Human Macrophage Colony-Stimulating Factor Induces Macrophage Colonies After L-Phenylalanine Methylester Treatment of Human Marrow

By Craig S. Rosenfeld, Carol Evans, and Richard K. Shadduck

Macrophage-colony stimulating factor (M-CSF) has well-known effects on murine bone marrow, but its colony stimulating activity for human bone marrow is controversial. After treatment of human bone marrow with L-phenylalanine methylester (PME), macrophage-colonies (CFU-M) were induced by M-CSF in a dose-dependent fashion. The optimal concentration of recombinant human-macrophage colony stimulating factor (rhM-CSF) was 1,000 U/mL. Purified human urine M-CSF had colony stimulating activity similar to rhM-CSF. Further studies were performed to determine the factors responsible for the enhanced CFU-M formation from PME treated marrow. Compared with nylon wool and carbonyl iron monocyte depletion methods, PME eliminated significantly more monocytes and myeloid cells. This observation suggested that these cells may release hematopoietic inhibitory factors for CFU-M. Low concentrations (1%) but not normal (10%) concentrations of blood monocytes were inhibitory (mean inhibition, 48%) to CFU-M. High concentrations of monocytes (50%) augmented CFU-M colonies. HL-60 conditioned media was used to simulate secretory products of early myeloid cells. HL-60 conditioned media (1%) inhibited CFU-M formation but not granulocyte macrophage or granulocyte colonies. We conclude that M-CSF has colony stimulating activity for human marrow that can be recognized after removal of inhibitory cells by PME treatment.

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Determination of cellular composition. After each manipulation, automated cell counts were performed (Coulter, Hialeah, FL) and viability assessed by trypan blue dye exclusion. Flow cytometry was performed as described previously. Monoclonal antibodies against T cells (Leu-4, CD 3) and monocytes (Leu-M3, CD 14) were purchased from Becton-Dickinson (Mountain View, CA). Cytochemical stains were performed on cells after cytocentrifugation (Shandon, Pittsburgh, PA). Myeloid cells were identified by staining with naphthol AS-D chloroacetate esterase and monocytes by α-naphthyl butyrate esterase (Sigma).  

Progenitor cell colonies. Granulocyte colonies (CFU-G), granulocyte-macrophage colonies (CFU-GM), and CFU-M were performed in agar gel as described previously. Colony stimulating activity was provided by recombinant human granulocyte colony stimulating factor (rhG-CSF) (Immunex Corp, Seattle, WA), recombinant granulocyte-macrophage colony stimulating factor (rhGM-CSF) (Immunex), rhM-CSF (Genetics Institute, Cambridge, MA), and huM-CSF. The specific activities of the rhM-CSF and huM-CSF were approximately 3 x 10^9 U/mg and 4 x 10^9 U/mg, respectively. Units of activity were assessed using murine bone marrow cells as described previously. One unit of activity was defined as stimulation of one colony as derived from the linear portion of the dose-response curve (20 to 100 colonies per culture plate). Assays for erythroid burst-forming unit (BFU-e) were performed in methylcellulose as described previously. One unit of erythropoietin (Amgen, Thousand Oaks, CA) was added to each erythroid culture. Burst promoting activity was provided by recombinant human IL-3 (Immunex). CFU-G were counted on day 7; other cultures were counted on day 14. Each culture was performed in triplicate. The effect of monocytes on CFU-M was evaluated with a double layer culture containing a 1 mL underlayer of 0.5% agar. Autologous monocytes were isolated on FCS-coated plastic Petri dishes. Cells isolated from this procedure were 90% to 95% monocytes as determined by butyrate esterase positivity. Cytochemical staining of agar cultures with naphthyl AS-D chloroacetate esterase and α-naphthyl acetate esterase (for myeloid cells) was performed as described previously.  

The effect of PME on stromal cells was determined by growth of fibroblast colonies (CFU-F). For CFU-F, 1 x 10^6 LDBMC cells were cultured in McCoy's 5A media with 15% FCS, 1 x 10^-4 mol/L hydrocortisone (Sigma), 1% glucose, and 1% antibiotic-antimyelocytic solution (GIBCO). The culture was incubated at 33°C with 5% CO_2 for 10 days. The plates were stained with Wright-Giemsa and aggregates of >50 cells were scored as colonies. All cultures were performed in triplicate.  

Preparation of HL-60 conditioned media. HL-60 cells (a human promyelocytic leukemic cell line) were incubated at 1 x 10^6 cells/mL in McCoy's media 5A (GIBCO) with 15% FCS, 1% glucose, and 1% penicillin-streptomycin-ampoterine at 37°C/5% CO_2. After 24 hours of incubation, the cells were removed by centrifugation and the media was filtered before use in the progenitor cell assays.  

Analysis and statistics. For comparisons, the total cell content after each manipulation was used. Recoveries were based on cells remaining after density gradient centrifugation. Statistical comparisons were performed using a two sided t-test. A p value <.05 was considered significant.  

RESULTS  

Macrophage colony formation. A representative dose response curve is shown in Fig 1. The results of three experiments indicated that 1,000 U (20 ng) of rhM-CSF resulted in maximal CFU-M formation (24 ± 2.7 CFU-M/2 x 10^5 PME treated cells). Typically, macrophage colonies were diffuse aggregates of cells containing 30 to 75 cells per colony. In situ cytochemical staining showed that the colonies were uniformly stained with α-naphthyl acetate esterase and negative for naphthyl AS-D chloroacetate esterase and luxol blue. Colony formation was detected from some density gradient marrows cultured with M-CSF. These M-CSF–induced colonies contained variable numbers of naphthyl AS-D chloroacetate positive colonies. Because colony formation had not been detected previously with huM-CSF in our laboratory, dose response curves were also performed with this source of M-CSF. Cultures were performed with equivalent activities of the two M-CSFs. Colony morphology and cytochemical staining of the cultures performed with huM-CSF were identical to those performed with rhM-CSF. In the dose range from 500 to 2,000 U/plate, there was no significant difference in colony formation between these two sources of M-CSF.  

Comparison of PME to other methods of monocyte depletion. Recovery of nucleated cells after PME was 44%. This recovery was significantly less than with carbonyl iron
CFU-M FROM PME-TREATED MARROW

(83%, \( P < .001 \)) but not nylon wool (57%). Viability after each cell manipulation was greater than 95%.

Elimination of \( \alpha \)-naphthyl butyrate esterase positive cells following PME treatment was 96% ± 1%. Elimination of monocytes by PME was significantly greater than with either carbonyl iron (50% ± 9, \( P < .05 \)) or nylon wool (23% ± 5, \( P < .01 \)). Recovery of myeloid cells was significantly less with PME (2% ± 1) than after carbonyl iron (96% ± 5, \( P < .005 \)) or nylon wool (72% ± 11, \( P < .05 \)).

There were no significant differences between the recoveries of CFU-G, CFU-GM, and BFU-e among the three monocyte depletion methods (Table 1). CFU-F were essentially eliminated by PME treatment. The carbonyl iron method preserved significantly more CFU-F (13 ± 2% \( P < .05 \)) than PME (0.1 ± 0.1%). The mean number of CFU-F recovered after nylon wool treatment (5 ± 5%) was intermediate between the other two methods of monocyte depletion. The mean number of CFU-M/2 \( \times 10^5 \) cells was 2.8 ± 1.9 for nylon wool and 4.6 ± 2.4 for carbonyl iron. Formation of endogenous colonies (without added colony stimulating factors) was eliminated by all three methods.

Surface marker analysis was performed in two experiments. Only the PME method eliminated CD 14 positive cells. The recovery of CD 14 positive cells following carbonyl iron (22% ± 16%) and nylon wool (21% ± 19%) were comparable. The mean recovery of T cells (CD 3 positive) exceeded 90% by all three methods.

**Effect of monocytes on CFU-M.** Results in the previous section suggested that complete removal of monocytes may be responsible for improved macrophage colony growth following PME incubation. Therefore, the effect of adding autologous blood monocytes to PME-treated marrow was evaluated. The number of monocytes added to the cultures ranged from 1% to 50% of the PME treated marrow cells. Cells were stimulated with rhM-CSF. Compared with a control without added monocytes, 1% added monocytes had a slight (mean, 48%), but significant, inhibitory effect on CFU-M formation. In contrast, the 50% concentration of monocytes was stimulatory to CFU-M. However, 10% added monocytes (a concentration comparable with that found in density gradient separated marrow) had no significant inhibitory or stimulatory effect.

**Effect of HL-60 conditioned media on CFU-M.** Another difference between PME and nylon wool or carbonyl iron treatment was the depletion of myeloid cells by PME. This suggested that early myeloid cells in the light density marrow fraction might be associated with inhibition of CFU-M. To test this hypothesis, HL-60 conditioned media (concentrations from 0.1% to 10%) were used to simulate secretory products of early myeloid cells. One of four experiments is shown in Fig 2. Concentrations of HL-60 conditioned media as low as 1% were strongly inhibitory to CFU-M. In contrast, HL-60 conditioned media did not significantly decrease CFU-GM and was stimulatory, or did not significantly alter, CFU-G. Media alone had no effect on CFU-M (not shown).

**DISCUSSION**

A paradoxical action of huM-CSF has been its preferential ability to stimulate CFU-M from murine marrow.\(^{19}\) However, data from in vitro colonies using purified human progenitors and in vivo administration of rhM-CSF indicates that rhM-CSF is indeed effective in stimulating human marrow.\(^{14,20}\) Data from this study indicate that induction of human CFU-M by M-CSF can be observed after PME treatment of the marrow. This was true for both huM-CSF and rhM-CSF. The colony size and cloning efficiency of CFU-M that we observed are similar to results reported by other investigators.\(^{24}\) Further experiments were performed to elucidate the method by which PME treatment of marrow induces CFU-M.

In order to determine the factors responsible for CFU-M formation from PME treated marrow, PME treatment was compared with nylon wool and carbonyl iron methods of monocyte depletion. All three methods reduced CFU-GM recovery in rough proportion to monocyte recovery. This is consistent with other observations that indicate monocytes can enhance the bone marrow progenitor cell response to GM-CSF by secretion of G-CSF.\(^{21,22}\) PME treatment of marrow resulted in lower cell recoveries than with nylon wool or carbonyl iron. However, the resulting concentration of cells (1.9-fold compared with carbonyl iron), cannot account for the five- to eightfold increase in CFU-M compared with these other methods of marrow processing.

The increased cloning efficiency of CFU-M appears to result from differences in cellular composition after PME treatment of the marrow. The elimination of CFU-F and monocytes by PME indicates that secondary production of M-CSF should not account for enhanced CFU-M growth following PME treatment.\(^{19}\) Furthermore, secondary production of lymphokines probably cannot account for differences in CFU-M since T-cell recovery was essentially complete for each of the marrow processing methods.

Monocytes are known to be inhibitory to colony growth under certain circumstances.\(^{23}\) This possibility was tested by adding autologous blood monocytes to PME-treated marrow. Low concentrations of monocytes inhibited CFU-M, whereas high concentrations augmented CFU-M formation. These

**Table 1. Cellular Recovery (%) After Monocyte Depletion**

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Nucleated cells</th>
<th>Myeloid cells</th>
<th>Monocytes</th>
<th>CFU-G</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-F</th>
<th>CD 14</th>
<th>CD 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon source</td>
<td>83 ± 2*</td>
<td>96 ± 5†</td>
<td>50 ± 9§</td>
<td>54 ± 15</td>
<td>117 ± 10</td>
<td>117 ± 10</td>
<td>13 ± 2§</td>
<td>22 ± 16</td>
<td>94 ± 21</td>
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<tr>
<td>Carbon source</td>
<td>57 ± 5</td>
<td>72 ± 11§</td>
<td>23 ± 6§</td>
<td>31 ± 6</td>
<td>44 ± 44</td>
<td>5 ± 5</td>
<td>21 ± 19</td>
<td>21 ± 19</td>
<td>108</td>
</tr>
<tr>
<td>Carbon source</td>
<td>44 ± 2</td>
<td>2 ± 1</td>
<td>4 ± 1</td>
<td>19 ± 0.3</td>
<td>108 ± 4</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>108</td>
<td>149 ± 24</td>
</tr>
</tbody>
</table>

*Compared with total cell content after density gradient centrifugation.
†As determined by cytochemical stains (see Methods).
§P < .005 compared with PME.
\( \Delta P < .01 \) compared with PME.
*Surface markers performed in two experiments; one experiment for CD3 after nylon wool procedure.
observations are compatible with the known ability of M-CSF stimulated monocytes to produce colony stimulating activity as well as potential inhibitors of hematopoiesis. However, concentrations of monocytes that would be found in density gradient separated marrow had no significant effect on CFU-M. These observations suggest that factors other than monocytes or their products may be responsible for the ability of PME-treated marrow to support CFU-M.

A striking difference between marrow treated with PME as compared with nylon wool or carbonyl iron was the near elimination of myeloid cells by PME. Therefore, we speculated that a product of early myeloid cells may be responsible for CFU-M inhibition in non-PME-treated marrow. HL-60 cells were used to simulate early myeloid cells in further studies. We realized, however, that HL-60 cells may produce substances that are not present in normal myeloid cells. HL-60 conditioned media was found to be strongly inhibitory to CFU-M. Furthermore, the inhibitory effect of HL-60 conditioned media was specific in that it did not inhibit CFU-G or CFU-GM. Ongoing studies are being performed to isolate and identify this CFU-M inhibitory substance.

In summary, huM-CSF can stimulate colony formation by PME-treated marrow. Thus, M-CSF has definite colony stimulating activity on human marrow. The inability to detect CFU-M from non-PME-treated marrow may be attributed to inhibitors produced by myeloid cells or monocytes. The methods described in this report may be a valuable aid in the study of normal monocyte-macrophage production and monitoring the effects of colony stimulating factors in vivo.

**REFERENCES**

3. Zhou YQ, Stanley ER, Clark SC, Hatzfeld JA, Levesque JP, Federici C, Watt SM, Hatzfeld A: Interleukin-3 and interleukin-la for CFU-M. These observations suggest that factors other than monocytes or their products may be responsible for the ability of PME-treated marrow to support CFU-M.

**Fig 2.** Effect of HL-60 conditioned media on CFU-C. Asterisks indicate a significant difference compared with the previous concentration. Media alone (not conditioned with HL-60 cells) had no effect on CFU-M.

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