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Recombinant Human Granulocyte-Colony Stimulating Factor and Recombinant Human Macrophage-Colony Stimulating Factor Synergize In Vivo to Enhance Proliferation of Granulocyte-Macrophage, Erythroid, and Multipotential Progenitor Cells in Mice

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Combinations of low dosages of purified recombinant human (rh) macrophage-colony stimulating factor (M-CSF; also termed CSF-1) and rh granulocyte-colony stimulating factor (G-CSF) were compared alone and in combination for their influence on the cycling rates and numbers of bone marrow and splenic granulocyte-macrophage, erythroid, and multipotential progenitor cells in vivo in mice pretreated with iron-saturated human lactoferrin (LF). LF was used to enhance detection of the stimulating effects of exogenously added CSFs. Concentrations of each CSF that were not active in vivo when given alone were active when given together, with the other CSF. The concentrations of rhM-CSF and rhG-CSF needed to increase progenitor cell cycling in the marrow and spleen were reduced by factors of 40–200 when these CSFs were administered in combination with low dosages of the other CSF. At the concentrations of rhM-CSF and rhG-CSF tested, synergism was not noted on absolute numbers of progenitor cells or total nucleated cell counts per organ or circulating in the blood. These findings may

Abbreviations: BFU-E, erythroid progenitor; CFU-GEMM, multipotential progenitor; CFU-GM, GM progenitor; CSF, colony stimulating factor; CSF-1, macrophage CSF; G, granulocyte; GM, granulocyte-macrophage; h, human; IL, interleukin; LF, lactoferrin; M, macrophage; n, natural; PWMSCM, pokeweed mitogen mouse spleen cell-conditioned medium; r, recombinant.

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have potential relevance when considered in a clinical setting where the CSFs might be used in combination with other biotherapy and/or chemotherapy.

Key words: colony stimulating factor, macrophage-colony stimulating factor, granulocyte-colony stimulating factor, in vivo action, synergism, hematopoietic progenitor cell, multipotential hematopoietic progenitor cell, granulocyte-macrophage progenitor cell, erythroid progenitor cell, lactoferrin

The genes for the hematopoietic colony stimulating factors (CSFs)—interleukin-3 (IL-3), granulocyte-macrophage (GM)-CSF, granulocyte (G)-CSF, and macrophage (M)-CSF (also termed CSF-1)—have been cloned and expressed and the CSFs purified [reviewed in 1–3]. Their actions in vitro have been well studied [1–3], and there are now numerous reports documenting the preclinical actions of the CSFs. IL-3 [4–14] and M-CSF [7,8,10–12,15–18] have thus far been studied in mice, while GM-CSF [7,10,12,19–22] and G-CSF [23–29] have been evaluated in mice and monkeys. Clinical trials with recombinant human (rh)GM-CSF [30–32] and rhG-CSF [33] are ongoing and it is only a short time before rhIL-3 and rhM-CSF will be evaluated in a clinical setting.

Cell regulation in vitro encompasses a complex set of biomolecule-cell interactions [1,2] and molecules can act together in an additive or synergistic manner to enhance hematopoietic progenitor cell proliferation [1,2]. While it is not always clear whether synergism noted with combinations of molecules is manifested by a direct action of the multiple molecules on the progenitor cell itself, there is some evidence that this does happen [34]. Synergism between molecules is an important concept since it allows for amplification of an effector function. We have recently reported that combinations of r murine (m) GM-CSF and rmIL-3, or rm GM-CSF and natural (n) mM-CSF, or rmIL-3 and nmM-CSF could synergize when administered to mice pretreated with human lactoferrin (LF) to enhance the cycling status and absolute number of bone marrow granulocyte-macrophage (CFU-GM), erythroid (BFU-E), and multipotential (CFU-GEMM) progenitor cells [12]. Additionally rm IL-3 and nmM-CSF synergized in vivo to enhance the cycling rates of bone marrow high proliferative potential colony forming cells [11]. LF was used to dampen steady-state myelopoiesis in the mice [8,35], thus making the detection of the actions of low dosages of CSFs in the mice more apparent [7,10].

Experiments were set up to evaluate the possibility that rhG-CSF and rh M-CSF might synergize in vivo when administered to mice for the following reasons. Clinical trials have begun with rhG-CSF [33], M-CSF is detectable in human serum [36–38], and both rhG-CSF [39] and rhM-CSF [40] can stimulate colony formation from murine CFU-GM in vitro. Mice were pretreated with LF to reduce endogenous levels of CSF [35] prior to addition of rhG-CSF and rh M-CSF, alone or in combination. Effects were assessed on the cycling rates and numbers of CFU-GM, BFU-E, and CFU-GEMM in the marrow and spleens of the mice.

MATERIALS AND METHODS

Mice

(C57BL/6 × DBA/2)F_1 (BDF_1) mice, 6–8 weeks old, were purchased from Cumberland View Farms (Clinton, TN).

Molecules

Recombinant hG-CSF [95% pure, specific activity $> 5 \times 10^7$ units/mg) [41] and rh CSF-1 from a truncated long clone (lot DCP-006, 95% pure, specific activity $>5 \times 10^7$ units/mg) [42] from Cetus Corporation (Emeryville, CA), were diluted in sterile pyrogen-free saline prior to use. Purified human milk LF, purchased from Sigma Chemical Co. (St. Louis, MO), was fully iron saturated [43] and depleted of endotoxin by removing the material that gelled in the presence of *Limulus* lysate (Sigma) [7]. The concentration of LF was measured by an immunoradiometric assay for LF [43]. Injections of factors to mice were given i.v. in volumes of 0.2 ml. LF was given 3 h prior to administration of the CSFs, and when both CSFs were given to mice, each CSF was injected separately with in a few minutes of the other CSF. By using the *Limulus* lysate test for endotoxin, which has a sensitivity range down to 0.5 ng/ml, no endotoxin (<0.1 ng per injection) was detected in the preparations of rhG-CSF, rhM-CSF, or LF.

Analysis of Hematopoietic Progenitor Cells In Vitro

The assays for CFU-GM, BFU-E, and CFU-GEMM were performed as previously described [7,12]. Granulocyte-macrophage colony formation (>50 cells cells/aggregate) was stimulated using 10% (vol/vol) pokeweed mitogen mouse spleen cell-conditioned medium (PWMSCM) as a stimulus. Erythroid and multipotential colony formation was stimulated with 1 unit erythropoietin (Hyclone, Logan, UT), 0.1 mM hemin (Eastman Kodak, Rochester, NY), and 1% PWMSCM. Bone marrow and spleen cells were plated at respective concentrations of 7.5×10^4 and 1.0×10^6 cells/ml. Cells were incubated in a humidified environment at lowered (5%) oxygen tension using an Oxyreducer (Reming Bioinstruments, Redfield, NY). Colonies were scored after 6–7 days of incubation.

Cycling Status of Hematopoietic Progenitor Cells

The proportion of progenitors in DNA synthesis (S-phase) was estimated as reported previously [7,12]. The high specific activity (20 Ci/mmol) tritiated thymidine (50 μCi/ml) (New England Nuclear, Boston, MA) kill technique is based upon calculation of the reduction in the number of colonies after pulse exposure of cells for 20 min to tritiated thymidine compared to control (McCoy's medium, Gibco, Grand Island, NY). A negative number, as seen in Table II, means that more colonies formed after cells were exposed to tritiated thymidine than after cells were exposed to McCoy's medium and that essentially no cells were in S-phase at that specific time of treatment. The percentage of cells in S-phase was based on control colony counts for CFU-GM BFU-E and CFU-GEMM that were, respectively, in the range 22–138, 9–45, 8–28 for marrow and 30–73, 10–148, and 8–60 for spleen.

Statistical Analysis

Three plates were scored for each CFU-GM sample and two plates were scored for each BFU-E/CFU-GEMM sample. The results are expressed as the mean $\pm$ 1 SEM, and these are derived from the averages of the colony counts from each of the individual mice within a group. The probability of significant differences between groups was determined with the use of Student's t-test.

RESULTS

Effects of CSFs on Hematopoietic Progenitor Cell Cycling Rates

Recombinant hM-CSF and rhG-CSF were assessed, alone or in combination, for their effects on the cycling status of bone marrow (Table I) and splenic (Table II) CFU-GM, BFU-E and CFU-GEMM in vivo. Mice were first pretreated i.v. with 100 μg human LF in order to reduce endogenous levels of CSFs and to dampen myelopoiesis [8,35]. Three hours later control mice were treated i.v. with sterile pyrogen-free saline and test mice were given rhM-CSF and/or rhG-CSF. Mice were sacrificed 22 hours later and femoral marrow and spleen from these mice evaluated in vitro. Less than or equal to 11% of marrow and splenic hematopoietic progenitor cells were in S-phase in mice receiving saline. The cycling rates of the hematopoietic progenitors were significantly enhanced ($P < 0.005$) by either 5,000–20,000 units rhM-CSF or 2,000–10,000 units rhG-CSF (Tables I, II). Lower concentrations of either CSF were ineffective in enhancing progenitor cell proliferation ($P > 0.05$). In contrast, administration of both CSFs together, at concentrations as low as 50 units each, was sufficient to enhance significantly ($P < 0.005$) the proliferative rates of the three progenitor cell compartments in the marrow (Table I) and in the spleen (Table II).

Effects of CSFs on Absolute Numbers of Hematopoietic Progenitor Cells and on Peripheral Blood Counts

Concentrations of 20,000 units rhM-CSF alone significantly ($P < 0.005$) enhanced absolute numbers of CFU-GM, BFU-E, and CFU-GEMM per femur, while 10,000 units rhG-CSF enhanced absolute numbers of marrow CFU-GEMM (Table III). Lower concentrations of either CSF alone, or concentrations of rhM-CSF plus rhG-CSF as high as 500 units each, had no significant effect on marrow progenitor cell numbers (Table III). Concentrations as high as 20,000 units rhM-CSF or 10,000 units rhG-CSF or the combination of 500 units rhM-CSF plus 500 units rhG-CSF had no influence ($P > 0.05$) on numbers of splenic progenitor cells (Table IV). No effect was noted on numbers of nucleated cells within the femoral marrow or spleen at any of the concentrations of CSFs assessed (Tables III, IV) and peripheral blood counts and differentials taken at 22, 48, or 72 h were not significantly different from control in any of the test mice (data not shown).

DISCUSSION

LF pretreated mice were used to enhance the capacity to detect the proliferative effects in vivo of low dosages of CSFs [7,8,35]. The reversible myelosuppressive effects of LF in vivo probably relate to the ability of LF to decrease the release of CSFs from cells of the mononuclear phagocytic lineage [44] and/or to decrease the release of a monokine [45–47], most probably IL-1 [48–51], which then triggers other cell types to release CSFs. In the LF-pretreated mice, rhM-CSF can synergize with rhG-CSF to enhance the percentage of marrow and splenic CFU-GM, BFU-E, and CFU-GEMM in S-phase of the cell cycle; 40–200-fold less of each CSF is needed in comparison to concentrations of each which induce the same conditions when each of the CSFs is administered only by itself (Table V). This study extends our previous reports [11,12] in which combinations of murine preparations of IL-3, GM-CSF, and M-CSF (CSF-1) manifest similar synergistic effects on marrow progenitors in the

TABLE I. Influence of Purified Recombinant Human M-CSF and Recombinant Human G-CSF, Alone or in Combination, on the Cycling Status of Bone Marrow Hematopoietic Progenitor Cells In Vivo After Mice Have Been Pretreated With Purified Human LF†

Test material given in vivo (units)	No. of mice	% Of progenitor cells in S-phase		
		CFU-GM	BFU-E	CFU-GEMM
Saline	19	11 ± 3	8 ± 5	6 ± 5
M-CSF (20,000)	6	55 ± 7*	61 ± 3*	67 ± 5*
M-CSF (10,000)	12	50 ± 4*	31 ± 8*	34 ± 7*
M-CSF (5,000)	12	34 ± 5*	12 ± 4	6 ± 4
M-CSF (2,000)	12	13 ± 5	3 ± 3	3 ± 3
M-CSF (1,000)	15	10 ± 5	13 ± 5	3 ± 4
G-CSF (10,000)	11	47 ± 5*	54 ± 6*	61 ± 7*
G-CSF (5,000)	12	28 ± 5*	43 ± 9*	41 ± 10*
G-CSF (2,000)	12	15 ± 3	14 ± 7	30 ± 9*
G-CSF (1,000)	15	11 ± 4	12 ± 6	16 ± 6
M-CSF (500) + G-CSF (500)	9	56 ± 3*	69 ± 4*	64 ± 5*
M-CSF (100) + G-CSF (100)	9	49 ± 5*	68 ± 5*	58 ± 4*
M-CSF (50) + G-CSF (50)	9	44 ± 3*	25 ± 8*	22 ± 7*
M-CSF (10) + G-CSF (10)	9	17 ± 5	12 ± 8	4 ± 7

†Mice were pretreated with 100 μg endotoxin-depleted iron-saturated human LF i.v. Pretreatment with LF placed the progenitors into a slow, or noncycling, state. Three hours later they were treated i.v. with sterile pyrogen-free saline or various concentrations of M-CSF or G-CSF, alone or in combination. Mice were sacrificed 22 h later.
*Significant change from saline group, P at least <0.005.

TABLE II. Influence of Purified Recombinant Human M-CSF and Recombinant G-CSF, Alone or in Combination, on the Cycling Status of Splenic Hematopoietic Progenitor Cells In Vivo After Mice Have Been Pretreated With Purified Human LF†

Test material given in vivo (units)	No. of mice	% Of progenitor cells in S-phase		
		CFU-GM	BFU-E	CFU-GEMM
Saline	13	6 ± 3	−4 ± 2	−4 ± 2
M-CSF (10,000)	6	30 ± 5*	51 ± 3*	54 ± 5*
M-CSF (5,000)	6	18 ± 6*	6 ± 3	0.3 ± 4
M-CSF (2,000)	6	9 ± 4	−3 ± 3	−3 ± 3
M-CSF (1,000)	9	12 ± 4	3 ± 3	−3 ± 4
G-CSF (10,000)	6	43 ± 4*	53 ± 4*	53 ± 3*
G-CSF (5,000)	6	14 ± 6	−4 ± 5	−10 ± 4
G-CSF (2,000)	6	7 ± 4	−6 ± 3	−0.3 ± 3
G-CSF (1,000)	9	13 ± 5	−7 ± 3	−0.3 ± 4
M-CSF (500) + G-CSF (500)	6	46 ± 1*	56 ± 3*	54 ± 3*
M-CSF (100) + G-CSF (100)	6	58 ± 4*	48 ± 1*	49 ± 3*
M-CSF (50) + G-CSF (50)	6	22 ± 7*	29 ± 5*	11 ± 7
M-CSF (10) + G-CSF (10)	6	11 ± 2	1 ± 3	−0.2 ± 4

†Mice were treated as described in the legend to Table I. A negative sign next to the No. means that there are few or no cells in S-phase.
*Significant change from saline group, P at least <0.005.

TABLE III. Influence of Purified Recombinant Human M-CSF and Recombinant Human G-CSF, Alone or in Combination, on Numbers of Bone Marrow Nucleated Cells and Hematopoietic Progenitors In Vivo After Mice Have Been Pretreated With Purified Human LF†

Test material given in vivo (units)	Nucleated cells (No. × 10^{-6} per femur)	Colonies, no. × 10^{-3} per femur		
		CFU-GM	BFU-E	CFU-GEMM
Saline	18.7 ± 0.7	14.4 ± 2.6	5.0 ± 0.5	2.5 ± 0.3
M-CSF (20,000)	21.1 ± 1.5	38.4 ± 11.6*	9.8 ± 1.6*	5.7 ± 0.7*
M-CSF (10,000)	17.9 ± 1.0	19.1 ± 4.9	4.7 ± 0.5	2.7 ± 0.3
M-CSF (5,000)	17.3 ± 0.8	15.2 ± 3.5	4.0 ± 0.4	2.3 ± 0.2
M-CSF (2,000)	18.6 ± 1.2	16.2 ± 3.8	4.6 ± 0.4	2.3 ± 0.2
M-CSF (1,000)	19.0 ± 0.8	14.8 ± 2.8	4.4 ± 0.4	2.0 ± 0.2
G-CSF (10,000)	17.8 ± 0.8	19.3 ± 5.4	5.8 ± 1.0	4.0 ± 0.8*
G-CSF (5,000)	18.6 ± 1.7	17.5 ± 4.5	5.4 ± 0.7	3.4 ± 0.5
G-CSF (2,000)	19.1 ± 1.0	16.2 ± 3.6	4.8 ± 0.4	2.8 ± 0.3
G-CSF (1,000)	18.8 ± 1.0	16.1 ± 3.1	5.1 ± 0.5	2.7 ± 0.2
M-CSF (500) + G-CSF (500)	18.0 ± 1.0	12.0 ± 1.6	6.7 ± 1.0	2.7 ± 0.5
M-CSF (100) + G-CSF (100)	19.0 ± 1.0	10.4 ± 2.1	5.1 ± 0.7	2.1 ± 0.2
M-CSF (50) + G-CSF (50)	17.0 ± 1.0	11.5 ± 0.5	4.2 ± 0.4	2.3 ± 0.2
M-CSF (10) + G-CSF (10)	20.0 ± 1.0	10.6 ± 1.7	4.7 ± 0.2	1.9 ± 0.4

†Protocol and No. of mice assessed are the same as those described in Table I.
*Significant change from saline group, P at least <0.005.

TABLE IV. Influence of Purified Recombinant Human M-CSF and Recombinant Human G-CSF, Alone or in Combination, on Numbers of Splenic Nucleated Cells and Hematopoietic Progenitors In Vivo After Mice Have Been Pretreated With Purified Human LF†

Test material given in vivo (units)	Nucleated cells (No. × 10^{-6} per spleen)	Colonies, no. × 10^{-3} per spleen		
		CFU-GM	BFU-E	CFU-GEMM
Saline	92.0 ± 9	5.1 ± 0.5	6.1 ± 1.4	1.6 ± 0.3
M-CSF (10,000)	79.0 ± 5	3.9 ± 0.4	4.3 ± 0.5	0.9 ± 0.4
M-CSF (5,000)	80.0 ± 4	4.1 ± 0.4	3.4 ± 1.4	0.9 ± 0.4
M-CSF (2,000)	81.0 ± 6	3.9 ± 0.6	4.6 ± 0.4	1.3 ± 0.1
M-CSF (1,000)	105.0 ± 10	6.3 ± 0.6	8.8 ± 2.2	2.0 ± 0.2
G-CSF (10,000)	102.0 ± 12	5.6 ± 0.9	6.1 ± 1.0	1.0 ± 0.2
G-CSF (5,000)	89.0 ± 8	4.8 ± 0.6	5.0 ± 0.5	0.9 ± 0.3
G-CSF (2,000)	88.0 ± 4	3.8 ± 0.7	4.7 ± 0.3	0.8 ± 0.5
G-CSF (1,000)	108.0 ± 9	5.8 ± 0.5	8.0 ± 1.8	2.2 ± 0.4
M-CSF (500) + G-CSF (500)	94.0 ± 8	6.2 ± 1.1	7.7 ± 1.8	2.3 ± 0.5
M-CSF (100) + G-CSF (100)	84.0 ± 7	5.8 ± 0.9	8.6 ± 2.3	2.2 ± 0.2
M-CSF (50) + G-CSF (50)	94.0 ± 11	5.2 ± 0.4	6.6 ± 1.6	1.4 ± 0.1
M-CSF (10) + G-CSF (10)	113.0 ± 7	5.9 ± 0.4	7.3 ± 2.9	2.2 ± 0.5

†Protocol is same as that described in legend to Table I and Nos. of mice assessed are the same as in Table II.

same model system, by evaluating recombinant preparations of human CSFs, assessing rhG-CSF, and determining effects in the spleen, as well as in the marrow.

The concept of synergism, roughly defined as the total effect's being greater than the sum of the two effects taken independently, is an intriguing one, especially in terms of the actions of biomolecules in vivo. Synergism adds further complexity to known biomolecule-cellular interactions uncovered through studies in vitro [1,2,34]. Synergistic interactions can reflect cascading effects mediated indirectly through

TABLE V. Lowest Concentrations of CSFs Having Activity for Marrow and Splenic Hematopoietic Progenitor Cell Cycling When M-CSF or G-CSF Are Administered, Alone or in Combination, to Mice Pretreated With LF

	CFU-GM			BFU-E			CFU-GEMM		
	CSF alone[a]	CSF + other CSF[b]	Fold change[c]	CSF alone[a]	CSF + other CSF[b]	Fold change[c]	CSF alone[a]	CSF + other CSF[b]	Fold change[c]
Bone marrow									
M-CSF	5,000	50	100	10,000	50	200	10,000	50	200
G-CSF	5,000	50	100	5,000	50	100	2,000	50	40
Spleen									
M-CSF	5,000	50	100	10,000	50	200	10,000	100	100
G-CSF	10,000	50	200	10,000	50	200	10,000	100	100

[a]Lowest concentration of CSFs having activity when administered alone.

[b]Lowest concentration of CSF having no activity alone, but having activity when administered with a dosage of the other CSF, that also has no activity when used alone.

[c]Fold change refers to differences between lowered CSF concentrations that are active when CSFs are given together compared to when CSFs are administered to mice in the absence of the other form of CSF.

accessory cells, effects mediated directly at both accessory and progenitor cell levels, or effects manifested entirely at the progenitor cell level. Our past [11,12] and present studies done in vivo don't allow us to distinguish which of the above possibilities apply, but we consider it likely that many of the effects noted, especially those of rhM-CSF and rhG-CSF on BFU-E and CFU-GEMM, are accessory cell mediated.

The results described here are also consistent with our previous findings that less CSF, alone or in combination with other CSFs, is required to enhance the cycling rates of hematopoietic progenitor cells than is required to increase the absolute numbers of these progenitors, and increases in numbers of progenitors per organ do not necessarily translate into increased total nucleated blood cell counts in the marrow, spleen or blood [7,12]. It is clear that multiple or continuous infusions of much higher concentrations of CSFs can increase circulating levels of blood leukocytes [20–23,52] and that single injections of CSFs, while having a proliferative effect on the progenitor cells, result in what appears to be ineffective myelopoiesis. Nevertheless, low dosages of CSFs may have potential clinical relevance. Clinical trials with rhGM-CSF [30–32] and rhG-CSF [33] have demonstrated efficacy in increasing circulating leukocyte levels, and we have noted that administration of rhGM-CSF to patients with the myelodysplastic syndrome and with cancer [30,31] place their slowly or noncycling marrow CFU-GM into a more rapid, but reversible, proliferative phase [53]. Marrow CFU-GM from the patients administered rhGM-CSF were increased in sensitvity to the suppressive effects in vitro of rh acidic isoferritin in two of eight cases, and to lower dosages of tumor necrosis factor-alpha in all cases evaluated [53]. Thus, it is possible that enhancement of the percentage of progenitors in S-phase of the cell cycle, without increases in progenitor or nucleated cell numbers, might be able to be utilized in a clinical setting in combination with other biotherapy and/or with chemotherapy.

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