Use of Cell Separation and Short-Term Culture Techniques to Study Erythroid Cell Development

By Jonathan Glass, Linda M. Lavidor, and Stephen H. Robinson

Cell populations highly enriched for the different stages of erythroid cell maturation were obtained by three sequential operations: harvesting of erythroid cells after induction of erythroid hyperplasia in the spleens of mice, elimination of the more mature erythrocytes by immunologic techniques, and separation of the residual nucleated erythroid cells as a function of size by the velocity sedimentation technique. The resulting cell fractions were studied both directly and after overnight incubation in the presence or absence of erythropoietin. In short-term culture, erythropoietin stimulated proliferation of pronormoblasts and basophilic normoblasts but probably not cells at later stages of differentiation. Erythropoietin also appeared to recruit increased numbers of pronormoblasts. In this experimental system, erythroid cell differentiation was able to proceed in the absence of erythropoietin, but without proliferation of these early erythroid cells. These techniques have provided a model system for the study of erythroid cells at different stages of maturation isolated from a uniform source at one point in time. The morphologic observations indicated that erythropoietin stimulates erythroid cell proliferation at several early stages of the maturation pathway.

MAMMALIAN ERYTHROPOIESIS has many advantages for the study of cell differentiation: erythroid cell differentiation is under hormonal control by erythropoietin, is associated with easily definable morphologic changes, and is characterized by the synthesis of a specific protein, hemoglobin. To develop a model system for studies in this area, several techniques of cell separation were combined to isolate murine erythroid cells at different stages of maturation. The cells could then be studied before and after short-term culture in the presence or absence of erythropoietin, with attention to changes in cell number and morphology.

MATERIALS AND METHODS

Techniques of Cell Separation

Hemolytic anemia was induced in CD-1 virgin female mice (Charles River Laboratories, Wilmington, Mass.) with intraperitoneal injections of phenylhydrazine, 30 mg/kg, on days 0, 1, and 3. Cell populations highly enriched for the different stages of erythroid cell maturation were obtained by harvesting spleens on day 4. Single cell suspensions were prepared by mincing the spleens with fine scissors, forcing the tissue through stainless steel mesh and then through 35-μm mesh Nitex cloth (Tobler, Ernst & Traber, Inc., Elmsford, N.Y.). Cells were routinely prepared in phosphate-buffered saline (PBS) containing 15%, fetal calf serum (FCS) and washed twice by centrifugation for 10 min at 4°C at 1500 rpm in an International Centrifuge model PR2.
Immunologic hemolysis was accomplished according to the method of Borsook et al. as modified by Cantor et al., using antiserum prepared in rabbits against mouse adult erythrocytes and guinea pig complement (BBL, Cockeysville, Md.). The titers of antiserum and complement were selected so as to hemolyze virtually all enucleated red cells, many orthochromatophilic normoblasts, and some polychromatophilic normoblasts. Usually a final antiserum titer of 1:2000 and a final complement titer of 1:35 to 1:40 were sufficient to provide the desired degree of hemolysis of suspensions containing $8 \times 10^7$ nucleated cells per ml. The erythroid cells in the resultant cell suspensions were comprised of pronormoblasts, basophilic normoblasts, polychromatophilic normoblasts, and some orthochromatophilic normoblasts. After hemolysis, cells were washed twice in PBS-5% FCS, resuspended in PBS-15% FCS, refiltered through 35-μ mesh Nitex cloth, and then fractionated by velocity sedimentation.

The velocity sedimentation technique employed was that described by Miller and Phillips. Using a Stuput Cell Separator (O. H. Johns Scientific Company, Toronto, Canada) with a bowl diameter of 18 cm, about $7 \times 10^6$ nucleated cells were loaded in 1 hr above a 1200-mI buffered step gradient comprised of 1%-2% bovine serum albumin (BSA Fraction V, Sigma Chemicals, St. Louis, Mo.). Cells were allowed to settle for 3 hr, and the chamber was then emptied at a rate of approximately 30 ml/min. The cone volume (250 ml) was discarded, and 60-mI samples were collected sequentially in sterile plastic tubes. The first three samples were pooled to form fraction I because of the limited number of cells. Cells were pelleted by centrifugation and prepared for study as described below.

**Techniques of Cell Culture**

The pelleted velocity sedimentation fractions were resuspended in modified McCoy's 5A medium containing 15% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Replicate samples were plated as either 0.2-mI aliquots in microtiter plates or as 2-mI aliquots in 30 x 15-mm tissue culture dishes (Falcon Plastics, Oxnard, Calif.) at concentrations of 2-6 x 10^6 cells per ml. Human urinary erythropoietin* was added to cultures at a concentration of 0.2 U/ml. Trial experiments indicated that this dose yielded near-optimal stimulation of cell proliferation and hemoglobin synthesis, similar to the changes observed with concentrations of 0.5 U/ml. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cell counts and differential counts were performed before and after culture for 18 hr.

**Cell Number and Differential Counts**

Cell counts were performed in hemocytometers with 3% acetic acid-1% gentian violet as diluent. Slides were prepared with a cytocentrifuge (Shandon Scientific Company, London, England) and stained with benzidine and Wright-Giemsa stain. Four hundred cells were counted per point. Enucleated red blood cells were enumerated per 100 nucleated cells during the differential counts.

**RESULTS**

The relative amounts of erythroid cells in the initial cell suspension and after immune lysis are shown in Table 1. Of the total cells isolated from the spleens, 76% were erythroid. For the purpose of the subsequent cell separation and the assays of cell function, a degree of controlled hemolysis was desired which would retain some of the more mature erythroid precursors. As shown in Table

*Human urinary erythropoietin (H-4-S4-6SL, 153.0 U/mg) was supplied by the National Institutes of Health. The erythropoietin was collected and concentrated by the Department of Physiology, University of the Northwest, Corrientes, Argentina, and further processed and assayed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, under Research Grant HE-10880.
As detailed in Materials and Methods, spleens were harvested 24 hr after a 3-day course of phenylhydrazine, single cell suspensions prepared, and mature erythrocytes removed by immunolysis in preparation for separation by sedimentation at unit gravity. Differential counts were performed on at least 400 nucleated cells; enucleated erythrocytes were counted simultaneously. Each category of erythroid cells is expressed as a percentage of the total number of erythroid cells. Results are expressed as the means ± SE for 12 experiments. Differential counts for the velocity sedimentation fractions are presented in Fig. 1.

The differential counts of the cell populations resolved from this suspension by the velocity sedimentation technique are shown in Fig. 1. As erythroid cells mature, cell volume diminishes. Since the rate of sedimentation is primarily a function of cell volume, the most rapidly sedimenting fraction, fraction I, contains the most immature cells, mainly pronormoblasts (68%) and basophilic normoblasts (18%), with only about 14% benzidine-positive cells. In the more slowly sedimenting fractions, progressively more mature cells are found such that fraction III contains primarily basophilic and polychromatophilic normo-
blasts, while fractions V and VI are composed largely of benzidine-positive cells. The recovery of each cell type after sedimentation tended to be better for the more mature cells, ranging from 15% for pronormoblasts to 55% for orthochromatophilic normoblasts. Contamination with nonerythroid cells ranged from 31%–43% in fractions I–IV to 64%–69% in fractions V and VI where many lymphocytes were found.

Cell Culture

Proliferation of erythroid cells was observed in all of the less mature velocity sedimentation fractions cultured for 18 hr in the presence of erythropoietin (Table 2). The total number of erythroid cells increased by 30%–70% in fractions I–IV cultured with erythropoietin, while erythroid cell number remained stable in the later, more mature fractions. In contrast, with cultures incubated in the absence of erythropoietin, the number of erythroid cells fell off moderately in fraction I and remained roughly constant in the higher, more mature fractions.

Table 2 also illustrates that erythroid cell maturation occurred on overnight culture of all of the velocity sedimentation fractions. The extent of differentiation in the presence of erythropoietin was such that the cells in fraction I matured to a level similar to that observed in fraction III prior to culture, and corresponding changes were seen in the other fractions. The differential counts of

Table 2. Effect of Erythropoietin on Erythroid Cell Number and Differentiation

<table>
<thead>
<tr>
<th>Velocity Sedimentation Fraction</th>
<th>Erythropoietin</th>
<th>Pronormoblasts</th>
<th>Basophilic Normoblasts</th>
<th>Polychromatophilic Normoblasts</th>
<th>Orthochromatophilic Normoblasts</th>
<th>Enucleated RBC</th>
<th>Total RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+</td>
<td>24.5 ± 1.0</td>
<td>6.6 ± 0.5</td>
<td>4.4 ± 0.9</td>
<td>0.7 ± 0.2</td>
<td>0</td>
<td>36.3 ± 1.3</td>
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<td></td>
<td>–</td>
<td>0.5 ± 0.5</td>
<td>2.0 ± 0.7</td>
<td>6.9 ± 0.7</td>
<td>13.7 ± 1.7</td>
<td>5.0 ± 1.3</td>
<td>28.0 ± 3.3</td>
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<tr>
<td>II</td>
<td>+</td>
<td>11.5 ± 1.3</td>
<td>9.6 ± 1.2</td>
<td>6.5 ± 1.3</td>
<td>0.8 ± 0.2</td>
<td>0</td>
<td>28.4 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>0.4 ± 0.2</td>
<td>2.2 ± 0.9</td>
<td>7.1 ± 1.2</td>
<td>16.5 ± 2.8</td>
<td>7.9 ± 1.4</td>
<td>34.2 ± 4.9</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>8.1 ± 2.3</td>
<td>19.2 ± 2.7</td>
<td>17.6 ± 1.7</td>
<td>3.7 ± 0.5</td>
<td>0</td>
<td>46.5 ± 4.0</td>
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<tr>
<td></td>
<td>–</td>
<td>0.1 ± 0.1</td>
<td>1.2 ± 0.3</td>
<td>8.9 ± 2.7</td>
<td>30.2 ± 7.3</td>
<td>14.6 ± 2.6</td>
<td>54.9 ± 10.3</td>
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<tr>
<td>IV</td>
<td>+</td>
<td>4.1 ± 1.0</td>
<td>15.8 ± 3.4</td>
<td>29.3 ± 5.1</td>
<td>10.2 ± 1.4</td>
<td>0.2 ± 0.1</td>
<td>60.6 ± 7.2</td>
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<td></td>
<td>–</td>
<td>0.04 ± 0.04</td>
<td>5.0 ± 3.7</td>
<td>22.6 ± 9.9</td>
<td>52.5 ± 14.4</td>
<td>20.9 ± 5.3</td>
<td>102.3 ± 14.4</td>
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<tr>
<td>V</td>
<td>+</td>
<td>0.3 ± 0.3</td>
<td>6.4 ± 2.7</td>
<td>42.9 ± 7.4</td>
<td>26.1 ± 7.8</td>
<td>0</td>
<td>75.7 ± 14.6</td>
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<td></td>
<td>–</td>
<td>0</td>
<td>0.9 ± 0.5</td>
<td>12.3 ± 3.0</td>
<td>29.8 ± 3.5</td>
<td>24.1 ± 6.8</td>
<td>67.4 ± 9.6</td>
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<tr>
<td>VI</td>
<td>+</td>
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<td>13.3 ± 5.3</td>
<td>28.1 ± 6.1</td>
<td>6.1 ± 4.8</td>
<td>47.9 ± 12.3</td>
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<tr>
<td></td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>3.9 ± 1.6</td>
<td>32.8 ± 13.1</td>
<td>25.1 ± 7.5</td>
<td>61.8 ± 18.1</td>
</tr>
</tbody>
</table>

Replicate aliquots of the velocity sedimentation fractions were cultured in the presence and absence of erythropoietin. Cell counts and differentials were performed prior to and after 18 hr of culture, and the absolute number of erythroid cells was calculated. The results are expressed as the mean cell number × 10⁵ ± SE for six experiments.
replicate samples cultured in the absence of erythropoietin showed a shift to an even greater degree of maturity. The absolute numbers of the more mature cells were similar for fractions cultured in the presence or absence of erythropoietin. Hence, the increase in cell number was due to greater numbers of early precursors (pronormoblasts and basophilic and polychromatophilic normoblasts) present in the cultures incubated with erythropoietin. However, in fraction I the absolute numbers of orthochromatophilic normoblasts and non-nucleated erythrocytes were greater in the cultures lacking erythropoietin, a finding that was present in each of the individual experiments.

**DISCUSSION**

The techniques described provide a means of studying erythroid cells at different stages of maturation, both by direct examination of cell fractions separated according to size by velocity sedimentation and by study of these same cell fractions as they differentiate in short-term culture. In studies to be reported separately these cell fractions were found to synthesize heme, hemoglobin, and globin mRNA actively; these functions are erythropoietin responsive.5,6

Erythroid cell development has been studied by a variety of techniques. Usually these studies have been directed toward obtaining the earliest precursor and observing subsequent differentiation in vivo or in vitro. For example, pronormoblasts may be obtained from livers of fetal mice by immune lysis2 or from bone marrows of rabbits recovering from actinomycin damage.7 Cells from both these sources will differentiate in vitro, although only minimal proliferation occurs with the latter system.7 The techniques described in the present study, utilizing a controlled degree of immune lysis and subsequent separation by velocity sedimentation, allow simultaneous isolation of erythroid precursors at the different stages of maturation. Hence studies may be performed at one time on cell populations representing nearly all stages of differentiation.

Other investigators have used these methods of cell separation. Borsook and co-workers1 used immune hemolysis and density gradient centrifugation to separate erythroid cells at different stages of maturation from rabbit bone marrow after phenylhydrazine-induced erythroid hyperplasia. In contrast to the present findings, the rabbit erythroid cells were resistant to immune lysis and separated poorly on density gradients.1 More recently, however, Clissold used immune serum raised in guinea pigs followed by density gradient centrifugation and was able to obtain populations of proerythroblasts and basophilic normoblasts from marrow of phenylhydrazine-treated rabbits.8 Velocity sedimentation at unit gravity has been used9,10 to study nucleic acid and protein synthesis in erythroid precursors11 and to separate both spleen colony-forming cells9 and mature cells capable of heme synthesis10 from immature erythropoietin-responsive cells. This method has not previously been used for examination of the morphologic and proliferative aspects of erythropoiesis.

The data reported here are consistent with the interpretation that erythropoietin stimulates cell proliferation at several early steps in erythroid cell maturation. Alternatively, it is conceivable that the higher cell numbers observed in the cultures containing erythropoietin were due to increased viability.
of the cultured cells. After overnight culture, the number of erythroid cells was higher when erythropoietin had been added to the medium in all velocity sedimentation fractions that contained significant numbers of pronormoblasts or basophilic normoblasts, i.e., fractions I–IV. The increase in cell number was at least as great in fractions II–V as in fraction I, despite the decreasing yield of pronormoblasts in these higher fractions, indicating that stimulation of proliferation by erythropoietin took place in the more mature basophilic normoblasts as well as in pronormoblasts. These findings appear to be in contrast to those of Djaldetti et al., who proposed that erythropoietin acts by stimulating the proliferation of only the pronormoblast; however, these authors apparently observed a small effect with basophilic normoblasts as well. No proliferation was observed in fraction V, which contained large numbers of polychromatophilic normoblasts, and it would appear that erythropoietin does not stimulate growth of cells at this level. However, one cannot exclude the possibility that the polychromatophilic cells that sedimented slowly, and hence were relatively small, were qualitatively different from the morphologically similar but larger cells that were present in the more rapidly sedimenting fractions III and IV.

Cell maturation and hemoglobin synthesis, as judged by morphologic criteria, took place in all cell fractions whether or not they were cultured with erythropoietin (Table 2). With the exception of fraction I, the absolute numbers of orthochromatophilic normoblasts and nonnucleated erythrocytes were similar in cultures incubated with or without this hormone. Thus, the most striking difference found in the cultures containing erythropoietin was the larger number of more immature precursors. This difference was particularly marked with the cultures of fraction I, in which clusters of 15–20 basophilic erythroid cells became conspicuous after 18 hr in culture. Although pronormoblasts decreased with culture, the larger number in the erythropoietin-treated cultures provides strong evidence that erythropoietin causes recruitment of pronormoblasts from a still earlier cell, or possibly stimulates self-renewal of the pronormoblast compartment itself. Unexplained is the observation, uniquely found with fraction I, that the number of the more mature cells was consistently lower in cultures containing erythropoietin.

In the absence of erythropoietin, cell number in the various fractions remained unchanged or increased slightly, but always to a smaller extent than in the presence of the hormone. Recruitment of the earlier precursors failed to occur, although erythroid cells were able to differentiate without erythropoietin. Clissold also reported that erythropoietin was not necessarily required for differentiation of rabbit pronormoblasts. These findings differ from those of Cantor et al., who observed a marked deterioration in erythroid cell number when early cells from fetal mouse liver were cultured in the absence of erythropoietin. Similarly, development of erythroid colonies with the plasma clot technique requires the presence of this hormone. The difference between these findings and those reported here may be related to the different systems under study. Alternatively, in the present experiments the behavior of cells placed in culture may have reflected prior stimulation by high levels of erythropoietin in the anemic donor mice. Such stimulation might be sufficient to permit subsequent in vitro differentiation but not proliferation of erythroid cells.
These methods offer a convenient means of studying morphologic and biochemical aspects of erythroid differentiation. The results of the present experiments and of recent studies of hemoglobin synthesis in similar cell fractions, taken in conjunction with other studies of iron uptake and hemoglobin synthesis, reflect effects of erythropoietin on the biochemical differentiation of erythroid cells at different stages of maturation.

REFERENCES

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