CONCISE REPORT

Interleukin 3 Promotes the Differentiation of Isolated Single Megakaryocytes

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Interleukin 3 (IL3) promotes the proliferation of murine hematopoietic progenitor cells, including the megakaryocytic colony-forming cell (CFU-MK). To determine whether IL3 influences more differentiated cells in the murine megakaryocytic lineage, IL3 was added to microcultures of single megakaryocytes isolated from CFU-MK-derived colonies. After two days of culture, cell diameter and DNA content were analyzed. Eighty-five percent of cells initially between 12 and 20 μm in diameter increased in size in response to IL3, while only 12% of these cells increased in size in its absence. The percentage of cells responding to IL3 varied inversely with the size of the initial cells. The culture procedure did not alter the relatively linear relationship between size and ploidy, suggesting that increments in cell size are accompanied by increased ploidy. After three days of culture, acetylcholinesterase (AchE) activity was assayed. A small but significant increment in AchE activity was observed in the presence of IL3. The data show that, at least in vitro, IL3 is a differentiation-promoting factor of the murine megakaryocytic lineage.

INTERLEUKIN 3 (IL3), a hematopoietic colony-stimulating factor, promotes the proliferation of multiple murine hematopoietic lineages, including hematopoietic cell lines[1]; pluripotent stem cells (CFU-S) of both limited or more extensive proliferative potential[2,3]; and more differentiated hematopoietic progenitors such as the myeloid, erythroid, mast cell, and perhaps pre-B cell.[4-8] Among the activities of IL3 is the ability to promote the proliferation of the megakaryocytic colony-forming cell (CFU-MK).[9]

Recent studies in our laboratory have shown that, in a serumless culture system, purified IL3 promotes the appearance of large polyploid megakaryocytes.[10] Acetylcholinesterase (AchE) production, a relatively specific marker of the megakaryocytic lineage in mice, is concurrently increased.[11,12] Although induction of proliferation of CFU-MK had been previously shown to be a property of IL3,[9] the positive influence of this factor on what are generally considered markers of differentiation (size, ploidy, and AchE content), suggested to us that IL3 might also promote this phase of megakaryocytopoiesis. Since previous studies had been performed using unfractionated marrow, the possibility that accessory cells may have provided differentiation-promoting activity could not be excluded. To determine whether IL3 promotes megakaryocytic differentiation directly, the present studies were performed using single megakaryocytes isolated from CFU-MK-derived clones. The advantage of this approach is that such clones contain both small, low-ploidy and large, high-ploidy cells, thereby allowing one to examine the effect of IL3 on megakaryocytes in varying stages of differentiation.

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MATERIALS AND METHODS

Culture of megakaryocytic colonies. Six- to eight-week-old specific pathogen-free $C_{57}Bl/6$ male mice obtained from Jackson Laboratories, Bar Harbor, Me, were used for all experiments. Bone marrow of the femur was flushed and resuspended through an 18 followed by a 22 gauge needle. Marrow was enriched for progenitor cells on a 1.070/1.077g/cm^3 two-step Percoll gradient and cultured in 35 mm dishes at 10^4 cells/mL in Iscove's modification of Dulbecco's medium (medium) containing 1% methylcellulose, 15% horse serum, 5% pokeweed mitogen-stimulated spleen cell conditioned medium, 50 μmol/L β-mercaptoethanol (β-ME), and 100 U/mL of penicillin-streptomycin. Cells were incubated under a humidified atmosphere of 5% of CO_2 in air at 37 °C.

Single cell culture of CFU-MK-derived megakaryocytes. Megakaryocytic colonies were identified in situ as previously described.[13] The appearance of megakaryocytic colonies in methylcellulose is similar to those grown in agar gel. After five days of culture randomly chosen colonies were selectively removed under an inverted microscope with a micropipette in 2 μL volumes and dispersed into 2 mL of medium containing 50 μmol/L β-ME. Fewer than five colonies were removed at a time to maximize the dilution of the initial culture constituents. Individual cells were removed in 2 μL volumes and cultured for 48 hours in 96 microwell plate covers in 35 μL of medium containing 3% bovine serum albumin, 50 μmol/L β-ME, 1% Nutricyte (J. Brooks Laboratories, Olivenhain, Calif); a serum-free medium supplement containing albumin, transferrin, and insulin ± 5 U/mL purified IL3. The final dilution of the initial culture constituents was at least 1:3,700.

Sizing of megakaryocytes. Cell diameter was measured on the same cells before and after the culture period with an inverted microscope equipped with an ocular micrometer.[14] An increase in diameter was defined as an increment of at least 0.5 μm. Four hundred and one single megakaryocytes were examined.

Ploidy analysis of megakaryocytes. After determining the diameter, individual cells were removed from the microwells, deposited on glass slides, and fixed with 70% ethanol (ETOH) for ten minutes. The DNA was stained with 1.7×10^{-5} mol/L chromomycin A_3 containing 1 mmol/L $MgSO_4$ for 30 minutes as described previously.[15] Ploidy was measured with a fluorescence microscope equipped with a photometer. Granulocytic cells were employed as 2N standards.

Assay of AchE activity of megakaryocytes. The single cell culture procedure employed for the AchE studies was similar to that described above with the exception that no β-ME was added and that the colonies were dispersed in 2 mL of medium containing 0.5 mmol/L diisopropylfluorophosphate for 20 minutes to inactivate endogenous cholinesterase prior to deposition of single cells into

Table 1. The Effect of IL3 on the Size of Single Megakaryocytes

Initial Cell Diameter (μm)	Estimated Ploidy (N)	Number of Cells Increasing in Diameter (%)*	
		−IL3	+IL3
12-20	<8	4/34 (12)	94/111 (85)
20-25	8-16	3/30 (10)	48/75 (64)
25-30	16-32	1/19 (5)	12/50 (24)
>30	32	0/14 (0)	3/68 (4)

*Of all cells increasing in size, the mean increment was 1.4 ± 0.9 μm in the absence of IL3 and 2.9 ± 2.0 μm in the presence of IL3. The range of increments was 0.5 to 15.7 μm.

wells.[11] After 72 hours of culture, 165 μL of a solution of 0.2% Triton X-100 in 1 mmol/L EDTA, 0.12 mol/L NaCl, and 50 mmol/L HEPES, pH 7.5 was added to each well containing a cell. Twenty μL of acethylthiocholine iodide was then added (final concentration 0.56 mmol/L). After six hours of incubation, 10 μL of 0.4 mmol/L coumarinphenylmaleimide[16] was added to 20 μL of the reaction mixture in borosilicate glass tubes, followed by the addition of 2 mL of 5 mmol/L sodium acetate (pH 5) containing 1 mmol/L EDTA and 0.2% Triton X-100. The fluorescence emission was measured with a filter fluorometer with an excitation filter of 390 nm and an emission filter of 450 nm. Cells of initial diameters of 15 to 25 μm were chosen for this assay.

RESULTS

Cell diameter. Cell diameter of 401 single megakaryocytes was measured at the outset of culture and 48 hours later. As demonstrated in Table 1, an increase in cell diameter occurred in response to IL3 addition, but this increase was dependent on the initial diameter of the examined cell. Eighty-five percent of cells initially between 12 and 20 μm in diameter increased in response to the factor. This percentage declined to 4% of cells initially greater than 30 μm in diameter. Conversely, in the absence of IL3, only 12% of cells initially between 12 and 20 μm in diameter increased in size.

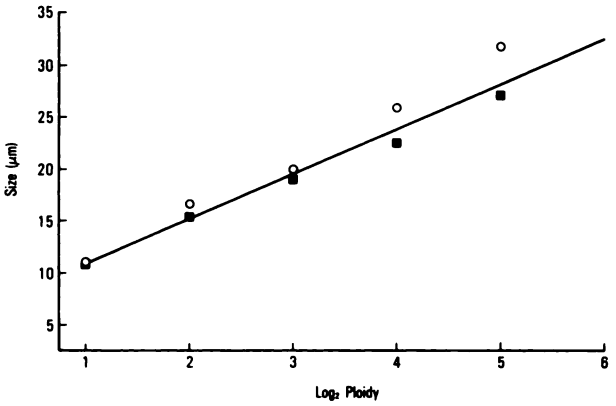

Fig 1. Composite linear regression of size and ploidy of single megakaryocytes before and after culture with IL3. The open circles represent the mean diameters before culture while the closed squares represent the mean diameters after culture in the presence of IL3. No significant differences in the relationship between size and ploidy were observed ($P > 0.05$). Thus, the ploidy of the cells at the outset of culture was estimated from the initial cell diameter.

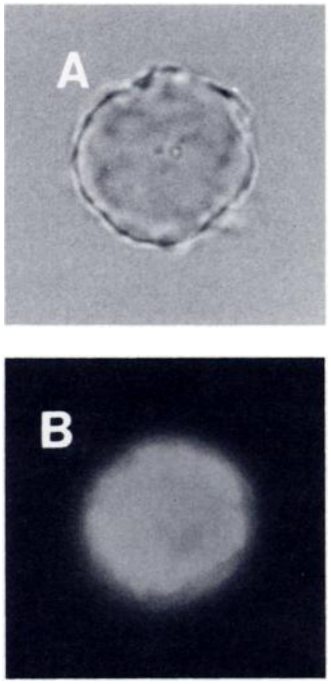

Fig 2. (A) A single megakaryocyte 48 hours after culture in the presence of 5 U/mL IL3 (×640). Initial diameter was 20.8 μm with an estimated ploidy of ≤16N. The final diameter prior to fixation was 36.5 μm. (B) The identical megakaryocyte stained with chromomycin A_3. Measured ploidy of this cell was 64N (×640).

Ploidy measurement. To determine if increments in cell size in response to IL3 were accompanied by increases in DNA content, the ploidy and size of 137 individual cells were measured prior to initiation of culture (n = 76) and after 48 hours of culture (n = 61). As shown in Fig 1, the relatively linear relationship between size and ploidy was unaffected by the culture procedure. These results indicate that the majority of the cells responding of IL3 (85% of cells 12 to 20 μm)

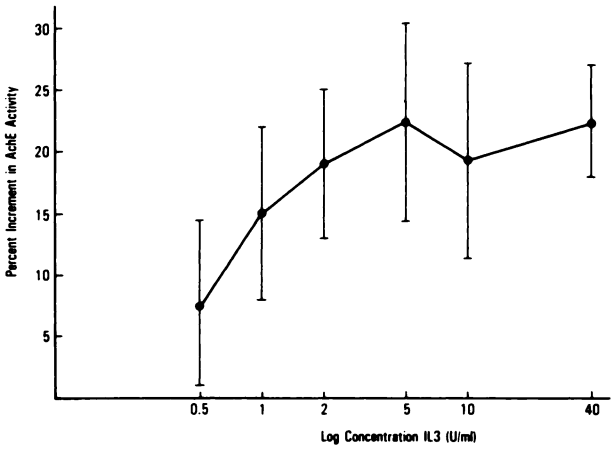

Fig 3. AchE activity of single cells as a function of IL3 concentration (mean percent increment ±1 SD). The increment in AchE activity is significant at all doses of IL3 as compared with cells cultured in its absence ($P < 0.01$; analysis of variance).

were ≤16N. These data also suggest that increments in cell size are likely to be associated with increments in DNA content, rather than cell spreading or isolated cytoplasmic maturation. Although cells greater than 32N were never observed, occasional cells of 64N ploidy were noted after culture with IL3 (Fig 2).

AchE Activity. AchE activity was assayed fluorometrically in cells initially 15 to 25 μm in diameter. Individual cells were cultured in microculture wells containing 35 μL defined medium with various concentrations of IL3. Figure 3 shows that a small but significant increment of this activity was observed in the presence of IL3.

DISCUSSION

These data demonstrate that IL3, a factor that promotes the proliferation of the CFU-MK, is sufficient to promote the differentiation of more distal cells in the megakaryocytic lineage as assessed by measurements of cell size, ploidy, and AchE content. Whether IL3 is necessary for the induction of megakaryocytic differentiation in vitro is unknown. The lesser influence of IL3 on large, high ploidy megakaryocytes may reflect a declining number of receptors for this factor as cells mature. Alternatively, increments in size, ploidy, or enzyme content may not be easily detectable in cells of greater than 16N ploidy and which are near the end of the maturation sequence.

The influence of IL3 on the latter stages of megakaryocytopoiesis may not be unique to this lineage. IL3 has been demonstrated to influence the expression of major histocompatibility antigens on thymus-dependent mast cells,[17] and to stimulate cell division and phagocytosis in single isolated peritoneal macrophages.[18] Another factor, the human granulocyte-macrophage colony-stimulating factor, has been shown to inhibit neutrophil migration.[19] Thus, since the range of effects of IL3 and other colony-stimulating factors may be more global than previously believed, the influence of these regulators of hematopoietic progenitor cells should also be considered in the latter differentiative stages of hematopoiesis, including, perhaps, the delivery of formed blood elements into the circulation.

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REFERENCES

1. Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D: Growth of factor-dependent hemopoietic precursor cell lines. J Exp Med 152:1036, 1980

2. Rennick DM, Lee FD, Yokota T, Arai K-I, Cantor H, Nabel GJ: A cloned MCGF cDNA encodes a multilineage hematopoietic growth factor: Multiple activities of interleukin 3. J Immunol 134:910, 1985

3. Spivak JL, Smith RRL, Ihle JN: Interleukin 3 promotes the in vitro proliferation of murine pluripotent hematopoietic stem cells. J Clin Invest 76:1612, 1985

4. Ihle JN, Keller J, Oroszlan S, Henderson LE, Copeland TD, Fitch F, Prystowsky MB, Goldwasser E, Schrader JW, Palaszynski E, Dy M, Lebel B: Biologic properties of homogenous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P cell-stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity. J Immunol 131:282, 1983

5. Bazill GW, Haynes M, Garland J, Dexter TM: Characterization and partial purification of a haematopoietic cell growth factor in WEHI-3 cell conditioned medium. Biochem J 210:747, 1983

6. Goodman JW, Hall EA, Miller KL, Shinpock SG: Interleukin 3 promotes erythroid burst formation in "serum-free" cultures without detectable erythropoietin. Proc Natl Acad Sci USA 82:3291, 1985

7. Nabel G, Galli SJ, Dvorak AM, Dvorak HF, Cantor H: Inducer T lymphocytes synthesize a factor that stimulates proliferation of cloned mast cells. Nature 291:332, 1981

8. Palacios R, Henson G, Steinmetz M, McKearn JP: Interleukin-3 supports growth of mouse pre-B-cell clones in vitro. Nature 309:126, 1984

9. Quesenberry PJ, Ihle JN, McGrath E: The effect of interleukin 3 and GM-CSA-2 on megakaryocyte and myeloid clonal colony formation. Blood 65:214, 1985

10. Burstein SA: Interleukin 3 promotes maturation of murine megakaryocytes in vitro. Blood Cells (in press)

11. Burstein SA, Boyd CN, Dale GL: Quantitation of megakaryocytopoiesis in liquid culture by enzymatic determination of acetylcholinesterase. J Cell Physiol 122:159, 1985

12. Jackson CW: Cholinesterase as a possible marker for early cells of the megakaryocytic series. Blood 42:413, 1973

13. Burstein SA, Adamson JW, Thorning D, Harker LA: Characteristics of murine megakaryocytic colonies in vitro. Blood 54:169, 1979

14. Burstein SA, Adamson JW, Erb SK, Harker LA: Megakaryocytopoiesis in the mouse: Response to varying platelet demand. J Cell Physiol 109:333, 1981

15. Chatelain C, Burstein SA: Fluorescence cytophotometric analysis of megakaryocytic ploidy in culture: Studies of normal and thrombocytopenic mice. Blood 64:1193, 1984

16. Parvari R, Pecht I, Soreq H: A microfluoremetric assay for cholinesterases, suitable for multiple kinetic determination of picomoles of release thiocholine. Anal Biochem 133:450, 1983

17. Wong GHW, Clark-Lewis I, Hamilton JA, Schrader JW: P cell stimulating factor and glucocorticoids oppose the action of interferon-inducing Ia antigens on T-dependent mast cells (P cells). J Immunol 133:2043, 1984

18. Clark-Lewis I, Crapper RM, Leslie K, Schrader S, Schrader JW: P cell stimulating factor: Molecular and biological properties, in Sorg C, Schimpl A (eds): Cellular and Molecular Biology of Lymphokines. Orlando, Fla, Academic, 1985, p 455

19. Gasson JC, Weisbart RH, Kaufman SE, Clark SC, Hewick RM, Wong GG, Golde DW: Purified human granulocyte-macrophage colony-stimulating factor: Direct action on neutrophils. Science 226:1339, 1984