

SYNTHESIS AND RELEASE OF ERYTHROID COLONY- AND
BURST-POTENTIATING ACTIVITIES BY PURIFIED
POPULATIONS OF MURINE PERITONEAL MACROPHAGES*

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The classical erythropoietic hormone, erythropoietin (Ep)¹ (1), has only recently become amenable to study *in vitro*. The recent development of semisolid colony-forming techniques (2, 3) similar to those used in the clonal detection of other hematopoietic precursors has greatly facilitated the investigation of the role of Ep and other humoral regulators in the control of at least two populations of committed erythroid precursors. In the presence of Ep *in vitro*, murine bone marrow gives rise to erythroid colonies after 2 d of incubation, deriving from a committed erythroid precursor termed the colony-forming unit-erythroid (CFU-E), and large bursts after 9 d incubation, deriving from a precursor called the burst-forming unit-erythroid (BFU-E) (4). Although it is now possible to demonstrate activity of Ep both *in vivo* and *in vitro*, the concentrations required *in vitro* are much higher than those detectable *in vivo*. This suggests that other host factors must potentiate the *in vivo* activity of Ep. Although the nature and origin of such erythroid-potentiating activities are not yet defined, recent studies by Iscove (5), Wagemaker (6), and Aye (7) have shown the existence of substances that promote the action of Ep *in vitro*. The variously named burst-promoting activities (BPA) or burst-feeding activities (BFA) may represent a class of molecules that are essential to the *in vivo* actions of Ep and may serve to explain some of the alterations seen in *in vivo* erythropoiesis despite the absence of measurable changes in the levels of Ep.

Numerous studies attest to the intimate physical relationship between macrophages or reticular cells and developing cells of the erythroid series (8). The macrophage has been proposed as a nurse cell that facilitates the transfer of iron to developing erythroblasts. We have used a variety of hematopoietic tissues and cell-separation procedures to demonstrate that the macrophage plays a role in the *in vitro* response of both CFU-E and BFU-E to Ep. This potentiation of *in vitro* Ep activity is independent of physical contact between macrophages and erythroid precursors, cannot replace an absolute requirement for Ep, is produced by macrophages in the absence of Ep, and is undetectable with any lymphocyte populations tested.

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¹ *Abbreviations used in this paper:* BFA, burst-feeding activity(ies); BFU-E, burst-forming unit(s)-erythroid; BPA, burst-promoting activities; CFU-E, colony-forming unit(s)-erythroid; CSF, colony-stimulating factors; Ep, erythropoietin; FCS, fetal calf serum; GM, granulocyte-macrophage; PGE, E-series prostaglandins.

Materials and Methods

Female B6D2F₁ mice (Cumberland View Farms, Clinton, Tenn.), were used at 2–3 mo of age to provide bone marrow, thymus, spleen, lymph node, and peritoneal cells.

Preparation of Cell Suspensions. Cells were prepared as previously described (9, 10). Briefly, bone marrow was obtained by flushing the femurs from at least three mice with ice-cold serum-free McCoy's 5A modified medium. The cells were pooled, a single-cell suspension was prepared, and the cells were washed three times by centrifugation. Spleen, thymus, axillary, mesenteric, and inguinal lymph nodes were excised, sliced into 1-mm fragments, and teased through stainless steel mesh. Large fragments of fat and connective tissue were removed, and the cells were washed three times, counted, appropriately diluted, and kept on ice until needed. Resident, noninduced peritoneal cells were collected by peritoneal lavage with ice-cold McCoy's 5A medium that contained 1 U/ml of preservative-free heparin, washed twice at 4°C, suspended in serum-free medium, and kept on ice until needed.

Cell Separations. Adherent cell-depleted populations were prepared by passage through columns of Sephadex G-10 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) with the method of Ly and Mischell (11), modified as previously described (10). Briefly, 2 ml of McCoy's 5A modified medium with 5% fetal calf serum (FCS) (Microbiological Associates, Walkersville, Md.) that contained 1×10^8 spleen or 2.5×10^7 peritoneal cells/ml was passaged through plastic columns of 10 ml of autoclaved Sephadex G-10 beads that were prewarmed to 40°C in Hanks' balanced salt solution that contained 5% FCS. Cells recovered in the column filtrate were predominantly lymphocytic and were washed three times, counted, and appropriately diluted. In other experiments, various numbers of washed peritoneal cells were allowed to adhere to 35-mm tissue culture dishes (Lux Scientific Corp., Newbury Park, Calif.) in McCoy's 5A medium with 15% FCS for 1.5 h at 37°C. The nonadherent cells were removed, washed, and saved. The cells that remained adherent after vigorous rinsing with serum-free phosphate-buffered saline comprised 15–20% of the initial inoculum. Morphologically, the adherent cells were exclusively macrophages; they all phagocytosed latex beads and stained positively with neutral red (12) and cytoplasmic nonspecific alpha-naphthyl acetate esterase (13). The nonadherent cells were predominantly lymphocytes, but also contained a few macrophages and granulocytic leukocytes.

Peritoneal cells were also separated by velocity sedimentation with a modification of the method described by Miller and Phillips (14). Fractions of cells were examined for the number of cells that adhered to plastic dishes as described above. The cells were also stained for nonspecific esterase on etched grids, as described previously (10, 15), cellular morphology was assessed by Wright-Giemsa staining of cytocentrifuged slide preparations, and phagocytosis by quantitating uptake of 0.8- μ m latex beads. The adherent cells were used as feeder layers in semisolid cultures designed to support the clonal growth of erythroid and granulocyte-macrophage precursors and B lymphocytes.

Culture and Scoring of Erythroid Colonies. Cells were cultured with the method of Iscove et al. (3). Briefly, 2×10^5 nucleated bone marrow cells were suspended in 1 ml of alpha medium (Flow Laboratories, Inc., Rockville, Md.) that contained 30 or 4% FCS, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, and 0.8% methylcellulose (Dow Chemical Co., Midland, Mich.). Various concentrations of Ep from several sources were added to the cultures. The culture mixture was layered over underlayers that contained lymphoid or macrophage cell populations. The underlayers, which were prepared by mixing cell suspensions with McCoy's 5A medium that contained 4 or 15% FCS and 0.5% agar (Difco Laboratories, Detroit, Mich.), were established 24 h before the methylcellulose overlayers were added. Cell-free agar underlayers were prepared where appropriate for controls, and in some experiments medium conditioned by peritoneal macrophages was incorporated into the underlayer. The cultures were incubated in a fully humidified atmosphere of 5% CO₂ in air at 37°C. Erythroid colonies and bursts that developed in the overlayers were scored *in situ* with an inverted microscope. Colonies were identified at 36–48 h by their characteristic appearance, as previously described (4), and contained eight or more hemoglobinized cells. Erythroid bursts were scored after 9 d of incubation.

Most of the experiments were performed with Ep kindly supplied by Dr. Norman Iscove (Basel Institute of Immunology, Basel, Switzerland) with a 63 U/mg sp act that had been

TABLE I
Effect of Lymphoid and Peritoneal Cell Feeder Layers on CFU-E and BFU-E

Feeder cells	Number of cells per feeder	CFU-E		BFU-E	
		-Ep	+Ep*	-Ep	+Ep
None	None	3 ± 2	105 ± 7	0	13 ± 2
	10 ⁵	5 ± 2	93 ± 6	0	13 ± 2
Spleen	5 × 10 ⁵	3 ± 1	93 ± 11	0	11 ± 2
	10 ⁶	3 ± 2	99 ± 10	0	11 ± 1
Spleen (Sephadex G-10 passaged)	10 ⁵	5 ± 2	103 ± 6	0	12 ± 2
	5 × 10 ⁵	3 ± 3	88 ± 5	0	13 ± 2
	10 ⁶	4 ± 2	99 ± 6	0	11 ± 1
	10 ⁵	5 ± 2	96 ± 5	0	12 ± 2
Lymph node	5 × 10 ⁵	3 ± 1	97 ± 8	0	11 ± 2
	10 ⁶	2 ± 1	97 ± 11	0	10 ± 1
	10 ⁵	4 ± 2	95 ± 8	0	12 ± 2
Thymus	5 × 10 ⁵	3 ± 2	90 ± 8	0	13 ± 2
	10 ⁶	2 ± 2	84 ± 6	0	12 ± 2
	10 ⁵	18 ± 2	159 ± 12	0	16 ± 2
Peritoneal lavage	5 × 10 ⁵	26 ± 4	283 ± 7	0	25 ± 5
	10 ⁶	54 ± 3	311 ± 23	0	32 ± 3
Peritoneal lavage (Sephadex G-10 passaged)	10 ⁶	ND	95 ± 7	ND	12 ± 2

* 2 U Ep/ml. 63 U/mg sp act.

prepared from the urine of patients with aplastic anemia. Some experiments were performed with Ep generously provided by Dr. Eugene Goldwasser (Department of Biochemistry, University of Chicago, Chicago, Ill.). This material had a 70,000 U/mg sp act and was pure by all available biochemical separative techniques (16).

Soft agar assays used for the detection of granulocyte-macrophage and B lymphocyte colony-forming cells, supported by feeder layers of macrophages or conditioned medium, were prepared as previously described (9, 10).

Results

Effect of Lymphoid and Peritoneal Cells on CFU-E and BFU-E. Methylcellulose erythroid cultures were overlaid on agar underlayers to examine the effect of lymph node, spleen, thymus, and peritoneal cells on the in vitro growth of CFU-E and BFU-E (Table I). In the absence of Ep, few CFU-E and no BFU-E were detectable in cultures prepared with a cell-free agar underlayer. The addition of 2 U/ml of Ep stimulated developing 2-d CFU-E and permitted the growth of 9-d BFU-E. Feeder layers that contained various concentrations of spleen, lymph node, and thymus cells had no effect on CFU-E and BFU-E in either the absence or presence of Ep. In contrast, feeder layers that contained peritoneal lavage cells potentiated both CFU-E and BFU-E in a dose-dependent fashion. Although peritoneal cells augmented CFU-E in the absence of exogenously added Ep, the same feeder layers could not replace the obligate requirement for Ep by BFU-E. Underlayers prepared from peritoneal cells passaged over columns of Sephadex G-10 lost all ability to enhance erythroid precursors. Underlayers prepared from spleen cells passaged over Sephadex G-10 had no effect on erythroid colonies, which indicated that the Sephadex-passaged cell suspensions were not inhibitory. Because mononuclear phagocytes represent a large component of peritoneal cell suspensions, and Sephadex G-10 passage depletes such

cells, these results suggested a role for diffusible products of macrophages in the potentiation of the proliferation of erythroid precursors *in vitro*.

The bone marrow cells used as the source of target CFU-E and BFU-E also contained appreciable numbers of granulocyte-macrophage (GM) precursors (CFU-GM) that underwent clonal proliferation in the presence of colony-stimulating factors (CSF) (GM-CSF) elaborated by the peritoneal cell underlayers. Myeloid colonies were distinguished from erythroid colonies by gross morphology, the absence of hemoglobinized cells, and by differences in times of appearance and persistence of CFU-E and erythroid colonies. Individual colonies were removed for histochemical identification to confirm their identification under the inverted microscope.

Potentiation of CFU-E and BFU-E by Adherent, but Not Nonadherent Cells. In addition to depleting adherent cells from peritoneal cell suspensions, we examined whether adherent peritoneal cells themselves were able to satisfy the unseparated peritoneal cell requirement to potentiate CFU-E and BFU-E. Erythroid colony and burst formation in methylcellulose were progressively stimulated by the addition of increasing concentrations of Ep over cell-free agar underlayers.

Underlayers that contained the plastic-adherent cells from 5×10^5 unseparated peritoneal lavage cells markedly augmented the numbers of both CFU-E and BFU-E stimulated by each concentration of Ep tested. In contrast, underlayers that contained 5×10^5 nonadherent cells had only a slight effect on CFU-E and no effect on BFU-E (Fig. 1). The small augmentation of CFU-E by the nonadherent peritoneal cells may be a result of the poor efficiency of plastic adherence in depleting adherent cells in comparison to Sephadex G-10. Because only ~20% of the noninduced peritoneal cells adhere to plastic in the presence of serum (10), the stimulatory effects of the adherent peritoneal cells represent the effects of only 1×10^5 cells that remained in the dish after removal of the nonadherent cells before the addition of the agar. At the highest concentration of Ep used, the adherent peritoneal cells were 35–40 times more potent than the nonadherent cells in promoting erythroid colony formation for equivalent cell concentrations. In the absence of added exogenous Ep, adherent cells potentiated CFU-E, but not BFU-E, thus indicating that the adherent peritoneal macrophages cannot replace the absolute Ep requirement for erythroid burst formation. The amount of potentiation of both CFU-E and BFU-E increased with increasing Ep concentrations.

Similar results were obtained with biochemically pure Ep (Fig. 2) (16). Despite the large difference in purity of the Ep preparations, the presence of adherent macrophages stimulated a much greater number of CFU-E and BFU-E at each Ep concentration; which indicated that both CFU-E and BFU-E become more responsive to Ep when cultured in the presence of macrophages.

Effect of Indomethacin on the Potentiation of CFU-E and BFU-E by Macrophages. The E-series prostaglandins (PGE) are reported to induce erythroid differentiation in Friend erythroleukemia cells (17), potentiate the action of Ep in the stimulation of [^{14}C]-glucosamine, and potentiate ^{59}Fe incorporation into newly formed erythrocytes (18). In view of the recent demonstration that monocytes and macrophages synthesize and release large amounts of PGE *in vitro* (19), we examined whether the elaboration of PGE may, in part, mediate the erythroid-potentiating activity of macrophages (Fig. 3). The addition of indomethacin, an inhibitor of prostaglandin synthesis (20), at concentrations that completely inhibit PGE synthesis by macrophages (19), was

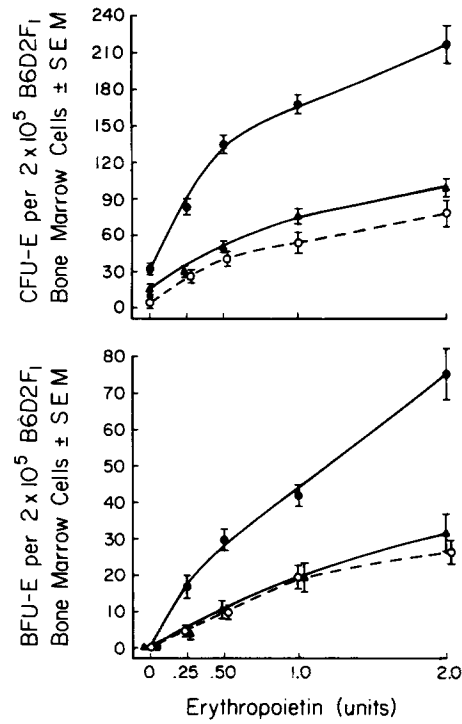


FIG. 1. Effects of adherent and nonadherent peritoneal cells on CFU-E and BFU-E. Methylcellulose cultures prepared with 2×10^5 normal B6D2F₁ nucleated bone marrow cells with the indicated concentrations of Ep were suspended over 0.5% agar underlayers that contained 5×10^5 unseparated washed peritoneal cells (▲) the plastic-adherent cells from 5×10^5 peritoneal cells (●), or control cell-free agar underlayers (○). The cultures were scored at 2 d for CFU-E (upper panel) and at 9 d for BFU-E (lower panel). Results are the mean of triplicate cultures \pm SEM.

without any effect on the enhancement of CFU-E and BFU-E by macrophages in response to Ep. Synthetically pure PGE₁ had a slight, but significant, enhancing effect on CFU-E and BFU-E (Fig. 4).

Promotion of CFU-E and BFU-E As a Function of Macrophage Concentration. We investigated the effects of varying numbers of adherent peritoneal macrophages on the growth of CFU-E and BFU-E in both the absence and presence of Ep (Fig. 5). In the presence of plateau concentrations of Ep, the numbers of CFU-E and BFU-E were progressively potentiated as a function of the number of adherent peritoneal macrophages in the underlayer. The enhancement of erythroid precursors approached a plateau with $>0.5 \times 10^6$ unseparated peritoneal cells. Because the nonadherent cells were removed before the addition of the agar and because $\sim 20\%$ of the peritoneal cells adhered to plastic, underlayers established with 1×10^5 peritoneal cells contained $\sim 2 \times 10^4$ adherent cells and potentiated CFU-E and BFU-E twofold. Plateau stimulation was seen with more than from 7.5×10^4 to 1×10^5 macrophages per underlayer.

In the absence of added exogenous Ep, no BFU-E could be stimulated by any of the macrophage concentrations tested. In contrast, the numbers of CFU-E that were detected in the absence of added exogenous Ep progressively increased with increasing

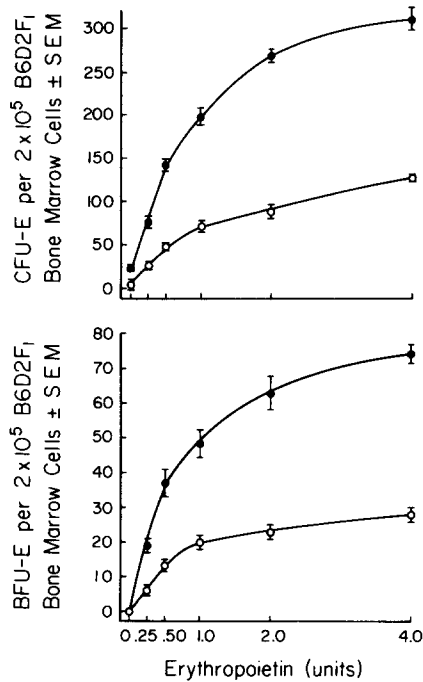


FIG. 2. Effects of adherent peritoneal macrophages on the responsiveness of CFU-E and BFU-E to purified Ep. Cultures were prepared as in Fig. 1, with 0.5% agar underlayers that contained the plastic-adherent cells from 5×10^5 peritoneal cells (●) or no cells (○). The methylcellulose overlayers contained the indicated concentration of purified Ep (79,000 U/mg). Results are the mean \pm SEM.

concentrations of macrophages in the underlayer. Within the range of macrophages used, there was no evidence of a plateau in this enhancing effect.

We investigated whether this macrophage stimulation of CFU-E in cultures prepared in the absence of deliberately added Ep was truly independent of Ep. Because Ep is a constituent of FCS, experiments were performed with the lowest possible concentration of FCS that would support erythroid colony formation in the presence of added Ep. By reducing the serum concentration from the usual 30% to 4% (Fig. 6), no CFU-E developed in the absence or presence of increasing concentrations of macrophages. The addition of 2 U of pure Ep alone stimulated low numbers of CFU-E. Although macrophages were unable to stimulate CFU-E in the absence of Ep, the presence of macrophages in the underlayers of cultures that contained pure Ep profoundly and progressively potentiated the numbers of developing CFU-E.

Detection of Erythroid-enhancing Activity in Macrophage Culture Supernate. Because macrophages promoted erythroid colony and burst formation only in the presence of Ep and were unable to stimulate CFU-E in the absence of Ep in cultures that contained low concentrations of serum, we examined whether the presence of Ep itself was essential for the production and/or release of the erythroid-enhancing activity by macrophages. Supernatant media were harvested at different times from liquid cultures that contained the adherent macrophages from 1×10^6 peritoneal lavage cells prepared in the presence of 2% FCS. These conditioned media were tested at a final concentration of 30% in cell-free agar underlayers for CFU-E-promoting activity in methylcellulose cultures stimulated by 2 U of Ep (Fig. 7). Erythroid-enhancing

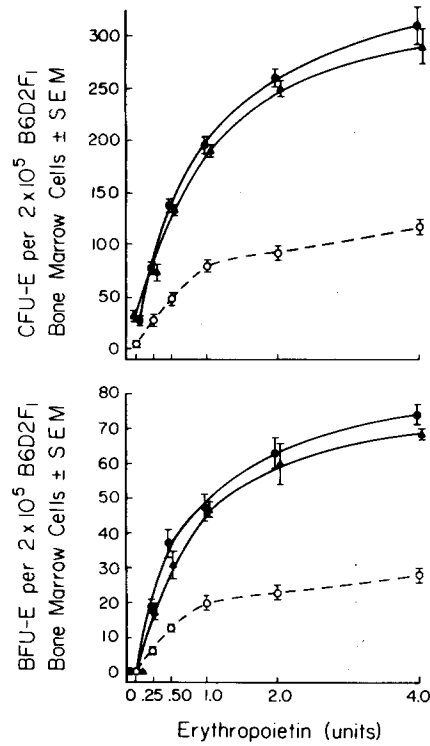


FIG. 3. Effects of indomethacin on macrophage enhancement of CFU-E and BFU-E. Cultures were prepared as in Fig. 2 with cell-free agar underlayers (open symbols) or underlayers that contained the plastic-adherent cells from 5×10^5 peritoneal cells (closed symbols) with the indicated concentrations of Ep. Replicate cultures were prepared in the presence of 1.4×10^{-7} M indomethacin (\blacktriangle) or control diluent (\bullet) and scored for CFU-E and BFU-E.

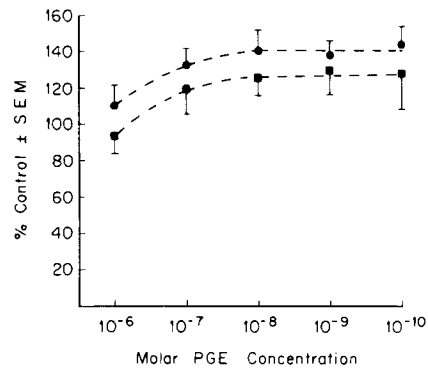


FIG. 4. Effect of synthetic PGE_1 on CFU-E (\bullet) and BFU-E (\blacksquare). Single-layer methylcellulose cultures of normal mouse bone marrow were prepared in the presence of the indicated concentrations of PGE. Results are expressed as a percentage of erythroid colony formation in cultures that contained a concentration of ethanol diluent identical to that in cultures that contained 10^{-6} M PGE.

activity was detected after 24 h of macrophage incubation, peaked at 48 h, and persisted over 144 h.

Sedimentation Velocity Characterization of the Cells That Elaborate Erythroid-enhancing Activity. Peritoneal lavage cells separated by sedimentation velocity revealed two

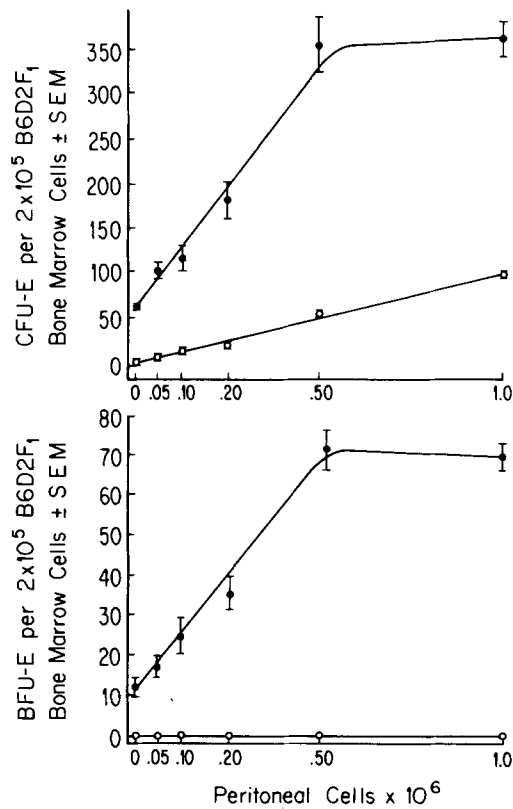


FIG. 5. Effects of varying macrophage concentrations of CFU-E and BFU-E. Agar underlayers were prepared by allowing the indicated numbers of peritoneal cells to adhere to plastic. Normal mouse marrow was used as a target in the methylcellulose overlays in the absence (○) and presence (●) of 2 U/ml of Ep.

major nucleated cell populations (Fig. 8 A). The first had a modal sedimentation velocity of 3.6 mm/h and consisted of small lymphocytes. The second peak, which sedimented at 6.3 mm/h, was composed of large mononuclear cells and macrophages. All the cells that adhered to plastic dishes also stained positively with alpha-naphthyl acetate esterase and actively phagocytosed 0.8- μ m latex beads. Such cells closely corresponded to the second major peak of nucleated cells that sedimented at 6.3 mm/h.

Media conditioned by each of the sedimentation velocity fractions of peritoneal lavage cells were tested for CFU-E-promoting activity in 30% underlayers with methylcellulose overlays that contained 2 U/ml of Ep (Fig. 8 B). The cells that elaborated the erythroid-enhancing activity were characterized by a modal sedimentation rate of 5.9 mm/h with a second peak of activity at 6.9 mm/h. The cells that produced and released myeloid colony-stimulating activity had a sedimentation rate of 5.9 mm/h and closely corresponded to the modal population of cells that elaborated activities that promoted CFU-E. In all instances, the cell populations that stimulated GM colony formation and enhanced CFU-E appeared, morphologically, to be macrophages and occurred among the adherent, phagocytic, and esterase-positive cells. In

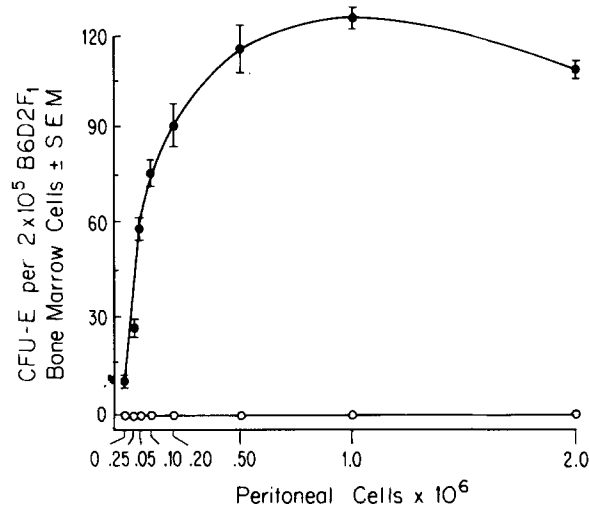


FIG. 6. Stimulation of CFU-E by macrophages and purified Ep in low-serum cultures. Cultures were prepared as in Fig. 5, with underlayers that contained the adherent macrophages from the indicated numbers of peritoneal cells. Both the agar underlayers and the methylcellulose overlayers were prepared with 4% rather than the usual 30% fetal calf serum. The methylcellulose overlayers were prepared in the absence (○) or presence (●) of 2 U/ml of Ep (79,000 U/mg).

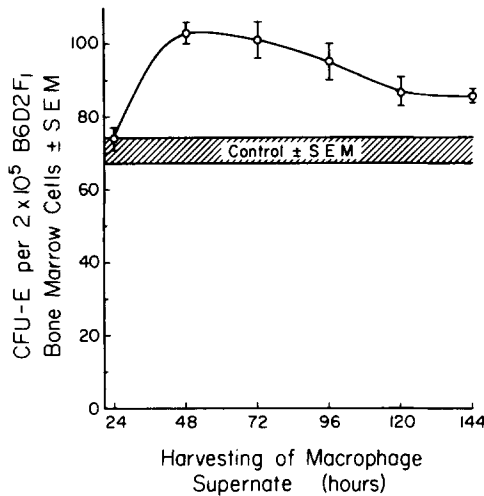


FIG. 7. Erythroid-enhancing activity in media conditioned by macrophages in the absence of Ep. The adherent cells from 1×10^6 peritoneal cells were allowed to condition media for the indicated period of time. The conditioned medium was used at 30% vol/vol to prepare agar underlayers. Methylcellulose overlayers were prepared with normal bone marrow cells and 2 U/ml of Ep. The shaded area represents the mean \pm 1 SEM erythroid colonies that developed in cultures prepared without conditioned medium.

contrast, the cells that elaborated murine B lymphocyte colony-stimulating activity (10) were more heterogeneous, having a wide sedimentation distribution that peaked at 5.4 mm/h, but in which the majority of activity was associated with the adherent and phagocytic cell population.

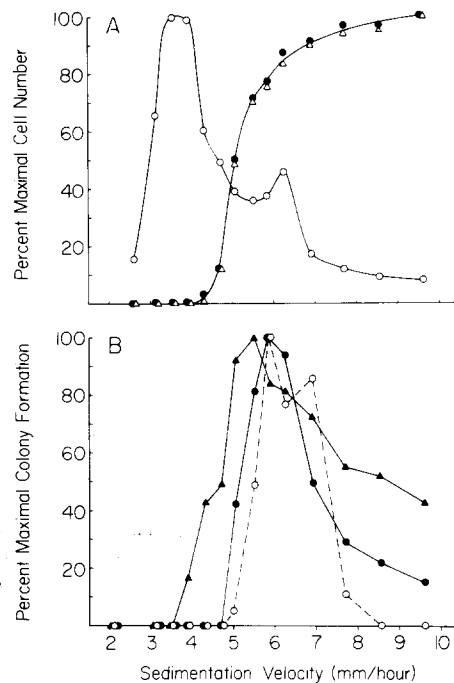


FIG. 8. Sedimentation velocity analysis of murine peritoneal cells for erythroid enhancing-activity. (A) Fractions of peritoneal cells separated by sedimentation velocity at unit gravity were examined for nucleated cells (○), plastic adherence and alpha-naphthyl acetate esterase positivity (●), and phagocytosis of 0.8- μ m latex beads (Δ). Results are expressed as percentage of respective peak value (nucleated cells, 17×10^6 ; adherent esterase-positive cells, 98.8%; phagocytic cells, 97.2%). (B) Agar underlayers were prepared from the plastic-adherent cells obtained from 8×10^5 cells from each fraction. Methylcellulose overlayers were prepared with normal mouse bone marrow in the absence of exogenously added Ep and assayed for CFU-E at 2 d (○). GM precursors (●) were assayed for in 0.3% agar overlayers. B lymphocyte colony-forming cells (\blacktriangle) were assayed in 0.3% agar overlayers that contained 5×10^4 lymph node cells that had been passaged over Sephadex G-10. The results are expressed as percentage of respective peak values for each fraction. The curves indicate the production of B lymphocyte CSF (\blacktriangle), GM-stimulating factor (●), or erythroid-enhancing activity (○) by peritoneal adherent cells of various sedimentation velocities. All cultures were prepared in a two-layer system with 0.5% agar underlayers.

Discussion

We report here that macrophages isolated from the peritoneal cavities of mice can enhance both CFU-E and BFU-E *in vitro*. In contrast to cocultivation techniques, our use of a two-layer methylcellulose-agar culture assay selectively defined macrophage influence on *in vitro* erythropoiesis mediated by the release of diffusible substances. This role for macrophages would therefore appear to be unrelated to that previously documented by morphological studies that depicted an intimate physical relationship between macrophages and developing erythroid cells in so-called "erythroblastic islands" (8).

In the presence of increasing number of adherent peritoneal macrophages, the incidence of both CFU-E and BFU-E proportionately increased. Of interest, macrophages appeared to stimulate the growth of CFU-E, but not BFU-E, in the absence of exogenous Ep. However, this apparent Ep independence was critically dependent

upon the concentration of FCS. Differing serum requirements for CFU-E and BFU-E in methylcellulose cultures supplemented with albumin, transferrin, and lipids have been reported by Iscove (21, 22). Reducing the FCS concentration from 30 to 4% completely abrogated the ability of macrophages to enhance erythroid colony formation in the absence of exogenously added Ep. The addition of apparently pure Ep (16) to low-serum cultures stimulated CFU-E and allowed enhancement of erythroid colony formation in response to increasing numbers of macrophages. Because Ep exists in normal sera, the concentration of Ep in those cultures that contain the higher concentration of FCS would presumably approach the concentrations normally seen *in vivo*. The fact that macrophages enhance erythroid colony formation under these conditions in which pharmacologic concentrations of Ep are not present supports a physiologic regulatory role for macrophages in erythropoiesis.

Ep was not required for the macrophage to synthesize and release the erythroid-enhancing activity, because serum-free media conditioned by macrophages alone contained significant levels of enhancing activity. From a regulatory standpoint it is important to conclude that macrophages are not obligate for erythroid colony and burst formation, but serve in a permissive capacity. The mechanism by which macrophages potentiate the growth of CFU-E and BFU-E is poorly understood. Titration experiments with two preparations of Ep, differing in their origin and purity, indicate that the presence of macrophages augments the responsiveness of *in vitro* clonable cells to a given concentration of Ep.

It has been reported that activities from lectin-stimulated murine spleen cells (5), bone marrow (6), and human leukocyte-conditioned media (7), promote erythroid burst formation in response to Ep. Such burst-promoting activities appear to reduce the *in vitro* requirement for Ep, and thus appear similar to the *in vitro* erythroid-enhancing activity produced by macrophages. Wagemaker (23) has reported that the cells responsible for BFA production in murine bone marrow are radioresistant and have a modal sedimentation velocity of 4.7 mm/h with a second peak of activity at 6.1 mm/h. Such sedimentation velocity characteristics are similar to the subpopulation of peritoneal cells that we have shown to be adherent and responsible for the erythroid-enhancing activity. There appears to be a physical similarity between those murine bone marrow cells that produce BFA and the peritoneal macrophage subpopulations that elaborate substances that influence Ep responsiveness. A similarly radioresistant cell in murine spleen has been found to potentiate ^{59}Fe incorporation into erythroid cells in response to Ep (24). The requirement for lectin in the production of BPA by murine spleen cells suggests some modulatory role for T lymphocytes (5). An *in vivo* role for macrophages in erythropoiesis is suggested by the observations of Seki (25), who reported that macrophage-coated cellulose acetate membranes provide a suitable environment for the focal proliferation and differentiation of erythroid as well as myeloid precursors.

These data are in contrast to the findings of Nathan et al. (26), which demonstrated that non-lectin-stimulated lymphocytes promoted erythroid colony formation *in vitro*. Although these investigators used plasma-clot culture techniques in the study of human peripheral blood BFU-E, it is difficult to envision any significant difference between the two culture assays, or indeed a functional difference between murine and human mononuclear phagocytes. Our study clearly documents that neither nonadherent, predominantly lymphocytic populations of resident peritoneal cells, nor

unseparated populations of lymphoid cells from thymus, lymph node, or spleen have any effect on erythroid colony formation in the absence of lectin stimulation. Furthermore, adherent-cell depletion of peritoneal cell suspensions by passage through columns of Sephadex G-10 led to a loss of all erythroid-enhancing activity. An explanation for the discrepancy between our findings and those of Nathan et al. (26) may lie in the contamination of their lymphocyte preparations by monocytes.

Analysis by sedimentation velocity analysis confirmed that the cells that enhance *in vitro* erythropoiesis are macrophages on the basis of morphologic, histochemical, and functional criteria, and can be satisfactorily separated from lymphocytes. The data also indicate that not all separated fractions of macrophages have the capacity to synthesize and/or release equal amounts of this activity under unstimulated conditions. This experimental evidence, which suggests mononuclear phagocyte heterogeneity, is of importance in view of the recent report that found that peripheral blood monocytes inhibit, whereas macrophages stimulate, erythroid colony formation *in vitro* (27). The purity of cell populations as well as concentration effects may well be critical. There appears to be no significant difference between cells that release the erythroid-enhancing activity and cells that produce GM colony-stimulating activity (15) and B lymphocyte (10) colony-stimulating activities by sedimentation velocity analysis. These findings suggest that the production and/or release of *in vitro* hematopoietic-potentiating substances by macrophages may be limited, at least under basal conditions, to a physically restricted subpopulation of macrophages.

The identity of the macrophage-derived substances that augment erythroid colony formation is, at present, unknown and awaits biochemical characterization. The cyclooxygenase inhibitor indomethacin was used to investigate a role for prostaglandin or products of arachidonic acid because of reports that the addition of synthetic PGE stimulate erythroid cells *in vitro* (17, 18). However, in the presence of indomethacin at concentrations that completely inhibit PGE synthesis by macrophages (19), there was no change in the enhancement of CFU-E or BFU-E by macrophages. It would therefore be difficult to substantiate any role for macrophage-derived PGE in stimulating the growth of CFU-E and BFU-E *in vitro*. Additional work needs to be done before any role for the oxygenation products of arachidonic acid metabolism can be dismissed from consideration as regulators of erythropoiesis *in vitro*.

This report documents yet another population of hematopoietic cells that can be cloned *in vitro* and is influenced by substances generated by mononuclear phagocytes. The detection of an activity similar to BPA in mouse serum (23), as well as in media conditioned by peripheral blood leukocytes, presumably implies that neither erythroid-enhancing activities nor the cells that give rise to them are restricted to the bone marrow or spleen. Although elevated levels of an erythroid-enhancing activity have reportedly been demonstrated in the serum of mice under conditions of perturbed erythropoiesis (23), it remains to be seen whether these activities play a genuine regulatory role in the intact animal.

Summary

We investigated the effects of murine resident peritoneal macrophages on the *in vitro* proliferation of erythropoietin (Ep)-sensitive committed precursors colony-forming unit-erythroid (CFU-E) and burst-forming unit-erythroid (BFU-E) with a two-layer cloning system of methylcellulose and semisolid agar. The addition of increasing

numbers of macrophages to the agar underlayer resulted in a progressive increase in the numbers of both CFU-E and BFU-E that proliferated in the presence of Ep. CFU-E, but not BFU-E, proliferated to form colonies in the absence of exogenously added Ep, and this proliferation was enhanced in a dose-dependent fashion by the presence of macrophages in the underlayer. The enhancing effects of a given number of macrophages and a given concentration of Ep were greater than the sum of the individual effects of macrophages and Ep alone. This erythropoietic synerism was more evident with BFU-E because burst formation was not seen in the absence of exogenously added Ep. Macrophage underlayers stimulated three to five times the number of erythroid bursts seen with Ep alone. Cell-free agar underlayers or agar underlayers prepared with nonadherent peritoneal cells or unseparated cells from thymus, lymph node, or spleen failed to augment Ep-dependent erythroid colony formation. No enhancement of CFU-E or BFU-E was demonstrable after depletion of adherent cells from peritoneal cell suspensions by passage over columns of Sephadex G-10. Analysis by sedimentation velocity of peritoneal cells confirmed that the cells responsible for elaborating the erythroid-enhancing activity were macrophages on the basis of morphologic, histochemical, and functional criteria. Serum-free media conditioned by macrophages in the absence of Ep contained the erythroid-enhancing activities, which indicated that Ep was not required for the elaboration of these diffusible substances. These studies indicate that although macrophages are not obligate for the growth of erythroid precursors, they serve as an important source of diffusible factors that reduce the in vitro requirement for Ep.

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