Megakaryocytic Maturation in Murine Long-Term Bone Marrow Culture: Role of Interleukin-6

By Rui-Lian Mei and Samuel A. Burstein

Megakaryocytic maturation was analyzed in long-term bone marrow cultures in the absence of added growth factors. Megakaryocytes could be observed for periods of up to 13 weeks in both the supernatant and stromal layer of these cultures. Using acetylcholinesterase staining for enumeration and sizing of megakaryocytes, and a novel rat antimmunoreactive platelet monoclonal antibody (MoAb) that detects only megakaryocytes in bone marrow, the number, volume, and ploidy of these cells were assessed microscopically and by flow cytometry. Correlation of these measurements with ambient interleukin-6 (IL-6) levels showed no relationship; conversely, the relatively high IL-6 bioactivity present during the first 2 weeks of culture was correlated with increased megakaryocytic size and ploidy, while the relatively lower IL-6 bioactivity present after week 3 corresponded to decreased megakaryocytic size and ploidy. Addition of neutralizing anti-IL-6 MoAb decreased megakaryocytic size and ploidy at times when ambient IL-6 levels were relatively high, while the addition of exogenous IL-6 increased size and ploidy at times when endogenous IL-6 concentrations were low. The data show that long-term bone marrow cultures can be used as a means to evaluate megakaryocytic maturation in vitro, and suggest that, to some extent, IL-6 plays a role in the maturation process in this system.

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MATERIALS AND METHODS

Mice and rats. Six- to 8-week-old specific pathogen-free C57B1/6 male mice obtained from Jackson Laboratories (Bar Harbor, ME) were used for all marrow culture experiments. SCID mice (provided by Dr Paul Kincade [Oklahoma Medical Research Foundation]) were used to produce ascites-derived monoclonal antibody (MoAb). Lou M1 rats (Charles Rivers Breeding Labs, Wilmington, MA) were used to produce MoAbs.

LTBMC. Cultures were prepared according to a modification of the method of Dexter et al. BM cells from two femurs were flushed with 8 mL of Iscove's modified Dulbecco's medium (IMDM; Irvine Scientific, Santa Ana, CA) into a 15-mL polypropylene tube and centrifuged at 250g for 10 minutes. The cell pellet was resuspended in 12 mL IMDM supplemented with 25% horse serum (GIBCO BRL, Grand Island, NY), 10−4 mol/L hydrocortisone (Sigma Chemical Co, St Louis, MO), 125 U/mL penicillin G, and 125 μg/mL streptomycin (GIBCO). Three milliliters of this cell suspension was inoculated into each well of a 6-well tissue culture plate (no. 25860; Corning Glass Works, Corning, NY). The plates were incubated in a 37°C, 5% CO2, humidified incubator. After 1 week, each culture was recharged by removing the supernatant and the nonadherent cells and adding fresh medium and the marrow cells from two femurs. The cultures were maintained by addition of one-half volume fresh medium weekly. Half of the nonadherent cell suspension was harvested from each group of three wells, pooled, and assayed for total cell number and type and megakaryocyte number, size, and ploidy. Cell-free supernatants were stored at −20°C for subsequent measurement of IL-6 bioactivity. The first week after recharging is termed week 1.

Experimental alteration of endogenous IL-6 levels. In some experiments, human recombinant IL-6 (10 ng/mL; R & D Systems, Minneapolis, MN) was added to the cultures weekly. Control wells contained the IL-6 diluent (0.1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]). To neutralize endogenous IL-6 bioactivity, purified monoclonal rat antiserum IL-6 IgG (68 μg/mL; cell line provided by Dr Jacques Van Snick [Ludwig Institute, Brussels, Belgium]) was added to the cultures. Preliminary experiments showed no differences between adding the antibody once per week or seven times per week in any of the measured parameters. Thus, the antibody was added once per week. Control cultures for these experiments contained rat Ig
(Sigma) in the same concentrations as 6B4. To test the possibility that immune complex formation (6B4-IL-6 complexes) might nonspecifically inhibit megakaryocytic growth, additional controls using a mixture of mouse IgG (5 μg/mL) and antimouse IgG (5 μg/mL) were used.

Nucleated cell counts and morphologic characterization. The cellular content of 50 mL of culture supernatant was determined weekly. Cell counts were enumerated at a 1:100 dilution with a hemocytometer. For morphologic characterization, the nonadherent cells were transferred onto slides by pipet. After staining with hematoxylin, the cells were analyzed microscopically, and graded as granulocyte, monocyte-macrophage, or other.

Megakaryocyte counts and size. Fifty microliters of cell suspension was added to each of four replicate wells of a 96-well microwell plate (no. 25860; Corning). The cells were fixed with 1% glutaraldehyde, washed with PBS, and stained for acetylcholinesterase (AchE) for 6 hours. All AchE-positive (+) cells were enumerated and their sizes measured microscopically. Megakaryocyte volume was calculated by first determining the geometric mean of two perpendicular diameters (d) of 20 AchE+ cells from each well, and subsequently calculating the volume according to the formula V = πd²/6. In some experiments, the number, size, and ploidy of megakaryocytes was determined in the adherent cell layer. After decanting of the nonadherent cells and extensive washing of the adherent cell layer with PBS, cells were liberated from within the stromal cell layer by adding 0.1% trypsin for 2 minutes at 37°C, followed by the addition of medium containing 25% horse serum. The detached cells were then transferred to a 96-well plate, centrifuged, and fixed with glutaraldehyde for AchE staining, or processed for flow cytometry. No residual megakaryocytes in the culture plates were observed after trypsinization.

Measurement of megakaryocytic ploidy. The relative DNA content of megakaryocytes in LTBMCS was determined by a modification of our previously described flow cytometric method. Each week the supernatant cells were concentrated by centrifugation and then incubated at 4°C for 30 minutes with 10 μg/mL of a fluoresceinated rat antirat platelet MoAb (4A5; see below). Subsequently, an equal volume of 1% sodium citrate solution containing 40 μg/mL propidium iodide, 30 μg/mL DNAse-free RNase (Boehringer Mannheim, Indianapolis, IN), and 0.1% Triton X-100 (Sigma) was added and incubated at 4°C for 15 minutes. Cells were analyzed with a Coulter Epics V flow cytometer (Coulter Instruments, Hialeah, FL). Small megakaryocytes exhibiting low fluorescence were distinguished from nonspecifically stained cells and autofluorescence by setting a gate at a level of fluorescence greater than that of cultured cells stained with a control antibody (fluoresceinated rat IgG). 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.

MoAb recognizing mouse megakaryocytes. Blood from 20 retired ex-breeder Balb/c mice was obtained by cardiac puncture, and the platelets prepared as described. Three cycles of low-speed centrifugation were used to deplete residual red and white cell contamination. After packing at 1,500g for 20 minutes, the platelets were suspended in Freund’s complete adjuvant and the emulsion was injected into multiple subcutaneous sites of Lou M1 rats. The rats were boosted three times with mouse platelets emulsified in incomplete Freund’s adjuvant. Antiplatelet antibody titer was monitored by an enzyme-linked immunosorbent assay (ELISA) method. Briefly, platelets were fragmented by sonication and bound to microwell plates. After blocking with 0.1% BSA in 0.05% Tween 20, dilutions of rat serum were applied for 2 hours. After multiple washes, bound antibody was detected photometrically at OD₄₉₀ using alkaline phosphatase-conjugated goat antirat IgG followed by addition of substrate. When a titer of 1:5,000 was observed, the rat was killed and the spleen fused in 0.05% polyethylene glycol (PEG) to Sp2 mouse myeloma cells as described. After hypoxanthine, aminopterin, thymidine (HAT) selection, positive clones were identified with the above described ELISA. Positive supernatants were tested for specificity to murine megakaryocytes by indirect immunofluorescence using fluorescence microscopy and flow cytometry of normal mouse marrow. One clone (designated 4A5) identified only mouse platelets in blood and megakaryocytes in marrow by this analysis. The ploidy distribution of freshly obtained normal mouse marrow exposed to this antibody was 7% 2N, 4% 4N, 9% 8N, 63% 16N, 17% 32N, and 9% 64N. After subcloning, ascites was made in Pristane-primed SCID mice. The antibody was shown to be an IgG₁ using a rat antibody subtyping kit (Serotec, Oxford, UK) and was purified from the ascites on a high performance protein G column (Genex, Gaithersburg, MD) after delipidation according to the instructions of the manufacturer. The IgG was fluoresceinated by standard techniques to an F/P ratio of 3:1.

Measurement of IL-6 activity in LTBMCS supernatants. The IL-6-dependent B9 cell line (provided by Dr Lucien Aarden [Central Laboratory of the Netherlands Red Cross Blood Transfusion Service]) was used to determine IL-6 bioactivity. B9 cells were grown in IMDM containing 5% fetal calf serum (FCS), 50 U/mL penicillin G, 50 μg/mL streptomycin, and 0.1 ng/mL human recombinant IL-6. For assay purposes, B9 cells were harvested by centrifugation and washed three times in IL-6-free medium. Twenty microliters of LTBMCS supernatant was added to 5,000 B9 cells in a final volume of 200 μL IMDM supplemented with 5% FCS. After incubation for 3 days, cell proliferation was determined by the MTT colorimetric assay. Fifty microliters of a 5 mg/mL stock solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to each culture well. After 2 hours of incubation at 37°C, the cells were centrifuged, the supernatant discarded, and 100 μL of a lysing buffer (20% sodium dodeyl sulfate, 50% dimethylformamide, pH 4.7) was added. After 4 hours of incubation at 37°C, the OD₄₉₀ was measured with a BioMek 1000 Automatic Laboratory Work Station (Beckman Instruments, Palo Alto, CA). One unit of activity is defined as the amount of IL-6 required to attain half-maximal stimulation. The specificity of the assay for IL-6 was assessed using rabbit antihuman IL-6 neutralizing antibody (provided by Dr David Hilbert [National Institutes of Health]) and for human IL-6 using rabbit antihuman IL-6 neutralizing antibody prepared in our laboratory.

Statistical analysis. Data were analyzed using Student’s t-test or by χ² analysis.

RESULTS

General observations of LTBMCS. Pilot experiments showed that long-term cultures containing FCS were incapable of supporting continuous production of mature megakaryocytes beyond 3 weeks of culture. However, in the presence of horse serum alone it was possible to observe maturing megakaryocytes for 3 months or longer. After recharging of the culture at week 1, the adherent layer became confluent. This layer contains endothelial cells, fibroblast-like cells, macrophages, and adipocytes as previously described. Cobblestone areas of active hematopoiesis were observed. Megakaryocytes were easily detectable in situ and were noted both in the medium and tightly adherent to the stromal layer (Fig 1). In general, numbers of megakaryocytes in the supernatant were proportional to the numbers in the adherent layer, with larger numbers of these cells noted in or on the adherent layer. In
situ observations of stained megakaryocytes suggested that those in the adherent layer were smaller than those in the supernatant, an observation confirmed by quantitative analysis (see below).

Quantitative determination of megakaryocyte number and size. Continuous production of megakaryocytes was observed for up to 13 weeks (Fig 2). Although the total number of nucleated cells decreased with time, megakaryocyte number in the supernatant increased from week 3 to about week 8 compared with the initial input of megakaryocytes. As many as 6% of all cultured cells were identified as megakaryocytes by AchE staining at week 7 in one experiment (Table 1). After week 13, AchE+ cells were no longer observed in the supernatant, although they were seen in the adherent layer until week 15 (data not shown). Conversely, granulocyte and macrophages continued to be produced in the culture after the disappearance of the megakaryocytes. In three experiments, the number of megakaryocytes in the adherent layer was compared with the number in the supernatant at week 1 and at week 5 by flow cytometric analysis of trypsinized stroma labeled with fluoresceinated 4A5. The number of stromal megakaryocytes exceeded supernatant cells in all three experiments (Table 2; P < .05). Figure 3 shows the volume of megakaryocytes produced in LTBMC. The volume of these cells increased significantly over the first week of culture (P < .001), decreased over the next 2 weeks, and was approximately stable thereafter. After trypsinization to remove the adherent cells at week 1, the mean megakaryocytic volume of adherent cells (300 cells in three experiments) was less than those in the same culture supernatant (3.8 × 10³ μm³ vs 8.6 × 10³ μm³, respec-
MEGAKARYOCYTES IN LTBMC

Table 1. The Frequency of Megakaryocytes in LTBMC

<table>
<thead>
<tr>
<th>Week</th>
<th>Nucleated Cells* (x 10^3)</th>
<th>Megakaryocytes</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
<td>80</td>
<td>24</td>
<td>0.03</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>18</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>1.25</td>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>22.5</td>
<td>70</td>
<td>0.3</td>
</tr>
<tr>
<td>4</td>
<td>23.8</td>
<td>103</td>
<td>0.4</td>
</tr>
<tr>
<td>5</td>
<td>3.76</td>
<td>57</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>1.88</td>
<td>77</td>
<td>4.1</td>
</tr>
<tr>
<td>7</td>
<td>1.25</td>
<td>74</td>
<td>6.0</td>
</tr>
</tbody>
</table>

The frequency of megakaryocytes was significant at all time points tested and in all experiments (P < .001) except for weeks 2 through 4 in experiment 1.

Abbreviation: --, not done.

* Nucleated cells and megakaryocytes were enumerated in 50 μL of culture supernatant at the indicated time-points.

Table 2. Comparison Between the Number and Volume of Adherent and Nonadherent Megakaryocytes

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Number*</th>
<th>Volume†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroma</td>
<td>4,710</td>
<td>6,196</td>
</tr>
<tr>
<td>Supernatant</td>
<td>1,385</td>
<td>1,785</td>
</tr>
</tbody>
</table>

The number of megakaryocytes in the supernatant and the stroma after trypsinization was determined as described in Materials and Methods.

† The difference between the number of megakaryocytes in the supernatant and the stroma after trypsinization was significant at all subsequent weeks (P < .05; t-test).

‡ The difference between the volume of megakaryocytes in the supernatant and in the stroma was significant (P < .05; t-test).

weeks 1 and 5. Compared with the supernatant megakaryocytes assayed from the same culture, the ploidy was shifted significantly more to the left, with modal ploidy classes of 2 to 8N in the stroma in each of the three experiments, compared with modal classes of 16 to 32N in the supernatant. These differences are more easily distinguished in Fig 4. Nevertheless, a substantial percentage of higher ploidy cells (22% to 28% ≥ 32N) was observed to be tightly adherent to the stromal layer.

Assay for IL-6 bioactivity. The IL-6 bioactivity was 0.88 U/mL in fresh marrow cell suspensions in culture medium at the outset of culture. Figure 5 shows the IL-6 bioactivity as measured weekly in culture. An increase was first noted at 3 hours after initiation of culture (15 U/mL) and peaked at the end of the first week (33 U/mL). Thereafter, a
Table 3. Ploidy Distribution of Nonadherent Megakaryocytes in LTBMC

<table>
<thead>
<tr>
<th>Week No.</th>
<th>Percentage of Megakaryocytes in Each Ploidy Class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2N</td>
</tr>
<tr>
<td>Normal†</td>
<td>828</td>
</tr>
<tr>
<td>Input*</td>
<td>1,089</td>
</tr>
<tr>
<td>1</td>
<td>677</td>
</tr>
<tr>
<td>2</td>
<td>322</td>
</tr>
<tr>
<td>3</td>
<td>710</td>
</tr>
<tr>
<td>5</td>
<td>894</td>
</tr>
<tr>
<td>6</td>
<td>923</td>
</tr>
<tr>
<td>7</td>
<td>104</td>
</tr>
</tbody>
</table>

*The absolute number of megakaryocytes assayed.
†The ploidy distribution of normal mouse marrow.
‡The ploidy distribution at the outset of culture on the day of recharging. The ploidy is left-shifted because of preferential destruction of the larger megakaryocytes during marrow suspension.

A gradual decrease was observed and stabilized after week 3 (~8 U/mL). Alterations in IL-6 bioactivity correlated strongly with the observed alterations in megakaryocyte size (Figs 3 and 5; r = .94; P < .02). In contrast, there was no correlation between IL-6 bioactivity and megakaryocyte number (r = -.35; P = .43).

Influence of alteration of endogenous IL-6 bioactivity on megakaryocytic maturation. To determine if IL-6 influenced the changes in megakaryocyte size and ploidy in LTBMC, a monoclonal rat antimouse IL-6 antibody was added to some cultures. In contrast to control cultures containing an equal concentration of rat IgG, a significant decrease in size (P < .05) and ploidy (P < .05) was observed at week 1 of culture (Table 5). This decrease was reversed by cessation of antibody addition and readministration of IL-6 (data not shown). The changes in ploidy correlated with the decrease in IL-6 bioactivity. To rule out the possibility that the decrement in ploidy was unrelated to inhibition of IL-6, but related rather to an inhibitory effect of immune complexes, an immune complex of mouse IgG and antimouse IgG was added to the cultures in separate controls. The results were similar to the control, with a slight, but not significant, increase in higher ploidy cells at week 1.

The addition of human IL-6 increased the size (P < .05 at weeks 1 through 3) and ploidy (P < .01 at week 3) of the megakaryocytes (Table 5, Figs 6 and 7). Although an increment in ploidy was not observed at week 1, the addition of exogenous IL-6 resulted in only a 21% increase in IL-6 bioactivity detected in the culture. In contrast, at week 3, almost a 16-fold increment in IL-6 was measurable in the culture, and this correlated with a marked rightward shift in ploidy.

Table 4. Comparison Between the Ploidy Distributions of Adherent and Nonadherent Megakaryocytes

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Percentage of Megakaryocytes in Each Ploidy Class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2N</td>
</tr>
<tr>
<td>1 Supernatant</td>
<td>601</td>
</tr>
<tr>
<td>Stroma</td>
<td>220</td>
</tr>
<tr>
<td>2 Supernatant</td>
<td>248</td>
</tr>
<tr>
<td>Stroma</td>
<td>180</td>
</tr>
<tr>
<td>3 Supernatant</td>
<td>316</td>
</tr>
<tr>
<td>Stroma</td>
<td>744</td>
</tr>
</tbody>
</table>

*The modal ploidy class is shown in boldface.

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Table 5. IL-6–Related Changes in Ploidy Distribution

<table>
<thead>
<tr>
<th></th>
<th>% Megakaryocytes in Each Ploidy Class</th>
<th>IL-6 Activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ 8N</td>
<td>16N</td>
</tr>
<tr>
<td>Week 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>157</td>
<td>24</td>
</tr>
<tr>
<td>Immune complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>173</td>
<td>15</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>285</td>
<td>24</td>
</tr>
<tr>
<td>IL-6</td>
<td>214</td>
<td>13</td>
</tr>
<tr>
<td>Week 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>519</td>
<td>81</td>
</tr>
<tr>
<td>Immune complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>175</td>
<td>75</td>
</tr>
<tr>
<td>Anti-IL-6</td>
<td>345</td>
<td>88</td>
</tr>
<tr>
<td>IL-6</td>
<td>112</td>
<td>56</td>
</tr>
</tbody>
</table>

Ploidy was determined 1 week after adding monoclonal anti–IL-6 antibody or IL-6 to LTBMC at the times shown.
*Total megakaryocytes analyzed.
†A significant change toward lower ploidy classes was noted after adding anti–IL-6 antibody \( (P < .05) \).
‡A significant change toward higher ploidy cells was seen after adding IL-6 \( (P < .01) \).

Influence of alteration of endogenous IL-6 bioactivity on total cellular and megakaryocytic frequency. Table 6 shows the effect of anti–IL-6 addition on the nucleated cells and megakaryocytes enumerated in culture. Following addition of anti–IL-6, no significant differences were observed at weeks 1, 3, and 6 on total nucleated cells when compared with control cultures. The frequency of megakaryocytes was similar on weeks 1 and 6 between the two groups, although an increase in megakaryocytic frequency of unclear biologic significance was observed at week 3 in this series of experiments. This increase may represent inherent variabil-

Fig 6. IL-6 and anti–IL-6 antibody effects on megakaryocyte size in LTBMC. The results shown are from three experiments (three replicate culture wells per group). Compared with control, the size after IL-6 addition was significantly increased \( (P < .05) \); t-test, week 1 through 3), and decreased in culture with anti–IL-6 antibody \( (P < .05) \); week 1). (○—○) Control; (●—●) IL-6; (△—△) anti–IL-6.

Fig 7. The difference in the size of megakaryocytes in the supernatant of cultures containing rat anti–IL-6 IgG (A), normal rat IgG (B), and IL-6 (C) is apparent after AchE staining.
ity, because a similar discrepancy was observed in other experiments at week 3 (Table 1, experiments 1 and 2). The addition of IL-6 did not substantially alter the total numbers of cells beyond week 1 of culture. However, a marked decrease in the frequency of megakaryocytes was observed at week 6.

**Effect of alteration of endogenous IL-6 bioactivity on the distribution of nonmegakaryocytes.** Table 7 shows the distribution of cell types assessed morphologically at weeks 1, 4, and 7 of LTBMC. Control cultures showed a progressive decrease in granulocytes, accompanied by an increase in macrophages and no change in other cells. After administration of anti-IL-6, no substantial changes were noted in granulocytes (although at week 1, a statistically significant decrease was observed). Macrophages increased at week 1 compared with control, and were relatively similar to control thereafter. Other cell types were not visibly affected by the antibody. Exogenous IL-6 produced a marked temporal decrease in granulocytes and an increase in macrophages, with no significant alterations of other cell types.

**DISCUSSION**

Recent studies of LTBMC have established two important functions of the cells in the adherent layer. One function is to modulate stem cell proliferation and differentiation by production of a number of polypeptide growth factors, while the other is to provide the initial reservoir of stem cells from which all nonadherent cells are subsequently derived.1,17,18,21-23 Previous studies have shown that megakaryocytic progenitor cells (colony-forming unit-megakaryocyte [CFU-MK]) can be maintained in LTBMC.1,6,20,22,23 Moreover, those studies also showed that more mature cells of the megakaryocytic lineage could be detected for up to 2 months. The present study suggests that the ability to produce maturing megakaryocytes in LTBMC appears to be a function of culture conditions, with horse serum providing factors necessary for megakaryocytic survival and maturation and/or lacking inhibitors of these processes perhaps present in FCS. Other studies demonstrating mature megakaryocytes also used horse serum and several reports showed that the concentration of hydrocortisone was a critical factor in the maintenance of these cells in LTBMC.1,32

In the present study, megakaryocytes were observed both adherent to the stromal layer and also suspended in the supernatant. Because the size of megakaryocytes in the adherent layer is smaller than those in the supernatant, one scenario for megakaryocytopoiesis in these cultures is that the progeny of proliferating megakaryocytic progenitor cells have cell surface adhesion molecules permitting tight binding to the stromal layer. These cells then synthesize the marker enzyme AchE while bound. At some point in the differentiation program, adhesion is disrupted via unknown mechanisms (proteolysis of adhesion molecules or a decrease in the rate of synthesis of such molecules by the maturing megakaryocyte). Subsequently, the released megakaryocyte continues to differentiate in the supernatant, with the maturation stimulus provided by the release of soluble growth factors from the stroma. However, we noted that about one-quarter of the megakaryocytes bound to the stroma were ≥32N. Thus, the release of megakaryocytes from the stroma does not appear to be related to the ploidy of these cells; rather, changes in the cell surface adherence characteristics of the megakaryocyte or the stromal cell may be more proximately associated with megakaryocytic detachment. Alternatively, the possibility exists that megakaryocytes that have been released from the stroma might be capable of rebinding under certain conditions that permit re-expression of cell surface adhesion molecules. The cell

### Table 6. Influence of Exogenously Added IL-6 or Anti-IL-6 on Total Nucleated Cells and Megakaryocytes in LTBMC

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Anti-IL-6</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleated Cells (x 10^9)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>35 ± 3</td>
<td>26 ± 5</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>Week 3</td>
<td>25 ± 5</td>
<td>27 ± 6</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>Week 6</td>
<td>6 ± 2</td>
<td>255 ± 12</td>
<td>8 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Anti-IL-6</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Megakaryocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>61 ± 32</td>
<td>82 ± 12</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Week 3</td>
<td>27 ± 6</td>
<td>121 ± 9</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>Week 6</td>
<td>255 ± 12</td>
<td>296 ± 18</td>
<td>4 ± 2</td>
</tr>
</tbody>
</table>

### Table 7. Influence of Exogenously Added IL-6 or Anti-IL-6 on Cell Morphology in LTBMC

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Control</th>
<th>Anti-IL-6</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Granulocyte</strong></td>
<td>86 ± 5</td>
<td>68 ± 3f</td>
<td>51 ± 6t</td>
</tr>
<tr>
<td><strong>Macrophage</strong></td>
<td>8 ± 6</td>
<td>23 ± 3f</td>
<td>28 ± 7</td>
</tr>
<tr>
<td><strong>Other</strong>†</td>
<td>6 ± 5</td>
<td>5 ± 3</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

The data represent the mean ± 1 SD of three separate experiments and are expressed as a percent of 200 cells analyzed after hematoxylin staining.

Anti-IL-6 and IL-6 were added as described in Materials and Methods.

*Includes both monocytes and macrophages.
†All other cells not identifiable in the granulocyte or macrophage categories.
‡Significantly different than control (P < .05)
types to which the megakaryocytes bind and the nature of the binding will be of particular interest for future studies.

In the present culture system, murine megakaryocytes could be detected for periods of up to 13 weeks in the absence of exogenous growth factors. The number, size, and ploidy of these cells were analyzed in a sequential fashion. Although the experiments reported are reproducible, the limited number of cells analyzed at each time point is a limitation of this study. Megakaryocyte numbers generally increased each week in LTBMC (except week 2) with a frequency of up to 6%, a 2-log enrichment compared with BM (Table 1). The etiology of this preferential enhancement of megakaryocyte numbers compared with normal murine marrow, and the subsequent decline and total loss of megakaryocyte production is unknown.

Several polypeptide growth factors are known to be involved in the complex regulation of proliferation and differentiation of myeloid progenitor cells in vitro. IL-6 is a 26-Kd glycoprotein produced by a number of cell types, including leukocytes, endothelial cells treated with various inducers, and fibroblasts stimulated with IL-1 or tumor necrosis factor. IL-6 has a wide range of biologic activities. Recent data have shown that IL-6 is a promotor of megakaryocytic maturation in culture, with little influence on the proliferation of megakaryocytic progenitors. The factor is synergistic with IL-3 in the production of megakaryocytic colonies. Moreover, in vivo administration of IL-6 is capable of increasing the platelet count in mice and primates.

LTBMC is useful in assessing the potential role of a growth factor, because exogenous growth factors are not required for hematopoiesis in culture. Our data show that changes in megakaryocyte numbers do not correlate with changes in endogenous IL-6 activity (Table 1, Fig 5). Conversely, these data show that the increases in megakaryocytic size and ploidy parallel IL-6 bioactivity in LTBMC (Figs 3 through 5). Adding the monoclonal anti-IL-6 antibody significantly decreases megakaryocytic size and ploidy, while addition of exogenous IL-6 shows that the megakaryocytes are capable of responding to this growth factor in a manner similar to that seen in short-term liquid marrow cultures. These data suggest that IL-6 has a role in megakaryocytic maturation in this culture system. Cells other than megakaryocytes, including granulocytes and macrophages, are also influenced by exogenous IL-6, as might be expected from previous studies in short-term cultures. The marked decrease in the percentage of granulocytes and the increase in macrophages observed on addition of exogenous IL-6 may represent an influence of IL-6 on the granulocyte-macrophage differentiation pathway; alternatively, deterioration of LTBMC is accompanied by similar changes, suggesting a possible toxic effect of exogenous IL-6 administered for a number of weeks. This latter possibility may be reflected by the marked decrease in the frequency and number of megakaryocytes observed under these conditions.

For a number of reasons, it is unlikely IL-6 is the only growth factor responsible for megakaryocytic maturation in LTBMC. First, after addition of anti-IL-6 antibody at week 1, neither size nor ploidy was decreased to the lowest levels observed at later weeks of culture. This finding was true irrespective of the frequency of addition of anti-IL-6 antibody (data not shown). This finding might be trivial, in that blocking of IL-6 action would not decrease the size or ploidy of existing megakaryocytes; rather, the addition of such an antibody might prevent the IL-6-related maturation of recently produced megakaryocytes, resulting in a decrease by dilution of the mean size and ploidy of the measured cells. Recent data suggesting that megakaryocytes produce their own IL-6 and IL-6 receptor point to the possibility that other growth factors could induce megakaryocytes to produce endogenous IL-6 in an autocrine fashion. Anti-IL-6 antibodies would be expected to neutralize accessible but not endogenous IL-6 under these circumstances. Second, the anti-IL-6 added to the cultures in these experiments did not completely neutralize IL-6 bioactivity (Table 5). In preliminary studies, even when the antibody was added up to seven times per week, complete neutralization of bioactivity was not observed. Third, other factors produced by the stromal cells may also have a role in megakaryocytic maturation. Among the defined hematopoietic growth factors that potentially could be involved are IL-1, -4, -7, and -11 and leukemia inhibitory factor. Other as yet undefined factors may also play a role, as could inhibition of maturation by factors such as transforming growth factor-β (TGF-β). The involvement of each of these factors could potentially be assessed using neutralizing antibodies as described.

In the present report, we describe a new antibody that may be useful in the analysis of murine megakaryocyteopoiesis. The antigen that this antibody recognizes is unknown and we have been unable to identify its molecular weight by Western blotting. However, this rat antimouse platelet MoAb appears to be specific to murine platelets and megakaryocytes in BM. The ploidy distribution of normal mouse marrow determined by flow cytometry (Table 3) is similar to that observed with polyclonal sera, showing a modal ploidy of 16N. Further characterization of the antigen and its relationship to the appearance of AchE will be of interest.

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