The response of haemopoietic cells to growth factors: developmental implications of synergistic interactions

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Summary

Haemopoietic cell growth factors are normally assayed using unfractionated marrow cells (NBM). However, using this population it is difficult to distinguish between direct *versus* indirect effects, because of the low incidence of colony forming cells (CFC) and the presence of possible accessory cells (which may themselves be acted upon by the growth factors and stimulated to produce other growth stimulatory or inhibitory molecules that influence the development of the CFC). Furthermore, NBM contain the whole spectrum of multipotent and lineage-restricted CFC and it is often difficult to determine precisely which populations are being stimulated to develop. This latter problem can be solved, in part, by using marrow from mice previously treated with 5-fluorouracil (5-FU): an agent that preferentially kills the more mature, actively cycling CFC but spares the proliferatively quiescent multipotent stem cells. Since the 5-FU-treated marrow also contains many possible accessory cells, however, it is again not clear whether or not the responses elicited by growth factors are due to direct or indirect effects upon the CFC. To circumvent this problem we have obtained a highly enriched population of multipotent stem cells (FACS-BM) that is free of accessory cells, and have compared the responses of these cells to NBM and to 5-FU-BM in the presence of a variety of growth factors. The data demonstrate that interleukin-1 (IL-1), which is not a growth factor by itself, can act synergistically with other growth factors on FACS-BM and 5-FU-BM but not on NBM. With FACS-BM, IL-1 can synergize with GM-CSF and with M-CSF but not with IL-3 or G-CSF. With 5-FU-BM, IL-1 can synergize with GM-CSF, M-CSF and IL-3. Furthermore, G-CSF, a poor growth stimulus when used alone, can synergize with GM-CSF and M-CSF, using either FACS-BM or 5-FU-BM, and can also synergize with IL-3 when using 5-FU-BM but not with FACS-BM. The data further indicate that extensive overlap occurs between the multipotential cells that respond to IL-3 alone and those that can respond to a combination of other growth-promoting stimuli such as IL-1 plus CSF-1 or G-CSF and CSF-1. This indicates that at least some multipotential cells have receptors for all the growth factors used in this study, but that combinations of growth factors sometimes need to be present in order to facilitate proliferation and development. Furthermore, the outcome of the response in terms of mature cell lineages produced is a reflection of the stimuli to which the multipotential cells are exposed. The developmental implications of these findings are discussed.

Key words: growth factors, stem cells, differentiation, synergism, haemopoiesis.

Introduction

All mature haemopoietic cells in the blood and tissues are derived from a population of pluripotent stem cells normally resident within the bone marrow. These cells can undergo proliferation to produce more stem cells or can become committed to a process of differentiation with accompanying proliferation. The end result of this process is the formation of monocytes and macrophages, platelets, basophils/mast cells, neutrophils, eosinophils and lymphocytes. *In vitro* the formation of mature cells, as colonies in soft agar, from single haemopoietic progenitor cells, is supported by a series of growth factors with a range of biological activities. At least four growth factors have been identified that can control the production of neutrophils and macrophages *in vitro*. These are: (1) the multilineage growth factor interleukin-3 (IL-3), which facilitates the survival and stimulates the proliferation of

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stem cells, and also the proliferation and development of committed progenitor cells, including those from the neutrophil/macrophage, eosinophil, erythroid, mast cell and megakaryocytic lineages (for review, see Moore, 1988): (2) granulocyte–macrophage colony stimulating factor (GM-CSF), which is also a multilineage stimulating factor but has a rather more limited range of activity than IL-3, stimulating primarily eosinophil, megakaryocytic and macrophage/neutrophil production in clonal assays (Metcalf, 1985): (3) macrophage colony stimulating factor (M-CSF or CSF-1); and (4) granulocyte colony stimulating factor (G-CSF), which stimulate macrophage and granulocyte colony formation, respectively, in soft agar assays (Metcalf, 1985; Stanley & Jubinsky, 1984).

Although GM-CSF has been shown to recruit at least *some* multipotential progenitor cells with some proliferation and development, the growth factors M-CSF and G-CSF were generally thought to have no direct growth-stimulatory effects on stem cell or multipotential cell populations *in vitro* (Metcalf, 1985); their target cells were believed to be confined to committed cells of the granulocyte–macrophage lineage. Recently, however, another factor, haemopoietin-1, has been shown to act synergistically with the apparent lineage-specific growth factor M-CSF, leading to the proliferation and development of primitive clonogenic cells present in the bone marrow of mice treated with 5-fluorouracil (5-FU) (Bartelmez & Stanley, 1985; Stanley *et al.* 1986; McNiece *et al.* 1986). In the absence of haemopoietin-1 no responsiveness to M-CSF alone was observed. Recently, haemopoietin-1 has been shown to be identical with IL-1α (Mochizuki *et al.* 1987).

There are, however, difficulties in the interpretation of possible mechanisms whereby IL-1α can enable primitive multipotent cells to respond to haemopoietic growth factors such as M-CSF. Treatment with 5-FU preferentially kills cycling cells, and since the majority of progenitor cells are cycling, while the primitive multipotent stem cells mainly consist of non-cycling cells (Bradley & Hodgson, 1979), treatment with 5-FU leads to a relative *enrichment* of these more primitive cells. Other non-cycling cells, however, also persist after 5-FU treatment, including macrophages, neutrophils and (presumably) connective tissue cells, which can and do release a wide variety of important cytokines, such as interleukins, prostaglandins, platelet-activating factor and haemopoietic growth factors (Houlihan & Saunders, 1986; Kurland *et al.* 1979; Thorens *et al.* 1987; Warren & Ralph, 1986). Some of these factors may be released during the course of a 7-day (or longer) *in vitro* colony forming assay by the action of IL-1α on the 5-FU-treated bone marrow cells, leading to the observed effects on synergistic induction of stem cell commitment and differentiation. For example, interleukin-1 is known to stimulate GM-CSF production in murine bone marrow cultures (Lovhaug *et al.* 1986). These problems are compounded by the relatively high cell density employed in these cultures of 5-FU-treated marrow cells (Stanley *et al.* 1986; McNiece *et al.* 1986; Mochizuki *et al.* 1987).

To investigate this further and to elucidate the possible mechanism whereby IL-1 may exert effects on primitive multipotential cells, we have isolated a population of normal bone marrow-derived 'stem' cells, using a technique employing a fluorescence-activated cell sorter (FACS). These cell are defined by their ability to form day 12 spleen colonies in irradiated mice (Lord & Spooncer, 1986). This purified population is highly enriched for multipotent cells capable of producing mixed myeloid colonies *in vitro* (CFC-Mix) (Lord & Spooncer, 1986) but is devoid of any mature cell types. We have used these cells to investigate the possibility that some of the synergistic effects of IL-1α on colony formation *in vitro*, observed using cells from 5-FU-treated mice, may in part be due to indirect effects of IL-1α. We have also compared the effects of combinations of purified colony stimulating factors on the clonogenic proliferation and development of highly purified FACS bone marrow and 5-FU-treated bone marrow cells, in order to assess and contrast the effects of haemopoietic growth factors on these two populations of cells.

Materials and methods

Isolation of cell populations

Cells were obtained from adult female B6D2F$_1$ mice aged between 8 and 12 weeks. Bone marrow cell suspensions were prepared by flushing femurs with medium as described (Lord *et al.* 1986), either from normal mice or from mice that had been treated wtih 5-FU (150 mg kg^{-1}) 24 h prior to use. Normal marrow cells highly enriched for day 12 CFU-S (colony forming unit-spleen) were obtained as described by Lord & Spooncer (1986). Routinely this population contained between 50% and 140% day 12 CFU-S when the spleen seeding efficiency was taken into consideration.

Colony assays

In vitro colony forming assays for the detection of granulocyte–macrophage CFC (colony forming cell) or mixed-myeloid CFC were performed as described (Spooncer *et al.* 1986). Briefly, cells were cultured in Iscove's medium supplemented with 20% (v/v) foetal calf serum, 1% (w/v) bovine serum albumin, 0·33% agar and the appropriate growth factors in 35 mm Petri dishes. In some experiments erythropoietin was also included to facilitate erythroid maturation. However, the effects of erythropoietin on colony forming efficiency were negligible. The plates were incubated in fully humidified conditions in an atmosphere of 5% CO_2 and 5% O_2. Cell aggregates containing more than 10 cells were counted at the appropriate time (usually at day 10) and were defined as colonies if they contained 50 cells or more. For morphological analysis, individual colonies were isolated and suspended in medium and slides were then prepared using a cytocentrifuge. These slides were stained with *O*-dianidisine and then May–Grunwald–Giemsa. 'Mixed erythroid' colonies contained erythroid cells plus cells of at least one and normally two or three other lineages, e.g. megakaryocytes, macrophages, eosinophils or neutrophils. CFU-S were assayed as described (Lord & Spooncer, 1986); colony counts were performed at day 12.

Growth factors

Purified native or recombinant growth factors were used in all the experiments. Recombinant murine IL-3 and GM-CSF were

supplied by Biogen; recombinant human G-CSF was supplied by Amgen; recombinant IL-1α was a kind gift from Dr M. A. S. Moore or supplied by Immunex. Immunex also supplied recombinant IL-1β. Recombinant human M-CSF was from Cetus. Native M-CSF was purified from L-cell conditioned medium to stage IV according to the procedure described by Stanley & Heard (1977). Purified human urinary erythropoietin (epo) was supplied by A. C. Eaves, Terry Fox Laboratories, Vancouver. All the growth factors were titrated against both normal bone marrow cells and FACS-sorted bone marrow cells to establish dose–response relationships. For the experiments here, the minimal concentration giving the maximal (plateau) colony formation was used. The final concentrations were as follows: IL-3 100 units ml^{-1}; GM-CSF 50 units ml^{-1}; G-CSF 1000 units ml^{-1}; IL-1α 10 units ml; IL-1β 10 units ml^{-1}; purified native M-CSF 20 units ml^{-1}; recombinant M-CSF 200 units ml^{-1}; epo 2 units ml^{-1}. The units of activity were extrapolated from data provided by our suppliers of the material, using their own respective assay systems.

Growth media and sera

Iscove's medium was supplied from Gibco (Paisley, UK). Bovine serum albumin was purchased from Sigma (Poole, UK). Foetal calf serum was obtained from Northumbria Biological (Cramlington, Northumberland, UK).

Results

Comparison of the relative plating efficiencies and CFU-S forming abilities of FACS-sorted bone marrow (FACS-BM) and 5-FU-treated bone marrow (5-FU-BM) with normal bone marrow (NBM)

NBM cells in the presence of IL-3 have an *in vitro* colony forming efficiency (number of colonies per cell number seeded) of 0·17 %. This colony forming efficiency (CFE) was markedly increased in the FACS-BM cell population (see Table 1) to 6·7 %, a 40-fold increase over normal bone marrow; in some experiments the CFE reached 40 %. With FACS-BM colony formation was linear over a range of cell concentrations plated (data not shown), indicating their clonal origin. However, the CFE of 5-FU-BM was only 0·018 %, markedly lower than that of NBM cells, presumably reflecting the removal of cycling IL-3 responsive cells by the 5-FU treatment. Even when optimal conditions were employed (see below), the CFE of 5-FU marrow cells only reached a maximum of 0·037 %, a value agreeing with previous work (Stanley *et al.* 1986). In all the subsequent studies reported here, the cell populations plated out were kept within the range of 5×10^2 to 1×10^3 for FACS-BM, 5×10^4 for NBM, and 5×10^5 for 5-FU-BM, for ease of scoring and statistical analysis.

5-FU-BM and FACS-BM (CFU-S enriched) both contained cells that were capable of forming day 12 CFU-S. In 5-FU-BM, these represented only 0·003 % of the cells injected (data kindly supplied by G. Molineux); while in FACS-BM about 10 % of the cells injected produced day 12 colonies (i.e. 95 % of the cells are potential spleen colony forming cells when the seeding efficiency into the spleen is taken into account; Lord & Spooncer, 1986). This common ability to produce colonies *in vitro* and also *in vivo* in the CFU-S assays indicates at least some overlap between the clonogenic cells present in the FACS-BM and the 5-FU-BM populations.

FACS-BM and 5-FU-BM show similar responses to haemopoietic growth factors

The responses of FACS-BM and 5-FU-BM to the growth factors IL-3, GM-CSF, G-CSF and M-CSF were assessed using *in vitro* clonogenic assays (Fig. 1). The results are expressed as the percentage of colonies developing in IL-3 alone (see Table 1) for the respective cell populations. When NBM cells were plated out with these growth factors, significant levels of colony formation with IL-3, GM-CSF, M-CSF and, to a much lesser extent, with G-CSF were obtained. FACS-BM gave the most colonies with IL-3, but GM-CSF and M-CSF gave approximately 30 % of the colony numbers produced in the presence of IL-3. 5-FU-BM also gave significant colony formation in the presence of IL-3 although, as described above, the incidence of CFC was much lower. GM-CSF stimulated about 30 % of the colony numbers produced by IL-3 (Fig. 1B), although stimulation with M-CSF produced <5 % of this value. These results indicate that FACS-BM and 5-FU-BM both represent populations where primitive progenitor cells have been selected from more lineage-restricted progenitor cells, but the incidence of colony forming cells in 5-FU-BM is very low.

The finding that FACS-BM displayed an ability to respond to M-CSF and G-CSF could indicate that this population contains a subset of more mature progenitor cells, rather than indicating that multipotential stem cells are responding to these 'downstream' growth factors. This will be discussed in more detail below. Recombinant M-CSF and purified M-CSF produced equivalent numbers of colonies when used either alone or in combination with other growth factors.

Responses of FACS-BM and 5-FU-BM cells to IL-1 and G-CSF

No colonies were produced when IL-1α was added to 5-FU-BM cells, FACS-BM cells or NBM (Fig. 1). In contrast, IL-3 induced significant colony formation by all three cell preparations; the co-addition of IL-1α gave no observable increase in colony numbers in FACS-BM or NBM, but induced a 60 % increase in colony numbers in 5-FU-BM (Fig. 2A–C). Colony formation stimulated by GM-CSF, M-CSF and G-CSF was in all cases significantly enhanced by the co-addition of IL-1α in the 5-FU-

Table 1. *Relative plating efficiencies of different sources of haemopoietic progenitor cells*

Source of cells	Number of cells per plate	Total number of colonies formed in interleukin-3	Colony forming efficiency (%)
FACS-sorted bone marrow (CFU-S-enriched)	1×10^3	67 ± 6	6·7
5-FU-treated bone marrow	5×10^5	87 ± 11	0·018
Normal bone marrow	5×10^4	87 ± 12	0·17

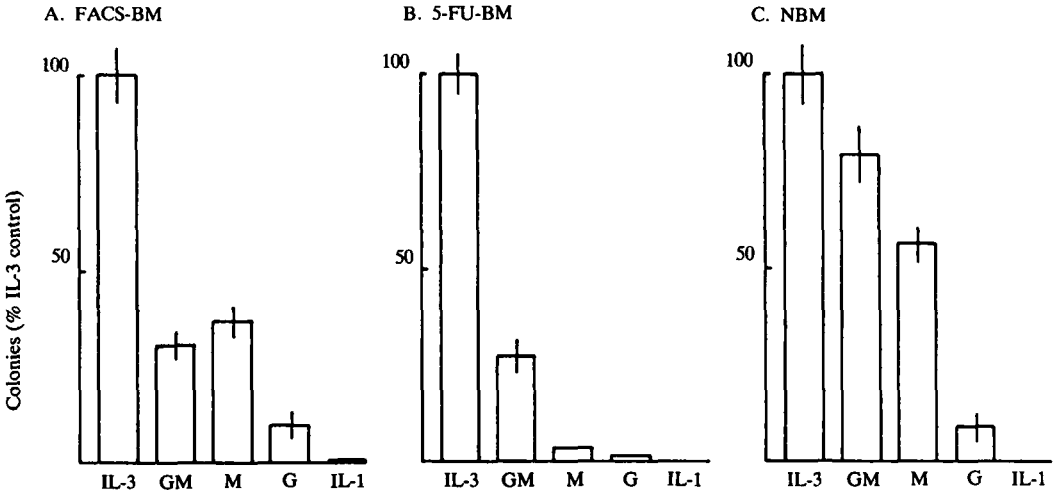

Fig. 1. The effects of highly purified growth factors on colony formation by: A, FACS-sorted population enriched for CFU-S; B, bone marrow from 5-FU-treated mice; C, normal bone marrow cells in colony forming assays. GM, GM-CSF; M, M-CSF; G, G-CSF. Results shown are the mean ± standard deviation of at least six observations expressed as a percentage of the colonies formed in the presence of IL-3.

BM and FACS-BM (Fig. 2A,B), the potentiation by IL-1α being more marked in the 5-FU-treated marrow cells. These increases in colony numbers were not simply due to a net increase in size of cell aggregates, i.e. converting some clusters into colonies; there was a significant increase in the *total* number of cell aggregates (i.e. colonies plus clusters) in all cases. NBM showed no significant increase in colony numbers on IL-1α co-addition: in fact a reproducible inhibition of colony formation was observed (Fig. 2C). When IL-β was employed instead of IL-1α very similar results were obtained in all the experiments performed (data not shown).

The addition of G-CSF in conjunction with other growth factors had remarkably similar effects to IL-1 (Fig. 2). IL-3-stimulated FACS-BM colony formation remained unaltered by the addition of G-CSF; whereas colony numbers stimulated by G-CSF, in combination with either GM-CSF or M-CSF, increased by 2·8-fold and 2·4-fold, respectively, the colony numbers seen with either GM-CSF or M-CSF alone (Fig. 2A). In comparison, the addition of IL-1 led to a 2·6-fold and a 2·15-fold increase in these two parameters. With 5-FU-BM cells, G-CSF enhanced IL-3-stimulated colony formation 1·48-fold compared to the IL-1 enhancement of 1·65-fold; enhanced GM-CSF-stimulated colony formation 2·1-fold compared with 3·07-fold for IL-1; and enhanced M-CSF-stimulated colony formation by 10·6-fold compared with 17-fold seen with IL-1 (Fig. 2B). A slight G-CSF-stimulated enhancement of colony formation stimulated by GM-CSF and M-CSF was also observed in NBM cells (Fig. 2C). Again, this increase in colony formation was not due simply to enhanced proliferative activity (i.e. converting cluster forming cells to colony forming cells), since a corresponding increase was seen in total aggregates (data not shown).

To investigate whether G-CSF and IL-1 enhance the formation of colonies from the same, overlapping or discrete populations of haemopoietic progenitor cells, the ability of G-CSF to modulate colony numbers in the presence of M-CSF and IL-1 was examined. The combination of G-CSF, IL-1 and M-CSF (see Fig. 3) gave a marked increase (greater than additive) in colony numbers in 5-FU-BM cells, indicating that G-CSF plus M-CSF was recruiting a different population of cells from those stimulated by M-CSF plus IL-1. However, when FACS-BM or NBM was used, a combination of G-CSF, M-CSF and IL-1 showed only a slight increase in colony number over that seen in G-CSF plus M-CSF or in IL-1 plus M-CSF, indicating that there is a significant degree of overlap in the responding cell populations.

Responses of FACS-BM and 5-FU-BM cells to combinations of IL-1, IL-3 and M-CSF

In order to establish whether the clonogenic cells responding to IL-3 are the same or different from the cells that respond to IL-1 plus M-CSF, we investigated the relative responses of 5-FU-BM cells and FACS-BM cells to combinations of these growth factors. With FACS-BM cells colony formation in cultures containing IL-3 plus IL-1 or IL-3 plus IL-1 plus M-CSF was not significantly different (Fig. 4A). Clearly, no additive effects are seen, indicating significant overlap between the IL-3 alone, IL-3 plus IL-1, and the M-CSF plus IL-1 population. It is of interest, however, that when IL-3 is combined with M-CSF, in the absence of IL-1, an additive effect is observed. This is perhaps indicating that M-CSF-responsive committed progenitor cells do indeed represent a minor contaminatory population of FACS-BM cells and that these cells are unresponsive to IL-3. Although the increase in colony numbers may be explained by the simple additive effect of these two growth factors on FACS-BM cells, it was also noticeable that the mean colony size was increased in cultures containing IL-3 plus M-CSF compared with IL-3 containing cultures (results not shown).

The substantial number of colonies formed in the presence of IL-1 plus M-CSF approaches that seen in IL-

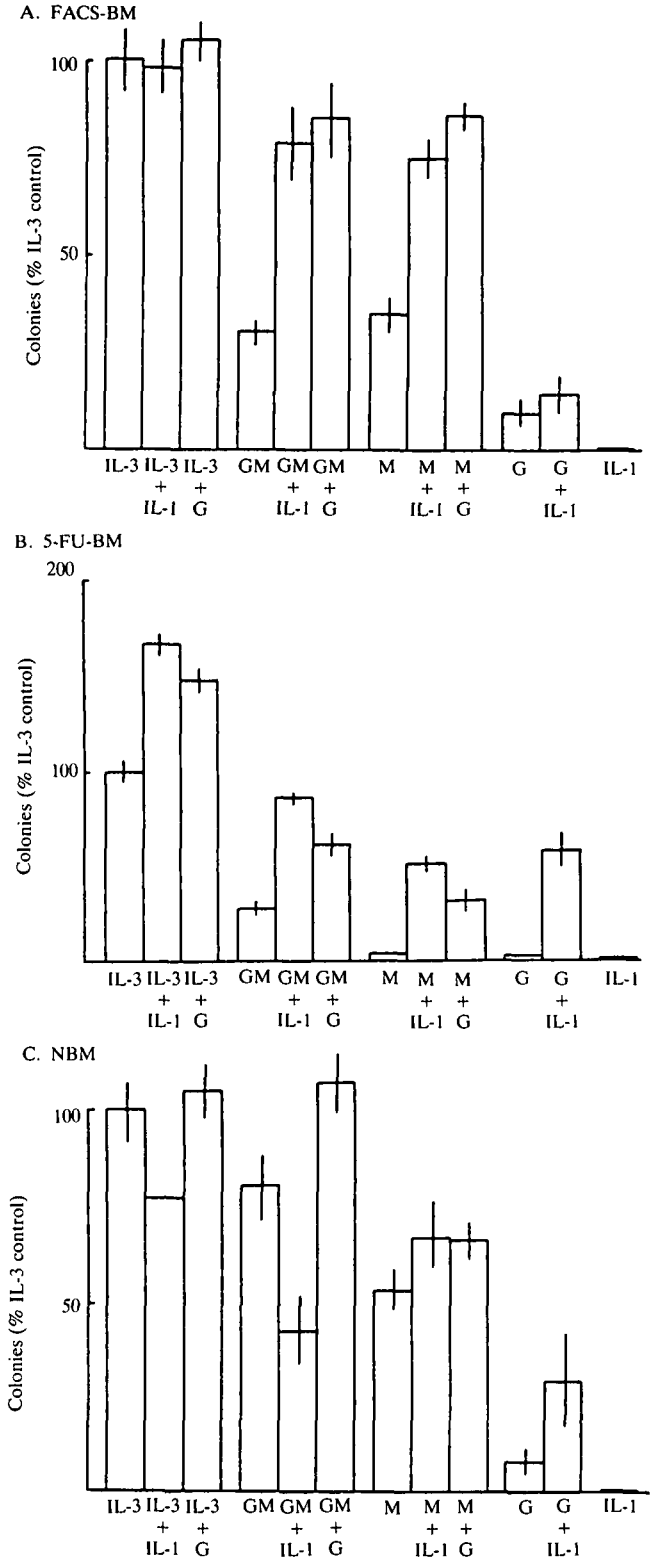

Fig. 2. The effect of interleukin-1 and G-CSF on colony formation by haemopoietic growth factors. Colony formation stimulated by IL-3, GM-CSF and M-CSF in the absence and presence of IL-1 or G-CSF is shown. A. Results for FACS-sorted bone marrow enriched for CFU-S. B. 5-FU-treated bone marrow cells. C. Normal bone marrow cells. Results are expressed as a percentage of the colonies formed in the presence of IL-3 and are the mean ± standard deviation from at least six observations.

3-stimulated colony-forming assays, indicating that a common set of target cells is acted upon by these two combinations of growth factors. It is of interest, however, that examination of colony morphology (see Table 2) revealed some distinct differences between cell types present in day 10 colonies. More than 95 % of the colonies stimulated by a combination of M-CSF plus IL-1 contained only macrophages or macrophage-like cells; but 60 % of the colonies from IL-3-stimulated FACS-BM contained blast cells, as well as erythroid cells with either mature macrophages or granulocytic cells, i.e. they are obviously derived from multipotential cells. The remaining 40 % of the colonies were single lineage erythroid or granulocytic colonies, or mixed granulocytes/macrophages. Pure macrophage colonies were comparatively rare. The addition of M-CSF, or M-CSF plus IL-1, to IL-3-containing cultures reduced the number of mixed myeloid colonies to 20 %. The majority of colonies produced contained only macrophages. Thus IL-3 and M-CSF plus IL-1 appear to have similar abilities to stimulate colony formation in a *common set* of target cells (many if not all of them multipotential), but the degree to which development into specific lineages occurs differs markedly in these two cases. Our observation that colony size is generally greater in IL-3 plus M-CSF compared to IL-3-stimulated cultures (see above) may perhaps be explained by the ability of M-CSF to act on some of the blast cells produced by the action of IL-3, leading to their proliferation and maturation to macrophages.

With 5-FU-BM similar effects on colony morphology and size may be seen (Fig. 4B). However, there are some differences in the numbers of colonies formed in response to the combinations of growth factors described above. M-CSF or IL-1, when used in combination with IL-3, enhanced colony formation by 54 % and 64 %, respect-

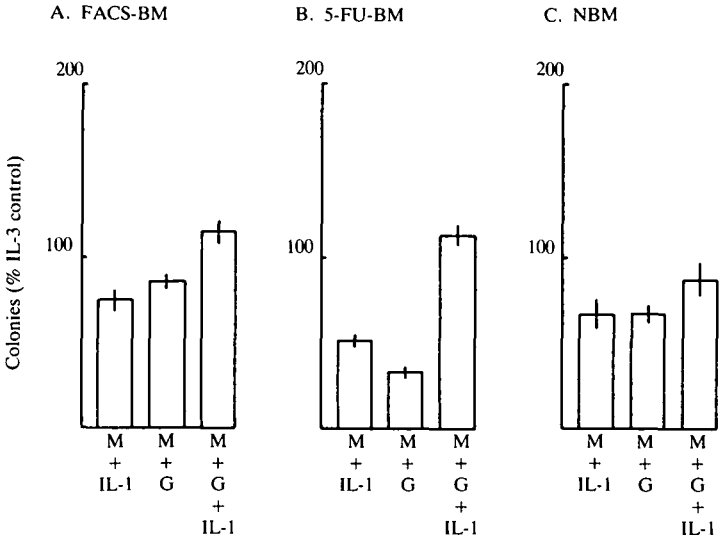

Fig. 3. The effects of combinations of IL-1, G-CSF and M-CSF on colony formation from populations of: A, FACS-sorted bone marrow enriched for CFU-S; B, 5-FU-treated bone marrow cells; C, normal bone marrow cells. Results are expressed as a percentage of the colonies formed in the presence of IL-3 and are the mean ± standard deviation from at least six observations.

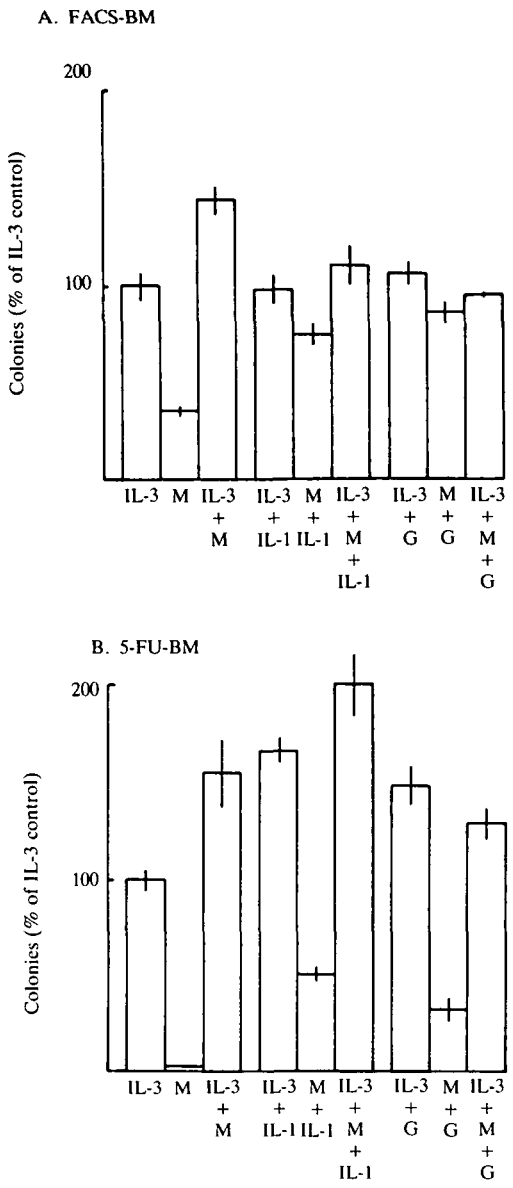

Fig. 4. The effects of combinations of IL-3 and M-CSF with IL-1 or G-CSF on colony formation by populations of: A, FACS-sorted bone marrow enriched for CFU-S; B, 5-FU-treated bone marrow cells. Results are expressed as a percentage of the colonies formed in the presence of IL-3 and are the mean ± standard deviation from at least six observations.

ively, compared with IL-3 alone. A combination of all three factors increased colony formation to 200% of that seen in IL-3 (added alone)-stimulated colony forming assays. These data indicate that some part of the 5-FU-BM population is not responsive to IL-3 or M-CSF alone (see Fig. 4B) but can respond to combinations of IL-1, IL-3 and M-CSF. As this is not the case with FACS-BM cells, the cells present in the 5-FU-BM may represent an IL-3-insensitive primitive cell that is not selected in FACS sorting.

Table 2. *Morphological analysis of colonies formed from FACS-sorted bone marrow cells in the presence of specific haemopoietic growth factors*

Growth factor(s) present	Colony type (%)				Colonies containing primitive cells
	G	M	GM	ME	
IL-3	20	5	15	50	60
CSF-1	0	92	8	0	0
G-CSF	34	66	0	0	0
IL-3 + IL-1	4	29	29	17	37
CSF-1 + IL-1	0	87	13	0	0
IL-3 + G-CSF	50	0	25	25	75
CSF-1 + G-CSF	0	50	50	0	0
IL-3 + CSF-1	0	30	50	20	20

G, granulocytic colonies; GM, granulocyte–macrophage colonies; M, macrophage colonies; ME, mixed/erythroid colonies as defined in Materials and methods. Primitive cells are defined as morphologically unrecognizable blast cells, promyelocytes or erythroblasts. When such cells comprise >20% of the cells in a colony that colony is defined as containing primitive cells. In some groups (IL-3, IL-3 + IL-1) a proportion of the colonies contained primitive cells *only*.

Responses of FACS-BM and 5-FU-BM cells to combinations of IL-3, M-CSF and G-CSF

A combination of M-CSF plus G-CSF produces significant synergy in colony formation from both 5-FU-BM and FACS-BM cells (see Figs 1 and 4). Using FACS-BM M-CSF plus G-CSF gave 84% of the colonies produced in the presence of IL-3 alone. To test whether the CFC that responded to a combination of M-CSF plus G-CSF were the same as, or different from, the cells responding to IL-3 alone, colony formation was assessed in cultures where all three factors were present. In these cultures, colony formation was not significantly increased over that seen in IL-3 alone, indicating that IL-3, and M-CSF plus G-CSF act on substantially the same set of colony forming cells. However, we also found that in the presence of M-CSF and IL-3, respectively, G-CSF appeared to inhibit colony formation, making interpretation of the relative interactions of these three growth factors somewhat difficult. In comparison, it is of some interest that 5-FU-BM cells are responsive to G-CSF plus M-CSF, but this combination stimulates only 32% of the colonies formed in the presence of IL-3 alone. IL-3, G-CSF plus M-CSF, and M-CSF plus IL-1 are capable of stimulating colony formation from only a proportion of the progenitor cells present in 5-FU-treated bone marrow. This is further evidence of a heterogeneous population of primitive colony forming cells in 5-FU-treated bone marrow.

Discussion

Interleukin-1 is a pleiotropic growth modulator that has a wide variety of effects on a multiplicity of tissues (see Dinarello, 1984). These include profound effects on the haemopoietic system where IL-1 can induce the production of colony stimulating factor (Lovhaug *et al.* 1986; Fibbe *et al.* 1988) and also facilitate the response of primitive cells to colony stimulating factors that normally

control the proliferation of more mature progenitor cells. The results presented here confirm previous findings that IL-1 is not a direct growth stimulus. The results also show that not only can IL-1 act synergistically with haemopoietic growth factors on the heterogeneous population of cells that constitute 5-FU-BM but also on preparations of FACS-BM cells. As the *in vitro* colony forming efficiency in this enriched population of multipotent cells averages about 6% (compared with only 0·02% for 5-FU-BM cells), and the number of potential spleen colony forming cells (CFU-S) averages between 50% and 140% (Lord & Spooncer, 1986), it is reasonable to infer that IL-1 is acting *directly* on the CFC present in the FACS-BM and that the effects are not mediated indirectly, due to the action of IL-1 on accessory cells present in the FACS-BM cell population. This is further supported by the observation that colony formation is linearly related to the number of cells present. Thus IL-1 can recruit primitive progenitor cells to a growth-factor-responsive state.

The response of FACS-BM cells

The rationale underlying the use of FACS-BM cells was based upon the premise that this population is highly enriched for multipotential cells. Although the yield of cells is low following this separation procedure (raising the possibility that a sub-population of stem cells is being examined) the majority of the cells can be characterized by their ability to produce day 12 CFU-2 (Lord & Spooncer, 1986). Thus, one of the questions we have tried to answer was directed at determining the response of multipotent 'stem' cells to a variety of purified myeloid cell growth factors, at least some of which were originally thought to be restricted in their target cells on the basis of previous *in vitro* analysis (Metcalf, 1985). Since IL-3 has previously been shown to stimulate the growth and development of CFC-Mix *in vitro*, it is perhaps not surprising that IL-3 stimulated significant colony formation from the FACS-BM cells. The colony efficiency, however, averaged only about 6% (although a colony forming efficiency of 40% has also been observed in some experiments). Since our data indicate that the day 12 CFU-S comprise the majority of the population of FACS-BM cells, the *in vitro* analysis indicates either that a sub-population of the day 12 CFU-S-enriched FACS-BM can respond to the proliferation/development stimulus provided by IL-3, or that the majority of cells possess the potential to respond to IL-3 but the culture conditions are not optimal for their growth. We are unable to distinguish between these two possibilities using the present assay procedures. Indeed, it is possible that the day 12 CFU-S present in the FACS-BM do not respond to the IL-3 and that we are detecting a minor population of 'contaminating' cells. While we consider this unlikely (because of the high colony forming efficiency seen in some experiments), we cannot rule out this possibility. Clearly, however, the cells responding to IL-3 are multipotent as determined by morphological analysis of the colonies formed. The situation with FACS-BM cells is, however, quite different from previously reported data using 5-FU-BM. In the latter case, extreme synergy was seen using combinations of IL-1 plus IL-3. In the experiments reported here using FACS-BM, this is not seen. We conclude, therefore, that in FACS-BM, IL-3 can act as a 'maximal stimulus' for recruitment of multipotent CFC. Whether the IL-3/IL-1 synergy seen with 5-FU-BM represents direct recruitment of a cell population not present in FACS-BM, or is the reflection of an indirect effect, will be discussed below.

This raises the question of the origin of colony forming cells that respond to GM-CSF, G-CSF or M-CSF when used alone. Since the growth and development of some multipotential cells in the presence of GM-CSF has been described before (Metcalf, 1985; Sieff *et al.* 1985), some degrees of colony formation by FACS-BM cells was, perhaps, to be expected. The data, however, clearly show that GM-CSF is not as powerful a stimulus for the development of these cells as is IL-3.

The response to G-CSF or M-CSF is more difficult to explain. To our knowledge, the *direct* response of multipotential cells to either of these two growth factors has not been reported previously. Because of this it may be suggested that the responsive cells present in the FACS-BM represent a minor contaminant of committed progenitor cells that shows little or no overlap with the cells responding to IL-3 or to GM-CSF. Certainly, the colony morphology would seem to support this, since the vast majority of colonies derived following stimulation with G-CSF or M-CSF contained only neutrophils or macrophages, respectively. This interpretation is too simplistic, however, since we have clearly shown that when IL-1 is combined with M-CSF (and to a lesser extent with GM-CSF) a significant synergy is seen, resulting in the recruitment of a number of colonies approaching that seen in IL-3. These colonies also consist of macrophages, suggesting that IL-1 has recruited progenitor cells restricted only to the macrophage lineage. Significantly, however, we found no additive colony formation when IL-1 and M-CSF were combined with IL-3: a combination that facilitated only the same number of colonies as that seen in IL-3 alone. This indicates that the cells that respond to IL-1 plus M-CSF overlap considerably with IL-3-responsive cells. Since the IL-3-responsive cells are multipotential (producing mixed lineage colonies), it follows that at least some of the multipotential cells possess receptors for, and can respond to, IL-1 plus M-CSF. Clearly, in this case, the colony morphology is determined more by the developmental stimulus present throughout the development of the colony than by an intrinsic lineage restriction present prior to the stimulation with growth factor. However, since a distinct additive effect is seen using a combination of IL-3 plus M-CSF (in the absence of IL-1) (i.e. the number of colonies developing is equal to the sum of the colonies obtained in IL-3 and M-CSF alone) it can be argued that some cells are present that can respond to M-CSF but not to IL-3. The additive response seen on the IL-3 plus M-CSF combination and the lack of such a response seen in the combination of IL-3 plus M-CSF plus IL-1 is a paradox for which we have no clear explanation.

What about G-CSF? Here the situation is again complex, since G-CSF alone was a poor stimulus for

clonal proliferation of the FACS-BM and no significant synergy was seen using a combination of IL-1 plus G-CSF. Significantly, however, we have now shown that G-CSF can synergize with either GM-CSF or with M-CSF, leading to the development of a number of colonies approaching that seen in IL-3 alone. Again, it is important to note that no additive effects are seen when IL-3 is combined either with G-CSF or with G-CSF plus M-CSF over that seen with IL-3 alone or IL-3 plus M-CSF, respectively. This again suggests extreme overlap between the multipotential IL-3-responsive cells and the cells that respond to the combination of G-CSF plus M-CSF, or G-CSF plus GM-CSF. By inference, then, this further suggests that the IL-3-responsive multipotential cells can respond to G-CSF under the appropriate conditions. The question now arises as to whether the receptors for G-CSF are expressed initially by the multipotential cells or are acquired during development. If the latter were the case then we would have to argue that, in the combination of M-CSF plus G-CSF, the initial developmental stages must be supported by M-CSF or by G-CSF. While we cannot rule this out, preliminary experiments using the delayed addition of G-CSF or M-CSF suggest that the G-CSF has to be present at the start of the cultures, along with the M-CSF, to elicit the growth and developmental response observed. Because of this we favour the suggestion that at least some multipotential cells do indeed possess receptors for G-CSF and M-CSF, but that a threshold level of stimulus for proliferation and development is provided only when the receptors for the M-CSF and the G-CSF are both occupied.

The response of 5-FU-BM cells

When exposed to IL-3, GM-CSF, G-CSF or M-CSF alone, 5-FU-BM showed a colony forming response similar to that previously reported: notably, reasonable numbers of colonies developing in IL-3, fewer in GM-CSF, while colonies stimulated by M-CSF and G-CSF were conspicuously rare. Even in the best assays, however, the maximal colony forming efficiency was only of the order of 0·04%.

The colonies developing in IL-3 were mainly of the mixed myeloid lineages and a marked synergism was seen with IL-1, unlike the effect seen with FACS-BM. These data, which agree with published findings (Stanley *et al.* 1986), suggest that 5-FU-BM contains a primitive population of cells that is refractory to the developmental stimulus of IL-3 alone, but can be recruited into development by culture in the presence of IL-1 plus IL-3. Clearly it is not possible to state if this represents a direct effect of IL-1 upon the target cells, suggesting that the 5-FU-BM contains a cell population that is not represented in FACS-BM, or is indirectly mediated. While colony number is apparently linearly related to the number of cells plated, the large cell number required for minimal colony formation does not preclude the possibility of an accessory cell effect.

Synergy was also seen in cultures of 5-FU-BM when IL-1 was combined with either GM-CSF or M-CSF. By analogy with our data from experiments with FACS-BM, it seems reasonable to infer that this represents IL-1-mediated recruitment of progenitor cells to respond to these two growth factors. However, unlike the effects seen with FACS-BM, when 5-FU-BM was exposed to a combination of IL-1 plus M-CSF plus IL-3 a more than additive effect was observed upon colony formation compared with the number of colonies developing in IL-3 and M-CSF alone. This indicates that the responsive cell populations do not overlap to the same extent as the FACS-BM-responsive cells. The data also indicate that the combination of IL-1 plus M-CSF plus IL-3 can recruit cells that cannot respond to IL-3 plus M-CSF, unlike the situation seen in FACS-BM, where IL-3 plus M-CSF is the 'maximal' colony forming stimulus. Whether or not this is a true heterogeneity of responsive cells or represents the result of interactive mediator molecules (elaborated by accessory cells present in the 5-FU-BM) has not been determined.

With 5-FU-BM cells, G-CSF can also synergize with M-CSF and GM-CSF, as with the effects of using FACS-BM. However, G-CSF can also synergize with IL-3 in 5-FU-BM cells, an effect that is not seen using FACS-BM. It should be noted that, although significant levels of synergy are found, the synergistic effects of G-CSF on 5-FU-BM are not as great as the effects elicited by IL-1. Clearly, the responses of 5-FU-BM and FACS-BM differ in some way.

The response of normal bone marrow cells

FACS-BM and 5-FU-BM both represent cell populations in which primitive progenitor cells have been selected. Normal marrow, on the other hand, contains the full range of haemopoietic progenitor cell types ranging from the earliest stem cell to the maturing precursor cells. The data clearly show that NBM contain cells that respond to the four myeloid cell growth factors studied. Significantly, however, no synergy is seen upon co-addition of IL-1. Neither are significant synergies found upon co-treatment with G-CSF plus the other growth factors. Indeed, the data indicate that, in the majority of instances, IL-1 exerts an inhibitory effect on the response of colony forming cells to the growth factors. These data suggest that the clonogenic cells recruited by IL-1 represent only the more primitive progenitor cells that are enriched in FACS-BM and 5-FU-BM, and which are presumably only present in low numbers in NBM. Alternatively, the cellular heterogeneity of NBM (the presence of putative accessory cells etc.) may be further modifying the response of potential colony forming cells. We favour the former suggestion in view of the work described by Williams *et al.* (1987), which demonstrated that little synergy was seen in the effects of different combinations of myeloid cell growth factors upon colony development of enriched bi- and uni-potent progenitor cells.

Implications for haemopoietic cell development

The process of differentiation is an enigma. Within the haemopoietic system, multipotent stem cells are present that are capable of generating progeny of the various haemopoietic lineages, but little is known of how the

decisions are taken. Two models have been proposed for lineage development. The first suggests that cell surface antigens, a class of proteins including growth factor receptors, are progressively acquired during development, so that the developing cells can then respond to specific lineage-restricted growth and developmental factors. The second model suggests that multipotent cells express receptors for all (or most) of the growth factors (albeit in low numbers) and that interaction of one (or more) of these receptors with the growth and developmental factors will then influence the subsequent pathway of lineage development (perhaps also leading to the expression of greater numbers of receptors) (Till & McCulloch, 1980). Our data support the second model in that at least some of the multipotent cells appear to have an ability to respond to IL-3, GM-CSF, G-CSF and M-CSF when used either alone, in combination or in the presence of IL-1. By inference, the data also suggest that the multipotent cells possess receptors for these different growth factors. Since, in many cases, combinations of growth-promoting agents are necessary to elicit a response, this may indicate that the numbers of receptors for individual growth factors may be very low and a combined stimulus is required. Alternatively, agents such as IL-1 may be working by modulating receptor affinity or altering the physiology of the cell in some other subtle way, so that it can now respond to a growth developmental factor.

Clearly, the existence of such synergistic actions has profound implications for our understanding of the regulation of haemopoiesis. Furthermore, the studies reported may have direct relevance in a variety of clinical situations in which myeloid cell growth factors are currently being used (Bronchud *et al.* 1987).

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