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The Effect of Erythropoietin on Colonial Growth of Erythroid Precursor Cells *In Vitro*

(fetal-mouse liver/cell culture/hemoglobin synthesis/differentiation/hormone)

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ABSTRACT **A method is described for the colonial growth, in semi-solid medium, of erythropoietin-responsive erythroid cell precursors. The erythroid cell precursors were isolated by immune hemolysis from fetal mouse liver. Both the number of precursor cells triggered to proliferate and differentiate, and the size of the erythropoietic colonies formed, are directly dependent upon the concentration of erythropoietin included in the culture.**

Erythropoiesis comprises an orderly progression of replication and differentiation starting from a pluripotential hemopoietic stem cell and concluding with the release of reticulocytes and erythrocytes into the circulation. Several *in vitro* culture methods have been described which transiently support erythropoiesis, either in suspension in liquid medium (1, 2) or as colonies in plasma clot (3). Recently, Cantor *et al.* (4) have described a method of cell fractionation by immune hemolysis that provides a population of erythroid precursor cells which respond to erythropoietin in suspension culture by proliferation and by differentiation (4, 5). In order to further characterize the effects of erythropoietin on the proliferation and differentiation of individual precursor cells it is necessary to employ techniques capable of examining the progeny of individual precursors. In this study, a technique for the colonial growth of precursor cells in semi-solid medium is described. This technique permits analysis of the responsiveness of single precursor cells to the hormone.

MATERIALS AND METHODS

Erythroid precursor cells from 12- to 13-day C57Bl/6J fetal-mouse livers were prepared by selective immune hemolysis of more mature erythroblasts and erythrocytes with rabbit anti-mouse erythrocyte antiserum in the presence of complement (4). These populations are contaminated with about 5% recognizable granulocyte precursors, macrophages, and hepatic epithelial cells.

The components of the culture medium (6) are provided in Table 1. After Millipore filtration (0.45-μm pore size) this medium was added to sterile (autoclaved) methylcellulose (4000 centipoise, Fisher, N.Y.) to a final concentration, in the complete medium with horse serum, of either 1.8% or 0.8% methylcellulose. This medium, without serum supplement, was stored frozen at $-20°$ in convenient aliquots. Horse serum (Microbiological Associates, Bethesda, Md.) was added immediately prior to use at a final concentration of 15%. The final viscous medium was transferred and distributed with a plastic syringe equipped with a 14 gauge 10-cm needle.

Erythroid precursor cells were suspended at concentrations between 5×10^4 and 8×10^5 cells per ml, in the final culture medium containing, in addition, 25 μl of a solution of erythropoietin‡ at various concentrations as indicated in the *text*. The suspension was mixed vigorously with the aid of a Vortex mixer, distributed in 1-ml aliquots into 35-mm plastic petri dishes (Falcon), and incubated at 37° in a humidified incubator gassed with 5% CO_2 in air.

The growth of erythroid colonies was scored by the examination of petri dish cultures, after selected periods of growth, with an inverted microscope equipped with a calibrated reticle. The compact form, and the small size of the individual cells in erythroid colonies serve to distinguish them from nonerythroid colonies in the culture (predominantly composed of macrophages and granulocyte precursors). These features were confirmed, initially, by the examination of colonies retrieved onto microscope slides and stained with benzidine–Wright–Giemsa (7) for light microscopy. Rapid scoring of culture plates is facilitated by *in situ* staining of erythroid colonies by flooding the petri dishes with a solution of 0.2% benzidine hydrochloride (Calbiochem, Los Angeles) in 0.5% acetic acid, containing 0.2 ml of fresh 30% hydrogen peroxide per 50 ml of staining solution. Within 3 min the erythroid colonies containing hemoglobinized cells, are stained deep blue (benzidine blue reaction; ref 7) and scoring is performed after 5 min of the staining reaction. Nonerythroid cells are completely unstained for at least 20 min. After that time a very faint blue stain, perhaps reflecting myeloperoxidase activity, can be identified in occasional nonerythroid cells.

In order to further confirm the features used for identification of erythroid colonies, entire culture plates were fixed *in situ* by flooding with 2 ml of 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 45 min at 4°. The cultures were then diluted with additional medium, transferred to a centrifuge tube and washed twice. The final pellet of fixed colonies

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‡ Human urinary erythropoietin was provided by the NIH Committee on Erythropoietin, from pool H-2-TaLSL, 20.2 units/mg, procured by the Department of Physiology, University of the Northwest, Corrientes, Argentina, processed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, for distribution by the National Heart Institute under Research Grant HE-10880.

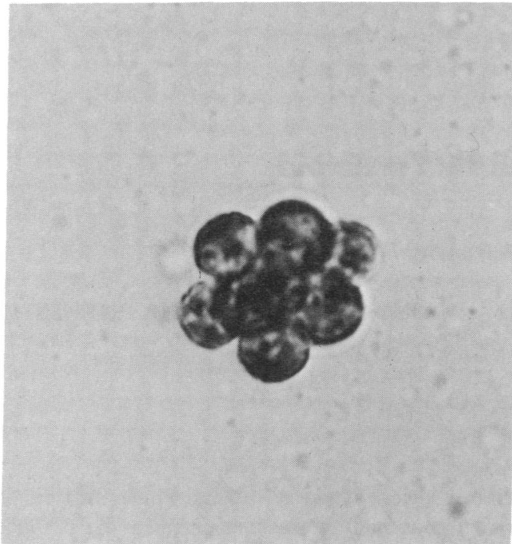

FIG. 1. Erythroid colony, from a 48-hr culture in methylcellulose-containing medium, fixed *in situ* with glutaraldehyde and stained with benzidine–Wright–Giemsa as described in the *text*. Magnification ×1000.

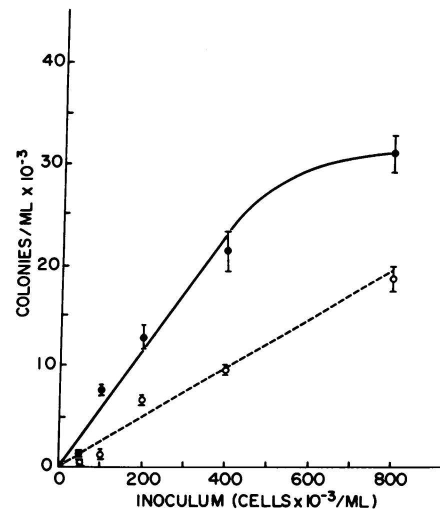

FIG. 2. Effect of inoculum size (cells/ml) on erythroid colony formation. Fetal liver erythroid precursor cells (13-day gestation) were cultured in semi-solid medium with 0.17 units/ml erythropoietin for 48 hr and the number of erythroid colonies scored by the *in situ* benzidine reaction. Cultures were made with 0.8% methylcellulose (●) and 1.8% methylcellulose (○). Each experimental point represents the mean of five 1-ml cultures ±SE of the mean. In this and the following figure, the experimental values have been multiplied by the indicated factor to obtain the numbers on the *axes*.

was resuspended in medium, deposited on microscope slides with the Cytocentrifuge (Shandon Instrument), refixed with methanol, stained with benzidine, lightly counterstained with Wright–Giemsa and examined in the light microscope.

The number of cells per colony was determined as follows: large drops of the culture medium, after specified periods of growth, were placed unfixed on glass slides, mixed with a drop of 0.5% toluidine blue (in 1% sodium borate), sealed under a cover slip and left flat for 3 days. During this period the colonies settle to the surface of the slide and the number of individual cells in each colony is readily ascertained by light microscopy.

RESULTS

Erythropoietin induces an orderly progression of erythroid cell proliferation and maturation in colonies formed in semi-solid medium. The inoculum, as previously described (4), consists predominantly of proerythroblasts and basophilic erythroblasts. Less than 1% of the cells are hemoglobinized. The inoculum is distributed uniformly and as single cells in the semi-solid medium, as verified by direct examination with the inverted microscope. The growth of erythroid colonies in the presence of erythropoietin is illustrated in Table 2, which provides data from one culture representative of five independent experiments. By 24 hr of culture, colonies containing at least three to four cells and even larger colonies are recognized. These colonies are composed of basophilic and polychromatophilic erythroblasts, in large part. Only the latter contain hemoglobin, hence the total count of erythroid colonies, defined by the benzidine reaction for hemoglobin, is underestimated at this early time point. By 48 hr the largest proportion of colonies contained at least nine to 16 cells, most of which are at the polychromatophilic erythroblast stage of maturation. Occasional basophilic cells are still recognized among the hemoglobinized forms. Fig 1. illustrates one ery-

TABLE 1. *Culture medium*

	ml
CMRL 1066 (2 S)†	400
Sodium pyruvate (100 mM solution)†	10
Eagle's MEM vitamins (100×)‡	4
Eagle's MEM amino acids (50×)‡	8
Eagle's MEM nonessential amino acids (100×)‡	4
Eagle's MEM glutamine (200 mM)‡	4
L-Serine (21 mg/ml)§	0.4
L-Asparagine (10 mg/ml)§	1.6
Penicillin and streptomycin*,‡	10

* Penicillin 1000 units/ml, streptomycin 5000 μg/ml

† GIBCO, Grand Island, N.Y.

‡ Microbiological Associates, Bethesda, Md.

§ Sigma, St. Louis, Mo.

TABLE 2. *Growth of erythroid colonies in semi-solid medium with erythropoietin*

Time in culture (hr)	Erythroid colonies/culture* (thousands of colonies)	Colony size (cells/colony)† 3–4	5–8	9–16	17–32	33–64	>65
		(% of total colonies).					
24	12.6	47	30	23	0	0	0
48	39.6	1	18	48	30	3	0
72	30.0	1	14	22	46	15	2
96	30.0	2	15	30	37	16	0
120	27.4	0	13	26	44	13	4

Erythroid precursor cells from 13-day fetal-mouse livers were cultured at 4 × 10^5 cells per ml in medium containing 0.8% methylcellulose and 0.17 units/ml of erythropoietin.

* Average number of benzidine-reactive colonies in triplicate cultures.

† Colony size scored as described in the *text*. One hundred random colonies were scored in each culture plate.

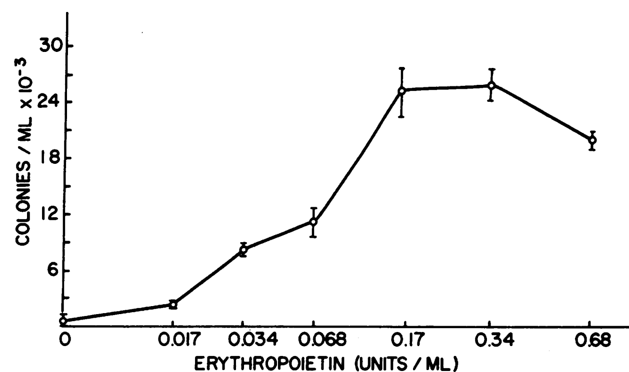

FIG. 3. Effect of erythropoietin concentration on colony formation. Erythroid precursor cells were cultured at 4 × 10^5 cells per ml in medium containing 0.8% methylcellulose. Cultures were scored at 48 hr; each point is the mean of five replicate plates ±SE of the mean.

throid colony at 48 hr. By 72 hr differentiation has, largely, progressed to the orthochromic erythroblast stage, characterized by advanced hemoglobinization and considerable nuclear pycnosis. Maturation is not wholly synchronous: in some colonies nonnucleated erythrocytes as well as persisting basophilic and polychromatophilic erythroblasts can be seen. By 96 hr orthochromic erythroblasts and nonnucleated forms predominate; macrophages which have ingested hemoglobinized erythroid cells are frequently numerous by this time. As may be noted in Table 2, there is no increase in either the number or size of erythroid colonies after 48–72 hr of culture. For this reason 48 hr was adopted as the standard time for scoring colony growth in subsequent studies.

The number of erythroid colonies formed in semi-solid culture is directly related to the number of cells inoculated (Fig 2). In 0.8% methylcellulose the colony-forming efficiency [100 × (number of colonies)/(number of cells inoculated)] is greater than in 1.8% methylcellulose at all cell concentrations tested. Nevertheless, whereas in 1.8% methylcellulose colony formation is linear throughout the range of cell concentrations tested, in 0.8% methylcellulose the colony-forming efficiency decreases at high precursor cell density (Fig 2). These observations suggest that high levels of colony formation (large inocula in optimal medium) may exhaust essential nutrients or factors necessary to sustain the growth of erythroid colonies. In order to determine whether, at high inocula, the concentration of erythropoietin used (0.17 units/ml) is limiting, the effect of erythropoietin concentration on colony formation was tested in cultures of 8 × 10^5 cells per ml of medium containing 0.8% methylcellulose (Table 3). These data indicate that at this high inoculum 0.17 units/ml of erythropoietin is not sufficient to achieve maximum colony formation. At cell densities of 4 × 10^5 cells per ml and below, the colony-forming efficiency in 0.8% methylcellulose is optimal at an erythropoietin concentration of 0.17 units/ml (see below) and ranged from 2.7 to 7.5% (mean = 4.8% ± 1.5%) in a series of eight independent experiments.

At concentrations of erythropoietin below 0.17 units/ml the number of colonies (colony-forming efficiency) is directly related to the concentration of erythropoietin added to the culture medium (Fig 3). Precursor cells inoculated at a concentration of 4 × 10^5 cells per ml were grown in concentrations of erythropoietin up to 3.4 units/ml. An effect of the

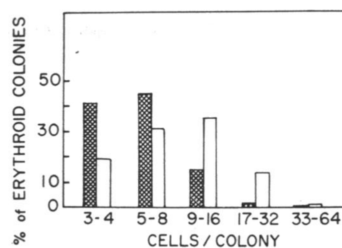

FIG. 4. Effect of erythropoietin concentration on colony size. Erythroid precursor cells were cultured at 4 × 10^5 cells per ml in 0.8% methylcellulose with 0.17 (*empty bars*) and 0.034 (*cross-hatched bars*) units/ml of the hormone. Colony size was scored at 48 hr of culture as described in the *text*.

hormone in stimulating colony formation can be detected with as little as 0.017 units/ml and the response reaches a plateau between 0.17 and 0.34 units/ml. Indeed, concentrations significantly above this level occasionally appear inhibitory to colony growth. In the absence of added erythropoietin a few small erythroid colonies are always detected. Whether these represent precursors already committed to differentiation or a response to low levels of the hormone in the serum component of the culture medium has not been determined.

The size of colonies developing in the presence of high (0.17 units/ml) and low (0.034 units/ml) concentrations of erythropoietin was examined after 48 hr of culture in semi-solid medium (Fig 4). At the higher concentration of hormone the average colony size (cells/colony) is distinctly larger than at the lower concentration. In the low concentration of erythropoietin, colony size does not change significantly after 48 hr, while at the higher concentration there is an increase in colony size between 48 and 72 hr (Table 4).

DISCUSSION

In these investigations a method has been developed which permits examination of the progeny of single erythroid cell precursors. It is shown that precursor cells, isolated from the fetal-mouse liver by immune hemolysis with antierythrocyte antiserum (4), are capable of initiating colonies of differentiating erythroid cells in the presence of erythropoietin. On the average, 5% of precursor cells are induced to form erythroid colonies in the presence of optimal concentrations of erythropoietin. Most of these cells undergo five to six cell divisions during the first 48–72 hr of culture and produce

TABLE 3. *Effect of erythropoietin concentration on colony formation at high cell density*

Erythropoietin (units/ml)	Colony formation* (colonies/culture)	Colony-forming efficiency† (%)
0	850 ± 221	0.1
0.17	39,886 ± 671	4.9
0.34	46,006 ± 1725	5.7
0.68	39,822 ± 2082	4.9

Thirteen-day fetal liver erythroid precursor cells inoculated at 8 × 10^5 cells per ml of culture medium containing 0.8% methylcellulose and the concentration of erythropoietin indicated.

* Mean of erythroid colony count in five 1-ml cultures ± SE of the mean, after 48 hr of growth.

† Colony-forming efficiency = 100 × (number of colonies)/(number of cells inoculated).

TABLE 4. *Effect of erythropoietin concentration on erythroid colony size*

Erythropoietin concentration (units/ml)	Time in culture (hr)	Colony size (cells/colony) 3–4	5–8	9–16	17–32	33–64
		(% of total colonies)				
0.034	24	60	39	1		
	48	15	53	31	1	
	72	18	48	32	2	
0.17	24	39	58	3		
	48	4	29	50	17	
	72	6	33	29	28	4

Erythroid precursors were cultured at 4×10^5 cells per ml in 0.8% methylcellulose and erythropoietin at the concentrations indicated. Colony size was scored as described in the *text*.

colonies containing up to 64 erythroblasts and erythrocytes. This proliferative rate compares favorably with the rate of proliferation of erythroid cells *in vivo* (8), and, indeed, seems more accurately to represent the response of precursors to hormone stimulation than the 2- to 3-fold total proliferation generally observed in liquid suspension cultures (2, 4). Independent measurements, employing the colchicine-induced mitotic accumulation rate (L. N. Cantor, unpublished observations), indicate an average generation time of 10–12 hr for erythroid precursor cells of fetal mice.

A principal effect of erythropoietin is the stimulation of proliferation of immature erythroid precursors and the associated differentiation of these cells (1, 4, 5, 9). Under the present conditions of colonial growth, hormone-stimulated precursors undergo cell division and those morphological changes characteristic of erythroid cell differentiation, including formation of heme proteins.

The rate of erythropoiesis, physiologically, appears to be regulated by the concentration of erythropoietin in the circulation (10). Two dose-related effects of the hormone *in vitro* are revealed by the present studies. The number of erythropoietin-sensitive cells stimulated to growth and differentiation (the colony-forming efficiency) is directly proportional to the concentration of hormone in the culture over a range of 0.017–0.17 units/ml. Higher concentrations of the crude hormone preparation have suppressive effects. These observations, taken together with previous radioautographic and biochemical studies (11) support the hypothesis that the primary effect of erythropoietin is on an immature precursor target cell. The size of individual erythroid colonies is also related to the concentration of hormone in the culture. Several possible explanations for this effect may be considered: (*1*) High-dose erythropoietin acts upon maturing erythroblasts, accelerating cell division and maturation so that a large population of mature erythroblasts is achieved rapidly; (*2*) High-dose erythropoietin stimulates precursor cell replication, so that colonial growth is sustained by inflow of early erythroblasts, known to be the stages most active in cell division (12); (*3*) High-dose erythropoietin stimulates a class of precursor cells programmed for more cell divisions than precursors responsive to lower concentrations of the hormone. These alternatives are not distinguished by the present data.

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1. Chui, D., Djaldetti, M., Marks, P. A. & Rifkind, R. A. (1971) *J. Cell Biol.* **51,** 585–595.
2. Paul, J. & Hunter, J. A. (1969) *J. Mol. Biol.* **42,** 31–41.
3. Stephenson, J. R., Axelrad, A. A., McLeod, D. L. & Shreeve, M. M. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 1542–1546.
4. Cantor, L. N., Morris, A. J., Marks, P. A. & Rifkind, R. A. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 1337–1341.
5. Terada, M., Cantor, L. N., Metafora, S., Rifkind, R. A., Bank, A. & Marks, P. A. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 3575–3579.
6. Robinson, W. A. & Pike, B. L. (1970) in *Symposium on Hemopoietic Cellular Proliferation*, ed. Stohlman, F. (Grune & Stratton, New York), pp. 249–259.
7. Barka, T. & Anderson, P. J. (1963) *Histochemistry* (Harper & Row, New York).
8. Stohlman, F., Jr. (1970) in *Regulation of Hematopoiesis*, ed. Gordon, A. S. (Appleton-Century-Crofts, New York), Vol. 1, pp. 317–326.
9. Maniatis, G. M., Rifkind, R. A., Bank, A. & Marks, P. A. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 3189–3194.
10. Filmanowicz, F. & Gurney, C. W. (1961) *J. Lab. Clin. Med.* **57,** 65–72.
11. Djaldetti, M., Preisler, H., Marks, P. A. & Rifkind, R. A. (1972) *J. Biol. Chem.* **247,** 731–735.
12. Monette, F. C., LoBue, J., Gordon, A. S., Alexander, P., Jr. & Chan, P. (1968) *Science* **162,** 1132–1134.