Effect of Recombinant Granulocyte-Macrophage Colony-Stimulating Factor on Murine Thrombocytopenosis In Vitro and In Vivo

By Toshiyuki Ishibashi, Hideo Kimura, Yayoi Shikama, Tatsumi Uchida, Shigeo Kariyone, and Yukio Maruyama

To investigate the effect of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) on murine megakaryocytopoiesis in vitro, the factor was added to both serum-free colony assays and liquid marrow cultures. GM-CSF had a significant megakaryocytic colony-stimulating activity. After 2 hours of preincubation with and without 10 ng/mL rGM-CSF, the percentage of megakaryocyte colony-forming cell (CFU-MK) in DNA synthesis was determined by tritiated-thymidine suicide using colony growth. The reduction of CFU-MK colony numbers in marrow culture was 47.5% ± 9.9%, 20.9% ± 5.2% (control), respectively, indicating that the factor affected cell cycle at CFU-MK levels. When acetylcholinesterase (AchE) production was measured fluorometrically after 4 days of liquid culture, rGM-CSF elicited an increase in AchE activity in a dose-dependent fashion. To determine if the hematopoietin acts directly on megakaryocytic differentiation, 2 ng/mL rGM-CSF was added to serum-free cultures of 295 single megakaryocytes isolated from CFU-MK colonies. An increase in size was observed in 65% of cells initially 10 to 20 μm in diameter, 71% of cells 20 to 30 μm, and 40% of cells greater than 30 μm. Conversely, in absence of GM-CSF, 17%, 31%, and 10% of cells in each group increased in diameter. These data suggest that rGM-CSF promotes murine megakaryocytopoiesis in vitro and that the response to the factor is direct. To determine if the factor influences megakaryocytic/thrombocytopenic lineage in vivo, 1 and 5 μg of rGM-CSF were administered intraperitoneally every 12 hours for 6 consecutive days. Although a two- to three-fold increase in peripheral granulocytes was observed, neither megakaryocytic progenitor cells or platelets changed. Histologic analysis of bone marrow megakaryocytes showed no increase in size and number. The in vivo studies demonstrated no effect of GM-CSF on thrombocytopenia. The discrepancies between the in vitro and in vivo effects of GM-CSF require additional investigations.

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MATERIALS AND METHODS

Marrow cell preparation. Six- to eight-week-old specific pathogen-free C57Bl/6 male mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were killed by cervical dislocation. Bone marrow from the femur was flushed and suspended into Iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories, Grand Island, NY). A monocellular suspension was made by repetitive expusion through an 18-gauge followed by a 22-gauge needle. Marrow cells were washed by centrifugation at 250 x g for 10 minutes at room temperature and resuspended in IMDM.

Serum-free liquid marrow culture. Whole marrow cells (1 x 10^7 per well) were cultured in a final volume of 0.2 mL of IMDM containing 1% Nutrieye (a serum-free medium supplement; J. Brooks Laboratory, San Diego, CA), 0.05% crystallized bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO) and 100 U/mL of penicillin-streptomycin (PS) in 96 microwell plates as previously described.22,23 Various concentrations of recombinant mouse GM-CSF4 (specific activity 5 x 10^6 U/mg; a gift of Sumitomo Pharmaceutical Inc, Osaka, Japan) were added to marrow cultures. Units of activity were defined by granulocyte-macrophage colony-forming cell (CFU-GM) colony formation in mouse bone marrow culture. Half-maximal colony formation was assigned to 50 U/mL.

Tritiated thymidine (3HdR) incorporation into serum-free liquid cultures. The effect of rGM-CSF on cell proliferation was determined by tritiated thymidine incorporation. Marrow cells were enriched for progenitor cells on a 1.065/1.077 g/cm^3 discontinuous Percoll gradient as previously described.22,24 After 2 days of culture, 0.5 μCi of 3HdR(methyl-3H thymidine, 25 Ci/mmol, Amersham International, Buckinghamshire, England) was added to each well. Sixteen hours later, the cells were harvested with an automatic multiple cell harvester (Labo Mash; Labo Science Co, Ltd, Tokyo, Japan) onto glass fiber paper, and the radioactivity measured with a scintillation counter.

Serum-free colony assays. To assess the influence of rGM-CSF on the detection of megakaryocytic (CFU-MK) and granulocyte-macrophage (CFU-GM) colony-forming cells, marrow cells were cultured at 1 x 10^5 cells/mL in agar in 1-mL final volumes in 35-mm culture dishes as previously described,25 with the exception that serum was replaced with 1% bovine serum albumin (BSA), 360 μg/mL human transferrin, and 0.98 μg/mL cholester. GM-CSF and heated material (100°C, 40 minutes) were added to each well at concentrations ranging from 0.05 to 10 ng/mL. After 7 days of culture, colonies were counted by the addition of crystal violet and the percentage of colonies was measured.

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culture, each dish was fixed with 2% glutaraldehyde onto glass slides. Acetylcholinesterase (AchE) staining was performed to detect CFU-MK colonies, followed by hematoxylin staining for CFU-GM colonies. Three to four replicate plates were counted for each experiment.

Progenitor cell cycle analysis. To examine the effect of rGM-CSF on the DNA synthetic phase (S phase) of progenitors, 'HTdR suicide studies were performed as previously described. Briefly, nonadherent marrow cells were preincubated with 10 ng/mL rGM-CSF at 37°C for 2 hours, washed three times, and exposed to 1 'HTdR (25 Ci/mmol, Amersham International) at 37°C for 20 minutes. Control cells were exposed to 1.2 µg/mL of cold thymidine. The cells were washed three times and cultured in colony assays using 7.5% pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) as a colony-stimulating activity.

Quantitation of AchE activity. AchE, a relatively specific marker of the megakaryocytic lineage in mice, was measured fluorometrically to quantitate proliferation and differentiation of murine megakaryocytosis in liquid cultures as previously described. Briefly, after 4 days of culture, the plate was centrifuged and the supernatant was discarded. Two-tenths milliliter of a solution of 0.2% Triton X-100 in 1 mmol/L EDTA (Sigma), 0.12 mol/L NaCl, and 50 mmol/L HEPES pH 7.5 (Flow Laboratories, Irvine, UK) was added to each well followed by the addition of 20 µL of acetylthiocholine iodide (final concentration 0.56 mmol/L). After 4 hours of incubation, 10 µL of 0.4 mmol/L coumarinphenylmaleimide (Molecular Probes Inc, Junction City, OR) was added to 10 µL of the reaction mixture, followed by 2 mL of diluent buffer containing 0.2% Triton X-100 in 1 mmol/L EDTA and 5 mmol/L sodium acetate pH 5.0. The fluorescence emission was measured with a filter fluorometer with an excitation filter of 390 nm and an emission filter of 450 nm.

Single cell culture of CFU-MK-derived megakaryocytes. Single megakaryocytes isolated from CFU-MK colonies were cultured as previously described. Briefly, bone marrow was enriched for progenitor cells on a 1.065/1.077 g/cm3 discontinuous Percoll gradient. Twenty-five thousand cells were cultured with various concentrations of rGM-CSF in serumless liquid cultures. Data are expressed as the mean ± SD of three experiments. The radioactivity of four replicate wells was measured for each experiment.

Table 1. Effect of rGM-CSF on Colony Growth

<table>
<thead>
<tr>
<th>GM-CSF (ng/mL)</th>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-MK colonies (1 x 10⁵ cells)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 ± 1</td>
<td>4 ± 2</td>
<td>7 ± 2</td>
<td>15 ± 2</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>CFU-GM colonies (1 x 10⁵ cells)</td>
<td>0</td>
<td>5 ± 1</td>
<td>31 ± 6</td>
<td>95 ± 14</td>
<td>141 ± 28</td>
<td>193 ± 12</td>
<td>203 ± 10</td>
<td>212 ± 11</td>
<td>221 ± 14</td>
</tr>
</tbody>
</table>

After 7 days of culture, CFU-MK colonies were detected by AchE staining, followed by hematoxylin staining for CFU-GM colonies. Results are expressed as the mean ± SD per 10⁵ cells of five experiments. Three to four replicated plates were enumerated for each experiment. One nanogram per milliliter of GM-CSF was required to support CFU-MK colony growth (P < .01).

RESULTS

Marrow cell proliferation. The influence of rGM-CSF on cellular proliferation was examined by 'HTdR incorporation assay in serumless liquid culture. As shown in Fig 1, a significant increase in radioactivity was observed at 0.05 ng/mL of rGM-CSF (P < .01), which appears more likely to

Fig 1. Effect of rGM-CSF on 1.065/1.077 g/cm² cut-off cells was assessed by 'HTdR incorporation. The cells were separated over a 1.065/1.077 g/cm² discontinuous Percoll gradient and cultured with various concentrations of rGM-CSF in serumless liquid cultures. Data are expressed as the mean ± SD of three experiments. The radioactivity of four replicate wells was measured for each experiment.
Table 2. Effect of rGM-CSF on the S Phase of Progenitor Cells

<table>
<thead>
<tr>
<th>CFU-MK</th>
<th>CFU-GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Reduction</td>
<td></td>
</tr>
<tr>
<td>GM (-)</td>
<td>GM (+)</td>
</tr>
<tr>
<td>GM (-)</td>
<td>GM (+)</td>
</tr>
<tr>
<td>Exp. 1</td>
<td>13.5</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>23.9</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>21.4</td>
</tr>
<tr>
<td>Exp. 4</td>
<td>24.9</td>
</tr>
</tbody>
</table>

M ± SD 20.9 ± 5.2 47.5 ± 9.9 22.0 ± 4.0 46.1 ± 6.2

To determine the effect of rGM-CSF on the frequency of the DNA synthetic phase in progenitor cells, 10 ng/mL of rGM-CSF was added to nonadherent marrow cells. After 2 hours of incubation at 37°C, H3Tdt suicide assay was performed as previously described. An apparent increase in the percentage of CFU-MK and CFU-GM in S phase was induced by the treatment of rGM-CSF (P < .01).

*Marrow cells were preincubated without rGM-CSF.

reflect the proliferation of granulocyte-macrophage lineage than megakaryocytic proliferation. Plateau incorporation induction occurred at an rGM-CSF dose of 2 ng/mL.

Colony assays. Table 1 shows the effect of rGM-CSF on progenitor cells. GM-CSF induced megakaryocytic colony formation at a concentration of 1 ng/mL (P < .01) with further stimulation at higher concentrations, while CFU-GM colony formation was stimulated by the factor in a dose-dependent fashion at concentrations greater than 0.05 ng/mL (P < .01). This observation was accompanied by an increase in cell number and size in individual CFU-MK colonies (data not shown). A heated GM-CSF did not support either CFU-GM or CFU-MK colony growth at any concentrations added.

Influence of rGM-CSF on the S phase of progenitors. As shown in Table 2, 2 hours of preincubation with 10 ng/mL of rGM-CSF induced CFU-MK into cell cycle, with 47% ± 10% of CFU-MK in S phase compared with 21% ± 5% in the absence of the factor (P < .01). As would be expected, the treatment with rGM-CSF increased the percentage of CFU-GM in S phase with 46% ± 6%, compared with 22% ± 4% in the absence of the factor (P < .01).

Effect of rGM-CSF on AchE activity in liquid marrow culture. AchE activity was measured fluorometrically after 4 days in serumless liquid culture. Figure 2 shows a significant increase in AchE activity at a concentration of 0.05 ng/mL (P < .01), with further increments noted at higher concentrations. Figure 3 demonstrates AchE staining for murine megakaryocytes in 96 well plates. The increase in the size and apparent number of megakaryocytes was observed in rGM-CSF-stimulated when compared with control cultures.

Single cell culture. To determine if rGM-CSF acted directly on megakaryocytic differentiation, 2 ng/mL of this factor was added to single megakaryocytes isolated from 5-day-old CFU-MK colonies. Cell diameter was measured on 295 megakaryocytes before and after culture. As shown in Table 3, an increase in size was observed in presence of rGM-CSF in 65% of cells initially 10 to 20 μm in diameter, 71% of cells 20 to 30 μm, and 40% of cells greater than 30 μm.
cyte number and size were determined on bone marrow from CFU-MK colonies before and after culture. An increase in size was observed for cells treated with and without rGM-CSF in each group is highly significant \( (P < .0001; \chi^2\text{-square analysis}) \).

In vivo studies. Table 4 shows that rGM-CSF administration elicited about two- to threefold increase in absolute granulocyte numbers, although injections of a control buffer had negligible effects on granulocyte levels. No significant increase in platelet counts was observed in mice injected with concentrations of both 1 and 5 \( \mu \)g of rGM-CSF. When a control solution or 1 \( \mu \)g of heated rGM-CSF was injected, platelet and granulocyte levels did not change. Megakaryocyte number and size were determined on bone marrow sections in each specimen of each group increased per 1 \( \mu \)g in mice injected with 1 \( \mu \)g of rGM-CSF and a control solution were injected ip every 12 hours for 6 days. A significant difference was observed between the granulocytes in mice injected with and without rGM-CSF (\( P < .01 \)), whereas there was no difference in platelet levels.

**Table 3. Effect of rGM-CSF on the Size of Single Megakaryocytes**

<table>
<thead>
<tr>
<th>Initial Cell Diameter (( \mu )m)</th>
<th>No. of Cells increasing in Diameter (%)</th>
<th>GM-CSF (-)</th>
<th>GM-CSF (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10–20</td>
<td>4/23 (17)</td>
<td>41/63 (65)</td>
<td></td>
</tr>
<tr>
<td>20–30</td>
<td>15/49 (31)</td>
<td>68/96 (71)</td>
<td></td>
</tr>
<tr>
<td>&gt;30</td>
<td>3/29 (10)</td>
<td>14/35 (40)</td>
<td></td>
</tr>
</tbody>
</table>

Cell diameter was measured on the same megakaryocytes isolated from CFU-MK colonies before and after culture. An increase in size was defined as an increment of greater than 0.5 \( \mu \)m. The difference between cells treated with and without rGM-CSF in each group is highly significant \( (P < .0001; \chi^2\text{-square analysis}) \).

Megakaryocyte proliferation was postulated to be controlled by two factors:29 a megakaryocyte colony-stimulating activity29,30 and a maturation-promoting, operationally termed thrombopoietin.29,31,32 Over the past decade, it has been shown that administration of IL-3 and Ep have an effect on mature megakaryocytes as well as megakaryocytic colony-forming cells.21,24 suggesting that these factors not only possess colony-stimulating activity but also maturation-promoting activity. In fact, Berridge et al.31 have shown that administration of Ep increases the platelet counts in mice. More recently, Ganser et al.32 have reported an increase in platelet numbers by IL-3 in vivo trial in humans.33 In addition, our laboratory has demonstrated that IL-6 induces a dramatic increase in platelets in vivo in mice.40

By several investigators have shown the stimulating activity of GM-CSF on megakaryocyte colony formation.41 However, neither the mechanism of its action or the effect on later stages of megakaryocyte development has been studied. The colony data shown here was consistent with other reports41 regarding CFU-MK colony formation. To determine if rGM-CSF-induced progenitors become into cells, the effect on the DNA synthetic phase was shown. Two hours of incubation with rGM-CSF increased the percentage of CFU-MK in S phase. These findings suggested that GM-CSF directly influenced the committed stem cells of the megakaryocytic lineage as well as the CFU-GM.

To determine if rGM-CSF promotes some aspects of megakaryocytic differentiation, AcE activity was measured in serumless liquid marrow culture. rGM-CSF increased AcE production at the same concentration (0.05 ng/mL) that the proliferation of granulocyte-macrophage lineage was primarily observed, with further increments at higher concentrations. However, this result does not preclude the possibility of mediation through accessory cells. Therefore, we measured the size of single megakaryocytes derived from CFU-MK colonies before and after culture under no interaction of other cells. An increase in size was observed in individual megakaryocytes with rGM-CSF when compared with control cultures, indicating that rGM-CSF directly influenced the differentiated megakaryocytes.

**Table 4. Peripheral Blood Counts in Mice Injected With rGM-CSF**

<table>
<thead>
<tr>
<th>Group</th>
<th>WBC (( \times 10^3/\muL ))</th>
<th>Granulocytes (( \times 10^3/\muL ))</th>
<th>Hematocrit (%)</th>
<th>Platelets (( \times 10^6/\muL ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (( n = 13 ))</td>
<td>2,440 ± 620</td>
<td>235 ± 110</td>
<td>45 ± 2</td>
<td>98.9 ± 13.4</td>
</tr>
<tr>
<td>1 ( \mu )g (( n = 11 ))</td>
<td>2,750 ± 730</td>
<td>508 ± 123</td>
<td>42 ± 3</td>
<td>86.8 ± 14.8</td>
</tr>
<tr>
<td>5 ( \mu )g (( n = 3 ))</td>
<td>3,400 ± 1,705</td>
<td>671 ± 266</td>
<td>47 ± 3</td>
<td>97.4 ± 7.0</td>
</tr>
</tbody>
</table>

A significant difference was observed between the granulocytes in mice injected with and without rGM-CSF \( (P < .01) \), whereas there was no difference in platelet levels.

**Table 5. Effect of rGM-CSF Administration on Bone Marrow Megakaryocytes**

<table>
<thead>
<tr>
<th>Group</th>
<th>Megakaryocyte No.*</th>
<th>Megakaryocyte Counts (( \times 10^3/\muL ))</th>
<th>Corrected Counts</th>
<th>Megakaryocyte Diameter (( \mu )m)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (( n = 6 ))</td>
<td>83.90 ± 11.92</td>
<td>10.86 ± 1.54</td>
<td>20.17 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>1 ( \mu )g (( n = 6 ))</td>
<td>86.82 ± 10.59</td>
<td>11.23 ± 1.48</td>
<td>20.15 ± 0.50</td>
<td></td>
</tr>
<tr>
<td>5 ( \mu )g (( n = 3 ))</td>
<td>81.99 ± 1.76</td>
<td>10.57 ± 0.32</td>
<td>20.27 ± 0.24</td>
<td></td>
</tr>
</tbody>
</table>

*One and five micrograms of rGM-CSF were administered intraperitoneally (ip) twice a day. Total 12 and 60 \( \mu \)g of rGM-CSF were injected over 6 consecutive days.

Humans and mice.43-31 We have demonstrated that IL-3 and Ep have an effect on mature megakaryocytes as well as megakaryocytic colony-forming cells.21,24 suggesting that these factors not only possess colony-stimulating activity but also maturation-promoting activity. In fact, Berridge et al.31 have shown that administration of Ep increases the platelet counts in rats. More recently, Ganser et al.32 have reported an increase in platelet numbers by IL-3 in vivo trial in humans.33 In addition, our laboratory has demonstrated that IL-6 induces a dramatic increase in platelets in vivo in mice.40

DISCUSSION

Megakaryocytopoiesis has been postulated to be controlled by two factors:29 a megakaryocyte colony-stimulating activity29,30 and a maturation-promoting, operationally termed thrombopoietin.29,31,32 Over the past decade, it has been shown that several known factors, including interleukin-3 (IL-3), erythropoietin (Ep), and GM-CSF have been involved in the regulation of megakaryocytopoiesis in vitro in humans and mice.43-31
EFFECT OF GM-CSF ON THROMBOPOIESIS

Although the effect of GM-CSF on granulopoiesis by studies in animals and clinical trials has been reported, the influence of the factor on platelet production has been variable. Nienhuis et al., Vadhan-Raj et al., and Antman et al. have shown some effect on platelet levels in primates and humans, while other investigators have not. Despite in vitro action of GM-CSF on megakaryocytopoiesis, administration of this hematopoietin to mice did not have a significant effect on megakaryocytic compartments, including CFU-MK, megakaryocytes, and circulating platelets. Several reasons why GM-CSF did not promote thrombocytopoiesis can be considered.

First, we injected GM-CSF intraperitoneally every 12 hours for 6 consecutive days. Metcalf et al. studied the serum levels of GM-CSF in mice injected with the factor intraperitoneally, demonstrating a half-life of 35 minutes, with a peak of 30 minutes after injection. Donahue et al. analyzed the circulating plasma clearance of \(^{35}\)S-methionine-labeled human GM-CSF in primates, showing a half-life of 7 minutes in \(\alpha\) phase and 80 to 90 minutes in \(\beta\) phase. These findings suggest that the injection schedule designed in this study might not maintain the plasma level of GM-CSF to induce a change in thrombocytopoiesis and that continuous administration may be more effective.

Second, GM-CSF in this study was nonglycosylated. Mayer et al. assessed the ability of two types of GM-CSF, one glycosylated and derived from CHO cells, and another nonglycosylated and extracted from *Escherichia coli*. Administration of both forms induced a granulocytosis of the same magnitude and duration in primates. Therefore, it is unlikely that the in vivo negative data might be due to the glycosylation of the growth factor.

Third, in vitro studies have shown that several factors have an effect on megakaryocyte proliferation and maturation. It is conceivable that GM-CSF alone might not be sufficient to promote the proliferation and differentiation of megakaryocytopoiesis and induce thrombocytosis. Administration of GM-CSF together with other hematopoietins may be necessary to demonstrate any potential thrombocytopoietic capacity.

In conclusion, there is a disparity between the in vitro influence of GM-CSF on megakaryocytopoiesis and in vivo effect. Additional data will be required to determine if the lack of influence of the factor in vivo is merely technical or indeed represents a fundamental difference between the in vitro and in vivo situation.

ACKNOWLEDGMENT

We are grateful to Dr. Samuel A. Burstein (University of Oklahoma, Oklahoma City) for reviewing the manuscript.

REFERENCES

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Table 6. Influence of rGM-CSF on the Bone Marrow and Spleen

<table>
<thead>
<tr>
<th>Group</th>
<th>CFU-MK (x10³)</th>
<th>CFU-GM (x10³)</th>
<th>Total Cells (x10⁵)</th>
<th>CFU-MK (x5 x10³)</th>
<th>CFU-GM (x5 x10³)</th>
<th>Total Cells (x10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg‡</td>
<td>27 ± 4</td>
<td>122 ± 26</td>
<td>28.6 ± 3.0</td>
<td>6 ± 1</td>
<td>30 ± 7</td>
<td>210 ± 26</td>
</tr>
<tr>
<td>Control‡</td>
<td>26 ± 4</td>
<td>135 ± 31</td>
<td>30.2 ± 2.5</td>
<td>1 ± 1</td>
<td>6 ± 1</td>
<td>133 ± 15</td>
</tr>
<tr>
<td>5 µg‡</td>
<td>33 ± 4</td>
<td>118 ± 8</td>
<td>31.7 ± 2.0</td>
<td>ND</td>
<td>ND</td>
<td>246 ± 56</td>
</tr>
<tr>
<td>Control‡</td>
<td>32 ± 2</td>
<td>120 ± 4</td>
<td>28.9 ± 3.5</td>
<td>ND</td>
<td>ND</td>
<td>159 ± 8</td>
</tr>
</tbody>
</table>

*One microgram, n = 13; control, n = 12; 5 µg, n = 3; control, n = 3.
†One microgram, n = 6; control, n = 3; 5 µg, n = 3; control, n = 3.
‡One and five micrograms of rGM-CSF and a control solution were injected every 12 hours for 6 days.
Effect of recombinant granulocyte-macrophage colony-stimulating factor on murine thrombocytopoiesis in vitro and in vivo

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