Proliferative Properties of Unfractionated, Purified, and Single Cell Human Progenitor Populations Stimulated by Recombinant Human Interleukin-3

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In this report, the biological properties of human recombinant interleukin-3 (rhIL-3) were studied. We investigated the range of unfractionated, purified and single cell human progenitors responsive to IL-3; compared the colony types observed with those obtained in the presence of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte-CSF (G-CSF). The results show that IL-3 directly stimulates the formation of colonies derived from eosinophil and, to a lesser degree, granulocyte and macrophage progenitors. In combination with erythropoietin, it supports the development of erythroid and mixed-erythroid colonies. Furthermore, the data show that IL-3 is a more potent stimulus for both erythroid and eosinophil progenitors than GM-CSF. Interleukin-3 stimulates the formation of both compact and dispersed colonies derived from eosinophil progenitors, whereas GM-CSF stimulates the formation of only the compact type. We conclude that some of the proliferative effects of IL-3 observed on unfractionated and semipurified bone marrow populations are indirect and most likely involve accessory cell interactions.

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WITH THE INTRODUCTION of semisolid culture techniques and the recognition that hematopoietic cells can proliferate in vitro when stimulated by appropriate specific colony-stimulating factors (CSFs), a functional identification of hematopoietic progenitor and multipotential cells as colony-forming cells has emerged[1,2]. The murine equivalent to the recently cloned gibbon interleukin-3 (gIL-3),[3] rMIL-3, has been shown to have a very broad spectrum of activities. It is known that rMIL-3 stimulates by direct action the proliferation of erythroid, mixed-erythroid, and all nonerythroid colony types in vitro,[4,5] as well as initiating proliferation of early hematopoietic cells (colony-forming unit-spleen; CFU-S) capable of giving rise to spleen colonies in lethally irradiated mice.[6] Recently, a number of studies have shown that recombinant gibbon interleukin 3 (rgIL-3) stimulates the proliferation and differentiation of human progenitor cells, giving rise to erythroid, mast, myeloid, mixed, and megakaryocytic lineages.[7,8] The human gene encoding IL-3 has been identified and cloned by using the gibbon sequence as a hybridization probe.[3]

We have studied the action of COS cell supernatants containing recombinant human IL-3 (rhIL-3) as well as purified rhIL-3 on nonadherent human bone marrow cells, highly purified human bone marrow progenitor cells, and single bone marrow progenitor cells. Our results indicate that some of the proliferative effects of rhIL-3 observed on unfractionated and nonadherent human bone marrow cell populations are indirect and possibly involve accessory cell interactions.

MATERIALS AND METHODS

Cells. Sternal bone marrow cells were obtained from individuals with no hematological disorders undergoing cardiac surgery. Informed consent was gained in accordance with guidelines established by the Ethics Committee of The Walter and Eliza Hall Institute of Medical Research (Victoria, Australia).

Marrow cells were washed in Eisen's balanced salt solution (EBSS) and layered over Ficoll-Hypaque (1.077 g/mL) and centrifuged for 20 minutes at 1,000 × g. Light density mononuclear cells were harvested from the interface, washed, and resuspended in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS).

Adherent cell removal. Adherent cells were removed from light density mononuclear cells by overnight incubation in plastic flasks (Falcon) containing IMDM and 20% FCS. The nonadherent cells were removed by gently shaking the flasks several times and collecting the supernatant. To remove any nonviable cells, nonadherent mononuclear cells were then layered over Ficoll-Hypaque (1.077 g/mL) and spun at 1,000 × g for 20 minutes. The interface cells were collected and washed three times, and cell numbers were determined.

Enrichment of bone marrow progenitor cells. Nonadherent mononuclear cells were labeled with the following panel of monoclonal antibodies (MoAbs): Leu-4 (T cells), Leu-7 (NK cells), Leu-11 (NK cells), and Leu-M3 (monocytes) from Becton Dickinson (Sunnyvale, CA); B1 (B cells), gift from Dr. A. Boyd (The Walter and Eliza Hall Institute of Medical Research); Wem G1 (granulocytes) and Wem G11 (promyeloctyes), gifts from Dr A. Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia); and anti-glycophorin A (nucleated red cells), a gift from Dr J. Griffin (Division of Tumor Immunology, Dana-Farber Cancer Institute, Boston, MA). Labeled cells were centrifuged (1,000 × g for 10 minutes) through FCS to remove unbound antibodies and then incubated with M-450 monosized, magnetic polymer particles (Dynal, Oslo, Norway) with goat anti-mouse immunoglobulin (Ig) attached to the surface, as described previously.[9]

Enriched bone marrow mononuclear cells (obtained by negative selection using the panel of MoAbs described above) were incubated with the MoAb RFB-1[10] (a gift from Dr M. Bodger, Cytogenetics Unit, Christchurch Hospital, New Zealand) at 4°C for 30 minutes. Cells were then washed through FCS to remove any unbound antibody, and incubated at 4°C for 30 minutes, with fluoresceinated F(ab')₂ rabbit anti-mouse antibody. After washing through FCS, cells were analyzed and sorted using a Becton Dickinson FACS II

instrument modified to sort on three parameters as described previously.[11] Cells were collected into 50% FCS in IMDM using siliconized collection tubes. Cells obtained in all experiments were greater than 95% viable, as determined by eosin exclusion. Approximately 60% to 80% of all cells sorted were recovered.

Cells with high RFB-1 fluorescence intensity were previously shown to contain a high percentage of colony-forming cells (CFCs) (5% to 30%),[11] and this population of cells was used for single cell sorting experiments as described previously.[12]

CSF preparations. All the recombinant growth factor preparations (recombinant human erythropoietin rEpo, rhG-CSF, rhGM-CSF, and rhIL-3) were kindly provided by Dr G. Wong, Genetics Institute, Cambridge, MA. The rEpo was purified from COS cell supernatants and had a specific activity of 236,000 U/mg by in vitro bioassay. The rhGM-CSF preparation was also pure and had a specific activity of 8×10^6 U/mg. Both the rhIL-3 and rhG-CSF preparations were obtained from transfected mammalian (COS) cell line supernatants. In some experiments purified, bacterially derived rhIL-3 was used, and it possessed a specific activity of 4×10^6 U/mg by in vitro bioassay.

Semisolid cultures. Semisolid cultures were performed in 35 mm Petri dishes as described previously.[11] Control medium (0.1 mL) or the required stimuli (0.1 mL) was added to the culture dishes prior to addition of agar-medium containing cells. Triplicate cultures were established with either medium alone, 2 units rEpo, 2 units rEpo plus rhG-CSF, 2 units rEpo plus rhGM-CSF, or 2 units rEpo plus rhIL-3. In all cultures, supramaximal concentrations of rhG-CSF, rhGM-CSF or rhIL-3 were used. For nonadherent bone marrow cultures, 400 units/mL of CSF activity were used; while for purified and single cell progenitors, 2,000 to 4,000 units/mL of CSF activity were required for maximal stimulation.

To test the effect of the delayed addition of Epo, 2 units of rEpo in 0.2 mL of IMDM were gently layered over recipient agar cultures after 3 days' incubation of cultures, initiated with either no stimulus or with a supramaximal concentration of rhIL-3. Cultures were examined after a total of 14 days' incubation as described above. In some experiments, methylcellulose (0.9%) was used as the immobilizing agent.

The number of cells cultured ranged from 3×10^4 to 5×10^4 per mL for nonadherent bone marrow and between 10^2 to 6×10^2 for FACS-fractionated bone marrow. After addition and mixing of cells, 1 mL aliquots of agar-medium or methylcellulose medium were placed in 35 mm Petri dishes and incubated at 37°C in a fully humidified incubator containing 5% CO_2 in air. To ensure the maintenance of the humidity, duplicate cultures were placed into 100 mm Petri dishes with an open third 35 mm Petri dish containing double distilled water.

Cultures were examined after 7 and 14 days' incubation with an Olympus SZ stereo dissection microscope with semi-indirect lighting, and pure erythroid, mixed-erythroid, and nonerythroid colonies were scored. Identification of these colony types was confirmed by glutaraldehyde fixaton of whole cultures, which were then stained with Luxol-Fast-Blue/hematoxylin or benzidine.[13]

Liquid cultures. Low numbers of purified bone marrow progenitor cells (3000 cells) were incubated in triplicate 1 mL cultures containing IMDM and 20% FCS with various stimuli, and aliquots of cells were removed after 21 and 42 days' incubation to count viable cells and make cytospin preparations.

Plate mapping. Cultures were mapped by using tissue culture dishes that had been marked to allow accurate alignment on a numbered reference grid. The localization and size of all clones of two or more cells were recorded in sample areas of the culture. Mapping was repeated at 2-day intervals during the 14 day observation period. After 14 days' incubation, colonies were picked off and typed as above.

Statistics. Where indicated, statistical analysis was performed using Student's *t* test.

RESULTS

Stimulation of nonadherent bone marrow mononuclear cells by recombinant CSFs. Media containing rhIL-3 stimulated the formation of nonerythroid colonies after 14 days' incubation of nonadherent bone marrow mononuclear cells (Table 1). This material also stimulated the formation of erythroid and mixed-erythroid colonies in the presence but not in the absence of Epo. When cultures were maximally stimulated with rhIL-3 plus Epo, the number of erythroid colonies was significantly higher (range, 1.4 to 2.1 times; n = 6; $P \leq .001$) when compared with the number maximally stimulated by GM-CSF and Epo. Similarly, the number of mixed-erythroid colonies stimulated by IL-3 and Epo was 2 to 4 times ($P < .005$) greater than the number stimulated by rhGM-CSF plus Epo. In combination with Epo, rhIL-3 was able to stimulate 2 to 5 times the number of day 7 erythroid colonies stimulated by rEpo alone (Table 1). Neither rhG-CSF nor rhGM-CSF was able to increase the number of day 7 erythroid colonies above that stimulated by Epo alone. The number of day 14 nonerythroid colonies, however, was not significantly different in rhIL-3 or rhGM-CSF stimulated cultures. Morphological examination of the stained agar cultures showed that rhIL-3 and rhGM-CSF stimulated similar percentages of granulocyte, macrophage, granulocyte-macrophage, and eosinophil colonies (Table 2). As expected, rhG-CSF stimulated only granulocyte, macrophage and granulocyte-macrophage colony formation. The number of day 7 nonerythroid colonies in cultures stimulated by rhIL-3 was generally lower than that stimulated by either rhG-CSF or rhGM-CSF (Table 1).

Stimulation of purified bone marrow progenitor cells by recombinant CSFs. In order to determine whether the stimulation by IL-3 of normal bone marrow cells was direct or mediated via accessory cells, the above experiments were repeated using highly purified hematopoietic progenitor cell populations. The results of the action of recombinant CSFs on purified progenitor populations obtained from the same three bone marrow samples referred to in Table 1 are shown in Table 3. There was no proliferation of progenitor cells in cultures without added CSF, indicating the virtual absence of accessory cells capable of producing endogenous CSF in vitro.

As observed with nonadherent bone marrow mononuclear cells, cultures of highly purified progenitor cells stimulated by the combination of rhIL-3 and rEpo contained increased numbers of day 7 and day 14 erythroid colonies, compared with cultures stimulated with rhGM-CSF plus Epo (range, 1.6 to 2.5 times; n = 3; $P \leq .005$), or Epo alone (range, 2.1 to 4.2 times; n = 3; $P \leq .001$). Furthermore, rhIL-3 in the presence of Epo also stimulated more mixed-erythroid colonies (1.5 to 3 times) than did rhGM-CSF plus Epo.

Although similar numbers of the various nonerythroid colony types were stimulated in nonadherent bone marrow cultures by either rhGM-CSF or rhIL-3 (Table 2), this similarity was not observed in cultures of highly purified progenitor cells (Table 2). Despite similar numbers of total

Table 1. Colony Formation in Cultures of NonAdherent Bone Marrow Mononuclear Cells

Experiment and Stimulus	No. of Cells/ Culture	Mean No. Colonies/10^5 Cells				
		Day 7		Day 14		
		Erythroid*	Non-erythroid	Erythroid†	Mixed Erythroid	Non-erythroid
1 None	5×10^4	0	0	0	0	4 ± 2
rhIL-3		0	4	0	0	64 ± 10
rEpo		64	0	100 ± 4	0	2 ± 2
rEpo + rhG-CSF		80	52	94 ± 4	0	50 ± 6
rEpo + rhGM-CSF		72	30	146 ± 12	4 ± 2	78 ± 8
rEpo + rhIL-3		260	8	234 ± 24	16 ± 4	58 ± 6
2 None	4×10^4	0	0	0	0	0
rhIL-3		0	3	0	0	65 ± 15
rEpo		183	0	85 ± 7	0	5 ± 2
rEpo + hG-CSF		176	15	90 ± 15	0	68 ± 8
rEpo + rhGM-CSF		208	10	130 ± 12	2 ± 0	83 ± 9
rEpo + rhIL-3		405	5	203 ± 12	8 ± 3	48 ± 24
3 None	5×10^4	ND	ND	0	0	4 ± 2
rhIL-3		ND	ND	0	0	42 ± 6
rEpo		ND	ND	42 ± 6	0	6 ± 4
rEpo + rhG-CSF		ND	ND	40 ± 8	0	24 ± 10
rEpo + rhGM-CSF		ND	ND	80 ± 8	2 ± 2	38 ± 6
rEpo + rhIL-3		ND	ND	116 ± 8	8 ± 2	44 ± 10

Nonadherent bone marrow mononuclear cells from three separate experiments were plated at 4 to 5 × 10^4/mL. Cultures were scored after 7 and 14 days of incubation. Where indicated, cultures were either unstimulated or maximally stimulated with rEpo, rhIL-3, rhG-CSF, and rhGM-CSF.

Abbreviation: ND, not done.

*Values are mean of duplicate cultures.

†Values are mean ± SD of triplicate cultures.

nonerythroid colonies from cultures of purified progenitor cells stimulated by either rhGM-CSF or rhIL-3, the percentages of the various nonerythroid colonies were different. Particularly, in each individual experiment, rhIL-3 stimulated on average three times as many eosinophil colonies to develop as did rhGM-CSF, and approximately half of these were dispersed in shape, unlike the usually compact eosinophil colonies observed with GM-CSF–stimulated cultures (Table 2). Interestingly, there was a corresponding reduction in the number of rhIL-3–stimulated neutrophil and macrophage colonies when compared to rhGM-CSF–stimulated

cultures. No significant differences in the pecentages of the various nonerythroid colonies were observed between cultures of nonadherent compared with purified progenitor cells stimulated by rhG-CSF.

Purified progenitors require increased concentrations of rhIL-3 for proliferation in vitro. Initially, rhIL-3 titration experiments were performed in parallel on both nonadherent and purified bone marrow cell populations, in order to determine the optimal concentration of rhIL-3 required for maximal stimulation. Figure 1 shows the result of one such typical titration experiment, where rhIL-3 was titrated on

Table 2. Nonerythroid Colony Differential Counts

Cells	Stimulus	Mean No. Colonies/10^5 Cells*				
		Neutrophil	Macrophage	Neutrophil-Macrophage	Eosinophil	Other
Nonadherent bone marrow cells (4-5 × 10^5/ plate)	rhG-CSF	18 ± 12 (56 ± 8)	13 ± 15 (34 ± 10)	3 ± 2 (10 ± 3)	0 (0)	0 (0)
	rhGM-CSF	13 ± 6 (36 ± 8)	13 ± 11 (36 ± 6)	5 ± 4 (12 ± 3)	6 ± 3 (16 ± 4)	1 ± 2 (2 ± 2)
	rhIL-3	11 ± 10 (30 ± 10)	15 ± 10 (38 ± 11)	3 ± 2 (7 ± 2)	9 ± 1 (22 ± 5)	1 ± 1 (3 ± 3)
Purified bone marrow cells (1-5 × 10^2/ plate)	rhG-CSF	1,500 ± 2,162 (72 ± 15)	815 ± 1,200 (26 ± 13)	117 ± 202 (2 ± 3)	0 (0)	0 (0)
	rhGM-CSF	1,070 ± 388 (33 ± 7)	1,025 ± 442 (30 ± 9)	176 ± 138 (5 ± 3)	1,000 ± 1,445 (30 ± 16)	0 (0)
	rhIL-3	433 ± 500 (8 ± 4)	440 ± 767 (5 ± 9)	110 ± 190 (1 ± 2)	3,013 ± 2,765 (86 ± 10)	0 (0)

Comparison of the number of percentage distribution of nonerythroid colonies stimulated by either rhG-CSF, rhGM-CSF, or rhIL-3 for nonadherent and purified bone marrow cells after 14 days incubation.

*Values represent mean number of colonies per 10^5 cells ± SD from three separate experiments. Values in parentheses indicate mean percentage distribution ± SD from three separate experiments.

Table 3. Colony Formation in Cultures of Purified Bone Marrow Progenitor Cells

Experiment and Stimulus	No. of Cells/ Culture	Mean No. Colonies/10^5 Cells				
		Day 7		Day 14		
		Erythroid*	Non-erythroid	Erythroid†	Mixed Erythroid	Non-erythroid
1 None	200	0	0	0	0	0
rhIL-3		0	8,000	0	0	6,000 ± 1,000
rEpo		11,500	0	10,000 ± 1,000	0	0
rEpo + rhG-CSF		10,000	2,000	9,500 ± 1,500	0	2,000 ± 1,000
rEpo + rhGM-CSF		9,500	2,500	15,000 ± 1,500	500 ± 500	5,000 ± 1,500
rEpo + rhIL-3		23,000	6,000	29,500 ± 1,500	2,000 ± 500	7,000 ± 1,500
2 None	100	0	0	0	0	0
rhIL-3		0	2,000	0	0	2,000 ± 1,000
rEpo		5,000	0	6,000 ± 1,000	0	0
rEpo + rhG-CSF		4,000	0	4,000 ± 1,000	0	0
rEpo + rhGM-CSF		4,000	0	10,000 ± 2,000	1,000 ± 1,000	4,000 ± 2,000
rEpo + rhIL-3		12,000	2,000	16,000 ± 0	3,000 ± 1,000	2,000 ± 1,000
3 None	500	ND	ND	0	0	0
rhIL-3		ND	ND	0	0	1,200 ± 600
rEpo		ND	ND	4,000 ± 400	0	0
rEpo + rhG-CSF		ND	ND	3,600 ± 400	0	600 ± 400
rEpo + rhGM-CSF		ND	ND	6,400 ± 800	200 ± 0	1,600 ± 400
rEpo + rhIL-3		ND	ND	13,000 ± 1,400	600 ± 200	1,000 ± 600

Purified bone marrow progenitor cells from three separate experiments shown in Table 1, were plated at 1 to 5 × 10^2 cells/mL. Cultures were scored after 7 and 14 days of incubation. Where indicated, cultures were either unstimulated or maximally stimulated with rEpo, rhIL-3, rhG-CSF, and rhGM-CSF.

Abbreviation: ND, not done.

*Values are mean of duplicate cultures.

†Values are mean ± SD of triplicate cultures.

nonadherent and purified bone marrow cells from the same sample. For both cell populations, the dose-response curves for erythroid and nonerythroid colony formation with rhIL-3 follows a sigmoid curve with a plateau.

In order to achieve 50% of maximal stimulation of erythroid or nonerythroid colonies for purified progenitor cell cultures, 4 or 12 times the concentration of rhIL-3 was required, respectively, compared with nonadherent mononuclear cell cultures (rhIL-3 titer of 1:960 compared with 1:3,840, or 1:960 compared with 1:80, respectively).

A number of experiments were performed using purified bacterially derived rhIL-3 (specific activity, 4 × 10^6 U/mg). In two separate experiments, the purified material had similar specificity by in vitro bioassay to the COS cell-derived crude hIL-3 (data not shown), with a molar concentration of 15 to 40 pmol/L required for half-maximal stimulation of nonadherent cultures.

Stimulation of mast cells by rhIL-3 in long term liquid cultures of purified progenitor cells. No mast cell colonies were observed in long term agar or methylcellulose cultures (14 to 42 days' incubation) of either unfractionated or purified bone marrow cells stimulated by rhIL-3.

In order to determine whether rhIL-3 stimulated the proliferation of mast cells, long term liquid cultures were established containing low numbers of purified bone marrow progenitor cells. The enriched progenitor population contained predominantly blast or lymphocyte-like cells (96%) with occasional monocyte-like or nucleated red blood cells (RBCs). These cells have been shown previously not to express cell surface markers for OKT3, OKT4, OKT8,

Leu-7, Leu-11, or B1 antigens. In particular, none of the cells stained positive with either Astra-Blue or Toluidine-Blue stains, which normally stain mast cell granules.[11]

When 3,000 cells from the purified progenitor cell fraction were incubated in liquid culture with either no stimulus, rhGM-CSF, or rhIL-3, proliferation was observed in the CSF-stimulated cultures but not the unstimulated cultures. After 21 days' incubation, cultures stimulated with rhGM-CSF yielded a mean of 1.5 × 10^6 viable cells, whereas rhIL-3–stimulated cultures yielded a mean of 9 × 10^5 cells. Examination of cytospin preparations did not reveal any Astra-blue or Toluidine-blue positive cells. However, after 42 days of incubation, approximately 30% of cells were Toluidine-blue positive in cultures stimulated with rhIL-3. In cultures stimulated by rhGM-CSF, no Toluidine-blue positive cells were detected after 42 days of incubation. The mean viable cell count after 42 days of incubation for rhIL-3 stimulated cultures was 2.5 × 10^5 cells, versus 2 × 10^4 for rhGM-CSF stimulated cultures.

Delayed addition of erythropoietin. A number of experiments were performed (n = 4) where the addition of rEpo was delayed for 3 days after initiation of cultures with rhIL-3. Table 4 shows data from two such experiments, where rEpo was added on day 3 of incubation. In Experiment 1, 6 × 10^2 purified bone marrow progenitor cells were incubated with either no stimulus, rEpo alone, rhIL-3 alone, or a combination of rEpo and rhIL-3. Cultures initiated without stimulus to which rEpo was added on day 3 of incubation did not give rise to any erythroid colonies after 14 days of incubation. However, cultures initiated with rhIL-3
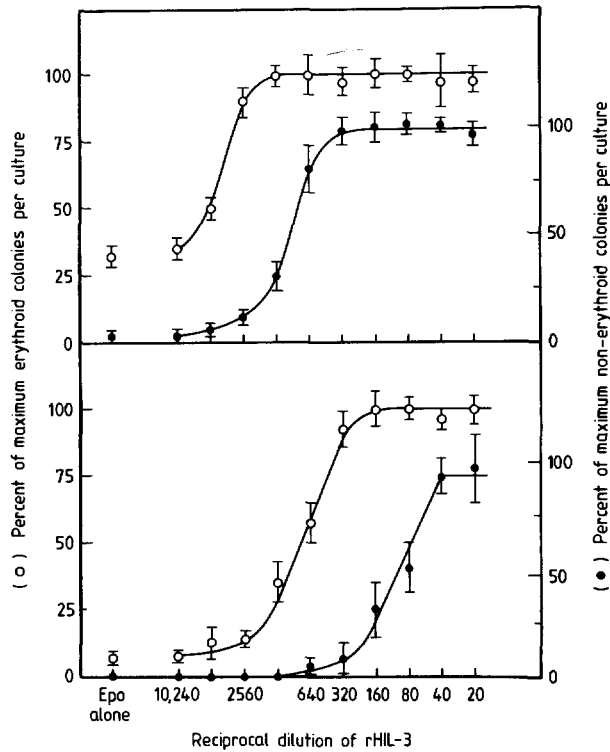
Fig 1. Top panel shows a typical titration of recombinant human interleukin-3 (rhIL-3) on nonadherent bone marrow cells (5 × 10⁴ mL) maximally stimulated with rEpo. Bottom panel shows a similar titration curve for rhIL-3 on purified bone marrow progenitor cells (3 × 10²/mL) from the same bone marrow sample. Points represent mean and standard deviation of triplicate determinations for erythroid (O) and nonerythroid colonies (●).

to which rEpo was added after 3 days of incubation gave rise to an average of five erythroid colonies per culture. The mean number of erythroid colonies initiated with both rEpo and rhIL-3 was 33 per culture. Similarly, for Experiment 2, delayed addition of rEpo to cultures initiated with no stimulus did not result in any erythroid colonies. For rhIL-3 initiated cultures, delayed addition of rEpo resulted in the formation of 13% of the total colony numbers maximally stimulated by both rhIL-3 and rEpo. In all experiments,

deprivation of rEpo for the first 3 days of incubation resulted in no formation of any mixed-erythroid colonies. Mixed-erythroid colonies were formed only in cultures initiated with both rhIL-3 and rEpo.

Fate of clones initiated in rhIL-3. In view of the reduced number of erythroid colonies observed with delayed addition of rEpo, a number of experiments were performed to determine the fate of clones initiated by rEpo, rEpo plus rhGM-CSF, or rhIL-3 plus rEpo. For this purpose, purified bone marrow progenitor cell fractions were plated at 100 and 300 cells per milliliter. The number, size, and eventual fate of developing clones was studied by plate mapping experiments.

Figure 2 shows the result of one such experiment in which 200 purified bone marrow progenitor cells were cultured in duplicate cultures. Cultures stimulated with rEpo only contained only erythroid colonies at day 14, with the majority of colonies (93%) initiated in the first 5 days. Thirty erythroid colonies were detected at day 14, for a total of 75 clones from duplicate cultures. For duplicate cultures stimulated with rhGM-CSF and rEpo, a total of 184 clones was detected from days 2 to 14, of which 128 formed colonies on day 14. A total of 58 erythroid colonies was formed, and 90% of these colonies began to proliferate in the first 3 days of culture. Mixed-erythroid colonies began to proliferate after 5 days but before 13 days of incubation. Neutrophil, macrophage, and neutrophil-macrophage colonies all began to proliferate in the first 5 days of incubation. Seventy-five percent of eosinophil colonies began to proliferate in the first 5 days, and the remainder proliferated before 11 days of incubation. The predominant types of nonerythroid colonies comprised either neutrophils or macrophages. Similar data were obtained for the various nonerythroid colonies initiated solely by rhGM-CSF (data not shown).

In cultures stimulated with rhIL-3 and rEpo, the number of total clones observed for duplicate plates between days 2 and 14 was greater than that observed for either rEpo alone or rEpo and rhGM-CSF (303 versus 75 versus 184, respectively). More than one third of the developing clones gave rise to erythroid colonies after 14 days of incubation. The majority of these colonies (89%) began to proliferate in the first 3 days. All the mixed-erythroid colonies began to proliferate between 7 and 13 days of incubation. As observed

Table 4. Delayed Addition of Erythropoietin to Cultures of Purified Bone Marrow Progenitor Cells

Experiment (No. Cells/Culture)	Initial Stimulus	Day of Addition of rEpo (2u)	Mean No. Colonies/Culture		
			Erythroid	Mixed-Erythroid	Nonerythroid
1 (600)	None	3	0	0	0
	rEpo	0	9 ± 2	0	0
	rhIL-3	3	5 ± 3	0	9 ± 3
	rEpo + rhIL-3	0	33 ± 4	2 ± 1	12 ± 4
	rhIL-3	ND	0	0	10 ± 2
2 (500)	None	3	0	0	0
	rEpo	0	25 ± 3	0	0
	rhIL-3	3	9 ± 4	0	27 ± 5
	rEpo ± rhIL-3	0	67 ± 6	4 ± 1	24 ± 6
	rhIL-3	ND	0	0	26 ± 4

Delayed addition initiated with either no stimulus, or rhIL-3 plus rEpo, or rhIL-3 alone. Triplicate 1 mL cultures were scored after 14 days of incubation. Abbreviation: ND, not done.
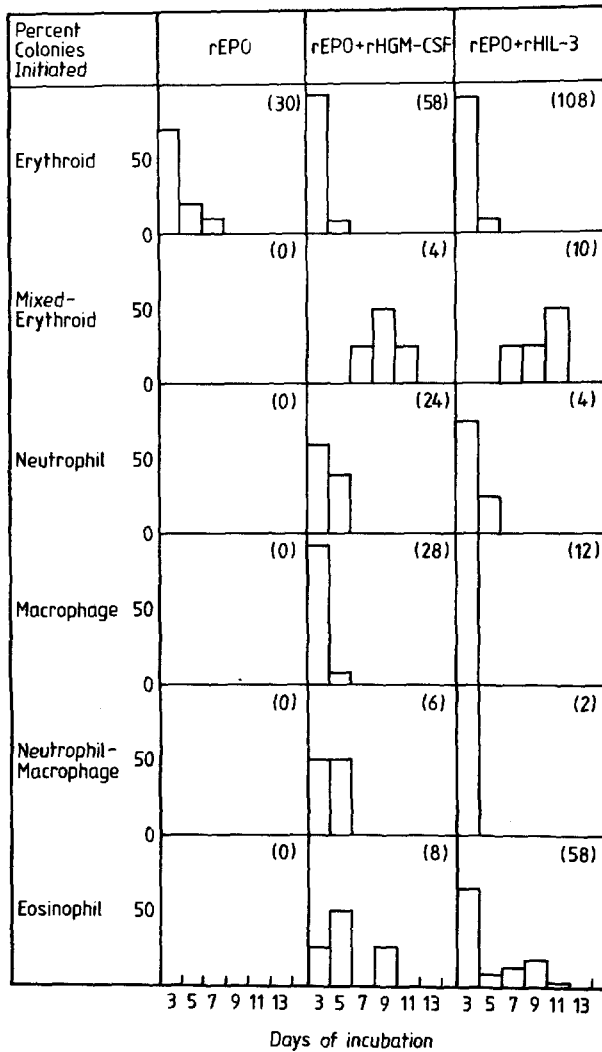
Fig 2. Plate mapping experiment from triplicate cultures containing 4 × 10² purified bone marrow progenitor cells (4 × 10²/mL). Where indicated cultures were maximally stimulated with rEpo, rhGM-CSF, or rhIL-3. The left hand column represents the type and percentage of colonies that were initiated after incubation. Numbers in parentheses indicate the total number of day 14 colonies.

in cultures stimulated with rhGM-CSF, the majority of nonerythroid colonies began to proliferate in the first 3 days. The majority of nonerythroid colonies comprised eosinophil colonies (58/76), with 67% of these colonies beginning proliferation in the first 3 days, and a significant but smaller proportion between days 5 and 11 of incubation. In the absence of rEpo, rhIL-3 was able to stimulate a similar percentage of the various nonerythroid colonies (data not shown).

Effect of rhIL-3 and rEpo on single progenitor cells. In order to exclude any accessory cell-mediated effects of rhIL-3 on progenitor cells, a number of single cell experiments were performed (Table 5). In Experiment 1, 480 single cells were sorted into 96-well microtiter plates containing agar-medium and rhIL-3 plus rEpo, or rEpo alone. The

combination of rhIL-3 and rEpo stimulated the formation of 12 erythroid and six nonerythroid colonies from single cells, as well as 23 clusters (3 to 40 cells). Five of the six nonerythroid colonies were eosinophil in type, confirming the observation that rhIL-3 predominantly stimulates the proliferation of eosinophil colonies from purified progenitor cell populations. In contrast, rhGM-CSF and rEpo stimulated the formation of four erythroid and four nonerythroid colonies from single cells, as well as 12 clusters. All of the nonerythroid colonies were magrophage in type. Similar data were obtained for Experiment 2: the majority of rhIL-3 stimulated nonerythroid colonies were eosinophil in type, whereas the majority of rhGM-CSF stimulated nonerythroid colonies were macrophage in type. Alone, rEpo was able to initiate the formation of erythroid colonies, albeit small colonies (≈40 cells), from single progenitor cells (1/12 from Experiment 1 and 1/7 from Experiment 2).

DISCUSSION

The biological properties of rMIL-3 have been extensively studied.[4,5] These studies have shown that rMIL-3 supports the proliferation of multipotent and committed progenitor cells in vitro, as well as supporting the proliferation of CFU-S.[4,5,6] The availability of the recently cloned gIL-3[3] and, subsequently, of rhIL-3[3] have now allowed studies on human hematopoietic cell populations to be performed. To date, rgIL-3 has been shown to have multipotential proliferative activity on unfractionated bone marrow cells,[7,8] as well as stimulating some functions of mature eosinophils.[7] Recently, Sieff et al[8] have demonstrated that gIL-3 stimulates the proliferation of more burst-forming unit-erythroid (BFU-E) and Mix-CFC than rhGM-CSF, when used in combination with rEpo. These investigators were also able to demonstrate stimulation of eosinophil, granulocyte-macrophage and megakaryocyte colonies by gIL-3 from culture containing enriched bone marrow and fetal liver progenitor cells.[8] However, even though these purified progenitor populations were plated at relatively low density (1,000 cells/mL), the possibility that gIL-3 may exert some of its effects indirectly via accessory cells cannot be excluded.[8] As has been recently shown for gIL-3, rhIL-3 was also shown to stimulate the proliferation of erythroid, mixed-erythroid, granulocyte, macrophage, granulocyte-macrophage and eosinophil colonies from cultures of nonadherent bone marrow mononuclear cells.

Recombinant human interleukin-3 was a better proliferative stimulus for day 14 erythroid and mixed-erythroid colony formation than rhGM-CSF. Similarly, rhIL-3 in the presence of erythropoietin was able to enhance the number of day 7 erythroid colonies compared with control cultures stimulated by erythropoietin alone or rhGM-CSF and erythropoietin. This is analogous to the situation observed in the murine system, where multi-CSF has a direct stimulatory role on colony forming unit-erythroid (CFU-E).[14] In the mouse it appears that Epo is not mandatory to observe this effect, whereas for human cells, Epo, as well as hIL-3, are required to observe enhancement of either day 7 or day 14 erythroid colonies. The increased number and size of erythroid and mixed-erythroid colonies stimulated by rhIL-3 in

Table 5. Cultures of Single Purified Progenitor Cells Stimulated by rhIL-3, rEpo, or rhIL-3 + rEpo

Experiment and Stimulus	Type of Culture		No. Day 14 Clones		
	Control (Cells/Culture)	Single Cells (Cells/Culture)	Clusters	Colonies	
				Erythroid	Nonerythroid (Type)
1 rhIL-3	300	—	33	0	20 (17 EO, 3M)
rEpo	300	—	2	3	—
rhIL-3 + rEpo	300	—	39	22	18 (15 EO, 2M, 1N)
rhGM-CSF + rEpo	300	—	38	7	9 (1 EO, 7M, 1N)
rhIL-3	—	480	18	0	7 (7 EO)
rEpo	—	480	3	1	—
rhIL-3 + rEpo	—	480	23	12	6 (5EO, 1M)
rhGM-CSF + rEpo	—	386	12	4	4 (4M)
2 rhIL-3	500	—	42	0	15 (9EO, 4M, 2N)
rEpo	500	—	7	1	0
rhIL-3 + rEpo	500	—	65	31	11 (6EO, 3M, 2N)
rhGM-CSF + rEpo	500	—	19	9	9 (2EO, 7M)
rhIL-3	—	480	55	0	10 (9EO, 1M)
rEpo	—	480	5	1	0
rhIL-3 + rEpo	—	384	54	7	7 (6EO, 1M)
rhGM-CSF + rEpo	—	576	9	2	8 (1N, 7M)

Data from two single cell experiments showing the fate of developing clones after 14 days incubation in 96-well microtiter plates. Triplicate control cultures were set up containing 3 to 5 × 10^2 purified normal bone marrow progenitor cells/mL, and where indicated were maximally stimulated with rEpo, rhIL-3, or rhGM-CSF.

Abbreviations: EO, eosinophil; M, macrophage; N, neutrophil.

comparison with those stimulated by rhGM-CSF suggests that IL-3 is able to stimulate a wider range of early hematopoietic cells than GM-CSF.

A number of differences were observed when rhIL-3 was tested on highly purified bone marrow progenitor cells compared with nonadherent mononuclear cells. First, higher concentrations of rhIL-3 (approximately fourfold for erythroid and eightfold for nonerythroid colony formation) were required for maximal stimulation; rhGM-CSF or rhG-CSF showed analogous differences.[15] Second, rhIL-3 appeared to predominantly stimulate eosinophil, erythroid, and mixed-erythroid colonies. Although granulocyte and macrophage colonies are also stimulated by rhIL-3, they appear to compose only a small percentage (less than 15%) of total nonerythroid colonies, in comparison with rhGM-CSF and rhG-CSF. In absolute numbers, rhGM-CSF and rhG-CSF stimulated two to three times as many granulocyte macrophage colonies per culture as did rhIL-3, whereas rhIL-3 stimulated three to five times as many eosinophil colonies per culture as did rhGM-CSF. This extra subset of eosinophil colonies consisted of dispersed cells and was morphologically distinct for the typical compact eosinophil colonies stimulated by rhGM-CSF. These data support the possibility that GM-CSF and IL-3 may regulate different subsets and/or aspects of eosinophil function. It is interesting that while IL-3 stimulated the formation of more eosinophil colonies than GM-CSF did, it appeared to do so at the expense of granulocyte and macrophage colony formation. These findings imply that either rhIL-3 has restricted proliferative activity on granulocyte-macrophage progenitor cells or that these progenitor cells may be "recommitted" in the presence of rhIL-3 to differentiate down the eosinophil lineage.

Further studies are warranted to distinguish between these two intriguing possibilities.

In order to explain the different effects of rhIL-3 on unfractionated and highly purified progenitor cells, we postulate that rhIL-3 either interacts with accessory cells to produce G-CSF and/or GM-CSF, or that endogenous G-CSF and GM-CSF produced by accessory cells in cultures interacts with rhIL-3 in a synergistic way to stimulate additional subsets of granulocyte-macrophage progenitor cells. Recently, studies by a number of investigators have demonstrated synergy between GM-CSF and rhIL-3 on serum deprived in vitro colony formation.[16-18] In addition, rhIL-3 also appears to stimulate the proliferation of mast cell progenitor cells in liquid culture but not in agar or methylcellulose cultures.

Delayed addition of erythropoietin has been used by a number of investigators[19-21] to study the erythroid potentiating activity of various CSF preparations. When highly purified human bone marrow progenitor cells were initiated in cultures with rhIL-3, only a small percentage (less than 15%) of day 14 erythroid colonies were observed when Epo addition was delayed until day 3 of incubation, compared with cultures initiated with Epo and rhIL-3. This finding suggested that either rhIL-3 initiated proliferation of some erythroid clones (and Epo was not required until day 3) or that some erythroid clones did not begin to proliferate until at least day 3. Plate mapping experiments confirmed that the majority of erythroid clones (75% to 90%) began to proliferate within the first 3 days of incubation, while 10% to 25% of erythroid clones remained dormant for up to 7 days of incubation. Clones initiated in rhIL-3 did not become hemoglobinated until rEpo was added.

In order to show definitely that rhIL-3 acts directly on bone marrow progenitor cells, we performed a number of experiments with single progenitor cells obtained by cell sorting. These data confirmed that single human progenitor cells could proliferate to give rise to colonies in vitro with appropriate stimuli. In cultures of single cells, rhIL-3 and Epo were able to stimulate the formation of erythroid, eosinophil, and occasional neutrophil/macrophage colonies.

Recent studies with rhIL-3 in the mouse have shown that this is a potent stimulus of progenitor cell proliferation in both normal and lethally irradiated mice.[4-7,22] These data support the possibility that particular CSFs, such as hIL-3, may be important in the treatment of cytopenias in man that are either acquired, such as postchemotherapy, postirradiaion, or postviral, or congenital in nature. Our studies suggest that while rhIL-3 appears to act on primitive hematopoietic progenitors in unfractionated and semipurified cell populations its ability to do so in cultures of highly purified progenitors or single cells appears to be restricted. However, despite the restricted proliferative activity of rhIL-3 on single human progenitor cells, its ability to stimulate the proliferation of a wide range of hematopoietic progenitor and multipotential cells from unfractionated cell populations makes this CSF a prime candidate, either by itself or in combination with other CSFs, in accelerating hematological recovery after bone marrow transplantation.

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