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Colony formation by primitive hemopoietic progenitor cells in serum-free medium

(multipotential/erythroid/granulocyte/macrophage/mouse bone marrow/erythropoietin)

JAMES F. ELIASON AND NICOLAS ODARTCHENKO

Swiss Institute for Experimental Cancer Research, Chemin des Boveresses, CH-1066 Epalinges sur Lausanne, Switzerland

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ABSTRACT **A serum-free medium has been developed for clonal growth of murine hemopoietic progenitor cells. In this medium, the number of nonerythroid colonies induced by factors produced by cloned T lymphocytes was 90 ± 10% of the number generated in serum-containing medium. Erythroid colony number in serum-free cultures containing the T-cell factors but no exogenous erythropoietin was significantly higher than that in cultures with serum, and the cloning efficiency was independent of cell concentration. Further addition of erythropoietin increased erythroid colony number ≈4-fold. Pure erythropoietin alone stimulated erythroid colony formation, but the cloning efficiency was highly dependent on cell concentration. Analysis of individual colonies generated in serum-free cultures containing the T-cell factors indicated that some contained cells of several hemopoietic lineages, demonstrating that multipotential progenitors can give rise to colonies in this system.**

The development of *in vitro* clonal assays for progenitor cells from mouse bone marrow has greatly facilitated investigations of the mechanisms controlling hemopoietic cell proliferation and differentiation. Clonal growth of hemopoietic cells is strictly dependent upon regulatory factors called colony-stimulating factors (CSFs), a number of which have been purified to apparent homogeneity, including GM-CSF (1), G-CSF (2), CSF-1 (3), erythropoietin (Epo) (4) and interleukin 3 (5). Several of these factors appear to be lineage-specific: namely, G-CSF, for production of granulocytes; CSF-1, for macrophages; and Epo, for erythrocytes. Bipotential progenitor cells are stimulated by GM-CSF to give rise to colonies composed of granulocytes and macrophages, whereas multipotential progenitors are stimulated by interleukin 3.

Unfortunately, the *in vitro* systems used to study the effects of purified CSFs generally contain substantial amounts of animal sera. Since serum may contain CSFs as well as substances capable of modulating CSF activity, it clearly would be of value to work with serum-free, hormonally defined media for clonal assay of hemopoietic progenitors. Advances in cell culture techniques have led to the development of serum-free media for growth of a wide variety of mammalian cells in liquid culture (6). For the hemopoietic system, Iscove *et al.* (7) have described a serum-free, methylcellulose-based medium for assay of mature erythroid progenitors from mouse bone marrow, which form colonies after 2 days of incubation. This medium did not, however, support colony formation by primitive progenitors (8). A modification of this method, using agar as the immobilizing agent, has been described for growth of granulocyte/macrophage colonies (9). More recently, growth of erythroid colonies derived from primitive progenitors in serum-free medium has been reported (10) but appears to be dependent upon contaminants present in impure bovine serum albumin (BSA) preparations.

In this paper, we describe a serum-free methylcellulose medium that supports growth of colonies derived from primitive erythroid and nonerythroid progenitors in the same cultures. We also present evidence that a proportion of the colonies generated in these cultures are derived from multipotential progenitors.

MATERIALS AND METHODS

Media. Preparation of enriched Dulbecco's medium (EMED) and modified Ham's F-12 nutrient mixture (FMED) has been described (11, 12). Nucleoside mixture was prepared as a 100-fold concentrated stock solution in EMED, and trace elements were 1000-fold concentrated as described previously (12). Two partially purified preparations of human urinary Epo with specific activities of 1140 units/mg of protein were used. One, CAT-1, was supplied by the National Heart, Lung, and Blood Institute (National Institutes of Health, Bethesda, MD). Pure α-Epo (70,000 units/mg of protein) and partially purified SP-7-16-76II were generously provided by E. Goldwasser (Univ. of Chicago).

Purified BSA (Behring, Marburg, F.R.G.) was treated with dextran/charcoal and deionized as described by Iscove *et al.* (7). Human transferrin, fully saturated with iron, was prepared as a stock solution of 80 mg/ml. Bovine pancreatic insulin was dissolved in Hanks' balanced salts solution (buffered with 10 mM Hepes, pH 7.8) to give a concentration of 3 mg/ml. Bovine hemin was dissolved in 0.2 M NaOH and diluted with EMED; the pH was adjusted to 7.8 and the solution was brought to a final concentration of 10 mM hemin. Methylcellulose was prepared as a 2% (wt/wt) solution in a 1:1 mixture of EMED and FMED as described by Worton *et al.* (13). Linoleic acid and cholesterol were dissolved in 95% ethanol to give concentrations of 2.8 mg/ml and 2.6 mg/ml, respectively. Fetal calf serum (lot 300068; KC Biological, Lenexa, KS) was selected on the basis of its capacity to support erythroid colony formation.

Preparation of Conditioned Media. The WEHI-3B cell line was passaged in serum-free medium for at least 2 weeks before the batch of conditioned medium (WEHI-3CM) used in these experiments was collected.

The non-cytolytic T-cell line C_{12} was cloned by single-cell micromanipulation from a C57BL/6 anti-DBA/2 mixed lymphocyte culture (14). The cells were grown for 2 months in serum-free medium consisting of a 1:1 mixture of EMED and FMED supplemented with 1% (wt/vol) BSA, 1% (vol/vol) nucleosides mixture, transferrin at 80 μg/ml, 0.7% (vol/vol) L-glutamine (200 mM), 0.1% (vol/vol) trace elements mixture, 0.1% (vol/vol) linoleic acid/cholesterol mixture, insu-

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Abbreviations: CSF, colony-stimulating factor; Epo, erythropoietin; BSA, bovine serum albumin; EMED, enriched Dulbecco's modified Eagle's medium; FMED, modified Ham's F-12 nutrient mixture; WEHI-3CM, medium conditioned by WEHI-3B cells; C_{12}CM, supernatant from activated, cloned T lymphocytes.

lin at 3 μg/ml, 20 μM ethanolamine, T-cell growth factor, and 10^6 irradiated (30 Gy) DBA/2 spleen cells per ml. To prepare the medium used in this study, the cells were washed with medium and subcultured at a concentration of 10^6 cells per ml in serum-free medium further supplemented with concanavalin A at 2.5 μg/ml. The medium (C_{12}CM) was harvested after 24 hr.

Cell Culture. Bone marrow cells from femurs of male or female BALB/c mice, 8–10 weeks old, were flushed into EMED using a 22-gauge needle. Nucleated cells were counted using a hemocytometer. Appropriate dilutions were made to give cell concentrations 10 times the final concentration. Unless otherwise stated, cells were cultured at a final concentration of 5×10^4 cells per ml. The cells then were diluted in the methylcellulose culture mixture, which was prepared from the stock solutions to give final concentrations as follows: methylcellulose, 0.9% (wt/wt); BSA, 10 mg/ml; transferrin, 320 μg/ml; nucleoside mix, 1%; L-glutamine, 1.4 mM; insulin, 9 μg/ml; hemin, 20 μM; linoleic acid/cholesterol mixture, 0.2% (vol/vol); and trace elements mixture, 0.1% (vol/vol). In most experiments, WEHI-3CM or C_{12}CM was added at a final concentration of 10% (vol/vol). Epo was added at 0.6, 2, or 5 units/ml.

Duplicate aliquots (1 ml) of the cells in the methylcellulose medium were plated in Petri dishes (Greiner Nürtingen, F.R.G.; no. 627102). The cultures were incubated at 37°C in 5% O_2/5% CO_2/90% N_2 atmosphere for 8–10 days. Colony counts were done using an inverted microscope with a magnification of ×63. Colonies were classified as erythroid or nonerythroid, based on the presence of visible clusters of small erythroid cells. Experiments were repeated at least twice and errors bars shown in the figures represent SEM.

Staining of Cells from Single Colonies. Individual colonies were removed from the cultures by drawing the cells into a capillary tube. Cells from colonies were placed directly on slides and fixed immediately with a drop of acid/alcohol (20% glacial acetic acid/80% absolute ethanol). After drying, the slides were covered with acid/alcohol for 10 min, then washed well with water, and finally air-dried before staining with benzidine and Giemsa stain.

RESULTS

Preliminary experiments were performed to compare the capacities of EMED and Iscove's modified Dulbecco's medium to support hemopoietic colony formation in cultures containing 5% fetal calf serum. Colony numbers were consistently higher in EMED, and best results were obtained using 75% EMED/25% FMED. The results of experiments examining the use of a low-oxygen mixture (5% O_2/5% CO_2/90% N_2) for incubating cultures indicated that colony numbers were not significantly increased in the low-oxygen atmosphere, but erythroid colonies appeared to be slightly larger and redder in color.

EMED/FMED (3:1) was capable of supporting colony formation in the absence of serum when supplemented with BSA, transferrin, fresh glutamine, nucleosides, hemin, insulin, trace elements, linoleic acid, and cholesterol (Fig. 1). To optimize this medium, the most important additions were tested by omitting individual components from the formulation. Albumin and transferrin previously have been shown to be required for serum-free growth of hemopoietic progenitors (7) and thus were not tested in this experiment. Deleting linoleic acid and cholesterol from the medium reduced the number of both erythroid and nonerythroid colonies to 20% of maximum or less (Fig. 1). Omitting insulin also led to significantly lower numbers of both colony types ($P < 0.05$). When nucleosides were omitted from the medium, nonerythroid colony formation was significantly ($P < 0.02$) reduced to 55–75% of maximum, whereas the decrease in erythroid

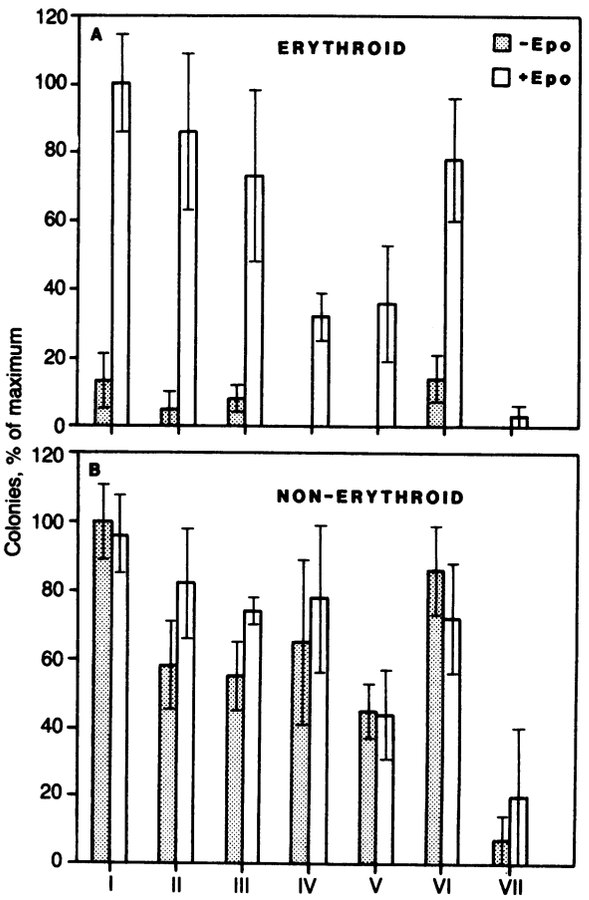
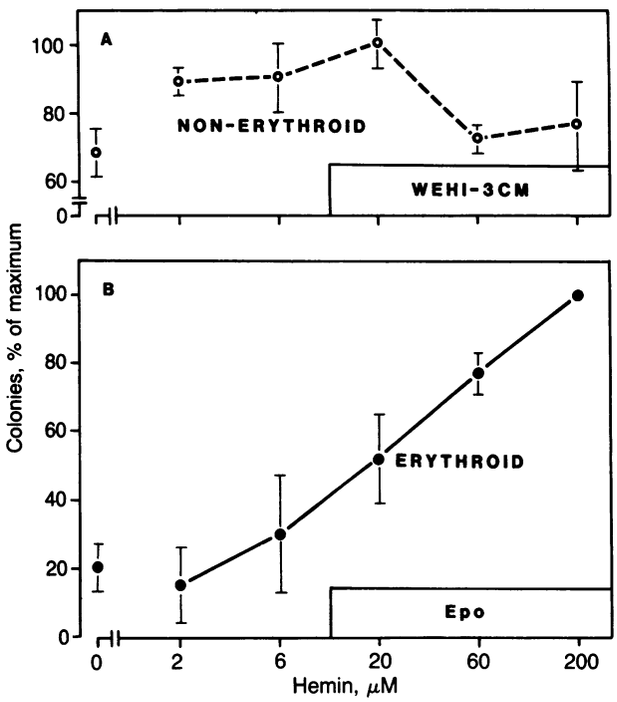


FIG. 1. Effects produced by omitting individual components from serum-free medium on erythroid (*A*) and nonerythroid (*B*) colony formation. Complete medium (I) is described in *Materials and Methods*. Epo (SP-7-16-76II) was added to half the cultures at a final concentration of 0.6 units/ml (open bars). The other cultures lacked Epo (stippled bars). All cultures contained 10% WEHI-3CM. The constituents omitted from complete medium for the experimental groups were additional L-glutamine (II), nucleoside mixture (III), hemin (IV), insulin (V), trace elements (VI), and linoleic acid/cholesterol (VII). Maximum colony numbers (I) were 29 ± 10 erythroid and 105 ± 44 nonerythroid colonies per 10^5 cells; 3 experiments.

colonies to 73% of maximum was not statistically significant. Removing hemin from the medium, on the other hand, led to a significant ($P < 0.02$) reduction in erythroid colony numbers, whereas the effect on nonerythroid colonies was not significant.

The addition of dipalmitoyl-L-α-phosphatidylcholine to the linoleic acid/cholesterol mixture decreased both erythroid and nonerythroid colony numbers when the lipid mixture was added at a concentration of 0.2%, which gave maximum colony numbers with linoleic acid/cholesterol alone. Insulin enhanced colony formation over a broad concentration range (0.9–90 μg/ml) with a maximum at ≈9 μg/ml. Titration curves for erythroid and nonerythroid colony numbers were similar.

Fig. 2*A* shows that the number of nonerythroid colonies stimulated by WEHI-3CM was enhanced by low levels of hemin (2–20 μM). At higher hemin concentrations, the numbers of colonies were similar to those in the controls. Erythroid colony formation stimulated by Epo increased with increasing hemin concentration up to 200 μM (Fig. 2*B*). The enhancing effect of increasing hemin concentration on erythroid colony number in cultures stimulated with both WEHI-3CM and Epo appears to be less pronounced, with a relatively large increase only between 60 and 200 μM (Fig. 2*C*).



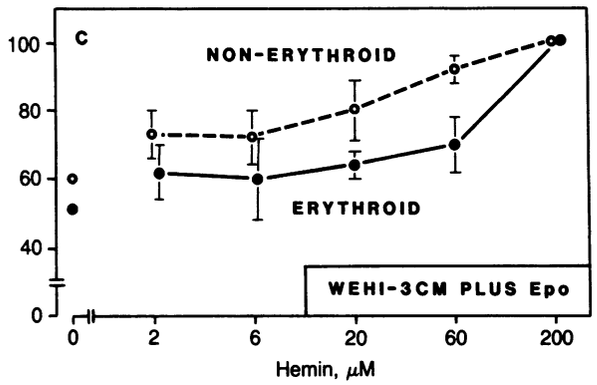


FIG. 2. Effect of hemin on hemopoietic colony formation in serum-free medium. Bone marrow cells were seeded in complete medium (see *Materials and Methods*) but with twice the linoleic acid/cholesterol mixture and with various concentrations of hemin. (*A*) Nonerythroid colonies in cultures containing 10% WEHI-3CM. Maximum = 71 ± 10 colonies per 10^5 cells; 5 experiments. (*B*) Erythroid colonies in cultures containing CAT-1 Epo at 2 units/ml. Maximum = 54 ± 8 colonies per 10^5 cells; 2 experiments. (*C*) Erythroid (closed circles) and nonerythroid (open circles) colonies in cultures containing 10% WEHI-3CM plus Epo at 2 units/ml. Maximum colony numbers were 81 ± 4 erythroid and 144 ± 34 nonerythroid colonies per 10^5 cells; 3 experiments.

Colony formation in the best serum-free formulation was compared to that in the same medium further supplemented with 10% fetal calf serum (Fig. 3). The concentration of $C_{12}CM$ was titrated in these experiments to compare colony numbers in cultures containing suboptimal amounts of stimulator(s). Nonerythroid colony numbers were slightly lower in the serum-free medium under these conditions, suggesting that the medium was not quite optimal for these progenitors. With the highest dose of $C_{12}CM$, however, colony numbers were similar in both media. In four experiments examining the effect of fetal calf serum in cultures containing 10% $C_{12}CM$, nonerythroid colony numbers in the serum-free medium were 90 ± 10% of those in cultures with serum. Since these cultures contained no exogenous Epo, it was surprising that significant numbers of erythroid colonies grew in the serum-free medium. Some of these colonies contained only erythroid cells. Furthermore, the number of such pure erythroid colonies was significantly higher in the serum-free medium than in serum-containing cultures (Fig. 3*A*). In four experiments, erythroid colony formation in the presence of fetal calf serum was only 27 ± 9% of that in the absence of serum.

The relationship between colony numbers and numbers of cells seeded was examined in this system. The numbers of erythroid colonies stimulated by $C_{12}CM$ (Fig. 4*A*) showed good fit to a straight line drawn through the origin, and significant numbers of colonies were detected in cultures containing only 5000 bone marrow cells. The slope of the regression line corresponds to a value of 17 colonies per 10^5 cells seeded. The addition of Epo to these cultures increased the frequency about 4-fold to 70 erythroid colonies per 10^5 cells and appeared to increase colony size as well. Nonerythroid colony number was also proportional to the number of cells plated, and there was no difference between cultures con-

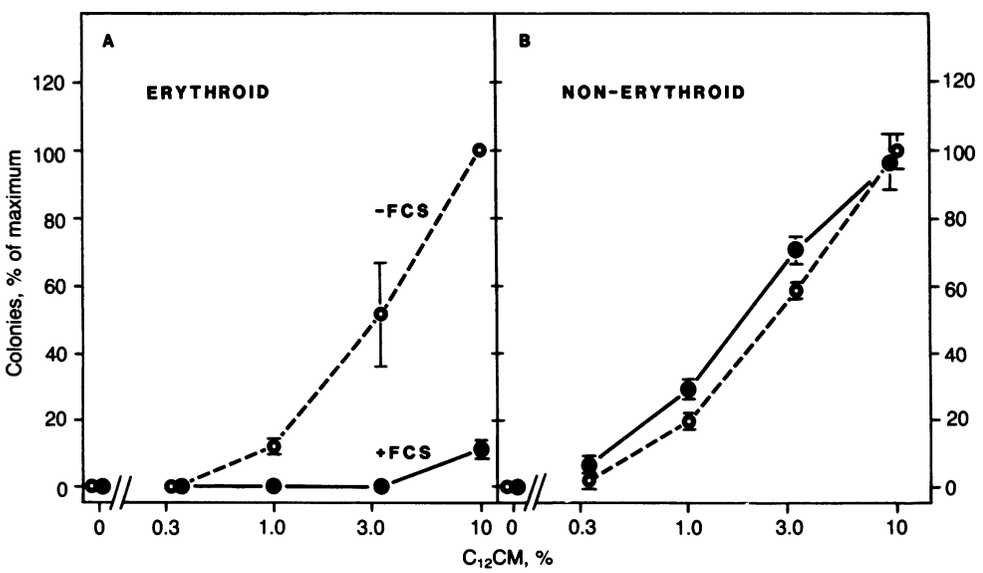


FIG. 3. Effect of fetal calf serum (FCS) on erythroid (*A*) and nonerythroid (*B*) colony formation in the presence of various concentrations of $C_{12}CM$. Marrow cells were plated in serum-free medium as described in *Materials and Methods* (open circles) and in the same medium plus 10% fetal calf serum (closed circles). Maximum colony numbers were 25 ± 17 erythroid and 161 ± 2 nonerythroid colonies per 10^5 cells; 2 experiments.

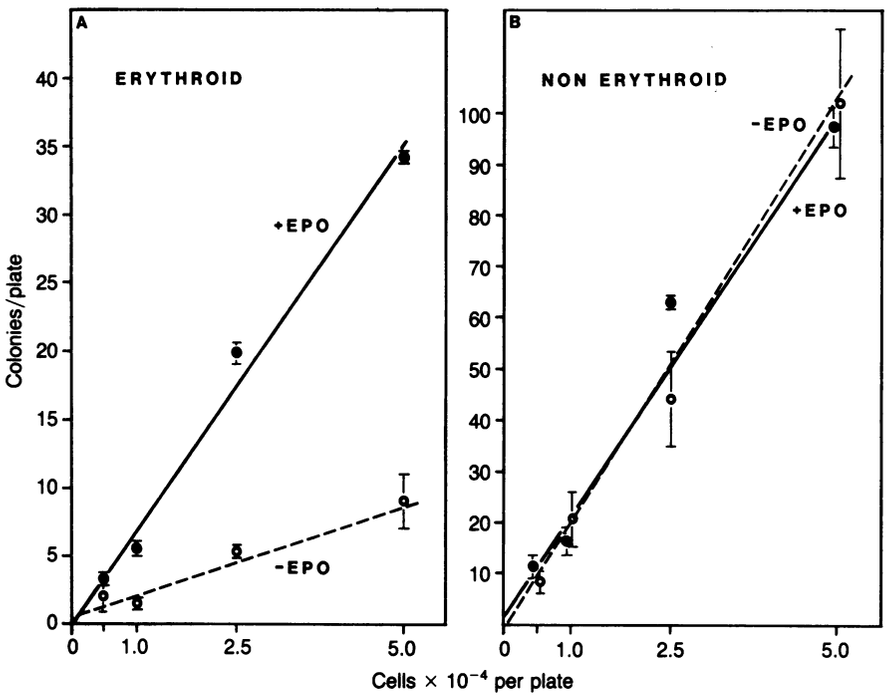


FIG. 4. Effect of cell concentration on erythroid (*A*) and nonerythroid (*B*) colony formation in serum-free cultures with (closed circles) and without (open circles) Epo. Marrow cells were plated in serum-free medium with 10% C_{12}CM and CAT-1 Epo at 2 units/ml. Results are expressed as mean colony numbers from two independent experiments.

taining C_{12}CM alone and those containing Epo and C_{12}CM (Fig. 4*B*).

Table 1 shows the results of a typical experiment from a series of three examining the linearity of colony formation in serum-free cultures containing only Epo. Pure Epo stimulated growth of a few megakaryocyte colonies, evident at the highest initial cell concentration. The frequencies of erythroid and megakaryocyte colonies were strictly dependent on the numbers of cells plated, and no colonies were present in cultures seeded with 2×10^4 cells. Similar results were obtained with the partially purified Epo, which appeared to contain contaminants capable of stimulating granulocyte/macrophage-cluster formation at higher cell concentrations.

The types of colonies generated in serum-free cultures were similar to those seen in serum-containing medium and included multilineage colonies. To obtain a more accurate estimate of the different colony types, all of the colonies generated in four plates, each seeded with 5000 cells and stimulated with 10% C_{12}CM, were removed and benzidine/Giemsa stained. Seven (16%) of the colonies contained erythroid cells, and four of these had cells of other lineages as well (Table 2).

Table 1. Effect of initial cell concentration on Epo-stimulated colony formation in serum-free cultures

Cells seeded	Epo preparation	Colonies per plate			Colonies per 10^5 cells		
		E	Mk	G/M	E	Mk	G/M
2×10^5	Pure	163	7	0	81.5	3.5	0
1×10^5	Pure	51	1	0	51	0.5	0
5×10^4	Pure	17	0	0	34	0	0
2×10^4	Pure	0	0	0	0	0	0
2×10^5	Cat-1	148	6	45	74	3	22.5
1×10^5	Cat-1	38	2	10	19	2	5
5×10^4	Cat-1	5	2	0	10	4	0
2×10^4	Cat-1	0	0	0	0	0	0

Epo was added at a final concentration of 5 units/ml to serum-free cultures. E, erythroid, erythroid plus megakaryocyte or macrophage; Mk, >4 megakaryocytes; G/M, granulocytes and/or macrophage colonies plus clusters (>10 cells).

DISCUSSION

We describe a serum-free medium that supports colony formation by mouse hemopoietic progenitor cells in response to purified Epo and/or serum-free conditioned media that contain specific colony-stimulating activities. Hemin is the only supplement in our system that is not commonly used for serum-free culture of nonhemopoietic cells (6). However, several studies using serum-containing medium have shown that hemin enhances colony formation by mouse (15–17) and human (18) erythroid progenitors. In our experiments, 200 μM hemin increased Epo-stimulated colony formation ≈5-fold, whereas colony numbers in the presence of both WEHI-3CM and Epo were increased only 2-fold. In fact, this apparent difference in enhancement was due to the lower numbers of colonies in control cultures with Epo alone. The actual increase due to hemin was approximately the same in both cases (40 colonies per 10^5 cells), suggesting that WEHI-3CM and hemin enhance erythropoiesis by independent mechanisms.

There is ample published evidence that hemin stimulates erythroid maturation in several ways, including enhancement of globin mRNA synthesis (19) and protein synthesis (20). Exogenous hemin also can be utilized directly to form

Table 2. Morphology of cells in individual colonies from serum-free cultures

	Morphologic type								
	E	E/M	G/E/M	G/E/M/Mk	G	M	G/M	B1	Total
No. of colonies	3	2	1	1	13	14	8	1	43
% of total	7	5	2	2	30	33	19	2	100

E, erythroid; G, granulocyte; M, monocyte/macrophage; Mk, megakaryocyte; B1, unclassifiable blast cells.

hemoglobin, thus bypassing the protoporphyrin biosynthetic pathway (21). Enhancement of nonerythroid colony formation is less simple to understand. Although the degree of enhancement was small, it was reproducible (Figs. 1 and 2), and others have reported similar enhancement in serum-containing culture systems (15, 21). It is conceivable that synthesis of protopophyrin is also rate-limiting *in vitro* for incorporation into cytochromes in these rapidly proliferating cells.

Several important earlier findings concerning the regulation of erythroid development have been confirmed in our serum-free system. The first is the dependence on cell concentration for colony formation stimulated by pure Epo, which strongly suggests that bone marrow cells produce another factor(s) required by progenitor cells (22, 23). Whether a specific regulatory factor is involved remains to be determined. An indirect role of Epo in regulating early erythroid development is compatible with these results (24, 25). The second point is the demonstration that supernatants from cloned lymphocytes stimulate erythroid colony formation. This extends previous reports of erythroid colony growth in the absence of exogenous Epo (26).

Since the present results were obtained in serum-free cultures, using medium conditioned by lymphocytes that had been passaged for several months in the absence of serum, the possibility of Epo contamination from serum can be ruled out. Another potential source of Epo contamination in the system is *in situ* production by macrophages (27). However, erythroid colonies were present in cultures with very low numbers of cells, making this unlikely. The remaining possibility is that the conditioned media may contain Epo or another factor with a similar activity. Such an activity, with chromatographic properties distinct from those of Epo, has been described in supernatants from activated lymphocytes (26). One candidate for this Epo-like activity is insulin at high concentrations (28) or insulin-like growth factor 1 at physiologic concentrations (29). Another candidate for this activity is interleukin 3, which has been shown to initiate early stages of erythroid differentiation up to an Epo-sensitive stage (8, 23–25).

It must be pointed out that our results do support an essential role for Epo in erythropoiesis. The numbers of erythroid colonies in cultures containing WEHI-3CM or C_{12}CM were substantially increased by the addition of Epo. Furthermore, colony numbers in cultures with C_{12}CM and Epo were independent of cell concentration, in agreement with models of erythropoiesis involving two regulatory factors (8, 22, 23).

In addition to erythroid cells, some colonies in cultures stimulated by WEHI-3CM or C_{12}Cm contained cells belonging to other hemopoietic lineages, demonstrating that multipotential progenitors can give rise to colonies in this medium. However, since unfractionated conditioned media were used, we cannot eliminate the possibility that substances other than specific colony-stimulating factors or interleukin 3, produced by lymphocytes or myelomonocytic leukemia cells, are required in addition. Studies with purified factors in this system should resolve this question and provide more detailed knowledge about the mechanisms controlling proliferation and differentiation of hemopoietic progenitor cells.

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1. Burgess, A. W., Camakaris, J. & Metcalf, D. (1977) *J. Biol. Chem.* **252**, 1998–2003.
2. Nicola, N. A., Metcalf, D., Matsumoto, M. & Johnson, G. R. (1983) *J. Biol. Chem.* **258**, 9017–9023.
3. Stanley, E. R. & Heard, P. M. (1977) *J. Biol. Chem.* **252**, 4305–4312.
4. Miyake, T., Kung, C. K.-H. & Goldwasser, E. (1977) *J. Biol. Chem.* **252**, 5558–5564.
5. Ihle, J. N., Keller, J., Oroszlan, S., Henderson, L. E., Copeland, T. D., Fitch, F., Prystowsky, M. B., Goldwasser, E., Schraeder, J. W., Palaszynsky, E., Dy, M. & Lebel, B. (1983) *J. Immunol.* **131**, 282–287.
6. Barnes, D. & Sato, G. (1980) *Anal. Biochem.* **102**, 255–270.
7. Iscove, N. N., Guilbert, L. J. & Weyman, C. (1980) *Exp. Cell Res.* **126**, 121–126.
8. Iscove, N. N. (1978) in *Hematopoietic Cell Differentiation*, eds. Golde, D. W., Cline, M. J., Metcalf, D. & Fox, C. F. (Academic, New York), pp. 37–52.
9. Kubota, K., Motoyoshi, K., Kajigaya, S., Suda, T., Saito, M. & Miura, Y. (1983) *Exp. Hematol.* **11**, 364–370.
10. Stewart, S., Zhu, B. & Axelrad, A. (1984) *Exp. Hematol.* **12**, 309–318.
11. Eliason, J. F. (1984) *Exp. Hematol.* **12**, 559–562.
12. Eliason, J. F., Fekete, A. & Odartchenko, N. (1984) *Recent Results Cancer Res.* **94**, 267–275.
13. Worton, R. G., McCulloch, E. A. & Till, J. E. (1969) *J. Cell. Physiol.* **74**, 171–182.
14. Kelso, A., Glasebrook, A. L., Kanagawa, O. & Brunner, K. T. (1982) *J. Immunol.* **129**, 550–556.
15. Holden, S. A., Steinberg, H. N., Matzinger, E. A. & Monette, F. C. (1983) *Exp. Hematol.* **11**, 953–960.
16. Porter, P. N., Meints, R. H. & Mesner, K. (1979) *Exp. Hematol.* **7**, 11–16.
17. Malik, Z., Bessler, H. & Djaldetti, M. (1979) *Exp. Hematol.* **7**, 183–188.
18. Lu, L. & Broxmeyer, H. E. (1983) *Exp. Hematol.* **11**, 721–729.
19. Ross, J. & Sautner, D. (1976) *Cell* **8**, 513–520.
20. Gross, M. (1979) *J. Biol. Chem.* **254**, 2370–2377.
21. Rothman, J., Hertogs, C. F., Malik, Z. & Pluznik, D. H. (1983) *Exp. Hematol.* **11**, 147–153.
22. Wagemaker, G. (1978) in *In Vitro Aspects of Erythropoiesis*, ed. Murphy, M. J., Jr. (Springer, Berlin), pp. 44–57.
23. Wagemaker, G., Peters, M. F. & Bol, S. J. L. (1979) *Cell Tissue Kinet.* **12**, 521–537.
24. Eliason, J. F., Testa, N. G. & Dexter, T. M. (1982) in *Experimental Hematology Today 1982*, eds. Baum, S. J., Ledney, G. D. & Thierfelder, S. (Karger, Basel), pp. 61–67.
25. Eliason, J. F. & Van Zant, G. (1983) *Cell Tissue Kinet.* **16**, 65–76.
26. Fagg, B. (1981) *Nature (London)* **289**, 184–186.
27. Rich, I. N., Heit, W. & Kubanek, B. (1982) *Blood* **60**, 1007–1018.
28. Kurtz, A., Jelkman, W. & Bauer, C. (1983) *Br. J. Haematol.* **53**, 311–316.
29. Kurtz, A., Jelkman, W. & Bauer, C. (1982) *FEBS Lett.* **149**, 105–108.