Biological Characterization of a Granulomonopoietic Enhancing Activity Derived From Cultured Human Lipid-Containing Macrophages

By Sheng-yuan Wang, Hugo Castro-Malaspina, Li Lu, and Malcolm A.S. Moore

We describe the biologic characteristics of an activity produced by human monocyte-derived lipid-containing cells (MDLCCs) that enhances the colony-forming capacity of granulocyte-macrophage progenitors (CFU-GM). Medium conditioned by well-developed MDLCCs (at day 21 to day 28 of cultivation) was added to bone marrow cultures containing GCT cell line-conditioned medium (GCT-CM) or other material as a source of granulocyte-macrophage colony-stimulating factors (GM-CSFs). MDLCC-conditioned medium (CM) had no detectable granulocyte-macrophage colony-stimulating activity (GM-CSA), but it contained an activity that enhanced the colony number in both day 7 and day 14 CFU-GM cultures. Dose-response curves for GCT-CM in the presence of MDLCC-CM demonstrated that this enhancing effect occurred at concentrations of GM-CSFs that stimulate maximal CFU-GM growth. This enhancing effect was seen with both granulocytic and monocytic progenitor cells. It was titratable and required the continuous presence of MDLCC-CM from initiation of culture. No enhancement was noted when MDLCC-CM was added 48 hours after plating. The enhancement still occurred when marrow cells were first incubated with MDLCC-CM and GCT-CM was added at later times. Neither the enhancing activity nor its production was dependent on horse serum contained in MDLCC culture medium. The enhancing effect was also seen when other sources of GM-CSA were used: medium conditioned by 5637 cell line, phytohemagglutinin-stimulated lymphocytes (PHAL), or placenta tissue. Furthermore, this enhancing activity appeared to be specific for CFU-GM. Addition of MDLCC-CM to mixed and erythroid cultures, stimulated by suboptimal and optimal concentrations of PHAL-CM did not modify the number of mixed colonies or erythroid bursts. This granulomonopoietic enhancing activity contained in MDLCC-CM was heat stable (56 °C and 75 °C for 30 minutes) and nondialyzable (3,500 and 14,000 molecular weight cut off tubing). Its production was increased by treating MDLCC with lipopolysaccharide (5 µg/mL) or zymosan (60 µg/mL) and inhibited by lactoferrin (10⁻⁷ mol/L). The production of a granulomonopoietic enhancing activity by MDLCCs represents the demonstration of another positive feedback regulator of myelopoiesis involving the monocyte-macrophage system.

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MACROPHAGES are found in human tissues in many forms, such as the alveolar macrophages of the lung, the liver Kupffer cells, the dermal Langerhans cells, the osteoclasts of bone, the histiocytes of connective tissues, peritoneal and pleural macrophages, and the free and fixed macrophages of hematopoietic tissues. Marrow macrophages are found near the vascular sinuses, where they have predominantly a phagocytic function, and at the center of erythroblastic islands, where they are presumed to have a regulatory function. Moreover, a small portion of the latter cells remain to be determined.

In vitro studies with isolated blood and marrow monocytes have demonstrated that these cells play a central role in the regulation of hematopoiesis. The proliferation and differentiation of cells of the granulocytic, monocytic, erythrocytic, megakaryocytic, and lymphocytic series are regulated by factors produced by monocytes-macrophages. Concerning the regulation of granulomonopoiesis, monocytes have been shown to have both a stimulatory and an inhibitory function. Blood monocytes and tissue macrophages have been identified as cells that are capable of producing granulocyte-macrophage colony-forming factors (GM-CSFs). Evidence also exists indicating that factors inhibiting colony formation, such as E-type prostaglandins and acidic isoferritins, are elaborated by monocytes-macrophages.

Studies in mouse and human long-term marrow cultures have shown that monocytes-macrophages are an essential component of the adherent layer supporting the self-replication and differentiation of hematopoietic stem cells. A small proportion of these cells are found as lipid-containing cells. We have recently described a method for culturing pure populations of monocyte-derived lipid-containing cells (MDLCCs), and have shown that these cells produce a new regulating activity that enhances the granulocyte-macrophage colony formation induced by GM-CSFs. In this study, we further describe the biologic charac-

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teristics and the production regulation of this granulocyte-macrophage colony-enhancing activity (GM-EA).

MATERIALS AND METHODS

Human Bone Marrow and Peripheral Blood Cells

Bone marrow and peripheral blood samples were taken from normal adult volunteers after informed consent had been obtained in compliance with hospital regulations established by the National Institutes of Health. Heparinized blood samples and bone marrow aspirates were centrifuged at 200 g for ten minutes. Buffy coat cells were pipetted off, washed twice, and resuspended in McCoy's 5A medium (Flow Laboratories, Rockville, Md).

Cell Separation Procedures

Density centrifugation. Washed buffy coat cells were separated by a "density cut" procedure35 into buoyant and dense fractions by centrifuging the cells in an isotonic Percoll solution (Pharmacia Fine Chemicals, Piscataway, N.J.) of density of 1.074 g/mL and 270 mosm at 3,500 g for ten minutes at 4 °C. Mononuclear cells devoid of red cells and mature granulocytes were recovered in the buoyant cell fraction, washed twice, and resuspended in growth medium.

Adherence. Low-density cells from peripheral blood or bone marrow were resuspended in McCoy's 5A medium supplemented with 15% fetal calf serum (Seralle System Inc., Logan, Utah) and allowed to adhere to the bottom of T25 or T75 culture flasks (Corning Glass Works, Corning, NY) or 35-mm Petri dishes (Lux Scientific Corp., Newbury Park, Calif.) at 37 °C. After 90 minutes of incubation, nonadherent cells were removed, washed twice with medium, and subsequently used for CFU-GM assay. Adherent cells were also washed twice with medium and cultured for MDLCCs.

Culture of Monocyte-Derived Lipid-Containing Cells

A detailed description of the method is given elsewhere.24 Briefly, adherent cells from blood or bone marrow were cultured in alpha medium containing 20% horse serum (HoS; GIBCO, Grand Island, NY) at 37 °C in a humidified atmosphere of 5% CO2 in air. The culture medium was totally changed every three to four days. To remove fibroblasts from the adherent layer derived from bone marrow cells at the seventh to tenth day of cultivation, adherent cells were treated with a solution of trypsin-EDTA (0.05% trypsin and 0.02% EDTA) (Flow Laboratories) for ten minutes at 37 °C. This treatment allowed the detachment of fibroblasts without detaching mononuclear cells from the plastic surface. After removal of fibroblasts, the remaining adherent cells were washed twice and cultured again in alpha medium containing 20% HoS. This remaining adherent cell population was composed of monocyteic cells and was free of fibroblastic cells and endothelial cells, as demonstrated by immunofluorescence staining with two monoclonal antibodies recognizing monocytic cells, antibodies against lysozyme, type III and type IV collagen, and factor VIII-associated protein.24,36 Adherent monocytic cells progressively enlarged up to seven to eight times their original size at days 21 through 28. This enlargement was associated with an increase in lipid content, as shown by oil red O staining and electron microscopy analysis.24

Preparation of MDLCC-Conditioned Medium

Serum-free alpha medium or alpha medium supplemented with 20% HoS was conditioned for three days by MDLCCs. The serum-free MDLCC-conditioned medium (MDLCC-CM) used in this study was prepared on days 21 through 23 of cultivation. After collection, the conditioned medium was passed through a Millipore filter (Millipore Corp., Bedford, Mass) and stored at -60 °C until assayed. Medium conditioned by pure populations of well-characterized passaged marrow fibroblasts,29 which was used in some experiments as a control, was prepared in the same manner.

To concentrate serum-free MDLCC-CM as well as GCT-CM, 50 mL of these conditioned media were placed into two separate dialysis membrane tubing (molecular weight [mol wt] cutoff 3,500) (Spectrum Medical Industries, Inc, Los Angeles), which were then covered with Carbowax polyethylene glycol 8,000 powder (Fisher Scientific Co, Springfield, NJ) and left at 4 °C. The concentrated volumes were then transferred to test tubes, and the volume was adjusted to 5 mL. The concentrated materials were Millipore-filtered and stored at -20 °C until testing.

To test the heat stability of activities contained in MDLCC-CM, aliquots were incubated in a water bath at 56 °C for 30 minutes, at 75 °C for 30 minutes, and at 100 °C for five minutes. To estimate the molecular size, aliquots were dialyzed against three changes of phosphate-buffered saline (PBS) at 4 °C using membrane tubing (Spectrum Medical Industries) with pores of different size (mol wt cutoff 3,500 and 12,000 to 14,000).

To assess the effect of lipopolysaccharide (LPS), zymosan, or lactoferrin (LF) on the production of enhancing activity, a final concentration of 5 µg/mL of LPS (DIFCO Laboratories, Detroit), 60 µg/mL of zymosan A (Sigma Chemical Co, St Louis), or 10-7 mol/L of LF (kindly provided by Drs H. Broxmeyer and J. Bognacki, Memorial Sloan-Kettering Cancer Center) was added to the freshly changed culture medium at day 20, and the medium conditioned by drug-treated MDLCCs was collected at day 23.

Sources of Granulocyte-Macrophage Colony-Stimulating Activity

Four sources of human granulocyte-macrophage colony-stimulating activity (GM-CSA) were used: medium conditioned by GCT human monocyte cell line (GCT-CM),28 medium conditioned by the 5637 cell line derived from a human bladder cancer (5637-CM),29 medium conditioned by human placenta (placenta-CM), and medium conditioned by 1% PHA-stimulated human blood lymphocytes (PHAL-CM).

Assay for Granulocyte-Macrophage Colony-Forming Cells (CFU-GM)

Fifty thousand nonadherent low-density human bone marrow cells were plated in a 1-mL layer of 0.3% agar (Difco Laboratories) in supplemented McCoy's 5A medium containing 10% heat-inactivated fetal calf serum. Colony formation was stimulated by the inclusion of 10% GCT-CM (vol/vol) or other sources of GM-CSA. MDLCC-CM, at a final concentration of 10% (vol/vol), was added directly to the culture dishes before the addition of the bone marrow cell suspension or as indicated. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air and scored for colonies and clusters after seven and 14 days of incubation.

Morphological examination of colonies and clusters was performed in situ after fixation with 5% glutaraldehyde, dehydration with methanol, and staining with Harris' hematoxylin.30

Counting of the Total Number of Nucleated Cells in CFU-GM Cultures

Eighty thousand nonadherent low-density bone marrow cells were resuspended in 1 mL of 1% methylcellulose culture medium containing 10% heat-inactivated fetal calf serum. After seven days of incubation, methylcellulose was dissolved in a solution of 0.05% EDTA in PBS and separated from the cells by centrifugation.31
cell pellet was then resuspended in medium and the cell number was determined in a hemocytometer.

**Assay for Multipotential (CFU-Mix) and Erythroid (BFU-E) Colony-Forming Cells**

The colony assay for CFU-Mix was carried out according to the procedure of Fauser and Messner as described. Bone marrow cells were plated in 35-mm Lux standard tissue culture dishes containing a 1-mL mixture of Iscove's modified Dulbecco's medium (GIBCO); 1% methylcellulose; 30% fetal calf serum; 1%, 2.5%, or 5% medium-conditioned by leukocytes from patients with hemochromatosis in the presence of 1% phytohemagglutinin (HA-15, Wellcome Research Laboratories, Detroit) and 5 x 10^{-3} mol/L 2-mercaptoethanol. MDLCC-CM in a volume of 0.1 mL was added to the culture dishes before the addition of the bone marrow cell suspension. Hemin, which is known to enhance CFU-Mix and BFU-E colony formation, was used in some experiments as a control. Bovine hemin (Eastman Kodak Co, Rochester, NY) was prepared in 0.5 mol/L NaOH and then neutralized with 1 N HCl, diluted with fresh culture medium, and used at 0.1 mmol/L final concentration. One unit of erythropoietin (Connaught Laboratories, Willowdale, Ontario, Canada) was added to each dish on day 0. Dishes were incubated at 37 °C in a humidified atmosphere flushed with 5% CO2 in air for 14 days. Colonies were scored with a fine pipette, putting on a glass slide, and staining with benzidine in the dishes or after picking out colonies with 5% medium conditioned by leukocytes from patients with hemochromatosis. BFU-E were scored from the same plates. In some cases, BFU-E were scored from plates cultured according to the method of Iscove and Sieber.

**RESULTS**

**Effect of MDLCC-CM on Granulocyte-Macrophage Colony Formation**

Figure 1 shows the average results of eight separate experiments in which the effect of MDLCC-CM on day 7 and day 14 granulocyte-macrophage colony formation was tested. No colony formation was induced by MDLCC-CM alone, but a net enhancement of the colony formation by both day 7 and day 14 granulocyte-monocyte progenitors was noted when MDLCC-CM was added together with GCT-CM. Plateau levels of GCT-CM were used in these experiments. The percentage of enhancement ranged from 50 to 150 in individual experiments, with a mean value of 80 ± 10 (mean ± 1 SEM). This enhancement was not seen when marrow fibroblast-conditioned medium was used instead of MDLCC-CM (results not shown).

To rule out that this enhancing activity was due merely to the use of suboptimal concentrations of the source of GM-CSF, or to the additive effect of low concentrations of GM-CSF contained in MDLCC-CM, dose-response curves for unconcentrated and tenfold concentrated GCT-CM, as well as experiments using tenfold concentrated MDLCC-CM, were performed. As shown in Fig 2, plateau levels of GM-CSF contained in GCT-CM, as well as tenfold concentrated GCT-CM (Δ) form were devoid of any GM-CSF activity. MDLCC-CM enhanced the CFU-GM colony-formation capacity of suboptimal and optimal concentrations of GCT-CM. Note that the GM-CSF capacity of 0.1 mL of unconcentrated GCT-CM (left panel) was already within the optimal concentration range. Moreover, use of tenfold concentrated GCT-CM (right panel) did not result in the abrogation of the enhancing effect of MDLCC-CM.
were used in our experiments with undiluted GCT-CM. Moreover, use of tenfold concentrated GCT-CM did not result in abrogation of the enhancing activity of undiluted MDLCC-CM. Similarly to the unconcentrated material, the tenfold concentrated MDLCC-CM was also devoid of any GM-CSF activity.

To confirm that MDLCC-CM enhanced the colony numbers and therefore the total number of nucleated cells per dish, similar experiments were performed in a methylcellulose culture system, which facilitates cell harvesting. Figure 3 shows the results of three experiments. An increase in the total cell number was seen in dishes that contained both CSF and MDLCC-CM.

To rule out the possibility that horse serum and not the conditioned medium contained some enhancing activities, experiments were done comparing MDLCC-CM with and without horse serum (Fig 4). Unconditioned media supplemented with horse serum as well as serum-free or horse serum-supplemented MDLCC-CM had no colony-stimulating capacity. Addition of unconditioned media supplemented with horse serum to cultures stimulated by GCT-CM did not result in an enhancement of granulocyte-macrophage colony formation. In contrast, colony numbers markedly increased after the addition of MDLCC-CM with or without horse serum. Moreover, the enhancing effect of serum-free conditioned media was not different from the ones containing serum.

Effect of MDLCC-CM on Different Sources of Human GM-CSA

The MDLCC-CM had an enhancing effect on the granulocyte-macrophage colony formation induced by several sources of GM-CSA. It enhanced not only the colony-stimulating activity of GCT-CM, but also that of 5637-CM, placenta-CM, and PHAL-CM. This enhancing effect was seen in both day 7 and day 14 colonies. The average percentage of enhancement for the different sources of GM-CSA ranged from 60 to 90% (Table I).

Analysis of Individual Colony Morphology

To determine whether the enhancement of the colony formation was related to alterations in the distribution of granulocytic, monocytic, and mixed colonies, the morphology of individual colonies was determined (Table 2). The colony type distribution for the various sources of GM-CSA was different. The percentage of granulocytic colonies (including eosinophil colonies) was about 60% in cultures stimulated by GCT-CM, whereas in cultures stimulated by 5637-CM or placenta-CM, it was greater (Table 2). There was no change in the distribution of colony types when MDLCC-CM was added in addition to each source of GM-CSA listed in Table 2. Thus, the enhancing activity of MDLCC-CM acted on both granulocytic and monocytic progenitors.
GRANULOMONOPOIETIC ENHANCING ACTIVITY

Table 1. Effect of MDLCC-CM on Human GM-CSA From Different Sources

<table>
<thead>
<tr>
<th>Sources of GM-CSA</th>
<th>Day 7</th>
<th>( \Delta % )</th>
<th>Day 14</th>
<th>( \Delta % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5637-CM</td>
<td>84 ± 4</td>
<td>-36 ± 4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5637-CM + MDLCC-CM</td>
<td>143 ± 7</td>
<td>+70</td>
<td>66 ± 6</td>
<td>+83</td>
</tr>
<tr>
<td>Placenta-CM</td>
<td>68 ± 4</td>
<td>-51 ± 4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Placenta-CM + MDLCC-CM</td>
<td>129 ± 11</td>
<td>+89</td>
<td>86 ± 7</td>
<td>+68</td>
</tr>
<tr>
<td>PHAL-CM</td>
<td>110 ± 5</td>
<td>-41 ± 4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PHAL-CM + MDLCC-CM</td>
<td>176 ± 6</td>
<td>+60</td>
<td>72 ± 5</td>
<td>+75</td>
</tr>
</tbody>
</table>

Nonadherent low-density human marrow cells were plated in a concentration of 5 \( \times \) 10^6 cells per dish.

*Percentage of change from CSA alone.

Table 2. Morphology of Individual Colonies

<table>
<thead>
<tr>
<th>Source of GM-CSA</th>
<th>Day 7 Colonies (%)</th>
<th>Day 14 Colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td>GCT-CM</td>
<td>57 ± 2</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>GCT-CM + MDLCC-CM</td>
<td>59 ± 2</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>5637-CM</td>
<td>71 ± 1</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>5637-CM + MDLCC-CM</td>
<td>69 ± 1</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>Placenta-CM</td>
<td>74 ± 3</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>Placenta-CM + MDLCC-CM</td>
<td>72 ± 3</td>
<td>17 ± 1</td>
</tr>
</tbody>
</table>

Results are expressed as means ± 1 SEM. G, granulocytic; M, monocytic; Mix, granulocytic and monocytic.

**Titration of the Enhancing Activity Contained in MDLCC-CM**

A typical dose-response curve was noted when dilutions of MDLCC-CM were added to CFU-GM cultures (Fig 5). The enhancing activity was detectable at fairly high dilutions (1:128), although a 50% reduction in activity was seen at 1:4 dilution.

**Relationship Between the Enhancing Effect and the Time of Addition of MDLCC-CM**

Aliquots of MDLCC-CM were added to CFU-GM cultures at the time of plating (0 hours) and at 12, 24, and 48 hours after plating. As shown in Fig 6, the enhancing effect on colony formation was maximum when MDLCC-CM was added at time 0, and it declined thereafter. No enhancing effect was noted when MDLCC-CM was added 48 hours after plating. This phenomenon occurred in both day 7 and day 14 colony formation.

To test whether the same enhancement of CFU-GM colonies occurred when marrow cells were first incubated with MDLCC-CM and then with GCT-CM added at later times, marrow cells were plated in agar culture medium with MDLCC-CM or medium control. Then GCT-CM was introduced to the cultures at time 0, 12, 24, and 48 hours later, and colonies were counted after seven more days of incubation. Results of a representative experiment are shown in Table 3. Although there was a progressive decline in the number of detectable CFU-GM, the enhancing influence of MDLCC-CM was still present 48 hours after initiation of cultures.

**Effect of MDLCC-CM on Mixed (CFU-Mix) and Erythroid (BFU-E) Colony Formation**

To test the effect of MDLCC-CM on the formation of mixed colonies and erythroid bursts by the putative multipotent hematopoietic stem cells (CFU-Mix) and early erythroid progenitors (BFU-E), respectively, aliquots of MDLCC-CM were added to methylcellulose cultures containing erythropoietin and suboptimal or optimal concentrations of PHAL-CM as a source of pluripoietin or burst-promoting activity (BPA). Results of a representative experiment are shown in Table 4. In absence of PHAL-CM, MDLCC-CM was not capable of stimulating mixed or erythroid colony formation, and when present, even at suboptimal con-
Fig 6. Relationship between the enhancing effect and the time of addition of MDLCC-CM. Aliquots of MDLCC-CM were added to CFU-GM cultures at the time of plating (0 hour), and at 12, 24, and 48 hours after plating. Colonies and clusters were scored at day 7 and day 14 of incubation. Results are expressed as the percentage of enhancement of CFU-GM formation.

Table 3. Enhancement of Granulocyte-Macrophage Colony Formation After Preincubation of Marrow Cells With MDLCC-CM in Ager Culture Medium

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Number of CFU-GM</th>
<th>Δ%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MDLCC-CM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GCT-CM</td>
<td>189 ± 12</td>
<td>100</td>
</tr>
<tr>
<td>MDLCC-CM + GCT-CM (T₁₂)</td>
<td>270 ± 11</td>
<td>43</td>
</tr>
<tr>
<td>Medium Control + GCT-CM (T₁₂)</td>
<td>143 ± 9</td>
<td>0</td>
</tr>
<tr>
<td>MDLCC-CM + GCT-CM (T₂₄)</td>
<td>200 ± 9</td>
<td>40</td>
</tr>
<tr>
<td>Medium Control + GCT-CM (T₂₄)</td>
<td>127 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>MDLCC-CM + GCT-CM (T₄₈)</td>
<td>182 ± 9</td>
<td>43</td>
</tr>
<tr>
<td>Medium Control + GCT-CM (T₄₈)</td>
<td>110 ± 7</td>
<td>0</td>
</tr>
<tr>
<td>MDLCC-CM + GCT-CM (T₄₈)</td>
<td>157 ± 15</td>
<td>41</td>
</tr>
</tbody>
</table>

Six x 10⁶ low-density nonadherent marrow cells per dish were plated. MDLCC-CM or medium control was incorporated into the cultures at the time of initiation of cultures. GCT-CM was added at the time of plating or 12, 24, or 48 hours later. Colonies and clusters were scored after seven days of incubation. Δ%, percentage of change from control cultures.

Table 4. Effect of MDLCC-CM on BFU-E and CFU-Mix

<table>
<thead>
<tr>
<th>Test Material</th>
<th>BFU-E</th>
<th>CFU-Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1% PHAL-CM</td>
<td>28 ± 1</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>2.5% PHAL-CM</td>
<td>89 ± 3</td>
<td>5.4 ± 0.9</td>
</tr>
<tr>
<td>5% PHAL-CM</td>
<td>141 ± 8</td>
<td>9.3 ± 0.9</td>
</tr>
<tr>
<td>Hemin</td>
<td>7 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>1% PHAL-CM + Hemin</td>
<td>28 ± 1</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>2.5% PHAL-CM + Hemin</td>
<td>89 ± 3</td>
<td>5.4 ± 0.9</td>
</tr>
<tr>
<td>5% PHAL-CM + Hemin</td>
<td>141 ± 8</td>
<td>9.3 ± 0.9</td>
</tr>
<tr>
<td>MDLCC-CM</td>
<td>10 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>1% PHAL-CM + MDLCC-CM</td>
<td>25 ± 2</td>
<td>0.3 ± 0.7</td>
</tr>
<tr>
<td>2.5% PHAL-CM + MDLCC-CM</td>
<td>52 ± 4</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>5% PHAL-CM + MDLCC-CM</td>
<td>75 ± 3</td>
<td>5.3 ± 0.7</td>
</tr>
</tbody>
</table>

Number of colony-forming cells expressed as mean ± SEM per 10⁶ cells plated in each dish. Low-density (<1.077 g/mL) normal marrow cells were used. Final concentration of hemin was 0.1 mmol/L and MDLCC-CM 10%.

*Significant at 5% level.

The enhancing activity of MDLCC-CM was relatively heat resistant. No changes occurred after heat treatment at 56 °C or 75 °C for 30 minutes, and only 15% of the enhancing activity was lost after exposure of MDLCC-CM at 100 °C for five minutes (Table 5).
Low-density human bone marrow cells were plated, and colonies and clusters were scored after seven days. Compare with control group: GCT + untreated MDLCC-CM.

Fig 7. Effect of IPS, zymosan (Zym), and LF on the production of enhancing activity by MDLCCs. MDLCC-CM were prepared in the absence (C-MDLCC-CM) or presence of IPS (IPS-MDLCC-CM), zymosan (Zym-MDLCC-CM), or LF (LF-MDLCC-CM). These CM were tested for enhancing activities in CFU-GM cultures. IPS, zymosan, or LF did not influence the effect of GCT-CM, but IPS (5 μg/mL) or zymosan (60 μg/mL) markedly increased the production of the enhancing factor. LF (10^{-7} mol/L) inhibited its production.

**DISCUSSION**

In this study, we have analyzed in vitro the role of MDLCCs in the regulation of myelopoiesis. Our data indicate that MDLCCs induced and maintained in liquid cultures produce an activity that enhances the granulocyte-macrophage colony formation induced by a wide variety of GM-CSF preparations. This enhancing influence is directed toward granulocytic as well as monocytic colony forming cells and results in increasing the number of both day 7 and day 14 CFU-GM. This enhancing activity appears to be specific for granulocyte-macrophage progenitor cells. It is nondialyzable and heat-stable. Its production can be increased by LPS or zymosan and inhibited by LF.

Cells of the monocyte-macrophage system have been shown to play an important role in the control and regulation of myelopoiesis. The production of CSF results in the clonal proliferation of myeloid cells, whereas the production of prostaglandin E constitutes a negative feedback mechanism inhibiting myelopoiesis. Our report on the production of a granulopoietic enhancing activity (GM-EA) represents the demonstration of another positive feedback regulator of myelopoiesis involving the monocyte-macrophage system.

The enhancing effect seen in CFU-GM cultures containing MDLCC-CM was not related to the use of suboptimal concentrations of GM-CSF or to the additive effect of low concentrations of GM-CSF contained in MDLCC-CM or GM-CSF produced by some cells constituting the target cell population. First, before using the GCT-CM as a source of GM-CSF, titration experiments clearly showed that the amount of GCT-CM used in our experiments provided CM-CSF levels that were capable of stimulating maximal CFU-GM growth. Furthermore, use of concentrated GCT-CM did not result in abrogation of the enhancing effect of MDLCC-CM. Second, bone marrow cells used for the CFU-GM assays were depleted of adherent cells to eliminate the endogenous CSF production. Third, the MDLCC-CM used in our experiments, which were collected between day 21 and day 28 of culture, were devoid of GM-CSF activity. This was confirmed using tenfold concentrated MDLCC-CM (Fig 2). Furthermore, we have preliminary evidence that partially purified MDLCC-CM collected at this time of cultivation has no GM-CSF activity (H. Castro-Malaspina, H. Welte, and E. Levy, Memorial Sloan-Kettering Cancer Center, unpublished observations, 1984). This lack of GM-CSFs in medium conditioned by monocytic cells, which are known to produce this growth factor, is rather surprising. However, we have previously shown that although the production of GM-EA by cultured monocytic cells progressively increases, the production of GM-CSF, which is maximal at the beginning of the incubation period, rapidly declines to undetectable levels. Moreover, Sullivan et al., studying the kinetics of the synthesis and secretion of GM-CSF by human monocytes stimulated by LPS,

<table>
<thead>
<tr>
<th>Conditioned Medium and Treatment</th>
<th>Number of CFU-GM/5 x 10^5 cells (Mean ± 1 SEM)</th>
<th>% Residual Activity (Exp/Control × 100)</th>
<th>P *</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCT</td>
<td>110 ± 3</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>MDLCC-CM, untreated</td>
<td>0.5 ± 0.2</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>GCT + MDLCC-CM, untreated (control)</td>
<td>188 ± 7</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>GCT + MDLCC-CM, dialyzed (mol wt cutoff 3,500)</td>
<td>178 ± 8</td>
<td>95 &gt;.1</td>
<td></td>
</tr>
<tr>
<td>GCT + MDLCC-CM, dialyzed (mol wt cutoff 12,000 to 14,000)</td>
<td>177 ± 7</td>
<td>94 &gt;.1</td>
<td></td>
</tr>
<tr>
<td>GCT + MDLCC-CM, heated 56 °C, 30 min</td>
<td>190 ± 9</td>
<td>101 &gt;.1</td>
<td></td>
</tr>
<tr>
<td>GCT + MDLCC-CM, heated 75 °C, 30 min</td>
<td>183 ± 6</td>
<td>97 &gt;.1</td>
<td></td>
</tr>
<tr>
<td>GCT + MDLCC-CM, heated 100 °C, 5 min</td>
<td>160 ± 6</td>
<td>85 &lt;.01</td>
<td></td>
</tr>
</tbody>
</table>

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Low-density human bone marrow cells were plated, and colonies and clusters were scored after seven days.

*Compare with control group: GCT + untreated MDLCC-CM.
found that the secretion of GM-CSF by these cells was initially copious, then steadily decreased after 24 hours, and ceased after 72 hours.

The granulomonopoietic enhancing activity present in MDLCC-CM was not related to enhancing factors present in the serum of the growth medium. It has been reported that the serum of human and certain other species contains a growth-promoting factor(s) for murine CFU-GM. The question thus arose as to whether the enhancing effect of MDLCC-CM was caused to some extent by serum. In our studies, horse serum was used for the culture of MDLCCs. Using GCT-CM as a source of CSA, the addition of horse serum into CFU-GM assays did not enhance the GCT-CM as a source of CSA, the addition of horse serum was used for the culture of MDLCCs. Using whether the enhancing effect of MDLCC-CM was initially copious, then steadily decreased after 24 hours, and ceased after 72 hours.

The enhancing activity of MDLCC-CM appears to be different from GM-CSFs. Several subclasses of CSFs are known to exist, notably, GM-CSF, macrophage CSF (M-CSF), neutrophil CSF (G-CSF), and eosinophil CSF (Eo-CSF). Using biochemical and immunologic methods, two distinct types of CSFs (CSF-I and CSF-II) have further been isolated from a variety of human tissues and cultured cancer cells. Whereas type II stimulates both day 7 and day 14 colonies, type I stimulates only the latter. It thus becomes important to dissociate GM-EA from the various CSFs, particularly CSF-I, the presence of which may not be detectable in routine CFU-GM assays. Nevertheless, it is unlikely that GM-EA is identical to any of the subclasses of CSFs, as the enhancement is not limited to any particular type of colonies and GM-EA itself has no colony-stimulating activity on day 7 or day 14 CFU-GM. Moreover, CSF-I is labile at 75 °C and totally inactivated by heating at temperatures over 90 °C, whereas GM-EA is stable at 75 °C and only partially loses its activity after exposure to 100 °C.

The MDLCC-derived enhancing activity appears to act specifically on the progenitors of granulocytes and monocytes without influencing the proliferation and differentiation of other hemic progenitor cells, particularly early erythroid stem cells and the putative pluripotent hematopoietic stem cells. Our results clearly show that MDLCC-CM does not stimulate erythroid and mixed colony formation. This indicates that MDLCCs do not produce erythroid BPA or the factor(s) promoting the growth of human mixed colonies or interleukin-3 (IL-3), and that the enhancing activity elaborated is specific for granulomonocytic colonies. Recent studies have shown that monocytes-macrophages are one of the sources of BPA. It was therefore intriguing that MDLCC-CM had no detectable BPA. We have previously shown that BPA can be detected in monocyte-conditioned medium at early stages of cultivation. Similar to GM-CSF production, it is apparent that BPA production declines progressively, and it is not detectable at the time GM-EA production reaches its peak.

The mechanism of action of GM-EA is yet to be determined, but several possibilities exist. It is conceivable that GM-EA either enhances the cloning efficiency of CFU-GM or increases the pool of granulocyte-macrophage progenitors responsive to GM-CSFs. GM-EA may enhance the responsiveness of the CFU-GM to the action of GM-CSFs by modifying the number or the affinity of CSF receptors on the cell membrane of CFU-GM. Our results suggest that this is not limited to a certain type of GM-CSF, since a similar enhancing effect was observed using several sources of this growth factor. Alternatively, GM-EA may increase the pool of CFU-GM responding to GM-CSFs by stimulating the proliferation and differentiation of an earlier progenitor. There is evidence in both the human and the murine systems that the transit through the CFU-GM pathway in culture require two distinct growth factors: the first one acting on a CFU-GM progenitor that appears not to respond in culture to GM-CSFs, and the second acting on progenitor cells expressing CSF receptors. Our results of preincubation experiments with MDLCC-CM suggest that GM-EA enhances the production of granulocyte-macrophage colonies by stimulating a "pre-CFU-GM." Studies are underway to delineate the actual mechanism of enhancement by GM-EA.

Several similarities are seen between the enhancing activity in human serum and the enhancing activity in MDLCC-CM. Both selectively enhance cells capable of forming granulocyte and macrophage colonies, and both are heat-stable and nondialyzable. It is likely that GM-EA produced by MDLCCs in vitro is the same as the enhancing activity seen in human serum. Thus, what is observed here in vitro may have significant in vivo relevance.

The present observations provide evidence that human lipid-containing macrophages produce a humoral factor that may play an important role in the regulation of human granulomonopoiesis. The availability of a human source of GM-EA should facilitate the biochemical characterization and purification of this new regulator and allow the characterization of the responding cell population.
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Biological characterization of a granulomonopoietic enhancing activity derived from cultured human lipid-containing macrophages

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