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Induction of Colonies of Hemoglobin-Synthesizing Cells by Erythropoietin *In Vitro*

(fetal mouse liver/erythropoietin/erythroid cells/^{59}Fe/granulocytes)

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ABSTRACT A culture method has been developed in which erythroid colonies are produced *in vitro* from hemopoietic cells from the livers of 13-day fetuses of C3H$_f$/Bi mice. Heme synthesis by the cultures was correlated with the presence of these colonies, and the hemoglobin produced was shown to be electrophoretically normal. The individual colonies were identified as erythroid since they were erythropoietin-dependent, positively stained by the histochemical 'Lepehne' procedure for hemoglobin, and labeled by ^{59}Fe radioautography. Evidence is presented that the development of these colonies is under separate control from that of granulocytic colonies found in the same cultures.

Hemopoietic differentiation along the erythroid line begins with a single pluripotent stem cell and ends with the production of a cohort of cells whose activities are directed almost exclusively to the synthesis of a single protein, hemoglobin. Because hemoglobin is a well-characterized and easily assayed protein, it has served as a useful marker for investigation of this process. Hemoglobin synthesis has been induced *in vitro* by the hormone erythropoietin in mass cultures of rat (1), rabbit (2), human bone marrow (3), and mouse fetal liver cells (4, 5). If colonies of hemoglobin-synthesizing cells could be obtained *in vitro*, analysis of the functions of individual cells in the erythroid differentiation process would be greatly simplified.

Methods of obtaining colonies of differentiating hemopoietic cells *in vitro* developed over the past few years have involved the use of semisolid agar (6–8), methylcellulose (9), or plasma (McLeod and Shreeve, to be published). Cell suspensions prepared from mouse spleen, bone marrow, and fetal liver and from rat and human bone marrow have been shown to give rise to both granulocyte and macrophage colonies. However, no evidence of erythroid colonies has been reported in any of these culture systems, even though in at least one case the medium was supplemented with erythropoietin (10).

Fetal hemopoietic cells have been shown to respond to erythropoietin *in vitro* by a burst of increased heme synthesis that reached a peak within 24–48 hr of exposure to the hormone (4, 5). If this response involved an increase in the number of heme-synthesizing cells, and if the progeny cells remained localized, discrete colonies of hemoglobin-synthesizing cells might be obtained in culture. Therefore, using a plasma culture technique, we systematically varied conditions in attempts to obtain erythroid colonies from cultures of hemopoietic cells from the livers of 13-day mouse fetuses.

In the present communication, we describe the production of colonies *in vitro* under the influence of erythropoietin, show that the cells in these colonies are engaged in hemoglobin synthesis, and present evidence that the induction of erythroid colonies in the cultures is controlled independently of the induction of granulocytic colonies.

MATERIALS AND METHODS

Mice

C3H$_f$/Bi Oci mice were bred in the Division of Laboratory Animal Science, University of Toronto. Fetuses of known age were obtained by leaving one male overnight with three or four females and on day 13 taking those mice found pregnant.

Preparation of cell suspensions

Liver cell suspensions were prepared from 13-day fetal mice by the method of Cole and Paul (4) except that the livers were trypsinized at 4°C for 2 hr instead of overnight. Bone marrow suspensions were prepared by the technique described by Till and McCulloch (11).

Culture method

Cells were suspended, at 2×10^5 cells per ml, in medium NCTC 109 (Microbiological Associates, Bethesda, Md.) containing the following: 10% heat-inactivated fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N.Y.), 0.24 U/ml erythropoietin (Step III, Connaught Medical Research Laboratories, Toronto, Canada), 0.02 mg/ml L-asparagine, 0.85% beef embryo extract (Grand Island Biological Co.), 1% bovine serum albumin, and 10% medium conditioned with mouse kidney tubules (to be described in a later publication). 1 ml of this cell suspension was added to a 35×10 mm Petri dish (Falcon Plastics, Los Angeles, Calif.) containing 0.1 ml of citrated bovine plasma (Grand Island Biological Co.), and the mixture was allowed to clot. Cultures were incubated for 4 days at 37°C in a humidified incubator with 5% CO_2 in air.

Combination stain for hemoglobin and peroxidase

For distinguishing erythrocytic and granulocytic colonies in the same culture, a histochemical procedure for peroxidase (12) was combined with a modification of the Lepehne technique (13) for hemoglobin.

The plasma clots were removed from the Petri dishes, spread out on a 50×75 mm glass slide, and blotted with

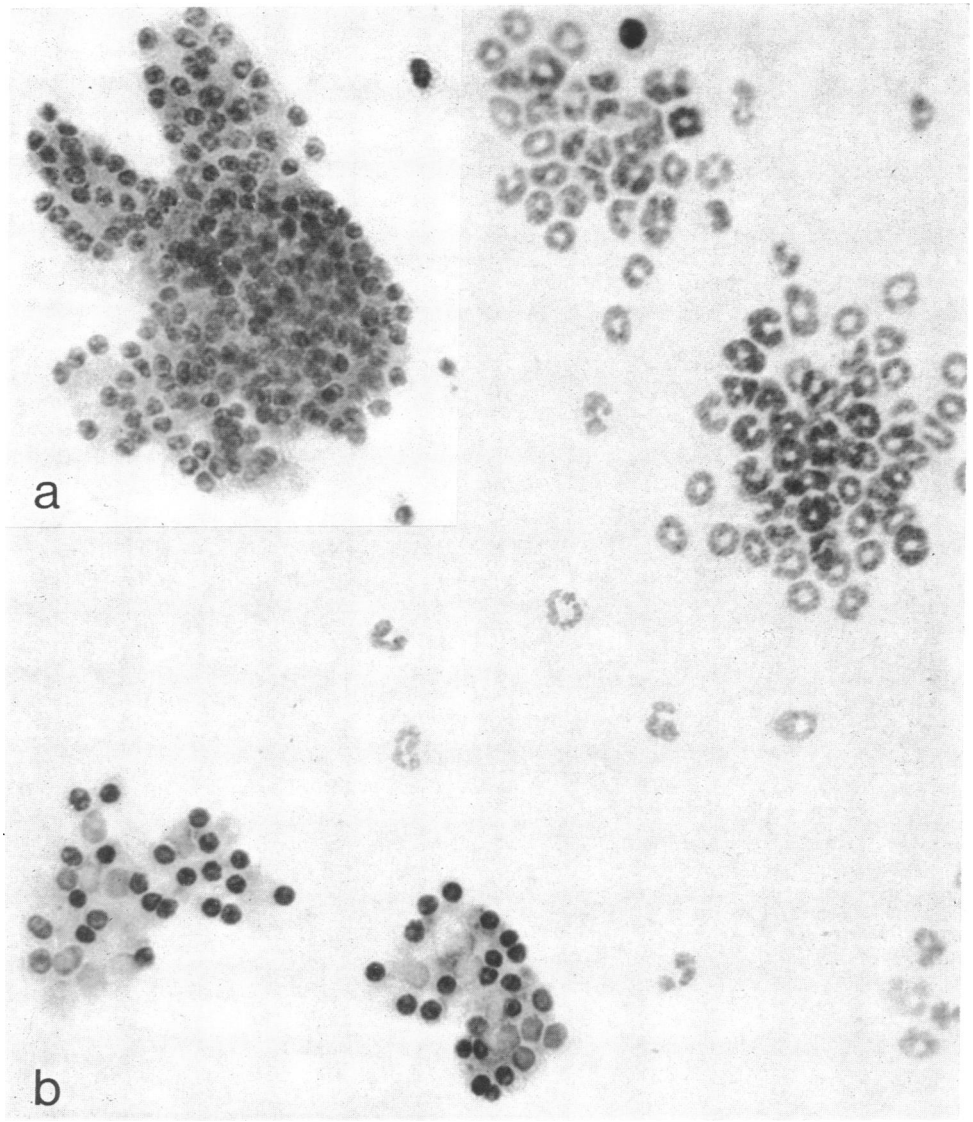

FIG. 1. Photomicrograph of 4-day culture of $C3H_f/Bi$ fetal liver cells. (*a*), Erythroid colony (×2700); (*b*), two granulocytic colonies on the right, two erythroid colonies on the left (×2240). Stained by the modified Lepehne procedure and counterstained with hematoxylin.

filter paper. Slides were air-dried, fixed in a 1:9 v/v mixture of neutral formalin and 95% ethanol, washed with 0.01 M phosphate buffer (pH 7), air-dried, treated with ether for 60 sec, and again rinsed with 0.01 M phosphate buffer. Staining for peroxidase was performed for 1–2 min in the peroxidase reagent of Rytömaa (12). Excess stain was rinsed off with buffer; slides were air-dried, treated with absolute methanol for 30 sec, and air-dried. Staining for hemoglobin was then carried out for 2 min in a 1% solution of 3,3′-dimethyl-oxybenzidine (Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.) in absolute methanol. Slides were immediately treated for 2 min in 30% hydrogen peroxide–70% ethanol 1:3, then rinsed for 15 sec in distilled water, counterstained for 15 sec with Harris Hematoxylin, and differentiated in running tap water. Permanent preparations were made by air-drying and mounting in Permount.

Determination of heme synthesis rates

1–2 μCi of transferrin-bound ^{59}Fe (14) was diluted in 0.5 ml medium NCTC 109 and added to culture plates after 78 hr of incubation. After a further 18 hr, 1 ml of 0.05% trypsin (3 × crystallized, Worthington Biochemical Corp., Freehold, N.J.) was added to each plate and cultures were incubated for 5 min at 37°C. The plasma clots were then transferred to 15-ml centrifuge tubes, an additional 1 ml of 0.05% trypsin was added, and after 15 min at 37°C, fetal calf serum was added to a final concentration of approximately 2%, and heme was extracted as described previously (5).

Radioautography

Plasma clots from ^{59}Fe-labeled cultures were transferred to slides, fixed, washed, and stained for hemoglobin, but not counterstained. Slides were then dipped in liquid emulsion (Nuclear Track Emulsion, NTB3 Eastman Kodak Co., Rochester, N.Y.), stored for 2–3 weeks, and developed. The resulting radioautographs were poststained with hematoxylin and made permanent.

Polyacrylamide gel electrophoresis

Cell lysates were prepared and hemoglobins separated by gel electrophoresis by the method of Barker (15). To ensure that only [^{59}Fe]heme was being measured, we cut out the

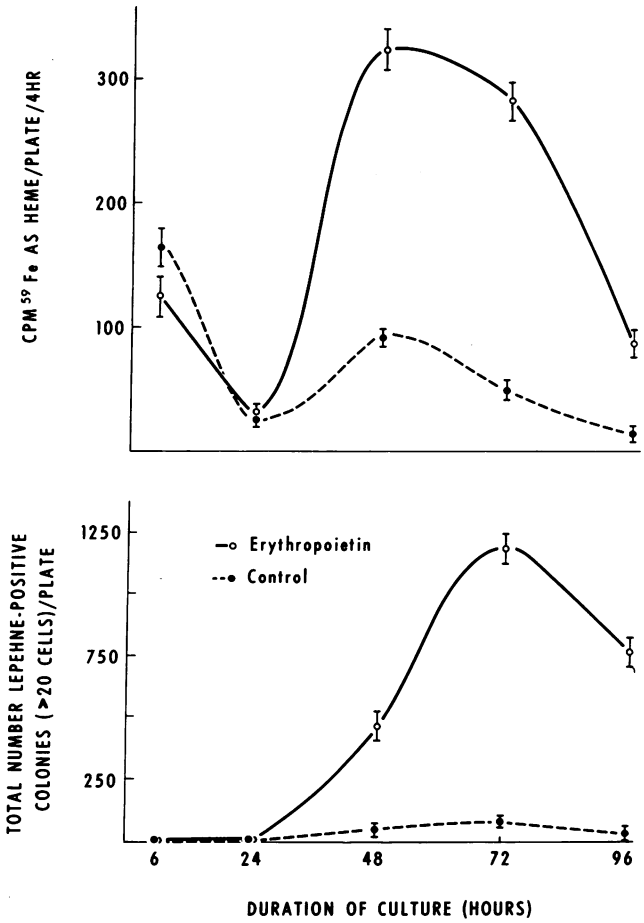

FIG. 2. Time course of the response to erythropoietin by cultured fetal liver cells of C3H$_f$/Bi mouse *in vitro*. Results of heme synthesis response (*top*) are expressed as mean ± SE (eight determinations). Each point indicates the middle of a 4-hr incubation with transferrin-bound ^{59}Fe. Counts of Lepehne-positive colony (*bottom*) are expressed as mean ± SE (five cultures).

regions of the gels corresponding to the visible hemoglobin bands, homogenized them, and eluted the hemoglobin at 4°C in 3–4 ml of distilled water for 24 hr. Heme was extracted from the aqueous supernatant, and the radioactivity was counted.

To prepare ^{59}Fe-labeled adult C3H hemoglobin, we injected [^{59}Fe]ferrous citrate, 10–15 Ci/g (Abbott Laboratories Ltd., North Chicago, Ill.), intraperitoneally into C3H$_f$/Bi mice at a dose of 3–4 μCi of ^{59}Fe (in 1 ml of phosphate-buffered saline) per animal. 24 hr later blood was collected, the cells were washed three times with cold phosphate-buffered saline, cell lysates were prepared and subjected to electrophoresis.

RESULTS

In cultures examined a few hours after initiation of the cultures, only single cells were found to be stained by the modified Lepehne procedure. By 2 days the number of single cells was reduced, and large numbers of colonies that consisted of 10–20 cells with the morphological appearance of basophilic erythroblasts and numerous mitotic figures were found. By 4 days the number of cells per colony had increased considerably; up to 60–70 cells were often present. Most of the cells had the appearance of normoblasts (orthochromatic erythroblasts) (Fig. 1); only an occasional mitotic figure remained. By the 7th day of culture, very few erythroid colonies could be seen.

CUT #	PERIPHERAL BLOOD CELL CONTROLS	FETAL LIVER CELL CULTURES
–	10.3 ± 3.2 %	6.1 ± 4.6
1		
2	13.9 ± 3.7	15.0 ± 2.6
	44.0 ± 3.2	47.7 ± 2.6
3		
4	28.6 ± 3.7	30.2 ± 4.6
+	2.9 ± 0.9	0.9 ± 0.3

FIG. 3. [^{59}Fe]heme distribution in polyacrylamide gels after electrophoretic separation of hemoglobin from 24-hr labeled lysates of adult C3H$_f$/Bi peripheral blood cells and 4-day cultures of C3H$_f$/Bi fetal liver cells. The diagram at the left of the table indicates the position in the gel of the adult C3H hemoglobin bands. The results are expressed as mean ± SE (four gels).

We then determined whether the Lepehne-positive colonies were in fact colonies of hemoglobin-synthesizing cells. The liver cells from 13-day fetuses were cultu- at 1×10^5 and 2.5×10^5 cells per plate. Erythropoietin (0.24 U/ml) was added to half of the cultures at each cell concentration. Eight erythropoietin-treated and eight control cultures at the higher cell concentration were treated at various times with 1–2 μCi of transferrin-bound ^{59}Fe for 4 hr, and heme extractions were performed. After 6–96 hr of culture, five erythropoietin-treated and five control cultures at the lower cell concentration were stained by the modified Lepehne technique. The total number of Lepehne-positive colonies with more than 20 cells was counted.

The rate of heme synthesis decreased during the first 24 hr of culture in both erythropoietin-treated and control cultures (Fig. 2, *top*). However, in erythropoietin-treated cultures, there was then a sharp increase in the rate of heme synthesis, which reached a peak by 48 hr and then gradually declined. In control cultures, the rate of heme synthesis fell and then remained low throughout. In the erythropoietin-treated cultures, the onset of heme synthesis preceded the appearance of Lepehne-positive colonies. Fig. 2 (*bottom*) shows that by 48 hr Lepehne-positive colonies were present in considerable numbers, and by 72 hr a peak of around 1200 colonies per plate was reached; the colony number then declined. In control cultures only very few Lepehne-positive colonies were ever found. These results establish the fact that the formation of Lepehne-positive colonies is an erythropoietin-dependent process.

To obtain independent evidence as to whether the heme synthesis detected in the cultures was performed by cells in the individual Lepehne-positive colonies, we cultured the liver cells at 1×10^5 cells per plate, and after 48 hr added 0.01–0.05 μCi of transferrin-bound ^{59}Fe to each culture. The cells were incubated for a further 48 hr, and radioautographs were prepared. Intense radioautographic reactions were found over the Lepehne-positive colonies, whereas very little label was found over other colonies or individual cells. The fact that the radioisotope was not washed out during fixation and washing indicates that the ^{59}Fe localized in the cells of the Lepehne-positive colonies was in macro-

molecular form. That its intracellular form in these colonies was hemoglobin was shown in the following mass culture experiment.

Several cultures of mouse fetal liver cells were incubated for 78 hr, 1–2 μCi of transferrin-bound ^{59}Fe was added per culture, and incubation was continued for a further 18 hr. Lysates were prepared from the pooled cultures, mixed with a small amount of unlabeled adult carrier hemoglobin, and subjected to polyacrylamide gel electrophoresis. Labeled normal adult C3H$_f$/Bi hemoglobin was also subjected to gel electrophoresis as control. The gel regions corresponding to the three hemoglobin bands from adult C3H were cut out, and the ^{59}Fe incorporated into heme extracted from these bands and from the regions above and below the bands was counted. As seen in Fig. 3, hemoglobin synthesized by fetal liver cells after 4 days of culture was electrophoretically indistinguishable from normal adult C3H hemoglobin. These results show that the Lepehne-positive colonies that develop in cultures of mouse fetal liver cells in the presence of erythropoietin are composed of cells that synthesize hemoglobin.

An attempt was next made to determine whether the production of erythroid colonies is related to, or independent of, the production of granulocytic colonies in the same cultures. It had been shown previously that the production of granulocytic colonies in hemopoietic cell cultures was stimulated by conditioned medium (8, 9, 17). The work described above had shown that the production of erythroid colonies was stimulated by erythropoietin, and that colonies of erythroid and granulocytic type could be clearly distinguished in the same cultures. Therefore, four groups of cultures were set up in which (*a*) standard medium was used as control, (*b*) erythropoietin was added but conditioned medium omitted, (*c*) conditioned medium but not erythropoietin was added, and (*d*) both erythropoietin and conditioned medium were added. Each group included eight plates at 2.5×10^5 cells and six plates at 5.0×10^5 cells per culture. After 78 hr, 1–2 μCi of transferrin-bound ^{59}Fe was added to each of the cultures at the higher cell concentration, and after a further 18 hr, heme synthesis was determined. At the end of 4 days of incubation, all cultures at the lower cell concentration were stained and the numbers of Lepehne-positive and peroxidase-positive colonies in each group were determined.

The pooled results from three such experiments are given in Table 1. In the control group in which neither conditioned medium nor erythropoietin was included, very few colonies were found, and most of them could not be classified. When erythropoietin was included, there was a great increase in the total number of colonies that developed, and most of these were erythroid. Concomitantly, there was a great increase in heme synthesis rate by the cultures. In the cultures with conditioned medium but no erythropoietin, the number and proportion of granulocytic colonies increased greatly, but there was no effect on erythroid colony number and only a slight effect on heme synthesis. When both erythropoietin and conditioned medium were included, there followed an increase in both erythroid and granulocytic colonies over controls. These results indicate that the production of erythroid colonies was virtually independent of the production of granulocytic colonies.

We next tried to culture adult mouse bone marrow under the conditions developed for production of erythroid colonies by mouse fetal liver cells. The Lepehne-positive colonies obtained were smaller than those produced by fetal liver cells, and the plating efficiencies were somewhat lower. However, these colonies were also erythropoietin-dependent. When bone marrow cells were obtained from mice that had previously been made hypoxic by exposure to a pressure of 0.5 atmosphere for 2 days, the number of erythroid colonies increased about 3-fold. The culture method described here thus appears to be useful for production of erythropoietin-dependent colonies of hemoglobin-synthesizing cells from adult as well as from fetal hemopoietic tissue.

DISCUSSION

A method has been presented for obtaining colonies of erythroid cells in cultures of hemopoietic cells from the liver of 13-day C3H$_f$/Bi mouse fetuses. The development of these colonies was shown to depend on the presence of the hormone erythropoietin in the medium, and the colonies were shown to be composed of hemoglobin-synthesizing cells. Moreover, the conditioned-medium dependency of granulocytic colonies (8, 9, 16) was confirmed.

TABLE 1. *Influence of erythropoietin and of conditioned medium on the formation of erythroid and granulocytic colonies, and on heme synthesis rates by 4-day cultures of C3H$_f$/Bi mouse fetal liver cells*

Treatment	Mean colony number*				Colony type expressed as percentage†			Heme synthesis (cpm/5 × 10^5 cells)‡
	Erythroid	Granulocytic	Unclassified	Total	Erythroid	Granulocytic	Unclassified	
Control	15	6	44	65	8	3	89	0.9 ± 0.3
Erythropoietin alone	432	39	132	603	76	9	16	67.0 ± 3.0
Conditioned medium alone	16	117	52	185	10	67	23	3.9 ± 0.4
Erythropoietin + conditioned medium	398	184	148	730	55	32	13	93.0 ± 5.4

* Mean values from three separate experiments with six plates per group in each experiment, rounded off to the nearest integer.

† Means of the percentages from three experiments.

‡ Mean ± SE (eight cultures per group in one of the three experiments).

Addition of erythropoietin did not result in a decrease in the number of granulocytic colonies, nor did addition of conditioned medium result in a decrease in the number of erythroid colonies. The production of erythroid colonies thus appeared to be independent of the production of granulocytic colonies in the same cultures. Although other possibilities exist, these data would be consistent with the hypothesis that the two types of colonies are derived from separate cells. Suspensions of liver cells from 13-day $C3H_f/Bi$ mouse fetuses thus may contain cells already committed as progenitors of erythroid colonies and cells already committed as progenitors of granulocytic colonies, each able to respond to its own appropriate stimulus—either erythropoietin or the active agent in conditioned medium, but not both. The response to each stimulus would be a burst of cell proliferation and differentiation of the respective class of cells.

At present, the term CFU-S is used to refer to the cell that gives rise to colonies in the spleens of supralethally irradiated mice after intravenous injection of bone marrow cells, while the term CFU-C is used in reference to hemopoietic cells that give rise to colonies (mainly granulocytic) in culture (17). With the evidence in the present paper that not only granulocytic but also erythroid colonies can be obtained *in vitro*, it may be operationally useful to replace the term CFU-C by two terms, CFU-G and CFU-E, the former to signify the cellular unit responsible for the production of granulocytic colonies, and the latter to refer to the cellular unit that gives rise to erythroid colonies, in culture.

Evidence from sedimentation velocity measurements (5) indicates that the CFU-S is not identical with the cell(s) that respond(s) to erythropoietin by an increased rate of heme synthesis in mass cultures of mouse fetal liver. Whether or not the CFU-E responsible for erythropoietin-dependent erythroid colony formation is identical with the erythropoietin-sensitive cell(s) in mass culture, and how the CFU-E is related to the CFU-S, are at present open questions.

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