Separation of Erythropoietin-sensitive Cells From Hemopoietic Spleen Colony-forming Stem Cells of Mouse Fetal Liver by Unit Gravity Sedimentation

By John R. Stephenson and Arthur A. Axelrad

An assay method for erythropoietin-sensitive cells has been developed based on the fact that cells of mouse fetal liver respond to erythropoietin in vitro by increased heme synthesis. To determine whether or not hemopoietic colony-forming stem cells are identical with erythropoietin-sensitive cells, C3H/Bl 13-day fetal liver cells were separated into fractions on the basis of size by unit gravity sedimentation through a 1–2 percent bovine serum albumin gradient. The cell fractions obtained were assayed for erythropoietin-sensitive cells by the present method and for spleen colony-forming cells by the method of Till and McCulloch. It was found that the modal sedimentation velocity of erythropoietin-sensitive cells was greater than that of the spleen colony-forming cells of mouse fetal liver, showing that these two classes of cells are distinct.

In the mammalian hemopoietic system, erythrocytes, granulocytes, and megakaryocytes are known to stem from a single pluripotent cell.1–3 It has been established that the production of erythrocytes, which can vary independently of the production of either granulocytes or megakaryocytes, is controlled by the hormone erythropoietin.4 However, the relationship between the cells on which erythropoietin acts and the pluripotent hemopoietic stem cells has yet to be clarified.

Originally, it was held that the cells which responded to erythropoietin were "stem cells" rather than differentiated cells, and that the hormone acted by inducing erythroid differentiation in them.4–6 Gurney et al.,7 in fact, used the response to erythropoietin as the basis of an assay method for stem cells, but the concept of stem cells still lacked clear definition at the time. The advent of the spleen colony assay method of Till and McCulloch1–8 in 1961 helped to sharpen the definition of "stem cells" and it also permitted the problem of the relationship between erythropoietin-sensitive cells and hemopoietic stem cells to be formulated in precise operational terms. Bruce and McCulloch9 demonstrated that hypoxia produced a differential effect on the kinetics of erythropoiesis and of colony-forming potential of spleen cells. These

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results seemed to suggest that erythropoietin-sensitive cells were distinct from pluripotent hemopoietic stem cells.

Two lines of evidence support this model. These include (1) a difference in the sensitivity of the erythropoietic response and of spleen colony-forming capacity to suicidal doses of $^3\text{H}$-thymidine, indicating that the cells responding to erythropoietin are in a state of much more rapid cycle than the hemopoietic stem cells, and (2) a dissociation between erythropoiesis and colony-forming cell proliferation in genetically anemic mice.

This evidence in favor of nonidentity of the two cell types is compelling; it cannot, however, be regarded as conclusive because it is all indirect. An unambiguous test of the model would be to see whether erythropoietin-sensitive cells can be physically separated from pluripotent hemopoietic stem cells.

A technique known as unit gravity sedimentation, which permits separation of cells on the basis of their size, has been used to separate colony-forming cells from other cells of mouse bone marrow, and to separate erythropoietin-sensitive cells from other cells of rat bone marrow. However, although a reliable method has been available for the detection of erythropoietin-sensitive cells of bone marrow in the rat, there has been no routine assay method for hemopoietic stem cells in that species. In the case of adult mouse hemopoietic tissues, the spleen colony assay method for stem cells has been well established, but until now there has been no satisfactory method for assaying erythropoietin-sensitive cells.

Cole and Paul have described a culture system in which exposure of mouse fetal liver cells to erythropoietin results in a readily detectable increase in their rate of heme synthesis. We have used this system as the basis of an assay method for erythropoietin-sensitive cells. The spleen colony assay method of Till and McCulloch has already been applied elsewhere to mouse fetal liver cells. We thus had the opportunity to ask experimentally whether in mouse fetal liver-cell suspensions, erythropoietin-sensitive cells could be physically separated from spleen colony-forming cells.

### MATERIALS AND METHODS

#### Mice

The mice used were C3Hf/Bi Oci, bred in the animal colony of the Division of Laboratory Animal Science, Medical Sciences Building, University of Toronto. Mice were housed in plastic cages and supplied freely with Rockland Mills Mouse Diet (Teklad, Monmouth, Ill.) and water.

#### Preparation of Cell Suspensions

Liver-cell suspensions were prepared by the method of Cole and Paul from 13-day fetal mice. Accurately dated fetuses were obtained by routinely leaving one male with three or four females overnight and then taking those mice found pregnant on day 13. Bone marrow suspensions were prepared by the technique described by Till and McCulloch.

#### Erythropoietin

Step III erythropoietin, with a potency of 8.5 U/mg, prepared from sheep plasma was
obtained from Connaught Medical Research Laboratories, Toronto, Canada. The erythropoietin was dissolved in CMRL 1066 (Connaught Medical Research Laboratories) at a concentration of 10 U/ml and stored at -20°C.

Isotope

$^{59}$FeCl$_3$ was obtained from New England Nuclear Corp., Boston, Mass., as a sterile solution, free of antibacterial agents. The concentration used was 8$\mu$g Fe per ml with a specific activity of 10 to 30 mCi/mg. This stock solution was diluted 8:1 with C3Hf/Bi Oci mouse serum and incubated for 12 hours at 37°C to allow binding of the $^{59}$Fe to mouse transferrin.$^{29}$ The results of preliminary experiments indicated that such prebinding greatly enhanced the incorporation of $^{59}$Fe into heme in our system.

Cell Culture

For the standard culture procedure, fetal liver cell suspensions were diluted to the desired cell concentration in CMRL 1066 plus 10 per cent fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N.Y., tested for toxicity by its effect on the colony-forming efficiency of murine hemopoietic cells in vitro), with an appropriate level of erythropoietin. Control suspensions without erythropoietin were prepared identically. Two ml of cell suspension were added to 35 x 10 mm disposable sterile Petri dishes (Falcon Plastics, Los Angeles, Calif.) and placed in a humidified 95 per cent air–5 per cent CO$_2$ incubator, at 37°C, for 24 hours. At the end of this period, 0.05 ml of the transferrin-bound $^{59}$Fe preparation described above was added to each culture, and the cells subsequently incubated for a further 12 hours, after which time $^{59}$Fe uptake into heme was determined.

In experiments to determine the time course of the response to erythropoietin (Fig. 1) cells were exposed to $^{59}$Fe for 2 hours rather than for the standard 12-hour period, and the length of exposure to erythropoietin varied.

Heme Extraction

Following culture, cells were washed three times with cold PBS and the heme was extracted by the method of McCool et al.$^{15}$ The extracted heme was counted in a

![Fig. 1.—Time course of heme synthesis response to erythropoietin by C3H/Bi mouse fetal liver cells in vitro. Results are expressed as mean ± 1 standard error (SE) based on six individual determinations. Each point indicates the middle of the 2-hour period of incubation with transferrin-bound $^{59}$Fe.](image-url)
well-type scintillation counter (Nuclear Chicago, Chicago, Ill.) and results expressed as cpm 59Fe incorporated into heme.

Assay for Spleen Colony-forming Cells (CFU)

C3H/Bi male mice, 6–8 weeks of age were irradiated with 950 rads at a dose rate of 115 rads/min in a 137Cs unit.21 Achromycin (American Cyanamid Co., Pearl River, N.Y.) was added to the drinking water of all irradiated mice. Bone marrow or fetal liver cells were suspended in CMRL 1066 with 10 per cent FCS at appropriate cell concentrations and injected intravenously in a total volume of 0.5 ml into irradiated mice.2 After 9 days, surviving mice were sacrificed, the spleens were removed and fixed in Bouin’s solution, and macroscopic colonies on the spleen surface were counted.

Cell Separation

Fetal liver cells, and also bone marrow cells, were separated on the basis of cell size by a slight modification of the method described by Miller and Phillips.13 The cell separator used consisted of a cylindrical plexiglass sedimentation chamber with an inside diameter of 21 cm, and a height of 12.5 cm (O.H. Johns Glass, Toronto, Canada). A conical section with a depth of 7.5 cm, and tapering from the full diameter of the chamber to that of an 18-gauge needle, was attached to the base of the chamber to allow loading and collection of fractions. A stainless steel baffle13 was placed in the bottom of the cone to prevent turbulence on loading and unloading.

To load the cell separator, first 120 ml of Hanks’ minimal essential medium (HMEM) was run into the chamber from the base, followed by 80 ml of 0.2 per cent bovine serum albumin (BSA) (Sigma Chemical, St. Louis, Mo.) in HMEM, in which a total of $3 \times 10^8$ cells were suspended. Subsequently, 60 ml of 0.33 per cent BSA in HMEM, and 3000 ml of a 1 to 2 per cent BSA in HMEM gradient were subsequently added. Cells were allowed to settle for 5 hours, measuring from the time of addition of the cell suspension to the separation chamber until collection of the first fraction.

After discarding the first 1000 ml, 50 fractions of 45 ml each were collected; the chamber was thus emptied at a rate of approximately 50 ml per min. Nucleated cell counts were done by hemocytometer on all fetal liver cell fractions, while for bone marrow, total and nucleated counts were done by an electronic cell counter (Coulter Electronics, Hialeah, Fla.). Each fraction was then centrifuged at 1200 rpm for 10 min, the supernatant poured off, and the cells resuspended in CMRL 1066 with 10 per cent FCS at appropriate cell concentrations for erythropoietin-sensitive cell and CFU assays.

RESULTS

Development of an Assay Method for Erythropoietin-sensitive Cells

It had been reported by Cole and Paul17 that fetal liver cells in vitro respond to erythropoietin with increased heme synthesis. In the course of repeating their work with C3H/Bi mice, we found that culturing the cells in Petri dishes rather than in tubes and replacing the mouse serum in the medium by FCS enhanced the heme synthesis response to erythropoietin of the fetal liver cell cultures. Cultures of 13-day C3H/Bi mouse fetal liver cells were prepared and incubated at $5 \times 10^5$ cells per plate in CMRL 1066 with 10 per cent FCS and 0.24 U/ml erythropoietin. At various times, 2–4 µCi transferrin-bound 59Fe were added to the erythropoietin-treated and control cultures, and after a further 2 hours of incubation, heme was extracted and counted. Six replicate plates were used in each group. As shown in Fig. 1, the rate of heme synthesis in control cultures without erythropoietin fell progressively with time and was virtually undetectable by 48 hours. In cultures
SEPARATION OF ERYTHROPOIETIN-SENSITIVE CELLS

Fig. 2.—Effect of erythropoietin concentration on the rate of heme synthesis by C3H/Bl mouse fetal liver cells in vitro. Results are expressed as mean ± 1 SE based on seven individual determinations for each erythropoietin concentration.

with erythropoietin, the rate of heme synthesis began to rise within several hours after the initiation of the cultures. By 30 hours the heme synthesis rate

Fig. 3.—Effect of variation in cell number on the heme synthesis response to erythropoietin by C3H/Bl mouse fetal liver cells in vitro. Erythropoietin-treated: \( Y = -0.110 + 0.649X \) over the range of \( 3.2 \times 10^4 \) to \( 2.5 \times 10^5 \) total cells; \( S_{y.x} = 0.33 \), Y-intercept not significantly different from zero \((p > 0.1)\). Control: \( Y = -0.031 + 0.061X \); \( S_{y.x} = 0.13 \); Y-intercept not significantly different from zero \((p > 0.1)\). Results are expressed as mean ± 1 SE based on six individual determinations per point.
had reached a peak, and thereafter it diminished rapidly, although it was still significantly greater than in controls at 60 hours.

Next, we undertook to determine the optimal conditions of response to the hormone in order to develop an assay method for the erythropoietin sensitivity of these cells. The results of the previous experiment had shown that the difference between the heme synthesis rate in the presence and in the absence of erythropoietin was greatest at 24 to 36 hours after the initiation of the cultures. Therefore, as the standard procedure in all subsequent experiments, transferrin-bound $^{59}$Fe was added after 24 hours of culture and heme extracted after a further 12 hours of incubation.

To determine the concentration of the hormone that would give a maximal response, cultures of fetal liver cells at $5 \times 10^5$ cells per plate were incubated with different concentrations of erythropoietin for the standardized times given above, and then assayed for heme synthesis. As shown in Fig. 2, the rate of heme synthesis rose with the concentration of erythropoietin and reached a plateau between 0.16 and 0.32 U/ml. Therefore a level of 0.24 U/ml of erythropoietin was used routinely thereafter. The reduced response seen at 0.40 U/ml is in agreement with a previous report of an inhibitory effect with high concentrations of Step III erythropoietin.22

To determine the effect of variation in the number of cells on the rate of heme synthesis, plates were prepared containing from $3 \times 10^4$ to $1 \times 10^6$ cells each. To one group of plates erythropoietin was added (0.24 U/ml), while the other group was kept as control. Conditions were otherwise as in

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**Fig. 4.**—Effect of variation in cell number on the heme synthesis response to erythropoietin by C3H/Bl mouse fetal liver cells in vitro. Each value on the ordinate represents the difference between the mean numbers of cpm $^{59}$Fe incorporated into heme per 12 hours in the absence of erythropoietin and in the presence of the hormone at a concentration of 0.24 U/ml. $Y = 0.04 + 0.46X$; $S_{y.x} = 0.15$; $Y$-intercept is not significantly different from zero ($p > 0.1$).
the previous experiment. It can be seen from the results in Fig. 3 that in the erythropoietin-treated cultures, over the range $3 \times 10^4$ to $5 \times 10^5$ cells per plate, the heme synthesis rate was directly proportional to cell number. In control cultures, the heme synthesis rate was also linearly related to cell number, in this case over the whole range examined, but the slope was much lower. The difference between the values with and without erythropoietin at any point above the abscissa represents the effect of a fixed dose of the hormone on the rate of heme synthesis by the cells at that point. This difference, which must be a measure of the erythropoietin-sensitivity of the system, is plotted against cell number in Fig. 4. It shows that over the range examined, $3 \times 10^4$ to $5 \times 10^5$ cells per plate, the sensitivity of C3H/Bi mouse fetal liver cells to erythropoietin was linearly related to cell number and this line extrapolated through the origin. The requirements of an assay method for erythropoietin-sensitive cells have thus been fulfilled in this system.

Separation of Erythropoietin-sensitive Cells from Spleen Colony-forming Cells

To attempt to answer the question as to whether cells possessing the function of sensitivity to erythropoietin are physically separable from the pluripotent hemopoietic stem cells of mouse fetal liver, the following experiment was done. Suspensions of $3 \times 10^6$ 13-day C3H/Bi fetal liver cells were separated into 50 fractions, on the basis of size, by unit gravity sedimentation. Duplicate cultures of $5 \times 10^5$ cells from each fraction were set up using the standardized conditions described above and 0.24 U/ml erythropoietin was added to one of each of the pairs of cultures. Incubation was carried out for 24 hours, transferrin-bound $^{59}$Fe was added to each culture, and incubation was continued for a further 12 hours, after which time heme extraction was carried out. The remaining cells in the fractions were then pooled in pairs and assayed at appropriate cell doses for spleen colony formation. In separate

![Fig. 5.—Sedimentation distribution profiles for erythropoietin-sensitive cells and colony-forming cells of 13-day C3H/Bi mouse fetal liver. CFU are expressed as mean ± 1 SE per fraction based on six to eight spleens counted per point.](image-url)
Fig. 6.—Sedimentation distribution profiles for total and nucleated cells and for CFU of adult C3H/Bi mouse bone marrow. CFU are expressed as mean ± 1 SE based on five to eight spleens counted per point.

experiments adult mouse bone marrow cells were similarly separated into fractions by unit gravity sedimentation and fractions assayed for spleen colony formation. As shown in Fig. 5, fetal liver cells that were sensitive to erythropoietin were found at a modal sedimentation velocity of 7.7 mm/hr and were thus clearly distinguishable from the fetal liver cells that were capable of forming spleen colonies and which had a modal velocity of 5.9 mm/hr. Comparison of Fig. 5 with Fig. 6 shows that the spleen colony-forming cells of fetal liver had a higher modal sedimentation velocity than those of adult bone marrow at 3.9 mm/hr. The modal sedimentation velocities of the spleen colony-forming cells, the cells sensitive to erythropoietin, and the total cells of fetal liver from a number of separate experiments are summarized in Table 1.

**Discussion**

The present work establishes the fact that the erythropoietin-sensitive cells are physically distinguishable from the pluripotent hemopoietic stem cells of mouse fetal liver.

The term "erythropoietin-sensitive cell" has generally been used in reference to the class of erythroid cells that become capable of heme synthesis only after being stimulated by the hormone erythropoietin. There is evidence, however, for another class of cells that respond to erythropoietin. These have been considered to be more mature than the former cells, and are already actively synthesizing hemoglobin even in the absence of erythropoietin. The hormone acts on these cells to increase their rate of hemoglobin synthesis. McCool et al. have succeeded in physically separating the cells of adult rat bone marrow into two populations, both of which were responsive to erythro-
separation of erythropoietin-sensitive cells

Poietin. They have shown directly that the major increase in heme synthesis in response to erythropoietin was due to the effect of the hormone on those cells (Fraction I) that were not actively engaged in heme synthesis prior to exposure to the hormone. The other population of cells (Fraction II), shown to be the progeny of the first, were actively synthesizing heme prior to exposure to the hormone and erythropoietin increased their rate of heme synthesis only slightly.

In the present study, the rates of heme synthesis by 13-day mouse fetal liver cells in the absence of erythropoietin were extremely low (Figs. 1, 3, and 5). Moreover, the sedimentation velocities of the cells that responded to erythropoietin (Fig. 5) were remarkably similar to those of Fraction I of McCool et al. in adult rat bone marrow, while no peak corresponding to the heme-synthesizing progeny cells of Fraction II was detectable. It therefore seems reasonable to conclude that the function of increased heme synthesis in response to erythropoietin in 13-day mouse fetal liver-cell suspensions belongs almost solely to the true “erythropoietin-sensitive cells” and not to more mature hemoglobin-synthesizing cells.

Application of the assay method for erythropoietin-sensitive cells and the spleen colony assay method to fractions obtained by unit gravity sedimentation of cells from the livers of 13-day fetal C3H/Bi mice revealed that the sedimentation velocities of the majority of erythropoietin-sensitive cells (range 6.5–7.7 mm/hr) were significantly greater than those of the majority of spleen colony-forming cells (range 4.9–6.3 mm/hr). Since sedimentation velocity is dependent primarily on cell size, the erythropoietin-sensitive cells of mouse fetal liver would appear to have greater diameters and therefore to be distinct from the pluripotent stem cells of mouse fetal liver.

A considerable degree of variation in the sedimentation velocities of erythropoietin-sensitive cells, spleen colony-forming cells, and total cells of mouse fetal liver was found from one experiment to the next (Table 1)—much more in fact, than is routinely found in adult bone marrow. It is known that the relative proportions of the component cell types of fetal liver change progressively with fetal age, and it has recently been shown that spleen colony-forming cells undergo a progressive decrease in size with increasing fetal age. The finding in the present study that the spleen colony-forming cells of fetal liver had higher sedimentation velocities than those of adult bone marrow is consistent with this observation. It is therefore possible that the variations in

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sedimentation velocities found in our work from one experiment to another might simply reflect slight variations in the developmental ages of the fetuses used.

It should be pointed out that the term "erythropoietin-sensitive cell" is a convenient designation for a population of cells having a particular measurable function. No strict homogeneity of this population with respect to either cellular structure or function other than sensitivity to the hormone is necessarily implied. The population, in fact, encompasses a fairly broad range of sedimentation velocities and, hence, of cell sizes. Although its mode is distinctly different from that of the spleen colony-forming cell population, the possibility nevertheless remains that some cells possessing the function of erythropoietin sensitivity might be identical with a minority of those capable of acting as pluripotent stem cells.

These results clearly indicate that erythropoietin does not act on the majority of pluripotent stem cells to induce erythroid differentiation in them. Rather they support the alternative model—that erythropoietin acts on cells that are already committed to the erythroid line of differentiation in the sense of having acquired the capacity to respond to the hormone by the synthesis of hemoglobin.

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