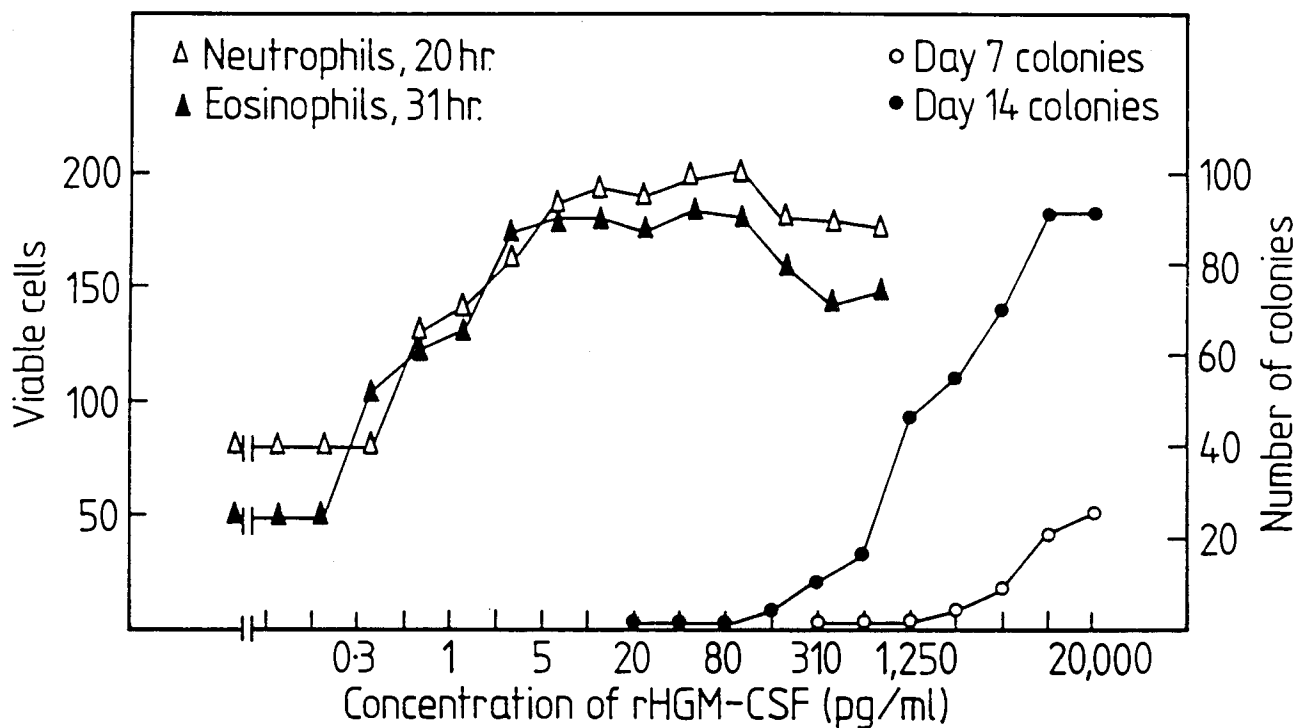
Fig. 9.5 Relative sensitivity of CSF assays during colony formation

or survival of mature cells stimulated by recombinant

human GM-CSF (rH GM-CSF). 2×10^4 marrow cells were

cultured per ml and colonies (of greater than 40 cells)

were scored on day 7 and day 14 of culture. The initial

concentration of rH GM-CSF was 18,200 pg/ml. The ability

of rH GM-CSF to enhance survival of mature neutrophils

and eosinophils is also shown. 200 cells were cultured

in 15 µl volumes and viable cells counted at 20 hours

(for neutrophils) and 31 hours (for eosinophils).

The initial concentration of rH GM-CSF was 670 pg/ml.

action on human cells. The rH GM-CSF on the other hand, was shown to stimulate enhanced survival of both neutrophils and eosinophils. The dual lineage activity of this molecule was also documented for progenitor cell populations (Chapter 3), thus making it analogous to the murine GM-CSF (Johnson and Metcalf, 1980). However, despite biological and sequence homologies, neither molecule appears to show cross-species reactivity (Chapter 3).

Several microassay systems have been described that are dependent upon proliferation of target cell populations to detect CSF. These systems are of value because of the small volumes of CSF required and because of the heightened sensitivity to CSF that such murine assays display (Schrader et al, 1981; Ihle et al, 1982; Tushinski et al, 1982; Burgess et al, 1982; Griffin et al, 1984). There is no evidence however that proliferation-dependent human microassays are more sensitive to CSF than traditional agar assays (Griffin et al, 1984) and this was also the case with the promyelocyte-myelocyte microassay described in Chapter 5.

The assay described here was thus unique in displaying an increased sensitivity to CSF between 10^2-10^3 times the sensitivity of agar assays. Furthermore, this assay provided a rapid result and retained lineage-specificity for the different CSF's.

The heightened sensitivity to CSF of this microassay compared with other human microassays or agar assays is likely to be the consequence of differences in both the target cell population employed and the endpoint of the assay. An heightened sensitivity to CSF is apparently associated with differentiation within progenitor cell compartments, as promyelocytes-myelocytes (cluster-forming cells) are more sensitive to CSF than blast cells (colony-forming cells) (Francis et al, 1981; Chapters 5,6). Furthermore, lower concentra-

tions of CSF are required for survival of progenitor cells than the levels required for proliferation (Burgess et al, 1982). This study has taken advantage of both these observations by employing terminally-differentiated (and therefore probably the most CSF-responsive) cells and examining cell survival rather than a functional endpoint.

It is also interesting that, for progenitor cells, the particular elicited response to CSF (i.e. survival vs proliferation vs differentiation) is dependent upon the concentration of CSF (Burgess et al, 1982; Metcalf, 1980; Johnson, 1983; Li and Johnson, 1984). It is possible that this is also true for mature cells, as the level of CSF required for the morphological change to occur within cells was approximately 10^2 times the concentration of CSF required for cell survival. It is not clear that the morphological change is associated with functional activation of the cells, however this result suggested a differential response to varying CSF concentrations by mature neutrophils and eosinophils.

The demonstration that mature cell survival is dependent on CSF has potential clinical applications particularly in areas such as granulocyte transfusion, where viability and function of transfused cells may be enhanced by the addition of CSF.

CHAPTER 10

GENERAL DISCUSSION

The activity of human and murine CSF's on human cells has been examined in this study. These molecules have been documented to stimulate proliferation, differentiation and survival, and possess activity for progenitor and mature cell populations.

The most fully characterized murine CSF's are Multi-CSF, GM-CSF, M-CSF and G-CSF and of these only G-CSF appeared to be a proliferative stimulus for normal human progenitor cells when both unfractionated marrow cells and fractionated populations of cells were examined. These results are in agreement with the reported action of murine CSF's in stimulating functional activation of mature human cells (Lopez et al, 1984). It was intriguing that murine GM-CSF demonstrated an inconsistant proliferative effect on normal human marrow cells but this activity was only evident in cultures containing a high proportion of macrophages. A direct proliferative action of this molecule therefore seems unlikely although it is possible that murine GM-CSF can stimulate human macrophages to elaborate a human-active CSF. It would be of interest to further examine the action of murine GM-CSF on purified human macrophages and determine its ability to compete for radio-iodinated rHGM-CSF binding-sites. In this regard the inactivity of rHGM-CSF on murine cells is also of interest, and this is despite the homology between the two molecules (Gough et al, 1984; Wong et al, 1985). Experiments using techniques such as site-directed mutagenesis or anti-CSF antibodies may provide further information regarding the relationship between the active-sites of these two molecules.

The experiments reported here have demonstrated rHGM-CSF possesses all the biological activities of the molecule previously described as CSF-α (Nicola et al, 1979). Based on similarities in biological and biochemical characteristics the human equivalent of

murine G-CSF appears to be CSF-β. This can be confirmed by studies examining nucleotide and amino acid homology between these proteins.

Another class of CSF was recognised following the demonstration that murine EDF is an Eo-CSF. The existence of a "lineage-specific" Eo-CSF is however not surprising (Metcalf, 1984) and argues strongly for the existence of other "lineage-specific" CSF's. Several important questions regarding the action of this molecule remain unanswered. Insufficient EDF (Eo-CSF) was available for dose-response relationships to be established, and in particular it is not clear whether human eosinophil progenitor cells are more or less Eo-CSF-responsive than their murine equivalent. Studies were not performed to examine the degree of cross-reactivity with other murine (or human) haemopoietic lineages. The presence of granulocyte-macrophage clusters (and occasional colonies) in all EDF (Eo-CSF) stimulated cultures suggests that such cross-reactivity exists. However the ability or otherwise of EDF (Eo-CSF) to support early stages of erythroid colony formation (as do GM-CSF and G-CSF; Metcalf, 1984) was not examined. Lineage cross-reactivity may be even more evident if higher concentrations of EDF (Eo-CSF) had been examined. It would also be of interest to examine the action of EDF (Eo-CSF) on human HL60 cells as these cells are reported to differentiate to eosinophils (Lu et al, 1981). Recently EDF (Eo-CSF) has been reported to also have B-cell growth factor activity (Sanderson et al, 1986).

Although the studies using rHGM-CSF and EDF (Eo-CSF) were performed using unfractionated cells, the direct action of these molecules on responding cells was established by clone transfer experiments.

The ability of normal human promyelocytes and myelocytes to proliferate clonally in vitro was documented in Chapter 5. Although these cells were useful in establishing a micro-assay system, the CSF-sensitivity of this assay was no greater than the agar culture assay. A more sensitive assay was however established and served to demonstrate CSF-enhanced survival of mature human cells in a "lineage-specific" manner.

The promyelocyte-myelocyte cells responded to stimulation by human CSF-α, CSF-β and murine G-CSF. This action was proven to be direct by clone transfer experiments and it was also demonstrated that the progeny of promyelocytes-myelocytes stimulated by one CSF (α or β) were also responsive to the other CSF. This was in contrast to earlier studies that had suggested that this was not the case and had therefore presented a dual-lineage model of granulocyte differentiation (Morstyn et al, 1981). Results presented here however suggest that these human cells are analogous to murine cells in being able to bind and respond to more than one type of CSF (Metcalf, 1985). It would be of interest therefore to examine a purified population of day 7 GM-CFC. Such a population could be obtained using for example antibodies WEM-G1 and WEM-G11. With the availability of purified rHGM-CSF and the identification of murine G-CSF as the equivalent molecule to human CSF-β, these studies could also now be performed using completely purified CSF's.

The possibility remains, suggested by experiments presented here, that a small and variable subpopulation of human promyelocytes-myelocytes are CSF-α-unresponsive. This may be the result of using impure CSF's and could now be re-examined using rHGM-CSF. It is of interest however that some leukaemic cell subpopulations also appeared unresponsive to either purified rHGM-CSF or purified murine

G-CSF although an equivalent concentration was used in all cultures (400 U/ml). This apparent unresponsiveness of some leukaemic cells could be further examined by clone transfer experiments or by auto-radiography using radio-labelled CSF preparations. This observation has been reported previously (Francis et al, 1980; Swart et al, 1982) and is of potential importance if these molecules are to be employed for therapeutic purposes in myeloid leukaemia.

The variability in CSF-responsiveness of fresh human leukaemic cells should also be examined in greater detail. The CSF-responsive-ness of particular leukaemic samples can now be examined and compared with results of binding studies using radio-labelled CSF's. This may provide information explaining the heterogeneity in CSF-respons-iveness in terms of altered number or affinity of CSF binding-sites.

The availability of large amounts of recombinant CSF's has allowed the study of the action of these molecules in vivo (Metcalf et al, 1986a; Kindler et al, 1986). One potential clinical applica-tion is in situations of infection as these molecules markedly increase the number and activate the functional activity of mature cells in vivo (Metcalf et al, 1986a). The possible role of these molecules in the myeloid leukaemias will probably also be under clinical evaluation but there are several important areas that require further study before this occurs. The action of CSF's to enforce differentiation-commitment and concomitant decreased self-renewal in fresh human leukaemic cells is not clear. The results of experi-ments using HL60 cells suggest such a role but further experiments should be performed using the technique of Buick et al. (1979) to examine possible suppression of clonogenicity by CSF. Differentia-tion-induction by CSF in murine leukaemic cells is linked to proliferation (Metcalf, 1982) and CSF's also stimulate the prolifera-

tion of human leukaemic cells. It would seem reasonable therefore that prior to clinical trials, murine-leukaemia models should be established to determine optimal timing of administration of these molecules in vivo. It also seems possible that CSF-unresponsive leukaemic cells may be selected by treatment with CSF. Such cells, as with murine leukaemic cells, might lack the receptor for a particular CSF (Nicola and Metcalf, 1984).

Despite these concerns however, the availability of large amounts of CSF will provide important information regarding the in vivo relevance of these molecules and will allow for example the development of monoclonal antibodies to the CSF's. Other exciting developments in this area will include an understanding of the nature of the receptors for these molecules and the role alterations in the CSF-genes (and their receptors) play in spontaneous human leukaemias.

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