

Human interleukin 6 is a direct promoter of maturation of megakaryocytes *in vitro*

(growth factor/hematopoiesis)

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ABSTRACT The response of cells of the murine megakaryocytic lineage to human interleukin 6 (IL-6) was assessed in serum-depleted cultures using a variety of biological assays. IL-6 alone had no influence on megakaryocytic colony formation but augmented the numbers of these colonies induced by the multipotent colony-stimulating factor interleukin 3. However, in liquid marrow cultures, IL-6 alone promoted marked increments in megakaryocytic size and the activity of acetylcholinesterase, a marker enzyme of the lineage. Moreover, IL-6 induced a significant shift toward higher ploidy classes when megakaryocytic DNA was quantitated by flow cytometry. To determine whether the influence of IL-6 on megakaryocytic maturation was direct, the factor was added to cultures of single megakaryocytes isolated from megakaryocytic colonies. Fifty-four percent of these cells increased in size compared with 19% of those grown without IL-6. The data show that human IL-6 is a potent direct-acting growth factor for murine megakaryocytes with activity promoting maturation of that lineage.

Megakaryocytopoiesis is a process that encompasses proliferation of committed megakaryocytic progenitor cells (CFU-MK) and cellular maturation comprising nuclear endoreduplication (polyploidization), cytoplasmic enlargement, and accumulation of lineage markers (1-3). This process appears, at least *in vitro*, to be stimulated by a number of cytokines. The multipotent colony-stimulating factor interleukin 3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin have been shown to promote the proliferation of megakaryocytic progenitors (4-6). Moreover, these growth factors can support not only proliferation, but also megakaryocytic maturation to some degree (7-9). These observations, nonetheless, do not exclude the idea that there exist growth factors that act predominantly to influence either proliferation or maturation. In this report the effects of recombinant human interleukin 6 (IL-6) on murine megakaryocytopoiesis are described. This 26,000 *M_r* glycoprotein with multiple biological activities has been purified to homogeneity from both murine and human sources and recently has been molecularly cloned (10-12). We now show that this cytokine significantly augments megakaryocytic maturation as assessed by size, acetylcholinesterase (AcChoEase) activity, and DNA content and that it synergizes with the megakaryocyte-growth-promoting activity of IL-3.

MATERIALS AND METHODS

Marrow Preparation. Marrow was flushed from the femurs of C57BL/6 mice with Iscove's modification of Dulbecco's medium (IMDM) supplemented with Nutridoma-SP (Beh-

ringer Mannheim), a serum-free medium supplement. For culture studies, a single-cell suspension was made by repetitive expulsion through progressively smaller needles. For flow cytometry, a monocellular suspension was made by gentle filtration through 100- μ m nylon mesh. In some experiments marrow cells were treated with 0.5 mM diisopropyl-fluorophosphate to inactivate endogenous AcChoEase (a marker enzyme of megakaryocytes in murine marrow; refs. 13 and 14). In other experiments adherent cells were removed to enrich the numbers of megakaryocytes or their progenitor cells. Up to 2×10^6 cells per ml were incubated in the presence of 10% (vol/vol) horse serum in IMDM for 45 min at 37°C in plastic tissue culture flasks, followed by repetitive washing of the nonadherent cells in IMDM to remove the serum.

Colony Assays. To determine the influence of IL-6 on colony formation with or without the presence of the multipotent colony-stimulating factor IL-3, megakaryocytic and granulocyte-macrophage colony assays were performed in a serum-depleted system. Nonadherent marrow cells (10^5) were cultured in 35-mm tissue-culture dishes in IMDM supplemented with 1% bovine serum albumin, human transferrin at 360 μ g/ml, and cholesterol at 0.98 μ g/ml and made semi-solid with 0.3% agar. After 7 days in culture at 37°C the agar discs were transferred to glass slides and fixed with 2% (vol/vol) glutaraldehyde. Colonies were enumerated after histochemical staining for AcChoEase and counterstaining with hematoxylin (13).

Liquid Cultures. Liquid marrow cultures were performed in serum-depleted conditions as described (7, 14). Nucleated nonadherent marrow cells (10^5) were set up in 96-well culture plates in 0.2 ml of IMDM supplemented with 1% Nutridoma in the presence of various concentrations of growth factors. After incubation at 37°C for 4-5 days the number and size of megakaryocytes were assessed after histochemical staining for AcChoEase (15). AcChoEase activity was measured by using a modification of our reported fluorometric method (9). One hundred and eighty microliters of a solution of 0.2% Triton X-100/1 mM EDTA/0.12 M NaCl/50 mM Hepes, pH 7.5, was added to each well, followed by the addition of 20 μ l of acetylthiocholine iodide (final concentration, 0.56 mM). After 3-hr incubation at room temperature 20 μ l of the reaction mixture from each well was transferred to the corresponding wells of a 96-well MicroFLUOR "B" plate (Dynatech). Twenty microliters of 0.4 mM 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (Molecular Probes) was then added followed by 160 μ l of diluent buffer (0.2% Triton X-100/1 mM EDTA/50 mM sodium acetate, pH

Abbreviations: IL-3, -4, -5, and -6, interleukin 3, 4, 5, and 6, respectively; AcChoEase, acetylcholinesterase; IMDM, Iscove's modification of Dulbecco's medium; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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5.0). Fluorescence emission was determined on a fluorometer capable of reading 96-well plates (MicroFLUOR; Dynatech). The DNA content of megakaryocytes grown in liquid culture was assessed by flow cytometry by using a modification of our described technique (16, 17). After 5 days in culture the contents of four replicate wells were incubated for 30 min at 4°C with a saturating concentration of fluoresceinated rabbit anti-mouse platelet globulin. The antiserum was prepared as described (18) and was extensively absorbed with mouse erythrocytes and platelet-poor buffy coat. The serum was treated by precipitating it twice with 50% (vol/vol) $(\text{NH}_4)_2\text{SO}_4$ and fluoresceinated to a fluorescein/protein ratio of 2.2:1 by standard techniques (19). Controls were incubated identically with fluoresceinated rabbit IgG. After immunofluorescent labeling the cells were stained with propidium iodide to assess DNA content (16, 20–22). Cells were analyzed with a Coulter Epics V flow cytometer with a 100- μm -diameter nozzle. Megakaryocytes were selected on the basis of membrane immunofluorescence by setting an electronic gate at a fluorescence level above that of the antibody control (16, 17). The ploidy distribution was determined by setting markers at the nadirs between peaks using the 2N and 4N peaks of the cells as internal standards (17).

Single Cell Cultures. To determine whether the influence of IL-6 was direct, isolated single megakaryocytes were set up in liquid culture as described (9). Marrow was enriched for progenitor cells on a 1.065/1.077 g/cm³ discontinuous Percoll gradient and cultured in methylcellulose for 5 days until megakaryocytic colonies were recognizable *in situ* (23). Individual colonies were removed under an inverted microscope with a micropipette in 2- μl vol and dispersed in 2 ml of IMDM. Individual megakaryocytes were removed in 1- μl vol and recultured for 24–36 hr in 35 μl of IMDM containing 3% (vol/vol) bovine serum albumin, 1% Nutridoma, 50 μM 2-mercaptoethanol, and IL-6 at 0 or 200 ng/ml in microwell plate covers. Cell diameter was measured at the outset and completion of culture.

Growth Factors. Murine recombinant IL-3, interleukin 4 (IL-4), interleukin 5 (IL-5), GM-CSF, and granulocyte colony-stimulating factor were purchased from Genzyme. Recombinant human IL-6 was expressed in *Escherichia coli* and was a gift of Ajinomoto (Kawasaki, Japan) or was purchased from R&D Systems (Minneapolis).

RESULTS

Effect of IL-6 on Colony Formation. Fig. 1 shows the influence of varying concentrations of added IL-6 on the appearance of megakaryocytic colonies. When added alone in concentrations up to 200 ng/ml, IL-6 did not promote megakaryocytic colony formation. However, the factor augmented the numbers of colonies detectable in the presence of either suboptimal (30 units/ml) or optimal (100 units/ml) concentrations of IL-3 (a dose-response analysis with IL-3 alone showed that concentrations of IL-3 ≥ 100 units/ml induced maximal colony formation). As has been shown by others, a small number of granulocyte-macrophage colonies was noted with IL-6 only at ≥ 50 ng/ml (data not shown; ref. 11).

One hypothesis for these synergistic effects of IL-6 on megakaryocytic colony formation is that the factor permits detection (by means of augmenting cell size or AcChoEase content) of a proportion of immature unrecognizable megakaryocytic colonies developing in response to IL-3. To study the effects of IL-6 on maturation more precisely, a liquid culture system was employed to measure the size, AcChoEase activity and ploidy of megakaryocytes.

Influence of IL-6 on Megakaryocyte Number and Size. When IL-6 was added to serum-depleted liquid cultures, individual megakaryocytes could be recognized *in situ* at day 2 of

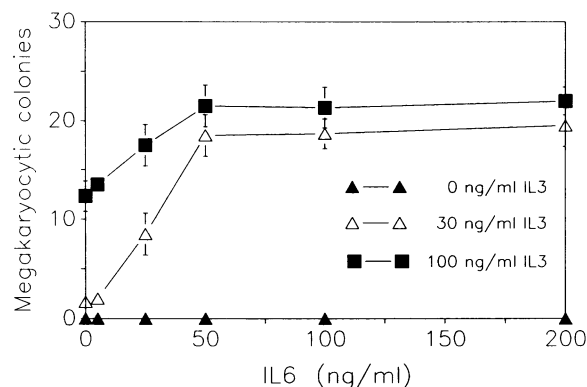


FIG. 1. IL-6 alone does not promote megakaryocytic colony formation. With IL-6 at up to 200 ng/ml, no megakaryocytic colonies were detected; however, in the presence of suboptimal (30 units/ml) concentrations of the multipotent growth factor IL-3, significant augmentation of megakaryocytic colony numbers was noted with IL-6 at 25 ng/ml ($P < 0.05$). Even at optimal concentrations of IL-3 (100 ng/ml), IL-6 at ≥ 25 ng/ml increased colonies ($P < 0.05$). One of four similar experiments done in serum-free conditions is presented.

culture and increased in size over the succeeding 3 days. On day 4 of culture the cells were stained for AcChoEase, after which they were counted and measured for size. Slightly more AcChoEase-positive cells were seen at IL-6 concentrations ≥ 100 ng/ml, but these increments were not significant. In contrast, a significant increase in megakaryocytic diameter was seen at IL-6 concentrations ≥ 1 ng/ml compared with cells cultured without the factor ($P < 0.01$; Table 1).

Fig. 2 shows the appearance of megakaryocytes stained for AcChoEase in liquid culture 4 days after adding IL-6, IL-3, or both factors. Fig. 2A shows that a few megakaryocytes appeared spontaneously in the absence of exogenously added factors. Fig. 2B shows the marked size increments seen when IL-6 was added. The multipoietin IL-3 promotes hematopoietic proliferation of many lineages, including the megakaryocytic, and the increased number of these cells together with other unidentified cells in the background was noted in Fig. 2C. When both factors were added together, a marked increase in both number and size of megakaryocytes was noted.

Effect of IL-6 on AcChoEase Activity. In contrast to the failure of IL-6 to increase significantly the numbers of megakaryocytes in liquid cultures, the factor increased AcChoEase activity compared to controls at all tested concentrations ≥ 0.5 ng/ml (Fig. 3). This fact suggests that the increase in megakaryocytic diameter in response to IL-6 is accompanied by accumulation of the marker enzyme (al-

Table 1. Influence of IL-6 on megakaryocyte number and size in liquid culture

IL-6, ng/ml	Megakaryocytes, no. per 10^5 marrow cells plated	Diameter, $\mu\text{m} \pm 1$ SD
0	83 \pm 5	20.4 \pm 4.8 (144)
1	81 \pm 8	25.1 \pm 8.2 (122)*
10	95 \pm 14	25.4 \pm 7.5 (143)
50	81 \pm 10	29.8 \pm 7.8 (113)
100	101 \pm 19	32.7 \pm 7.3 (124)
200	104 \pm 19	34.1 \pm 8.5 (112)

The diameter represents a geometric mean of two perpendicular diameters; number of cells measured is indicated in parentheses.

*At all concentrations of IL-6 ≥ 1 ng the differences in diameter compared to control were significant ($P < 0.01$).

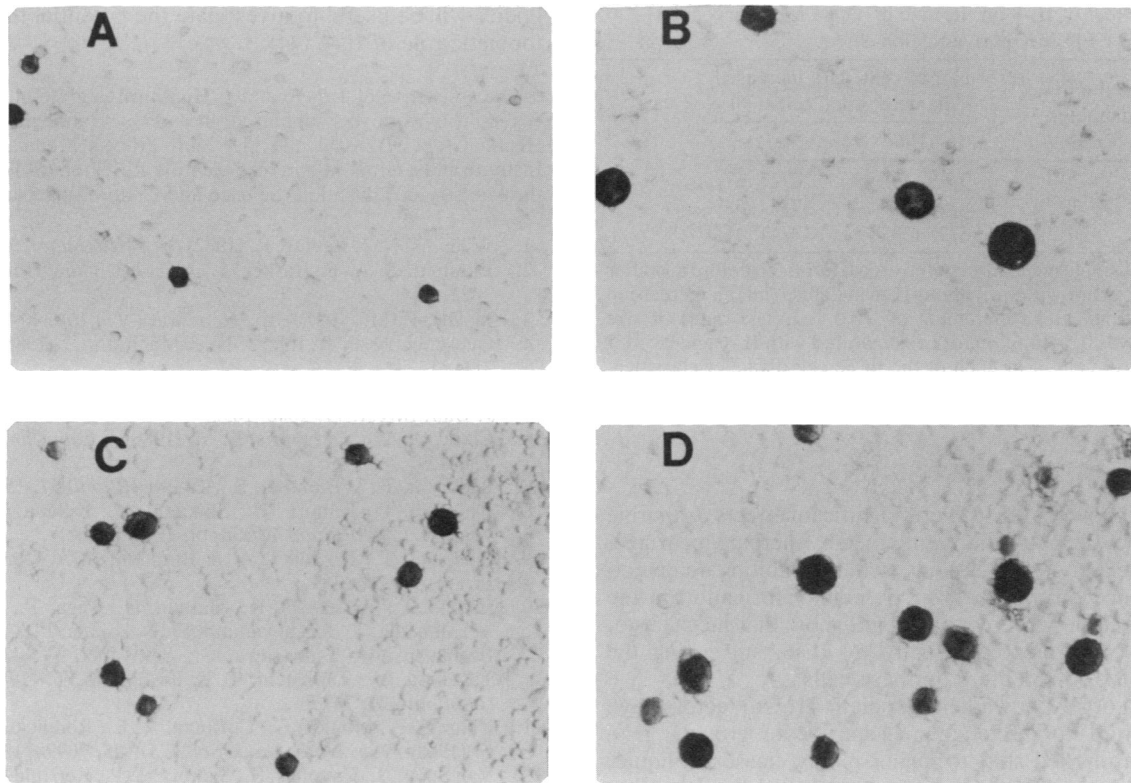


FIG. 2. IL-6 augments megakaryocyte size. Marrow cells were stained for AcChoEase after 5 days in liquid culture. The dark stained megakaryocytes seen in the control culture (A) are increased in size when the cultures are supplemented with IL-6 at 10 ng/ml (B). Cultures treated with IL-3 at 100 units/ml show a generalized increase in cellular proliferation and in the number and, to some degree, the size of megakaryocytes (C; ref. 7). When IL-6 at 10 ng/ml is added to IL-3-containing cultures, additional size increments are seen (D). (Bright-field $\times 320$.)

though an increase in the specific activity of AcChoEase is also possible).

Influence of IL-6 on Ploidy. Table 2 shows the ploidy distribution of megakaryocytes derived from liquid marrow cultures stimulated by various hematopoietic growth factors. The modal ploidy of megakaryocytes grown in culture for 5 days in the absence of exogenous growth factors was 16N (where N represents the haploid number). In comparison to the effect of maximally stimulating concentrations of IL-3, GM-CSF, granulocyte colony-stimulating factor (G-CSF), IL-4, or IL-5, only IL-6 markedly shifted the ploidy distribution to the right, with 41% of all megakaryocytes $\geq 32N$.

Stimulation of Isolated Single Megakaryocytes. Because marrow comprises heterogeneous populations of cells, the observed effects of IL-6 could have been mediated indirectly. To determine whether the influence of IL-6 was direct, isolated single megakaryocytes were set up in serum-free culture (7, 9). Table 3 shows that the factor markedly enhanced the percentage of cells that increased in size. Although the effect of IL-6 was greatest on cells with an initial diameter $< 20 \mu\text{m}$ (67% increased in size compared to 16% of control cells), 41% of large megakaryocytes $> 30 \mu\text{m}$ in diameter increased in size compared with 20% of cells grown without IL-6.

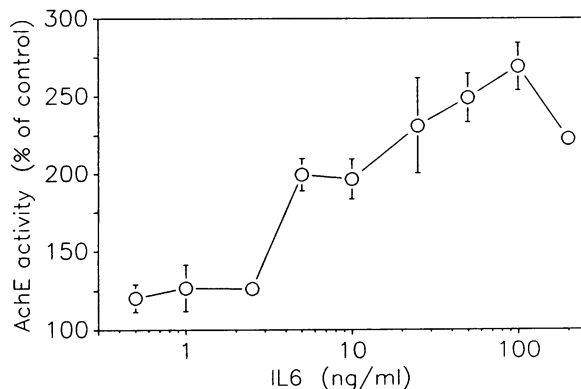


FIG. 3. IL-6 augments AcChoEase (AchE) activity. Nonadherent marrow cells (10^5) were cultured for 4 days with different concentrations of IL-6 and assayed fluorometrically for AcChoEase activity. A significant activity increase was seen with IL-6 at 0.5 ng/ml ($P < 0.05$), whereas maximal activity was seen at ≥ 5 ng/ml. The data represent the means of four replicate experiments.

Table 2. The effect of various growth factors on the ploidy distribution of megakaryocytes in culture

Factor (n)*	Ploidy class					
	2N	4N	8N	16N	32N	64N
Control (2073)	12 [†]	15	18	34	18	3
G-CSF (729)	20	8	21	42	9	0.1
GM-CSF (1520)	30	17	10	20	21	2
IL-3 (2072)	20	15	12	34	17	2
IL-4 (1472)	28	13	16	32	11	0.3
IL-5 (959)	28	4	18	42	8	0
IL-6 (1450)	15	13	13	18	34	7

Ploidy was assessed by flow cytometry on megakaryocytes derived from marrow cultured in a serum-depleted system stimulated by no added growth factor (control), granulocyte colony-stimulating factor (G-CSF) at 200 units/ml, GM-CSF at 100 units/ml, IL-3 at 100 units/ml, IL-4 at 200 units/ml, IL-5 at 100 units/ml, or IL-6 at 10 ng/ml.

*Absolute number of megakaryocytes analyzed.

[†]The percentage of megakaryocytes in each ploidy class. The frequency of the modal class is boxed.

Table 3. Effect of IL-6 on the size of single megakaryocytes isolated from CFU-MK-derived colonies

Cell initial diameter, μm	No. of cells with increased diameter/total cells (%)	
	- IL-6	+ IL-6
10-20	3/19 (16)	18/27 (67)
20-30	7/34 (21)	37/64 (58)
>30	5/25 (20)	18/44 (41)

The size of single megakaryocytes was determined before and after 24-36 hr in culture with or without IL-6 at 200 ng/ml; an increase in size was defined as an increment of $\geq 0.5 \mu\text{m}$. The range of size increments was 0.5-10.6 μm for cells cultured with IL-6 and 0.5-5.7 μm for control cells. For each of the three cell-diameter classifications the percentage of cells that increased in size was significantly greater in the IL-6-treated group than in controls ($P < 0.01$).

DISCUSSION

These data show that IL-6 exerts significant effects on murine megakaryocytic maturation as defined by increments in size, AcChoEase activity, and ploidy, while exhibiting no detectable influence on megakaryocytic colony formation in the absence of IL-3. Moreover, the data show that megakaryocytic size is augmented directly by IL-6, suggesting the presence of IL-6 receptors on these cells.

Although other known hematopoietic growth factors have been reported to influence megakaryocyte growth, IL-6 promotes a marked shift to higher ploidy classes in liquid, serum-depleted culture, an effect not shared by five other growth factors examined (Table 2).

Promotion of megakaryocytic maturation delineates an additional biological activity of this multifunctional cytokine that includes stimulation of B- and T-cell differentiation, granulocyte-macrophage colony formation, hepatocyte stimulation, and support of hybridoma growth (10, 11, 24-27). More recently, the factor has been shown to act synergistically with interleukin 3 in the proliferation of early hematopoietic progenitor cells, to induce differentiation of murine leukemia cells, and to inhibit the growth of human breast carcinoma and leukemia cell lines (28-30).

The hypothesis that several growth factors influence megakaryocytopoiesis, some at the proliferative stage and others at the maturational stage, has been fostered by a number of investigators (1-3, 31-34). Data from several laboratories have suggested that there are growth factors (designated "potentiators", thrombopoiesis-stimulating factor and thrombopoietin) that synergize with defined colony-stimulating factors to promote megakaryocytic colony formation (although these factors do not primarily induce colony formation), and/or promote megakaryocytic maturation (33-41). Because these factors have not been purified, their relationship to IL-6 is unknown. However, it is intriguing that preliminary data suggest a popular source of thrombopoiesis-stimulating factor, a human embryonic kidney cell line, expresses IL-6 mRNA (42, 43). Because IL-6 or IL-6 mRNA is present (or can be induced) in macrophages, T cells, and various macrophage and marrow stromal cell lines, it is also of interest that these cell types have been used as sources of megakaryocyte potentiator activity (11, 35-38). Studies using neutralizing antibodies to IL-6 may help determine the degree to which IL-6 contributes to these activities.

Although IL-6 promotes maturation of megakaryocytes *in vitro*, its influence on megakaryocyte growth and, ultimately, platelet production *in vivo* is unknown. Recent data indicating the factor may be elevated in patients with rheumatoid arthritis, an inflammatory disorder often associated with thrombocytosis, suggests the plausibility of a role for IL-6 in platelet production (44, 45). The availability of both the natural and recombinant factor together with anti-IL-6 anti-

bodies will be useful to investigate the potential thrombocytopoietic role of IL-6 (44).

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