# Detection of the Target Progenitor Cells of Granulomonopoietic Enhancing Activity

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**Macrophage-derived granulomonopoietic enhancing activity (GM-EA) is a novel mediator that amplifies colony formation of myeloid progenitor cells (CFU-GM) in conjunction with colony-stimulating factors (CSFs), and is distinct from other hematopoietic synergizing factors such as interleukin (IL)-1, IL-4, and IL-6. In the present study, we try to ascertain whether or not there is a GM-EA–specific responsive myeloid progenitor cell population. Human bone marrow cells deleted of adherent cells and T lymphocytes were separated by velocity sedimentation into three subpopulations with respective sedimentation rates (millimeters per hour) of 7.4 ± 0.4, 6.0 ± 0.6, and 4.7 ± 0.3. These subpopulations corresponded to the day 7 CFU-GM, day 14 CFU-GM, and the earlier myeloid progenitor cells, pre–CFU-GM, respectively. Pre–CFU-GM failed to respond to the colony-inducing effect of GM-CSF but could be stimulated by GM-EA alone to generate small clusters (5 to 25 cells) in soft agar after 14 days of incubation. Correspondingly, suspension preculture of the fractionated bone marrow cells also showed that only the progenitor cells with low sedimentation rate (4.7 mm/h) could be activated by GM-EA to generate CFU-GM. Taken together, our results suggest that the specific target cell of GM-EA is the pre–CFU-GM, and that GM-EA acts on these cells as a growth/maturation factor, but on the day 7 and day 14 CFU-GM as a synergistic growth factor.**



MONOCYTOID CELLS can constitutively produce colony-stimulating factor(s) (CSFs) that induce the proliferation and/or differentiation of the myeloid progenitor cells (CFU-GM),[1,2] and they can also secrete negative feedback mediators such as prostaglandin E and acid isoferritins, which are potent inhibitors of CFU-GM colony formation.[3,4] In addition, other monokines such as interleukin-1$\beta$ (IL-1$\beta$)[5,6] IL-6,[7-9] and tumor necrosis factor-$\alpha$[10,11] also play important roles in the regulation of myelopoiesis.

Recently, we have shown that pure population of human monocyte-derived lipid-containing macrophages (MDLMs) produce a novel myelopoietic regulator designated granulomonopoietic enhancing activity (GM-EA),[12] which can promote the clonal growth of CFU-GM in the presence of various sources of CSFs.[13] GM-EA is a glycoprotein with an apparent molecular weight of 73 to 74 Kd.[14] This enhancing factor is distinct from other hematopoietic synergizing factors (IL-1, IL-4, and IL-6) in biochemical and functional properties, and its production and function are regulated by different mediators involved in myelopoiesis.[14,15] The action of GM-EA is myeloid lineage-specific and has no effect on either erythroid (BFU-E) or pluripotent (CFU-GEMM) progenitor cells.[13] In this study, we further extend our investigation to elucidate the nature and properties of specific target cells of GM-EA, and our results suggest that GM-EA acts on an earlier subpopulation of myeloid progenitor cells, pre–CFU-GM, as a growth/maturation factor, and on day 7 and day 14 CFU-GM as a growth-promoting factor.

## MATERIALS AND METHODS

*Reagents.* Recombinant human (rh) CSFs, including rG-CSF (activity: $1 \times 10^9$ CFU/mg), rM-CSF (activity: $1 \times 10^8$ CFU/mg), rGM-CSF (activity: $5 \times 10^7$ CFU/mg), and rIL-3 (activity: $1 \times 10^8$ CFU/mg) were all purchased from Genzyme Corporation (Boston, MA). Step III preparation of sheep erythropoietin was obtained from Connaught Laboratories (Willowdale, Ontario, Canada). rhIL-1$\beta$ (activity: $1 \times 10^8$ U/mg), IL-4 (activity: $1 \times 10^8$ U/mg), IL-6 (activity: $1 \times 10^7$ U/mg), polyclonal rabbit anti-human IL-1 (1.25 mg/mL), anti–IL-4 (1 mg/mL), and anti–IL-6 (1 mg/mL) were also provided by Genzyme. All reagents were stored at −70°C in small aliquots until use.

*Preparation of LNAT$^-$ bone marrow cells.* Normal human bone marrow cells were separated by density gradient centrifugation in a Ficoll-Hypaque solution (1.077 g/mL) at 400*g* for 30 minutes. Low-density cells recovered at the interface were resuspended in $\alpha$-minimal essential medium ($\alpha$-MEM) containing 15% fetal calf serum (FCS) and then cultured in $T_{75}$ culture flasks (Corning Glass Works, Corning, NY) at 37°C. After 90 minutes of incubation, the nonadherent cells were collected, washed, and further depleted of T lymphocytes by rosetting with aminoethylisothiuronium bromide (AET)-treated sheep red blood cells.[16] The low-density nonadherent and T-cell–depleted (LNAT$^-$) bone marrow cells obtained usually contained less than 4% T cells as determined by immunofluorescence using monoclonal antibodies OKT3 and OKT11 (Ortho Diagnostic System, Raritan, NJ).

*Preparation and purification of GM-EA.* Conditioned medium from MDLMs (source of GM-EA) was prepared by culturing day 21 to 24 human macrophages in serum-free medium as described previously.[12,13] The GM-EA containing supernatants were collected after incubation at 37°C in 5% $CO_2$ for 3 days, and stored at −70°C until use.

Purification of GM-EA was performed by anion-exchange chromatography and reverse-phase high performance liquid chromatography as described in detail elsewhere.[14] The purified GM-EA is a glycoprotein with an apparent molecular weight of 73 to 74 Kd. Its activity is about $10^6$ U/mg protein. One unit of activity is defined as the amount of GM-EA that can induce 20% enhancement of granulocyte-macrophage (GM) colony formation in cultures plated with $5 \times 10^4$ low-density nonadherent bone marrow cells in the presence of 100 U of rGM-CSF.

*Preparation of rabbit anti–GM-EA antiserum.* Aliquots of purified GM-EA (200 $\mu$g/0.1 mL) were emulsified in equal volumes of Freund's complete adjuvant and injected intramuscularly into three rabbits. The animals were boosted with two additional injections of GM-EA in Freund's incomplete adjuvant at the fourth and

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sixth weeks after the primary immunization. One week following the last injection, antisera were collected and partially purified by diethyl aminoethyl anion-exchange chromatography. One milligram of anti–GM-EA immunoglobulin G [IgG] could neutralize approximately 10,000 U of GM-EA activity in the CFU-GM assay.

*Reactivity of anti–GM-EA to various synergizing factors.* The cytokine specificity of anti–GM-EA was determined using a solid-phase enzyme-linked immunoassay (EIA). Briefly, 10 pg/well of each of the synergizing factors (GM-EA, rIL-1, rIL-4, and rIL-6) was incubated with various dilutions of anti–GM-EA (1 mg/mL), and nonimmuned rabbit serum was included as the negative control. After 1 hour of incubation at 37°C, a second antibody of alkaline phosphatase-conjugated goat anti-rabbit antiserum (Sigma Co, St Louis, MO) was added, and the results were then read with an enzyme-linked immunosorbent assay reader (Bio-Tek Instrument, Winooski, VT).

In addition, rabbit antisera against IL-1, IL-4, and IL-6 were allowed to react with GM-EA in parallel in the same manner.

*Assay for IL-1, IL-4, and IL-6.* Crude and purified GM-EA (2 to 4 U/mL) preparations were assayed for the presence of IL-1, IL-4, and IL-6 by commercially available assay kits (Genzyme) using a solid-phase EIA as described by the manufacturer.

*Assay for CFU-GM.* CFU-GM was assayed using a soft agar culture method as previously described.[13,14] Briefly, 5 $\times 10^4$ low-density and nonadherent human bone marrow cells were plated in a 1-mL layer of 0.3% agar in McCoy's 5A medium (GIBCO Lab, Grand Island, NY) containing 10% heat-inactivated FCS, essential and nonessential amino acids, vitamins, and pyruvate. Colony formation was induced by the addition of 100 U/dish of human rGM-CSF (or rG-CSF/rM-CSF/rIL-3) in the presence or absence of purified GM-EA (4 U/dish). Cultures were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Colonies and clusters were scored after 7 and 14 days of incubation.

In some experiments, enhancement of G-M colony formation was induced by combinations of rGM-/rG-CSF (100 U/mL) with rIL-1/rIL-4/rIL-6 (100 U/mL), and the blocking effects of various anti-enhancing factor antisera were assessed by preincubation of the enhancing factors (4 U/mL of GM-EA, 100 U/mL of rIL-1/rIL-4/rIL-6) with their respective antibodies (5 NU/mL of anti–GM-EA, 100 NU/mL of anti–IL-1/anti–IL-4/anti–IL-6) at 37°C for 90 minutes before addition to the CFU-GM assay cultures.

Morphologic observation of colonies and clusters were performed in situ as described previously.[13]

*Assay for CFU-GEMM.* CFU-GEMM was assessed by clonal culture as described by Lu et al.[17] Colony formation of pluripotent progenitor cells was induced by the addition of various concentrations (20 to 100 U/dish) of rIL-3 in the presence or absence of GM-EA (4 U/dish). One unit of erythropoietin was added to each dish on day 0. The cultures were incubated at 37°C in a humidified 5% $CO_2$ incubator for 14 days, and then scored for mixed colonies with an inverted microscope.

*Velocity sedimentation.* Velocity sedimentation was used to separate the different subpopulations of CFU-GM according to procedures described by Jacobsen et al.[18] LNAT$^-$ human bone marrow cells ($5 \times 10^7$) were suspended in 40 mL of 0.2% bovine serum albumin (BSA) in isotonic phosphate-buffered saline (PBS) and allowed to sediment at 1*g* at 4°C through a gradient of 0.4% to 2.0% BSA in PBS. After 3.5 hours, 35-mL fractions were collected, and the fractionated cells were then harvested, washed, and assayed for CFU-GM in the presence or absence of rGM-CSF (100 U/mL) and/or purified GM-EA (4 U/mL) at 5 $\times 10^4$ cells/mL/plate.

*Suspension preculture.* The early developed myeloid progenitor cells, pre–CFU-GM, were obtained using suspension preculture according to the methods described by Jacobsen et al[19] and Broxmeyer et al.[20] Briefly, LNAT$^-$ bone marrow cells were fractionated into different subpopulations by means of velocity sedimentation. Cells corresponding to three peak fractions with respective sedimentation rates (millimeters per hour) of 7.4, 6.0, and 4.7 were collected, washed, and resuspended at $5 \times 10^5$ cells/mL in McCoy's 5A medium containing 10% FCS. One-milliliter aliquots of the cell suspension from each fraction were then cultured separately in the presence of rGM-CSF (100 U), GM-EA (4 U), or medium alone. After incubation at 37°C in 5% $CO_2$ for 7 days, the cells were harvested, washed, and resuspended in 1 mL of McCoy's 5A medium without counting, and 0.1-mL aliquots of the cell suspension were then assayed for CFU-GM using soft agar culture in the presence of rGM-CSF (100 U/dish).

## RESULTS

*Effect of GM-EA on the colony formation of CFU-GM and CFU-GEMM.* Colony formation of day 7 and day 14 CFU-GM was assayed in the presence or absence of different CSFs (rGM-CSF, rG-CSF, rM-CSF, and rIL-3). Results in Fig 1 show that GM-EA alone has no stimulatory effect on G-M colony formation, but can markedly promote the colony-stimulating effect of all the CSFs tested. The percent enhancement of colony formation by GM-EA was similar for day 7 CFU-GM and day 14 CFU-GM (Fig 1), with mean values of 70% to 80% and 55% to 70% for rGM-CSF and rG-CSF/rM-CSF/rIL-3, respectively. However, the profile of colony typing remained unchanged in the presence of GM-EA with each of the four CSFs (data not shown).

When the clonal growth of pluripotent progenitor cells was assayed in the presence of rIL-3 (multi-CSF) at 20 to 100

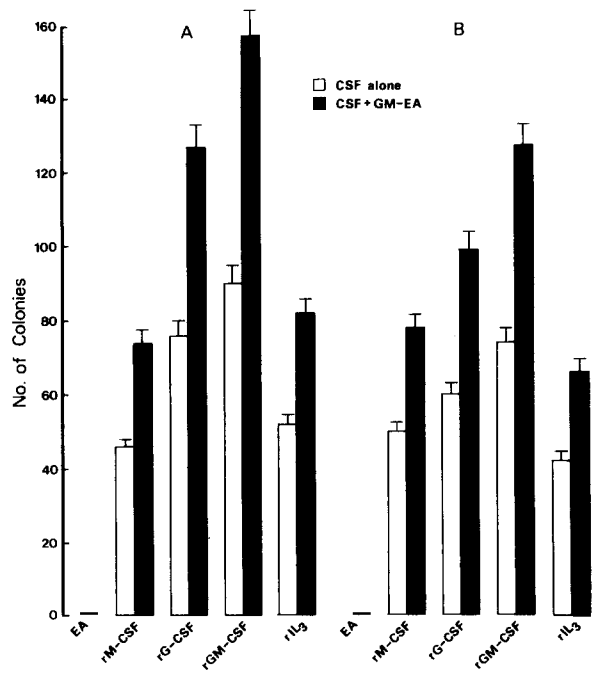


**Fig 1. Effect of GM-EA on colony formation of day 7 (A) and day 14 (B) CFU-GM. Low-density nonadherent human bone marrow cells were plated at 5 $\times$ $10^4$ cells in a 1-mL layer of soft agar culture in the presence or absence of 100 U of different CSFs (rGM-CSF, rG-CSF, rM-CSF, and rIL-3) with or without highly purified GM-EA (4 U). Colonies were scored after 7 and 14 days of incubation. Results are expressed as mean value ± SEM.**

**Table 1. Enhancement of G-M Colony Formation by Various Hematopoietic Synergizing Factors and the Effects of Their Specific Antibodies**

| Source of CSF | Synergizing Factors | Antibody Specificity* | Colony Enhancement (% of CSF alone†) |
|---|---|---|---|
| GM-CSF | GM-EA | — | 176 ± 5 |
| GM-CSF | IL-1 | — | 98 ± 2 |
| G-CSF | IL-4 | — | 195 ± 6 |
| GM-CSF | IL-6 | — | 171 ± 5 |
| GM-CSF | GM-EA | Anti-GM-EA | 108 ± 3 |
| GM-CSF | GM-EA | Anti-IL-1 | 171 ± 5 |
| GM-CSF | GM-EA | Anti-IL-4 | 180 ± 6 |
| GM-CSF | GM-EA | Anti-IL-6 | 174 ± 4 |
| GM-CSF | IL-1 | Anti-IL-1 | 96 ± 3 |
| GM-CSF | IL-1 | Anti-GM-EA | 103 ± 3 |
| G-CSF | IL-4 | Anti-IL-4 | 120 ± 4 |
| G-CSF | IL-4 | Anti-GM-EA | 191 ± 6 |
| GM-CSF | IL-6 | Anti-IL-6 | 112 ± 3 |
| GM-CSF | IL-6 | Anti-GM-EA | 173 ± 5 |

Low-density nonadherent human marrow cells were plated at 5 × $10^4$/mL in a soft agar culture containing 100 U/dish of rGM-CSF/rG-CSF. Enhancement of colony formation was induced by the addition of various synergizing factors (4 U/mL of GM-EA or 100 U/mL of rIL-1/rIL-4/rIL-6). Results are expressed as the mean percent enhancement ± SEM of three separate experiments.

*Rabbit anti-GM-EA (5 NU/mL), anti-IL-1 (100 NU/mL), anti-IL-4 (100 NU/mL), or anti-IL-6 (100 NU/mL) was preincubated with various synergizing factors at 37°C for 90 minutes before addition to CFU-GM assay cultures. Antisera alone had no effect on CSF-induced colony formation.

†The numbers of G-M colonies induced by rGM-CSF and rG-CSF were 88 ± 5 and 75 ± 4 per 5 × $10^4$ marrow cells, respectively.

U/mL, a dose-dependent response in mixed colony formation was noted after 14 days of incubation, and GM-EA when either used alone or in combination with IL-3 had no detectable stimulating or enhancing effect (data not shown).

*Effect of anti-enhancing factor antisera on colony enhancement.* To rule out the possibility that GM-EA was related to other hematopoietic enhancing factors such as IL-1, IL-4, and IL-6, G-M colony enhancement was induced by rGM-/rG-CSF in combination with various enhancing factors in the presence or absence of their specific antibodies. As shown in Table 1, enhancement of G-M colony formation was seen when GM-EA, rIL-4, or rIL-6 was added to CFU-GM cultures in the presence of rGM-CSF/rG-CSF, whereas rIL-1 had no synergism with either GM-CSF (Table 1) or G-CSF (data not shown). Anti-GM-EA antiserum could neutralize the enhancing activity of GM-EA but had no effect on IL-1, IL-4, and IL-6. In parallel, antisera specific for IL-1, IL-4, and IL-6 were unable to reduce the colony-enhancing activity of GM-EA (Table 1). These results suggest that GM-EA is distinct immunologically and biologically from IL-1, IL-4, and IL-6.

*Distinct properties of GM-EA compared with other enhancing factors.* An overview of the comparative characteristics of GM-EA with other hematopoietic enhancing factors (IL-1, IL-4, and IL-6) is summarized in Table 2. GM-EA is distinct from these mediators based on the following criteria. First, GM-EA is constitutively secreted by the fully mature macrophages but not by the other cell types. Second, the molecular weight of GM-EA (73 to 74 Kd) as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis is much higher than the other ILs. Third, based on EIA, specific anti-GM-EA antiserum has no detectable cross-reactivity with any of the ILs, and GM-EA preparations have no detectable level of ILs 1, 4, and 6. Finally, the colony-enhancing profile of GM-EA is quite different from all of the ILs tested. Taken together, GM-EA is a novel enhancing factor that is specific for the myelopoietic progenitor cells.

*Response of cell fractions from velocity sedimentation to GM-CSF/GM-EA.* As indicated in Fig 2, three major subpopulations of myeloid progenitor cells were obtained from velocity sedimentation with respective peak sedimentation rates (SRs) of 7.4 ± 0.4 mm/h (fraction A), 6.0 ± 0.6 mm/h (fraction B), and 4.7 ± 0.3 mm/h (fraction C). Progenitor cells in fractions A and B were characterized by their clonogenic response to GM-CSF, while cells in fraction C were defined by their ability to generate small clusters (5 to 25 cells) in the presence of GM-EA alone at day 14 postincubation (Table 3). It is noteworthy that the efficiency of enhancement of GM-CSF by GM-EA is invariably higher using fractionated cells (greater than 80%) in comparison to assays with unfractionated cells because of the presence of large amounts of contaminating nonprogenitor cells in the crude preparations. Morphologic examination showed that the cells in fraction C consisted mostly of small lymphoid-like cells with diameters of 6 to 9 μm, and the GM-EA–induced clusters composed of mostly myelocytes and metamyelocytes/

**Table 2. Comparison of Biologic and Biochemical Properties Between GM-EA and Other Hematopoietic Enhancing Factors**

| | GM-EA | IL-1 | IL-4 | IL-6 |
|---|---|---|---|---|
| Cell Source[7,8,12,15,21] | | | | |
| T lymphocytes | − | − | + | + |
| Neutrophils | − | + | − | − |
| Macrophages | + (FM) | + (LM) | − | + (LM) |
| Fribroblasts | − | − | + | + |
| Molecular weight (Kd)[7,14,21] | 73-74 | 17-33 | ~20 | 21-26 |
| Antigenic reactivity* | | | | |
| Anti-GM-EA | 1,600 | <10 | <10 | <10 |
| Anti-IL-1 | <10 | 400 | ND | ND |
| Anti-IL-4 | <10 | ND | 800 | ND |
| Anti-IL-6 | <10 | ND | ND | 800 |
| Colony-enhancing profile[6,9,11,13,22,23] | | | | |
| CFU-G† | + | − | + | + |
| CFU-M | + | + | − | + |
| CFU-GM | + | − | − | + |
| BFU-E | − | − | + | − |
| CFU-Meg | − | − | + | − |
| CFU-GEMM | − | − | − | + |

Abbreviations: FM, fully mature macrophages; LM, less mature macrophages; ND, not done.

*Antigenic reactivity was determined based on EIA. Data represented the reciprocal of the highest dilution of antisera that yielded optical density readings of twofold over the control.

†G, granulocyte; M, macrophage; GM, granulocyte and macrophage; E, erythroid; Meg, megakaryocyte; GEMM, granulocyte-E-macrophage-megakaryocyte.

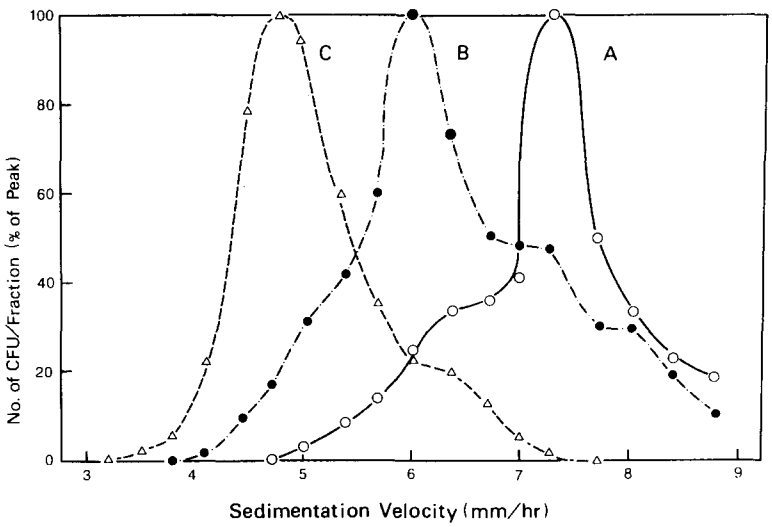


**Fig 2. Velocity sedimentation profile of myeloid progenitor cell subpopulations from normal human bone marrow. A total of 5 × $10^7$ LNAT$^-$ bone marrow cells were separated into different fractions by means of velocity sedimentation. Cells from each fraction were then assayed for CFU-GM using soft agar culture in the presence of rGM-CSF (100 U/dish) and/or purified GM-EA (4 U/dish). Results from three separate experiments were expressed as mean CFU/fraction as percent of peak. Respective average numbers of CFU-GM in the peak fractions were: 11,182 on day 7 (fraction A), 6,643 on day 14 (fraction B), and 2,040 for GM-EA responsive CFU (fraction C) on day 14.**

young monocytes, with only a limited number of immature blast cells and mature segmented granulocytes (data not shown).

Results in Table 3 show that GM-EA alone had no stimulating effect on day 7 and day 14 CFU-GM, but it markedly promoted the colony formation of both myeloid progenitor subpopulations in the presence of GM-CSF. Furthermore, progenitor cells with low-velocity SR (less than 4.7 mm/h) failed to respond to GM-CSF alone, but could form colonies after treatment with GM-CSF plus GM-EA.

**Table 3. Clonogenic Response of Different Fractions of Bone Marrow Cells to GM-CSF and/or GM-EA**

| SR (mm/h) | GM-CSF Responsive CFU | | GM-EA Responsive CFU* | |
|---|---|---|---|---|
| | Day 7 | Day 14 | Day 7 | Day 14 |
| 8.79 | 1,710 (3,192)† | 741 (1,630) | 0 | 0 |
| 8.43 | 2,433 (5,138) | 1,318 (2,548) | 0 | 0 |
| 8.08 | 3,700 (6,660) | 1,924 (3,944) | 0 | 0 |
| 7.73 | 5,600 (9,856) | 1,893 (3,713) | 0 | 0 |
| 7.38 | 11,182 (21,835) | 3,133 (4,762) | 0 | 40 |
| 7.04 | 4,399 (8,122) | 3,189 (5,719) | 0 | 110 |
| 6.07 | 3,404 (5,920) | 3,271 (6,156) | 0 | 310 |
| 6.37 | 3,420 (6,916) | 4,940 (8,694) | 0 | 430 |
| 6.04 | 2,800 (4,970) | 6,643 (10,296) | 0 | 490 |
| 5.71 | 1,505 (2,736) | 4,113 (7,558) | 0 | 710 |
| 5.38 | 1,056 (2,244) | 2,831 (7,030) | 0 | 1,240 |
| 5.06 | 480 (840) | 2,184 (5,678) | 0 | 1,940 |
| 4.74 | 0 (406) | 1,056 (2,110) | 0 | 2,040 |
| 4.43 | 0 | 585 (1,871) | 0 | 1,590 |
| 4.12 | 0 | 211 (839) | 0 | 450 |
| 3.81 | 0 | 0 (512) | 0 | 110 |
| 3.50 | 0 | 0 (291) | 0 | 60 |
| 3.20 | 0 | 0 (62) | 0 | 20 |

LNAT$^-$ human bone marrow cells (5 × $10^7$) were allowed to sediment at 1*g* at 4°C through a gradient of 0.4% to 2.0% BSA in PBS. After 3.5 hours, 35-mL fractions were collected and the cells were counted and assayed for colony formation in soft agar in the presence of rGM-CSF (100 U/dish) and/or purified GM-EA (4 U/dish). Results from three experiments are expressed as mean number of colony-forming units (CFU) per fraction.

Abbreviation: SR, sedimentation rate.

*The cell aggregates induced by GM-EA alone were small clusters (5 to 25 cells), and no colony formation could be observed.

†The number in parentheses is colonies induced by rGM-CSF plus GM-EA.

*Effect of GM-EA on suspension preculture of myeloid progenitor cell subpopulations.* To further substantiate the differentiation status of the GM-EA target cells, we re-examined the response of enriched subpopulation of pre–CFU-GM to GM-EA by suspension preculture. Results in Fig 3 show that preculture of progenitor cells from fraction A (SR of 7.4 mm/h) invariably failed to generate myeloid colonies in subsequent CFU-GM assay, and only a few colonies could be induced from cells in fraction B (SR of 6.0 mm/h). However, the number of inducible colonies in fraction C (SR of 4.7 mm/h) was markedly higher than in the other two subpopulations. Furthermore, cells in fraction C treated with GM-EA generated fivefold more colonies than those treated with either GM-CSF or medium alone (Fig 3). In addition, related hematopoietic regulators such as IL-1, IL-4, and IL-6 had not been detected in the supernatants of the day 7 suspension precultures (data not shown). These results suggest that the specific target cells of GM-EA are the "earlier" myeloid progenitor cells, pre–CFU-GM.

## DISCUSSION

We have shown in a series of studies that GM-EA is a novel monokine produced by the well-developed macrophages.[12-15] This mediator is a myeloid lineage-specific growth factor that acted on the promotion of G-M colony formation induced by GM-CSA from various sources, but had no effect on the proliferation of erythroid or pluripotent progenitor cells.[13] These findings were further substantiated in the present study using highly purified GM-EA and recombinant CSFs. Accordingly, GM-EA can enhance the colony formation of both day 7 and day 14 CFU-GM (Fig 1), but has no effect on CFU-GEMM. However, it remains uncertain whether GM-EA can act directly on certain population(s) of CFU-GM progenitor cells. Therefore, the aim of this study is to ascertain the effect of GM-EA on

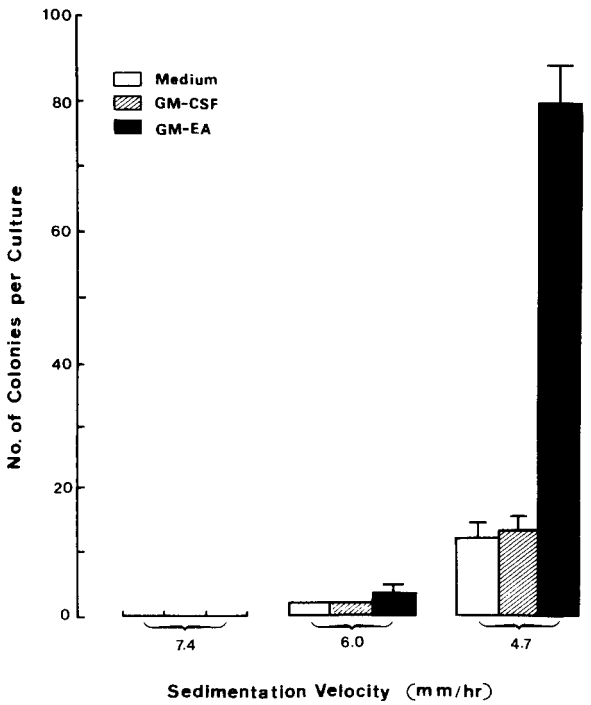


**Fig 3. Effect of GM-EA on suspension preculture of three subpopulations of myeloid progenitor cells. LNAT$^-$ bone marrow cells were fractionated into different subpopulations by means of velocity sedimentation. Cells corresponding to the three peak fractions with respective SRs (mm/h) of 7.4, 6.0, and 4.7 were collected. One-milliliter aliquots of cells suspension (5 × $10^5$ cells) from each fraction were precultured separately in the presence of rGM-CSF (100 U), GM-EA (4 U), or medium alone. After 7 days of incubation, the cells were harvested, washed, and resuspended in 1 mL of medium, and 0.1 mL aliquots of the cell suspension were then assayed for CFU-GM in the presence of 100 U/dish of rGM-CSF. Results are expressed as the mean number of colonies/dish ± SEM.**

different subpopulations of myeloid progenitor cells obtained by velocity sedimentation with or without suspension preculture.

There is ample evidence to suggest that between the pluripotent stem cells and the CFU-GM there exists a subpopulation of progenitor cells, which is intermediate at its stage of maturation.[20,24-27] This subpopulation has been referred to as CFUs in diffusion chambers (CFU-D)[18,19,27-29] or as pre–CFU-GM derived from suspension preculture of bone marrow cells.[19,20,24-30] Our observed velocity sedimentation profile of normal bone marrow cells is similar to that described by Jacobsen et al[18] and shows three physically separable myeloid progenitor subpopulations corresponding to the day 7 CFU-GM, day 14 CFU-GM, and the "earlier" CFU-GM (Fig 2). Among them, only the GM-CSF unresponsive "earlier" myeloid progenitor cells can be activated by GM-EA to proliferate in soft agar culture. It is unlikely that the action of GM-EA on the "earlier" CFU-GM is mediated indirectly through induction of other enhancing factors such as IL-1, IL-4, and IL-6, since before cell sedimentation we have removed the adherent cells and the T lymphocytes, which are the most probable candidates capable of exerting a feeder cell effect. Furthermore, the marrow stromal cells are relatively larger in size than the "earlier" myeloid progenitor cell (pre–CFU-GM), and most, if not all, of these contaminating cells would be expected to be removed after fractionation by velocity sedimentation. In addition, GM-EA has unique functional and antigenic properties that are distinct from ILs 1, 4, and 6 (Tables 1 and 2), and the ILs have no apparent effect on the ability of GM-EA to induce the proliferation of the pre–CFU-GM target cells (data not shown).

The GM-EA responsive progenitor cells are reminiscent of the CFU-D since they share the same velocity SR (4.7 to 5.2 mm/h). However, the two subpopulations differ in that CFU-D give rise to progeny cells of other types in addition to cells of granulopoietic lineage.[28] This difference may arise from the fact that GM-EA is myeloid lineage-specific and the GM-EA responsive cells may be the counterpart of the neutrophilic CFU-D, which are regarded by some investigators as pre–CFU-GM since both of them can generate day 14 CFU-GM and express Ia-like (HLA-DR) antigen.[24,27,28] Results of our study with suspension preculture, which preferentially enriches pre–CFU-GM, also suggest that the specific target cells of GM-EA are indeed the pre–CFU-GM. Thus, only progenitor cells derived from the less mature subpopulation (SR of 4.7 mm/h) can generate a significant number of G-M colonies in soft agar culture while the more mature progenitors yield little or no colonies after suspension preculture (Fig 3).

Coincidentally, Broxmeyer et al[20] have reported that adherent mononuclear bone marrow cells can release a pre–CFU-GM stimulating activity in suspension preculture, and such activity is most likely to be GM-EA. A similar phenomenon was noted by Bol and Williams[31] that some enhancing activities in postendotoxin serum could potentiate the growth of CFU-GM. Taken together, GM-EA reactive cells may be a subpopulation of early committed myeloid progenitor cells referred to by others as pre–CFU-GM. However, the nature of their interaction remains to be determined and deserves further study.

In recent years, CSFs have been applied to clinical trials, and the preliminary results show that rG-CSF/rGM-CSF has promising effects on the treatment of some refractory diseases.[32] Combination of chemotherapy with CSFs to prevent or reduce drug-induced myelosuppression is considered a hopeful approach to cancer therapy. [33] Our studies suggest that GM-EA may also have clinical application in conjugation with CSFs to improve the myelopoietic function of patients with marrow suppression.

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