**CONCISE REPORT**

**Interleukin 3 Promotes the Differentiation of Isolated Single Megakaryocytes**

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

Recent studies in our laboratory have shown that, in a serumless culture system, purified IL3 promotes the appearance of large polyploid megakaryocytes. Acetylcholinesterase (AchE) production, a relatively specific marker of the megakaryocytic lineage in mice, is concurrently increased. Although induction of proliferation of CFU-MK had been previously shown to be a property of IL3, 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 megakaryocytosis. Since previous studies had been performed using unfraccionated 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 C57Bl/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-gauge needle. Marrow was enriched for progenitor cells on a 1.070 to 1.077 g/cm³ two-step Percoll gradient and cultured in 35-mm dishes at 10⁵ 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% CO₂ in air at 37 °C.

**Single cell culture of CFU-MK-derived megakaryocytes.** Megakaryocytic colonies were identified in situ as previously described. 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 at 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. 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 x 10⁻⁴ mol/L chromomycin A₃ containing 1 mmol/L MgSO₄ for 30 minutes as described previously. 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

<table>
<thead>
<tr>
<th>Initial Cell Diameter (µm)</th>
<th>Estimated Ploidy (N)</th>
<th>Number of Cells Increasing in Diameter (% Increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12–20</td>
<td>&lt;8</td>
<td>- IL3: 4/34 (12) + IL3: 94/111 (85)</td>
</tr>
<tr>
<td>20–25</td>
<td>8–16</td>
<td>- IL3: 3/30 (10) + IL3: 48/75 (64)</td>
</tr>
<tr>
<td>25–30</td>
<td>16–32</td>
<td>- IL3: 1/19 (5) + IL3: 12/50 (24)</td>
</tr>
<tr>
<td>&gt;30</td>
<td>32</td>
<td>- IL3: 0/14 (0) + IL3: 3/68 (4)</td>
</tr>
</tbody>
</table>

*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. 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 acetylthiocholine iodide was then added (final concentration 0.4 mmol/L) coumarin-phenylmaleimide 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.

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 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 38.5 µm. (B) The identical megakaryocyte stained with chromomycin A3. Measured ploidy of this cell was 64N (×640).

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 $\leq 16N$. 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 $\mu$m in diameter. Individual cells were cultured in microculture wells containing 35 $\mu$L 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 AchE content may not be easily detectable in cells of greater than 16N ploidy and which are near the end of the maturation sequence.

AchE activity was assayed fluorometrically in cells initially 15 to 25 $\mu$m in diameter. Individual cells were cultured in microculture wells containing 35 $\mu$L 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.

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REFERENCES

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