

REGULATION OF MACROPHAGE AND
GRANULOCYTE PROLIFERATION
Specificities of Prostaglandin E and Lactoferrin*

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Clonal macrophage and/or granulocyte expansion and differentiation from committed hematopoietic progenitor cells (CFU-c)¹ can be detected by proliferation in semisolid agar culture, and require the continuous presence of specific regulatory molecules termed colony-stimulating factor(s) (CSF) (1-3). Stimulating activities, which differ in molecular weight and specificity of colony formation can be derived indirectly from cellular underlayers (4, 5) or, alternatively, directly provided by the addition of CSF-containing cell-free preparations (6-10). Colony formation *in vitro* is sensitive to the presence of bioregulatory molecules and, therefore, permits the investigation of events controlling cellular proliferation and differentiation.

The physiological control of myelopoiesis *in vivo* is not adequately explained by models of regulation based solely on variations in CSF levels. Increasing evidence indicates that regulatory negative control mechanisms operate to limit excessive and inappropriate proliferation, particularly after exposure to microbial antigens. Two mechanisms for myelopoietic regulation have been reported. Monocyte/macrophage populations have been identified as major producers of CSF (11-13) and have been implicated in a dualistic feedback mechanism, whereby the stimulation of myelopoiesis is limited by the coincident production of prostaglandin E (PGE) (5, 12, 14). In a second regulatory mechanism, normal polymorphonuclear neutrophils release a colony-inhibiting activity (15), identified as lactoferrin (LF) (16), which decreases production and/or release of CSF from monocytes and macrophages. Although both feedback mechanisms provide for the limitation of myelopoiesis, the exact nature and specificity of control has not been elucidated.

Recent evidence has demonstrated that the differentiation of myeloid progenitor cells into populations of macrophages or granulocytes can be independently examined by using CSF preparations which are more selective in stimulating colonies of defined

* Supported by grants CA-19052 and CA-23528 from the National Cancer Institute and National Research Service Award F32 CA-05904 (to L. M. Pelus) from the National Cancer Institute, Department of Health, Education, and Welfare.

‡ Scholar of the Leukemia Society of America, Inc.

¹ *Abbreviations used in this paper:* CFC, colony-forming cell(s); CFU-c, granulocyte-macrophage colony-forming cell; CSA, colony-stimulating activity; CSF, colony-stimulating factor; FCS, fetal calf serum; LC-CSF, mouse L-cell-derived colony-stimulating activity; LF, lactoferrin, fully iron-saturated; M, molar concentration; N, neutrophil; PC, resident murine peritoneal cells; PGE, prostaglandin E.

morphology (8–10). In the present study, the roles of the reported myelopoietic inhibitors PGE and LF in the regulation of morphologically distinct clonal proliferation were investigated. Evidence is presented which indicates that PGE, particularly macrophage-derived PGE, functions as a selective inhibitory molecule with a high degree of specificity for the regulation of macrophage proliferation and clonal expansion. Furthermore, induction of macrophage PGE synthesis is a property of CSF preparations which stimulate macrophage but not neutrophil colony formation. In addition, the defined role of LF on the inhibition of macrophage CSF production is confirmed and extended to include the inhibition of CSFs with stimulatory activity for both macrophage and neutrophil proliferation.

Materials and Methods

Animals. Female B6D2F₁ mice, 2–3 mo old (The Jackson Laboratories, Bar Harbor, Maine) were used as a source of both bone marrow and peritoneal macrophages. All mice were acclimated for a minimum of 1 wk before use and permitted continuous access to food and water.

Isolation of Peritoneal Cells. Noninduced resident peritoneal cells were isolated by peritoneal lavage with 8 ml ice-cold McCoy's 5A modified medium (Memorial Sloan-Kettering Institute Media Laboratory), supplemented with 2% fetal calf serum (FCS) (Flow Laboratories, Inc., Rockville, Md.), antibiotics and 2 U/ml heparin (Abbott Diagnostics, Diagnostic Products, North Chicago, Ill.). Average cell yields were $3\text{--}7 \times 10^6$ cells/mouse consisting of 31% morphologically identifiable macrophages, determined by microscopic examination ($\times 960$) of Wright's stained cytocentrifuge preparations. The resident peritoneal cells (PC) were diluted to the appropriate cell concentration in McCoy's 5A medium containing 15% FCS. Various numbers of PC were seeded into 35-mm culture dishes (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) and allowed to adhere for 1½ h at 37°C in a fully humidified 5% CO₂ atmosphere. After incubation, the nonadherent cells were removed, and the adherent monolayers washed three times with mouse tonicity (310 mosM) phosphate-buffered saline (PBS) and fresh 15% FCS-supplemented McCoy's 5A medium added.

Bone Marrow CFU-c Assay. The proliferation of mouse macrophage-granulocyte progenitor cells in response to exogenously added CSF was tested in semisolid soft agar culture. Mouse bone marrow was isolated by perfusing the femurs from a minimum of three mice with ice-cold McCoy's 5A modified medium. Single cell suspensions were obtained by gentle refluxing, washed, and resuspended to a concentration of 5×10^4 or 7.5×10^4 nucleated bone marrow cells/ml in McCoy's 5A medium supplemented with 15% FCS, essential and nonessential amino acids, vitamins, sodium pyruvate, L-glutamine, serine, asparagine, and 0.3% Bacto Agar (Difco Laboratories, Detroit, Mich.) as described in (17). 1-ml aliquots of bone marrow-agar suspensions were dispensed into 35-mm tissue culture dishes (Lux Scientific, Corp., Newbury Park, Calif.), allowed to gel, and incubated in a fully humidified 5% CO₂ atmosphere. Colonies (>50 cells) were scored at $\times 25$ magnification on day 7 of culture. In the single-layer agar culture used to test soluble supernates, colony proliferation was stimulated by the addition of 0.1 ml of various concentrations of CSF. The effects of adherent resident peritoneal macrophages on mouse bone marrow progenitor cell proliferation was also investigated using a two-layer soft agar culture technique. Varying numbers of resident PC harvested as described above were allowed to adhere to 35-mm culture dishes (Falcon), washed three times with PBS, and overlaid with 1.0 ml of cell free 0.5% agar in supplemented McCoy's 5A medium. Murine bone marrow target cells at a concentration of 7.5×10^4 cells/ml in 0.3% agar-McCoy's medium were subsequently added on top of the 0.5% underlayers. The layer of 0.5% agar serves as a physical barrier between adherent macrophage monolayers and bone marrow target cells, yet allows for the diffusion of stimulatory and inhibitory molecules.

Colony-Stimulating Factors. Concentrated conditioned medium (CM) from the myelomonocytic leukemia cell line (WEHI-3) was used as a source of murine-active CSF (18). Serum-free WEHI-3 CM was further purified by ion-exchange chromatography on DEAE Sephadex A25 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). The DEAE break-

through (G-CSF) and DEAE-eluate (M-CSF) fractions obtained were enriched in their ability to stimulate morphologically distinct colony types. At concentrations which maximally stimulated colony formation, the DEAE-G-CSF preparation stimulated 12% macrophage, 49% mixed macrophage-neutrophil, and 38% pure neutrophil colonies, whereas the DEAE-M-CSF fraction stimulated 58% macrophage, 40% mixed macrophage-neutrophil, and 2% pure neutrophil colonies. Procedures for cell culture and collection of WEHI-3 CM and the partial characterization of separable colony-stimulating activities have been described (8). L-cell-CSF (LC-CSF) was provided by a 72-h conditioned medium harvested from log-growth phase L-929 cells grown in McCoy's 5A medium supplemented with 2% FCS. Conditioned medium was partially purified by Sephadex G-50 chromatography and concentrated fivefold. This CSF preparation stimulates 93% pure macrophage and 7% mixed macrophage-neutrophil colony proliferation. No pure neutrophil colony formation is observed. Concentrations of all CSFs which maximally stimulated CFU-c proliferation when tested at 10% vol/vol on 7.5×10^4 mouse bone marrow cells were designated as having a concentration of 1.0.

Single Colony Morphology. Single colonies on day 7 of culture were transferred to marked glass slides with the aid of a micropipet and allowed to air dry. When dry, a cover slip was placed over the colonies, 0.6% orcein in 60% acetic acid run under, and the edges sealed. Slides prepared in this manner can be kept for long periods of time without overstaining of colonies. Routinely, 40–50 sequential colonies for each sample were examined, and each colony classified as being macrophage, mixed macrophage-neutrophil, or pure neutrophil in composition.

Radioimmunoassay of PGE. Radioimmunoassay measurements of PGE were performed on cell-free supernates according to the method of Levine et al. (19). All samples were extracted with acidified ethylacetate-isopropanol and dried under a stream of air at 37°C. Dried extracts were resolubilized and applied to silicic acid columns (BioSil A, 100–200 mesh, BioRad Laboratories, Richmond, Calif.) for the chromatographic separation of the various prostaglandin classes. The columns were sequentially eluted with benzene-ethylacetate and increasing concentrations of methanol. The PGE containing column fraction II, eluted with benzene-ethylacetate-methanol (60:40:5) was dried under air, resolubilized in Tris-isogel buffer (pH 7.4), and alkaline hydrolyzed to convert PGE₁ and PGE₂ to PGB (20, 21). The specificity of the silicic acid chromatography is such that PGE is clearly separated from PGA, PGB, and PGF, therefore, alkaline hydrolyzed samples contain PGB resulting solely from the conversion of PGE. Competitive binding between ³H-PGB, authentic PGB standard or test supernate and a specific PGB antibody was measured using a commercially available radioimmunoassay kit (Clinical Assays, Inc., Div. of Travenol Laboratories, Inc., Cambridge, Mass.). All PGE measurements were corrected for extraction efficiency and chromatographic recovery.

Reagents. Indomethacin and prostaglandins E₁, E₂, and F_{2 α} were purchased from Sigma Chemical Co. (St. Louis, Mo.). Prostaglandins were diluted to a concentration of 10⁻² M in absolute ethanol (ETOH) and stored at -20°C. Indomethacin was prepared fresh before use as a 10⁻²-M solution in ETOH. Prostaglandins and indomethacin were diluted to the required final concentration in McCoy's 5A medium supplemented with 15% FCS. Ethanol concentrations of 0.1% or lower were without effect on colony formation. Commercial LF obtained from Metallo Protein Laboratories Ltd. (Windsor, Ontario, Canada) or Calbiochem-Behring Corp., American Hoechst Corp., (San Diego, Calif.) was fully iron saturated as previously described (15).

Results

Effects of PGE₁ on CFU-c Proliferation and Colony Morphology. The WEHI-3 myelomonocytic cell line, grown in suspension culture, produces colony-stimulating activities which stimulate the proliferation of macrophage (22–24%), mixed macrophage-neutrophil (40–51%), and pure neutrophil (27–38%) colonies from mouse bone marrow cells in vitro.

The effects of the dose wise addition of PGE₁ on CFU-c formation, stimulated by the CSF(s) present in WEHI-3-conditioned medium, were tested in agar culture (Table I). PGE₁ at concentrations of 10⁻⁵–10⁻⁸ M resulted in a dose-dependent

TABLE I
Effects of PGE₁ on Proliferation and Morphology of CFU-c Stimulated by WEHI-3 CSF

PGE ₁ (concentration)	CFU-c/culture*	Colony Morphology‡		
		MΦ	MΦ-N	N
0	102 ± 4.6	24 —	40 —	38 —
10 ⁻⁵ M	42 ± 2.6 (59%)§	2 (92%)§	12 (70%)§	28 (26%)§
10 ⁻⁶ M	60 ± 4.5 (41%)	4 (84%)	22 (45%)	34 (11%)
10 ⁻⁷ M	78 ± 7.4 (24%)	10 (59%)	34 (15%)	36 (5%)
10 ⁻⁸ M	88 ± 1.0 (15%)	12 (50%)	38 (5%)	36 (5%)
10 ⁻⁹ M	98 ± 4.8 (4%)	14 (42%)	42 (0)	42 (0)
10 ⁻¹⁰ M	96 ± 4.6 (6%)	20 (17%)	39 (0)	37 (0)
10 ⁻¹¹ M	100 ± 4.0 (0)	24 (0)	40 (0)	38 (0)
10 ⁻¹² M	100 ± 4.6 (0)	24 (0)	40 (0)	38 (0)

N, neutrophil.

* Total colonies (>50 cells)/7.5 × 10⁴ mouse bone marrow cells. $\bar{X} \pm SE$.

‡ Single colony morphology of 50 sequential colonies transferred to microscope slides and examined by aceto-orcein staining. Data is expressed as actual number of colonies of specified morphology.

§ Percent inhibition of total or morphologically specified colony formation.

inhibition of total colony formation. Morphological analysis of single colonies on day 7 of culture indicated that macrophage colonies were significantly more sensitive to inhibition by PGE₁ than both neutrophil colonies and mixed colonies of macrophages and neutrophils. In comparison to macrophage colonies, \cong 1,000-fold greater concentrations of PGE₁ were required for the equivalent inhibition of mixed colonies. Furthermore, a shift in the percentage of cells within mixed colonies displaying mononuclear macrophage morphology was observed. In the presence of PGE₁, mixed colonies were predominantly neutrophilic in composition, whereas mixed colonies in control cultures contained equivalent numbers of macrophages and neutrophils. However, some mixed colony macrophages persisted, albeit at reduced levels, even at concentrations of PGE₁ that totally inhibit pure macrophage colony proliferation. Neutrophilic colony formation was least sensitive to the effects of PGE with inhibition observed only at the highest concentration of PGE tested (10⁻⁵ to 10⁻⁶ M). It should be noted that comparison of total colony inhibition and inhibition of pure macrophage colonies are not coincident. Because macrophage colonies comprised at best 23% of total colonies, changes in the number of macrophage colonies result in much smaller changes in total colony incidence. No compensatory rise in the absolute number of mixed or neutrophil colonies in response to PGE₁ was detected.

The preferential inhibition of macrophage colony formation by PGE was further tested by employing CSF preparations which differ in their ability to stimulate morphologically distinct colony types. The addition of PGE₁ within a dose range of 10⁻⁵ to 10⁻¹² M was tested for its effect on the proliferation of mouse bone marrow progenitor cells exposed to concentrations of mouse L-cell-derived (LC)CSF and the DEAE breakthrough G-CSF fraction of WEHI-3-conditioned medium which maximally stimulated colony formation (Fig. 1). Macrophage colonies stimulated by LC-CSF were extremely sensitive to inhibition by PGE. A linear increase in colony inhibition was observed over log increases in PGE concentrations between 10⁻¹¹ and 10⁻⁶ M. Significant inhibition of macrophage colony formation was observed with as little as 10⁻¹⁰ M PGE ($P < 0.05$). 50% inhibition of macrophage colony formation

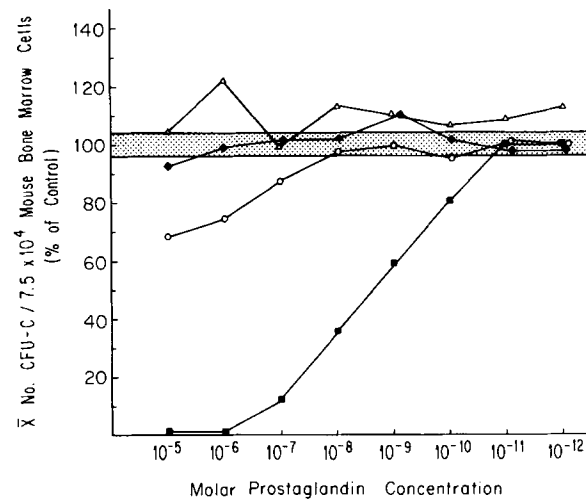


FIG. 1. Proliferation of murine bone marrow CFU-c stimulated by LC-CSF (closed symbols) and G-CSF (open symbols) in the presence of varying concentrations of PGE₁ (■, ○) and PGF_{2_a} (◆, △). Triplicate day 7 cultures were scored for each point. Data is expressed as a percent of control cultures (shaded area, $\bar{X} \pm SE$).

occurred between 10^{-8} and 10^{-9} M PGE (five experiments). Equivalent results were obtained using PGE₂ (not shown). In contrast to that observed using LC-CSF, CFU-c stimulated by G-CSF were resistant to all but 10^{-5} and 10^{-6} M concentrations of PGE₁. However, morphological analysis of G-CSF-stimulated colonies indicated that the inhibition of total colony formation, observed at these levels of PG, resulted from the inhibition of a small percentage of macrophage colonies (7–16%) stimulated by this CSF preparation. PGF_{2_a} was without inhibitory effect on total colony formation stimulated by either G-CSF, LC-CSF (Fig. 1) or WEHI-3 CSF (not shown), however a small enhancement (5–20%) in total proliferating colonies stimulated by G-CSF was observed. No differential effect on colony type was evident. Enhancement of WEHI-3 CSF-stimulated colony formation by PGF_{2_a} has been reported previously (17).

The sensitivity of colony formation to inhibition by PGE was not dependent on CSF concentration. The ability of 10^{-8} M PGE₁ to affect colony inhibition was tested over the extent of the dose-response curves for both G-CSF and LC-CSF. At all levels of G-CSF tested, PGE₁ was without significant inhibitory effect on colony formation (Fig. 2A). In contrast, colony formation stimulated by LC-CSF was inhibited by PGE₁ at all points where colony formation occurred (Fig. 2B).

Delayed Addition of PGE. The ability of PGE₁ to inhibit CFU-c proliferation, when added at various times after culture initiation, was investigated (Fig. 3). Regardless of whether 10^{-8} M PGE₁ was added simultaneously with or up to 72 h after culture initiation, no effect on the clonal growth or expansion of colonies stimulated by G-CSF was observed. In contrast, colony formation, stimulated by LC-CSF, was sensitive to PGE₁ when added as late as 72 h after the start of agar culture. Furthermore, colony size was also reduced by PGE addition, indicating an effect on both macrophage cloning efficiency and clone size. The DEAE eluate fraction of WEHI-3 CM, M-CSF, which stimulated predominantly macrophage (58%) and mixed (40%) colo-

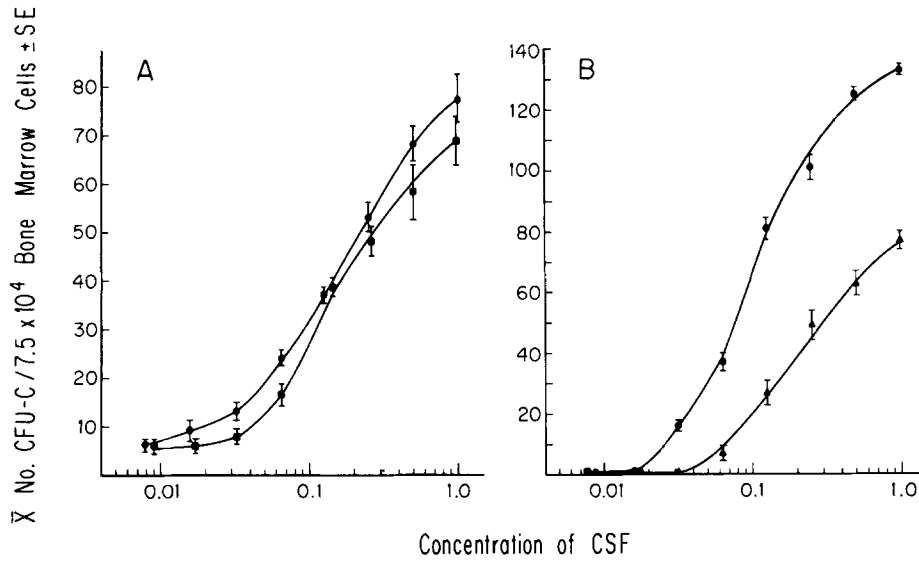


FIG. 2. Dose-response curves for G-CSF(A) and LC-CSF(B) preparations in the absence (●) and presence (■, ▲) of 10^{-8} M PGE_1 . Data is expressed as \bar{X} colonies (> 50 cells) \pm SE of triplicate day seven cultures.

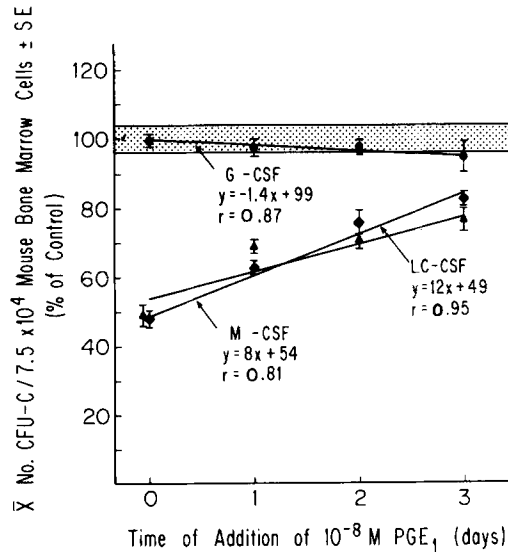


FIG. 3. Inhibition of colony (> 50 cells) proliferation stimulated by G-CSF, M-CSF, and LC-CSF preparations as a function of the time of addition of 10^{-8} M PGE_1 . Quadruplicate cultures were scored for each point on day 7 of culture and are expressed as $\bar{X}\% \pm$ SE of control cultures (shaded area). Colony inhibition was analyzed by least squares linear regression analysis and the coefficient of correlation (r) for each line indicated.

nies, was also tested for PGE_1 sensitive colony formation. A linear response between time of addition of 10^{-8} M PGE_1 and colony inhibition was observed, which was virtually identical to that observed using LC-CSF. The fact that the linear inhibition profiles are the same for both M-CSF and LC-CSF preparations suggest that the

TABLE II
PGE Production by Resident Murine Peritoneal Macrophages after Stimulation by WEHI-3 Colony-Stimulating Activities

CSF dilution	PGE*		
	WEHI-3CM	DEAE break-through (G-CSF)	DEAE elute (M-CSF)
Control	65 ± 11	65 ± 11	65 ± 11
1:2 (1.0)‡	2,030 ± 12	186 ± 112	4,093 ± 711
1:4	1,504 ± 180	19 ± 16	2,485 ± 78
1:8	1,199 ± 76	23 ± 12	1,793 ± 93
1:16	1,131 ± 64	24 ± 24	1,445 ± 174
1:32	897 ± 13	24 ± 24	1,801 ± 1

* Radioimmunoassay measurements of PGE in cell-free 24-h supernates. The results are expressed as mean concentration of PGE (picograms/milliliter) ± SE elaborated by adherent macrophages derived from cultures of 2.5×10^5 BDF₁PC.

‡ Concentration of CSF which maximally stimulates CFU-c proliferation.

population(s) of colony-forming cells (CFC) stimulated by both of these CSF are similar and equally sensitive to PG-mediated inhibition.

CSF Stimulation of Prostaglandin Synthesis. Production of PGE by mouse macrophages is thought to be linked to their surveillance of endogenously produced/released CSF. Experimentally, exposure to exogenously added CSF results in induction of macrophage PGE biosynthesis (5, 12). Evidence that different CSF stimulate colony formation differing in PG sensitivity suggested that CSF preparations may also differ in their capacity to stimulate PG production. To test this hypothesis, the unseparated and partially purified CSFs present in WEHI-3 CM were tested for their ability to stimulate PGE production by resident murine peritoneal macrophages in liquid culture (Table II). Radioimmunoassay measurements on cell-free 24-h supernates demonstrated that increasing concentrations of unseparated WEHI-3 CSF progressively stimulated macrophage PGE production. The DEAE G-CSF which stimulates predominantly mixed and neutrophilic colony formation in agar culture of mouse bone marrow cells had no effect on mouse macrophage PGE production. In contrast, the DEAE M-CSF preparation, which stimulates macrophage and mixed colony formation, was extremely active in stimulating PGE synthesis by resident mouse macrophages at all concentrations tested. Furthermore, the ability of DEAE M-CSF to stimulate PGE synthesis was enhanced in comparison to unseparated WEHI-3 CSF. No PGE production was observed using DEAE fractions devoid of colony-stimulating activity (not shown). In similar experiments, a preparation of highly purified L-cell CSF (22) was also found to stimulate PGE biosynthesis (430 ± 152 pg/ml, control; $1,687 \pm 309$ pg/ml per 280 U CSF, stimulated) when tested on adherent macrophage monolayers derived from 2.5×10^5 resident BDF₁ PC. Activity of this CSF preparation has been defined as 4.5×10^7 U/mg protein (22).

Macrophage Control of CFU-c Proliferation. The macrophage has been shown to be a constitutive and inducible producer of both CSF and PGE (5, 12, 23). The roles of CSF and PGE on CFU-c proliferation and morphology of resulting colonies were investigated using a two-layer soft agar culture system (Fig. 4 A). Increasing numbers of resident peritoneal macrophages were used as feeder layers for mouse bone marrow

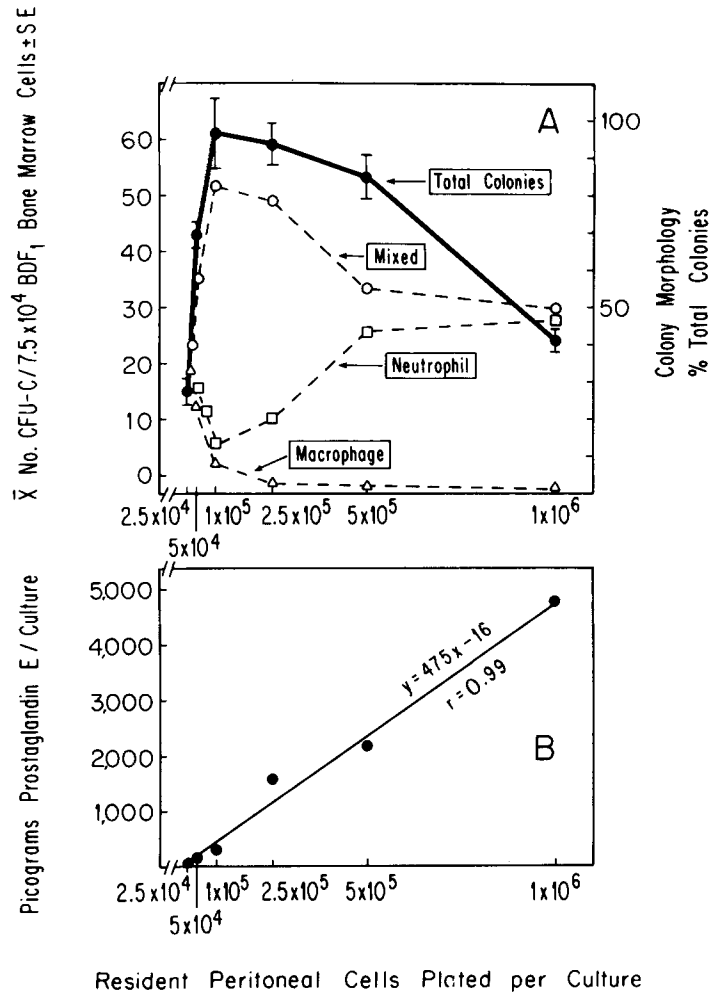


FIG. 4. (A) Morphological analysis of CFU-c proliferation stimulated by increasing concentrations of adherent resident murine macrophages used as feeder layers. Total colonies (> 50 cells) were determined for quadruplicate cultures on day 7 of culture and the results are expressed as \bar{X} CFU-c \pm SE. Single colony morphology was determined for each point by analysis of 40 sequential colonies transferred to microscope slides and stained with aceto-orcein. (B) Constitutive PGE production, measured by radioimmunoassay, was determined on 24-h cell-free supernates harvested from replicate liquid cultures containing the identical number of adherent macrophages as the agar underlayers. The results are expressed as total picograms per 24 h.

cells. In the absence of exogenously added CSF, macrophages stimulated the proliferation of bone marrow CFU-c, with maximal colony formation observed with adherent macrophages derived from 1.0×10^5 peritoneal cells. Increasing the number of peritoneal cells beyond this concentration resulted in a progressive decline in total colony growth.

Analysis of individual colony morphology demonstrated that increasing the number of feeder layer cells beyond the macrophage equivalent of 5×10^4 PC per culture resulted in a progressive decline in the percentage of macrophage colonies. The percentage of mixed colonies seemed to parallel that of total colonies, being stimulated

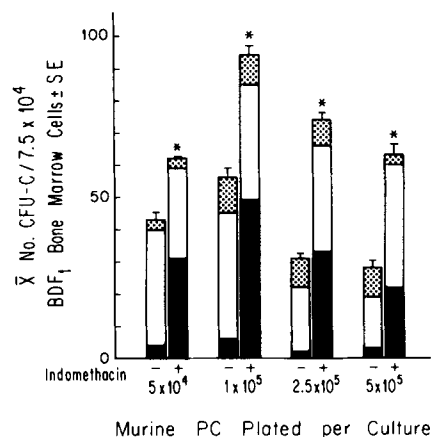


FIG. 5. Modulation of CFU-c proliferation and differential morphology by indomethacin. Target cell suspensions at 7.5×10^4 mouse bone marrow cells in 0.3% agar were plated above underlayers, containing varying numbers of adherent murine macrophages, prepared in the absence and presence of 10^{-6} M indomethacin. Quadruplicate seven day cultures were scored for each point and the results are expressed as total proliferating colonies \pm SE. Differential colony morphology, determined by analysis of 50 sequential colonies, is expressed as the number of colonies identified as being neutrophilic granulocyte (▨), mixed macrophage-neutrophil (□) or macrophage (■) in composition. Statistical significance, $P < 0.0005^*$.

by low numbers of adherent cells and inhibited by high numbers. In addition, after a small initial decline, the percentage of neutrophil colonies progressively increased as mixed and macrophage colonies declined.

Radioimmunoassay measurements of PGE levels were performed simultaneously on parallel cultures of adherent macrophages derived from the identical number of peritoneal cells used to prepare the cell-feeder layers. A progressive increase in PGE production was observed with increasing numbers of adherent peritoneal macrophages (Fig. 4 B). The synthesis of PGE by adherent macrophages in liquid culture suggested that the decrease in total colonies, and more specifically macrophage colonies, observed with increasing numbers of adherent peritoneal cells used in agar underlayers was a result of the accumulation of PGE.

To test the effects of PGE synthesis by macrophages used as agar feeder layers on the cloning efficiency of morphologically distinct colony types, similar experiments were performed in the absence and presence of 10^{-6} M indomethacin, a specific inhibitor of prostaglandin production (Fig. 5). As shown in the previous experiment, in the absence of indomethacin maximal CFU-c proliferation occurred at adherent macrophage concentrations equivalent to 1×10^5 PC, whereas a decline in total colony proliferation occurred with adherent cells above this concentration. The percentage of total colonies morphologically identifiable as macrophage was 9–11%. Mixed colony inhibition was evident only at PC concentrations of 2.5×10^5 and 5×10^5 , whereas the percentage of neutrophilic colonies increased steadily from 7% of total colonies at 5×10^4 PC/culture to 32% at 5×10^5 PC culture in a similar fashion to that observed in Fig. 4 A. The addition of indomethacin to the agar feeder layers at the start of culture resulted in the significant augmentation ($P < 0.0005$) of colony formation at all concentrations of feeder cells tested and greatly reduced the decline in colony numbers observed at high PC concentrations. More dramatic than

TABLE III
Effects of Lactoferrin on Macrophage-Derived CSF Stimulation of CFU-C Proliferation and Colony Morphology in the Presence of Indomethacin

Experiment	Addition	CFU-c/culture*	Colony morphology‡		
			MΦ	MΦ-N	N
I	Control +I§	87 ± 3.8	45	38	4
	lactoferrin +I	46 ± 1.8 (47%)¶	24 (47%)¶	20 (47%)¶	2 (50%)¶
II	Control +I	57 ± 2.4	20	24	14
	lactoferrin +I	23 ± 2.1 (60%)	10 (50%)	12 (50%)	5 (64%)
III	Control +I	93 ± 3.3	23	60	7
	lactoferrin +I	57 ± 3.0 (40%)	19 (18%)	34 (43%)	4 (43%)
IV	Control +I	106 ± 3.3	21	69	16
	lactoferrin +I	64 ± 3.0 (40%)	18 (14%)	38 (45%)	8 (50%)
V	Control +I	77 ± 1.5	36	34	6
	lactoferrin +I	50 ± 2.2 (37%)	26 (28%)	23 (33%)	1 (83%)
x̄% Inhibition ± SE range		(45 ± 4.1%) (37-60%)	(31 ± 7.4%) (14-50%)	(44 ± 2.9%) (33-50%)	(58 ± 7%) (43-83%)

* Total colonies (>50 cells)/7.5 × 10⁴ BDF, Bone marrow cells.

‡ Single colony morphology based upon examination of 40-50 sequential colonies transferred to microscope slides and stained with aceto-orcein. Data is expressed as actual number of colonies of specified morphology.

§ 10⁻⁶ M final indomethacin concentration.

|| 10⁻⁸ M 100% Fe-Saturated Lactoferrin.

¶ % inhibition as a result of lactoferrin treatment.

the effect of indomethacin on total colony incidence was the effect on macrophage colony formation. Inhibition of adherent PC feeder cell PG synthesis resulted in 6.8 (5 × 10⁴)-, 7.2 (1 × 10⁵)-, 15.5 (2.5 × 10⁵)-, and 6.3 (5 × 10⁵)-fold increases in the number of macrophage colonies proliferating at the PC concentrations indicated. Mixed colonies were markedly less sensitive to the effects of indomethacin, with 57 and 137% increases in colony formation observed at PC concentrations of 2.5 × 10⁵ and 5 × 10⁵, respectively, with no increases observed at lower PC concentrations.

Effects of LF on Macrophage Colony-Stimulating Activity Production. A granulocyte derived inhibitor of myelopoiesis, recently identified as LF, decreases macrophage CSF production and has no inhibitory effect on the proliferation of CFU-c stimulated by cell-free sources of CSF (15, 16). The role of LF on macrophage production of CSF and analysis of the resulting colonies was investigated (Table III).

Previous results (Figs. 4 and 5) demonstrated that constitutive macrophage production of PG over 7 d in culture was sufficient to dramatically reduce pure macrophage colony proliferation. These results suggested that the inhibition of macrophage colonies observed as a result of LF might be complicated by coincident macrophage PGE production. Eilayer agar experiments were therefore performed using macrophage feeder layers treated with both LF (10⁻⁸ M) and indomethacin (10⁻⁶ M) at the start of culture (Table III). In the presence of indomethacin, macrophage stimulation of CFU-c proliferation was reduced 45 ± 4.1% by the addition of LF. The effects of LF were observed on all proliferating colony types, however, macrophage-derived CSF, which stimulates neutrophil colony formation, was more sensitive to inhibition by LF (*P* < 0.025) than macrophage-derived CSF

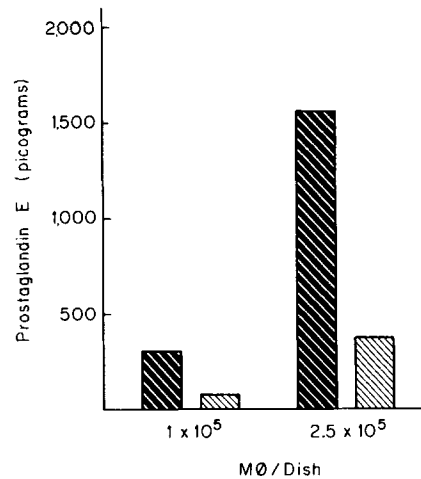


FIG. 6. Constitutive PGE production by 1×10^5 and 2.5×10^5 resident adherent murine peritoneal macrophages in the absence (▨) and presence (▩) of 10^{-8} M 100% iron-saturated lactoferrin. Data is expressed as picograms per milliliter PGE produced in 24 h, as measured by radioimmunoassay.

which stimulates macrophage colony formation. In the absence of indomethacin, LF reduced total colony formation $47 \pm 7.4\%$ with similar effects on specific colony types (not shown). These results suggest that the effects of LF are independent of the effects of PGE.

Inhibition of Macrophage PGE Production. We have previously demonstrated that the granulocyte-derived colony-inhibitory activity (CIA) was capable of inhibiting macrophage PGE production (5). Similarly, LF was tested for its ability to reduce constitutive macrophage PGE production in liquid culture. Cell-free 24-h supernates, harvested from cultures of adherent macrophages derived from 1×10^5 and 2.5×10^5 resident mouse peritoneal cells that were cultured in the absence or presence of 10^{-8} M LF, were measured for PGE content by radioimmunoassay (Fig. 6). PGE production by adherent macrophages derived from 1×10^5 and 2.5×10^5 peritoneal cells, which maximally stimulate CFU-c proliferation, was reduced 74 and 76%, respectively.

Discussion

Hemopoietic colony-forming cells (CFC) are not homogeneous, but consist of subpopulations of progenitor cells at different stages of differentiation and maturation. Mouse bone marrow CFU-c which give rise to macrophage and neutrophil progeny are heterogeneous in their physical characteristics (24, 25) and responsiveness to different molecular species of CSF (26). We now demonstrate that CFU-c populations committed to macrophage differentiation are differentially inhibited by prostaglandins of the E series. Moreover, a hierarchy of sensitivity to PGE₁ was observed among CFU-c stimulated either by exogenously added CSF preparations, or proliferating in response to colony-stimulating activity provided by adherent macrophage feeder layers.

PGE-mediated regulation of macrophage colony formation is demonstrated most dramatically using adherent peritoneal macrophage feeder layers. Simultaneous

analysis of colony morphology on bilayer cultures and PGE measurements performed on cell-free supernates conditioned by mouse macrophages, indicate that a dramatic decline in macrophage colonies, as well as total CFU-c, observed with increasing macrophage concentrations, occurs coincident with a linear increase in PGE production.

Inhibition of adherent macrophage PGE production by indomethacin results in enhanced total colony formation and prevents the decline in colony formation observed at high macrophage concentrations. More specifically, morphologic analysis of proliferating colonies demonstrated that macrophage colonies were selectively enhanced by inhibition of PG synthesis. Total absolute numbers of mixed and neutrophil colonies were relatively unchanged, however an increase in the percentage of neutrophil colonies was observed with increasing macrophage concentrations in the absence of indomethacin. This may be explained by the fact that as macrophage colonies are selectively inhibited, the remaining noninhibited neutrophil colonies therefore represent a greater percentage of total colonies. In addition, selective inhibition of the monocytoid component of mixed colonies may have resulted in the conversion of some mixed colonies into pure neutrophil colonies, thereby resulting in a small increase in the absolute numbers of neutrophil colonies (Figs. 4 and 5).

The effects of PGE on mixed colony formation were intermediate between that observed for colonies of pure macrophages or neutrophils. In comparison to macrophage colonies, $\cong 3$ log greater concentrations of PGE₁ are required for equivalent inhibition of mixed granulocyte-macrophage colony formation. Although the growth of mixed colonies was significantly less sensitive to inhibition by PGE, the number of mononuclear cells in the mixed colony was dramatically reduced. In a similar fashion, a decrease in the monocytoid component of mixed colonies is observed with increasing numbers of peritoneal macrophages used as feeder layer cells. In the presence of indomethacin, the macrophage and granulocyte composition of mixed colonies was equivalent. These results support the differential sensitivity of macrophage colony forming cells to the effects of PGE. The persistence of macrophages within mixed colonies even at concentrations of PGE which can inhibit pure macrophage colony formation suggests that the macrophages observed within mixed colonies may be derived from a more resistant macrophage CFC.

Prostaglandins of the F series had little effect on myeloid colony proliferation, although a small enhancement in total colony formation was observed. The inability of PGF_{2 α} to inhibit macrophage proliferation is further supportive of the specific role of PGE in the control of the macrophage stem cell compartment.

The cloning efficiency of macrophage CFU-c stimulated by exogenously added LC-CSF was sensitive to inhibition by PGE concentrations as low as 10^{-9} to 10^{-10} M. Levels of 10^{-10} M PGE are routinely detected in human peripheral blood (27). At this concentration only moderate inhibition of macrophage differentiation would be observed. However, in circumstances resulting from trauma, inflammation, or infection, localized levels of PGE as high as 1×10^{-7} M can be obtained (28). Under these conditions, significant inhibition of macrophage proliferation could occur. In contrast, no inhibition of colony forming cells which give rise to neutrophilic granulocytes was observed below 10^{-6} M PGE concentrations. It is highly unlikely that PGE levels of this magnitude could occur in vivo.

We have recently reported that CSF plays an important regulatory role for macrophage prostaglandin production, whereby surveillance of the levels of consti-

tive, inducible or exogenously added CSF results in and may be necessary for induction of macrophage/monocyte prostaglandin biosynthesis (5, 12). Direct measurements of PGE biosynthesis in response to unpurified, semipurified, and highly purified CSF preparations confirms and extends this hypothesis. The ability of CSF to initiate macrophage PGE mediated feedback regulation is coincident with its ability to specifically stimulate clonal macrophage proliferation. These results indicate that a specific compartmental regulatory mechanism unique within the hemopoietic system may exist, whereby mature tissue macrophages regulate the proliferation of their specific progenitor cells by production of CSF and PGE. Colony formation by specific macrophage progenitor cells present in thioglycollate-induced peritoneal populations (CFU-PM) have also been demonstrated to be extremely sensitive to inhibition by PGE (17). Furthermore, the growth and differentiation of the WEHI-3 myelomonocytic cell line is inhibited by PGE and may reflect the retention of many macrophage characteristics by this cell line (29). Progenitor cells which give rise to megakaryocyte and eosinophil (30) or erythroid colonies (J. Kurland and P. Meyers, unpublished observations) are not inhibited by PGE. The inhibition of B-lymphocyte colony formation by macrophage-derived PG (31) would appear to contradict the uniqueness of macrophage CFC to control by PGE, however the B-lymphocyte assay does not detect progenitor cell proliferation but rather measures the clonal potential of mature B lymphocytes (32). It is well known that PGE is highly inhibitory to mature B-cell function (33).

Specific control of macrophage differentiation by PGE indicates that a second feedback mechanism may exist for the control of granulocyte formation. Studies on the mechanism of action of lactoferrin now indicate that it suppresses the production by macrophages of colony-stimulating activities for macrophage, mixed macrophage-granulocyte and neutrophilic granulocyte colony proliferation, as well as reducing constitutive macrophage PGE production presumably by decreasing CSF production.

The *in vitro* regulatory influences of lactoferrin and PGE defined in this paper suggest one model which may explain both the steady-state control of macrophage and granulocyte formation *in vivo* and the response of the host to bacterial infection or transient endotoxemia. In the unperturbed state, macrophage and granulocyte differentiation is governed by the balance between the positive stimulus provided by CSF and the negative influences of macrophage-derived PGE and polymorphonuclear granulocyte-derived lactoferrin. In addition, lactoferrin may serve as a safeguard against excessive macrophage-derived PGE-mediated limitation of monocytopenesis. Bacterial infection or endotoxemia leads to increased myeloid progenitor cell proliferation as a result of increasing CSF concentrations, which overcome steady-state control by LF (15) and PGE (12). In the presence of high CSF levels, macrophage PGE production increases progressively and specifically limits monocytopenesis while allowing granulopoiesis to proceed. Accumulating neutrophils release increasing quantities of LF which have little inhibitory effect in the continuing presence of the stimulus for CSF production (bacterial lipopolysaccharide) (15). However, upon elimination of the source of infection, LF serves to reduce CSF production, and coincidentally macrophage PGE synthesis, thereby facilitating a return to steady-state conditions.

Summary

Hemopoietic colony-forming cells committed to macrophage differentiation (M-CFC) are selectively and differentially inhibited by prostaglandin E (PGE). A

hierarchy of sensitivity was observed among murine CFC stimulated by colony-stimulating factors (CSF) which differ in their ability to initiate proliferation of morphologically distinct colony types, or stimulated by CSF provided by macrophage feeder layers. Inhibition of macrophage colony formation to 50% levels occurred with PGE concentrations between 10^{-8} and 10^{-9} M, and was still evident at 10^{-10} – 10^{-11} M PGE concentrations. The growth of mixed colonies containing both macrophages and neutrophils was less sensitive to the inhibitory effects of PGE, however, the monocytoid component of these colonies was reduced in the presence of PGE. Neutrophil progenitor cell proliferation was not influenced by PGE concentrations below 10^{-6} M, regardless of time of addition of PGE, whereas clonal macrophage expansion, as well as clone size, was sensitive to inhibition by PGE when added as late as 3 d after culture initiation. Prostaglandin $F_{2\alpha}$ was not inhibitory to colony formation. Experimental evidence for a selective role of macrophage PGE in the regulation of macrophage colony formation was directly provided by utilizing resident peritoneal macrophages as a source of CSF for bone marrow target cell overlays. Simultaneous morphological analysis of colonies proliferating in bilayer culture in response to increasing concentrations of macrophages, and direct measurements of PGE synthesized by an identical number of macrophages maintained in liquid culture demonstrate that a specific decline in macrophage colony formation occurs coincident with a linear increase in macrophage PGE synthesis. Inhibition of macrophage PGE synthesis by indomethacin results in the specific enhancement of macrophage colony formation. Furthermore, macrophage PGE synthesis is induced by CSF preparations with the selective capacity to differentially stimulate macrophage proliferation, but not by those which preferentially stimulate granulocyte colony formation. In comparison to the effects of PGE on M-CFC, polymorphonuclear granulocyte-derived lactoferrin (LF) reduces macrophage production of colony-stimulating activities for macrophage, mixed macrophage-neutrophil and neutrophil colony formation. The ability of LF to reduce macrophage PGE synthesis, presumably by decreasing CSF production, suggests that LF and PGE can interact in the control of macrophage and granulocyte proliferation.

The authors wish to thank Dr. Neil Williams and Richard Eger for providing WEHI-3 CSF preparations, and Dr. R. Shadduck for purified L-cell CSF. The excellent secretarial assistance of Ms. Harriet Shields and Ms. Carol Beeghly is gratefully acknowledged.

Received for publication 12 April 1979.

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