

# Isolation and Characterization of the Erythroid Progenitor Cell: CFU-E

WILLEM NIJHOF and PIET K. WIERENGA

*Laboratory of Physiological Chemistry, State University, Bloemensingel 10, 9712 KZ GRONINGEN, The Netherlands*

**ABSTRACT** Erythroid progenitor cells, CFU-E (colony-forming-unit-erythroid), were isolated to practical homogeneity by a combination of three enrichment procedures. CFU-E were generated in large amounts in spleens of mice previously bled and treated with the erythropoiesis-suppressing drug thiamphenicol. The average CFU-E concentration in spleens from mice 4 d after the thiamphenicol-treatment was 10%. These CFU-E were separated from lymphocytes, erythrocytes, and granulocytes and their progenitor cells by centrifugal elutriation and Percoll density gradient centrifugation. A three- to five-fold enrichment was obtained by elutriation, leading to a CFU-E concentration of 45%. With the Percoll gradient another twofold enrichment was achieved, providing us with a 80–100% CFU-E cell population. The overall recovery of CFU-E was 60–70%. This is a cheap, rapid, and highly efficient method of obtaining large quantities of viable CFU-E. The sequential formation of two-, four-, and eight-cell colonies from CFU-E cultured *in vitro* was studied. These cells enable us to study the biochemical changes occurring in the differentiation process of an erythroid progenitor cell induced by the hormone erythropoietin. The morphological and some physical and biological properties of these cells are presented.

The study of the hormone-induced differentiation processes in hemopoietic stem cells is hampered by the fact that these processes *in vivo* cannot be described in simple terms because of the complex cell-composition of hemopoietic tissues. Another major problem is the low incidence of stem cells in their biological environment (1).

One of the goals of many research efforts has been the isolation of stem cells to obtain a well defined, highly enriched cell preparation. Several methods have been used, based on differences in size, density, or specific surface properties of the cells. Large amounts of cells can be processed with the velocity sedimentation method of Miller and Phillips at unit gravity (2). This is, however, a time-consuming process (4 h) and the resolution as well as the enrichment is poor (3). Continuous or discontinuous BSA density gradients have the disadvantage of a low cell load and/or separation artifacts at the interfaces (4, 5). Isolation of cells by means of specific surface markers (cell sorters) is potentially perhaps, the most effective way. However, the equipment is still very expensive and processing time is long (6, 7). Until now, none of these methods were successful in obtaining highly enriched stem-cell preparations. We present

a cheap, rapid, and efficient method for isolating large quantities of the erythroid progenitor-cell CFU-E (colony-forming-unit-erythroid) practically devoid of other cell contaminants. This cell belongs to the erythroid stem-cell compartment. It is the target cell for the hormone erythropoietin (EPO).

## MATERIALS AND METHODS

### *Treatment of Animals*

Male or female RPTV mice (inbred mice strain, wild type X C57 bl, from the Department of Radiopathology, State University, Groningen) weighing 20–25 g and 10–14 wk old were used in these studies. Mice were bled and treated with thiamphenicol (TAP) via a dialysis bag, subcutaneously implanted in the neck as previously described (8). After 4 d the bag was removed and the mice were allowed to recover from the severe suppression of erythropoiesis for a chosen period, varying from 1 to 6 d depending on the type of the experiment.

### *Preparation of the Cell Suspension*

Spleens from two mice killed by cervical dislocation were disrupted by pressing with a spatula through a stainless steel screen (100 mesh) into 4 ml of  $\alpha$ -medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 10 mM HEPES, pH 7.2. This cell suspension was aspirated several

times through 18- and 25-gauge needles to disperse cell clumps. Cell counts were performed on a Coulter Counter-Model ZF (Coulter Electronics Inc., Hialeah, FL).

### Separation of Cells by Centrifugal Elutriation

Cells were loaded into an alcohol-sterilized Beckman elutriator separation chamber in a JE-6 rotor of a Beckman J2-21 centrifuge (Beckman Instruments, Inc., Fullerton, CA) running at 2,000 rpm. Up to  $10^8$  nucleated cells were applied. The counter flow was set at 20 ml/min. The cells were elutriated with  $\alpha$ -medium supplemented with 5% fetal calf serum (Gibco Laboratories). The procedure occurred at 20°C. When the cells entered the separation chamber,  $5 \times 35$ -ml fractions were collected in sterile tubes at 0°C. Then the counter flow was increased to 30 ml/min and another five fractions were collected. During this procedure, cells gradually precipitated on the bottom of the chamber. At last, the counter flow was stopped and the remaining cells precipitated. The fractions recovered at a counter flow of 20 and 30 ml/min were centrifuged in an International Centrifuge PR-6 (International Equipment Co., Damon Corp., Needham Heights, MA) at 1,500 rpm for 10 min at 4°C. The five pellets obtained at both counter flow speeds were combined into 2 ml of  $\alpha$ -medium (fraction I at 20 ml/min, fraction II at 30 ml/min). The precipitated cells in the separation chamber were resuspended into 4 ml of  $\alpha$ -medium (fraction III).

The diameter of the cells was calculated from the equations for the sedimentation of a particle in a gravitational field and Stokes' law. The sedimentation velocity  $V = 0.536 \times 10^{-4} \frac{FR}{(RPM/1,000)^2}$  (9) and  $V = \frac{2}{9} r^2 \frac{(\rho_1 - \rho_2)g}{\eta}$ , where  $FR$  = flow rate in  $\text{ml} \cdot \text{min}^{-1}$ ,  $RPM$  = revolutions per minute,  $r$  = radius of cell in cm,  $\rho$  = density of particle (1) and medium (2) [(1) = 1.070; (2) = 1.0056 g/ml],  $\eta$  = viscosity of medium in poise (0.0105 P), and  $g$  = gravitational acceleration in  $\text{cm} \cdot \text{sec}^{-2}$ .

### Separation of Cells by Percoll Gradient Centrifugation

Cell suspensions (2 ml) obtained after centrifugal elutriation were mixed with 32 ml of Percoll medium (54% Percoll, Pharmacia Fine Chemicals, Uppsala, Sweden, 15% fetal calf serum, 10 mM HEPES pH 7.2 in  $\alpha$ -medium). The density of this medium was 1.072 g/ml. The cell suspension was centrifuged in a Ti 60 rotor of a Beckman L5-65 centrifuge for 30 min at 15,000 rpm. The temperature was 20°C. In a parallel tube, density marker beads (Pharmacia Fine Chemicals) suspended in the same medium were run. The covered range of densities was between 1.018 and 1.141 g/ml. After the run, 2-ml fractions were collected from the top of the gradient and diluted with 2 ml of  $\alpha$ -medium. The cells were centrifuged for 10 min at 1,500 rpm in a Homef centrifuge. Ultimately, the cell pellets were resuspended in 0.5 ml of  $\alpha$ -medium.

### Colony Assays

The committed stem cells BFU-E (burst-forming-unit erythroid), CFU-E (colony-forming-unit-erythroid), and CFU-GM (colony-forming-unit granulocyte, macrophage) were assayed with the method described by Iscove (10). In short:  $2-20 \times 10^4$  nucleated cells were plated on 35-mm petri dishes in 0.8% methylcellulose (Fluka), 30% fetal calf serum (Gibco Laboratories),  $10^{-4}$  M mercaptoethanol (Merck & Co., Inc., Rahway, NJ), 1% bovine serum albumin (Gibco Laboratories), 100  $\mu\text{g}$  streptomycin/ml (Mycopharm, Delft), 100 U penicillin/ml (Gist Brocades, Delft) in  $\alpha$ -medium. For CFU-E culture, 0.5 U EPO/ml was added. (Human urinary erythropoietin CAT-1 (1,140 U/mg) prepared by Dr. E. Goldwasser (University of Chicago) as provided by the Division of Blood Diseases and Resources of the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD). A linear relationship between plated cells and formed colonies was observed during all purification steps. BFU-E and CFU-GM were measured in the same culture. The cultures were supplied with 2 U EPO/ml and 10  $\mu\text{l}/\text{ml}$  of a pokeweed mitogen-stimulated spleen cell conditioned medium containing BPA (burst-promoting-activity, necessary for BFU-E growth) and CSF (colony-stimulating factors, necessary for growth of granulocyte/macrophage colonies) (11). These amounts were optimal under our conditions. The plates were incubated at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere. The colonies were scored after 2 d for CFU-E or 8 d for BFU-E and CFU-GM derived colonies. Discrimination of the latter is possible after benzidine staining (12).

### [<sup>3</sup>H]Thymidine Incorporation into DNA of CFU-E

The cells ( $10^6/\text{ml}$ ) were mixed with the complete methylcellulose culture medium as described above. 0.2 ml of this suspension was plated in duplo in micro titer wells of 0.35 ml (Greiner, Nürtingen). The plates were incubated at

37°C in 5%  $\text{CO}_2$  and at selected times 1  $\mu\text{Ci}$  [<sup>3</sup>H]thymidine (23.8 Ci/mmol) was added. The incubation was continued for 30 min. The cells were harvested by diluting and washing with ice-cold  $\alpha$ -medium. To facilitate precipitation with 5% TCA, 25  $\mu\text{l}$  of 10% BSA was added. After washing with TCA the precipitate was dissolved in 0.5 ml of Lumasolve (Lumac, Schaesberg) and counted for radioactivity.

### Morphology of Cells

**LIGHT MICROSCOPY:** Cells were centrifuged and resuspended in fetal calf serum. A cell smear was made fixed with May-Grünwald and counter-stained with Giemsa solution.

**ELECTRON MICROSCOPY:** Immediately after isolation the cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at room temperature. After washing in phosphate buffer the cells were postfixated in 1%  $\text{OsO}_4$  and 1.5%  $\text{K}_4\text{Fe}(\text{CN})_6$  in 0.1 M phosphate buffer pH 7.4 (13, 14). After dehydration in alcohol the cells were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate. The samples were examined in a Philips EM201 electron microscope.

## RESULTS

### The Generation of CFU-E in Spleens of TAP-treated Mice

Whereas normal bone marrow contains about 0.1–0.3% CFU-E and normal spleens even much less, these numbers can be greatly enhanced as we showed previously (8). Especially, the incidence in the spleen can be high. Fig. 1 shows the relation between the spleen weight, obtained on different days after drug removal, and the concentration of CFU-E. Immediately after the treatment no CFU-E could be detected in the spleen. After 1 d the spleen is rapidly gaining weight concomitant with an increase in the CFU-E concentration. On the basis of their nucleated cell content, spleens between 250 and 400 mg were found to contain ~10% CFU-E. Occasionally, extremely high values above 20% were observed. The average

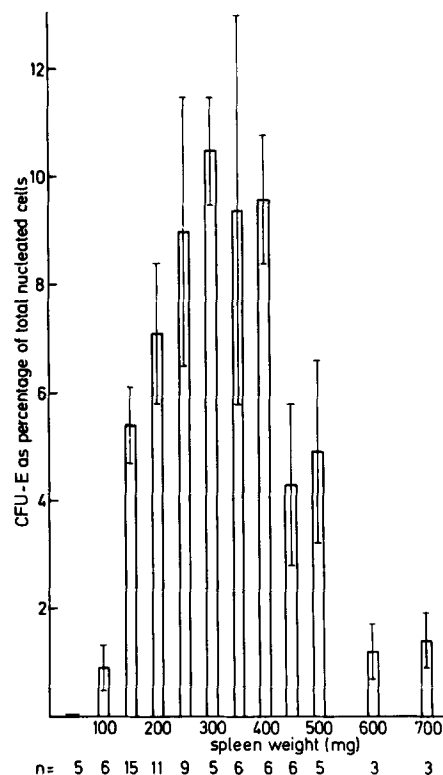


FIGURE 1 The generation of CFU-E in spleens from mice pretreated with thiamphenicol (the numbers in spleens of 0–50, 50–100 mg etc. were averaged; percentages  $\pm$  SEM  $>$  500  $\pm$  SD).

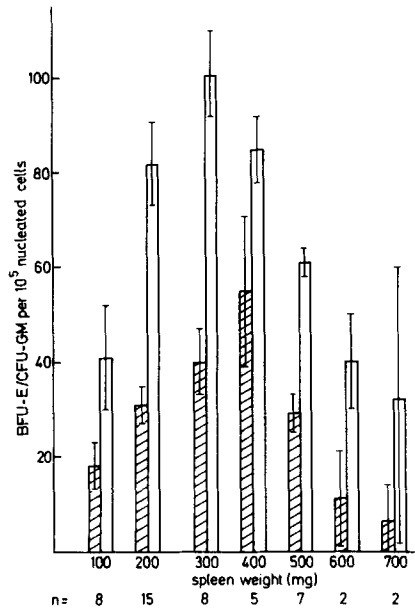


FIGURE 2 Occurrence of BFU-E and CFU-GM in spleens from mice pretreated with thiamphenicol. (The numbers in spleens of 0-100, 100-200 mg etc. were averaged;  $\pm$  SEM;  $>500$  mg  $\pm$  SD).  $\square$  BFU-E;  $\square$  CFU-GM.

TABLE I  
Cellular Composition of Spleens from Mice after a TAP-Treatment

| Spleen weight<br>mg | Lymphoid | Granuloid | Erythroid |
|---------------------|----------|-----------|-----------|
|                     |          | %         |           |
| 25 (n = 2)          | 90       | 10        | 0         |
| 52 (n = 2)          | 84       | 14        | 2         |
| 144 (n = 3)         | 78       | 14        | 8         |
| 283 (n = 3)         | 41       | 10        | 50        |
| 547 (n = 2)         | 6        | 4         | 90        |

of 10% means a 100-fold enrichment in CFU-E compared with the normal bone marrow concentration.

The concentration of other hemopoietic progenitors BFU-E and CFU-GM was maximal in spleens of 200-400 mg (Fig. 2).

The relative amounts of hemopoietic cells from spleens of TAP-pretreated mice were very different (Table I). Small spleens mainly consisted of lymphocytes. Large spleens were mainly late erythroid (polychromatic and orthochromatic erythroblasts). Intermediate spleens contained all types of hemopoietic cells.

### CFU-E Enrichment by Centrifugal Elutriation

In the elutriation procedure cells are separated according to cell mass. As density differences of hemopoietic cells are small, separation occurs mainly by volume differences. The percentage of nucleated spleen cells recovered in the three main fractions was dependent on the spleen weight. Cells from a small spleen were recovered mainly in fraction I and consisted of lymphocytes. Erythrocytes were also concentrated in this fraction. With increasing spleen weights, a shift in the cell recovery to the other fractions occurred. This was due to the increase of erythroid cells in the larger spleens. The cellular composition of the three elutriation fractions from 335 mg spleens is shown in Table II. Fraction I was mainly lymphoid, whereas the majority of the cells in fraction II and III were erythroid. It must be noted that the composition of fraction III

was influenced by cells adhering to the chamber walls. Precipitation occurred when the counter flow was stopped. This is the reason why the percentage of lymphocytes and granulocytes increased again in this fraction.

The numbers of CFU-E in these fractions are shown in Fig. 3. 65% of the recovered CFU-E was present in fraction II, eluted at 30 ml/min.  $\sim 10\%$  was obtained in fraction I and 25% in fraction III. The concentration of CFU-E also was highest in fraction II. Up to 45% CFU-E preparations could be obtained. The average enrichment factor for the CFU-E was three- to fivefold. The majority of the BFU-E and CFU-GM appeared in fraction I with minor amounts in fractions II and III (Table III). During the elutriation, some loss or inactivation of the progenitor cells occurred (Table IV). Whereas the total cell recovery was  $>80\%$ , recovery of the progenitor cells remained between 60 and 70%.

The calculated values of the diameter of the cells in fraction II were between 9 and 11  $\mu$ m.

### CFU-E Enrichment by Percoll Gradient Centrifugation

Cells of elutriation fraction II were subjected to a Percoll gradient centrifugation. During centrifugation a density gradient was formed with slowly increasing densities in the middle of the tube, which permitted a high resolution power in the separation of cells with minor density differences. A cell profile as depicted in Fig. 4 was obtained. A minor cell peak was present at a density of 1.065 g/ml and a major peak at 1.070 g/ml. Occasionally, a shift in the cell numbers to the lower

TABLE II  
Cellular Composition of the Elutriation Fractions from Spleens of  $335 \pm 26$  mg \*

| Elutriation fraction | Lymphoid    | Granuloid   | Erythroid   |
|----------------------|-------------|-------------|-------------|
|                      |             | %           |             |
| I                    | 73 $\pm$ 15 | 14 $\pm$ 9  | 14 $\pm$ 8  |
| II                   | 6 $\pm$ 3   | 6 $\pm$ 0.6 | 88 $\pm$ 3  |
| III                  | 13 $\pm$ 8  | 13 $\pm$ 8  | 74 $\pm$ 14 |

\* n = 3;  $\pm$  SD

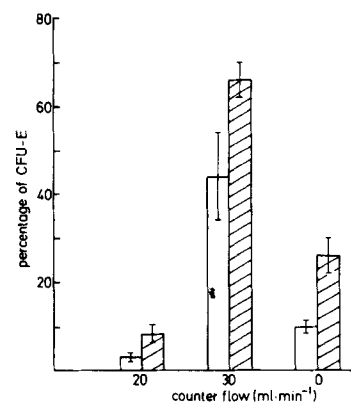


FIGURE 3 Incidence of CFU-E in the fractions collected during elutriation. ( $\pm$  SEM; n = 6); ( $\square$ ) Percentage of total nucleated cells; ( $\square$ ) Percentage of total recovery.

TABLE III  
Percentage of Total Recovered Progenitor Cells in the Elutriation Fractions ( $\pm$  SEM)

|                | I          | II         | III        |
|----------------|------------|------------|------------|
| BFU-E (n = 10) | 82 $\pm$ 9 | 10 $\pm$ 9 | 8 $\pm$ 6  |
| CFU-GM (n = 6) | 77 $\pm$ 3 | 13 $\pm$ 6 | 10 $\pm$ 5 |

density occurred, leading to a 3:1 ratio in the cell numbers instead of the common 1:4. Not only the cell numbers, but also the percentage of CFU-E in both fractions could differ greatly. The main peak was constant between 80 and 100%. The concentration in the minor peak varied between 1 and 70%. The recovery of CFU-E from a Percoll gradient was high, even better than the total nucleated cell recovery and BFU-E and CFU-GM (Table V). The contamination with other progenitors in the pure fraction was negligible. Only 0.03% BFU-E

TABLE IV  
Cell Recovery in the Elutriation Procedure

|                       | % ± SEM |        |
|-----------------------|---------|--------|
| Total nucleated cells | 83 ± 3  | n = 23 |
| CFU-E                 | 61 ± 4  | n = 21 |
| BFU-E                 | 56 ± 7  | n = 10 |
| CFU-GM                | 68 ± 10 | n = 6  |

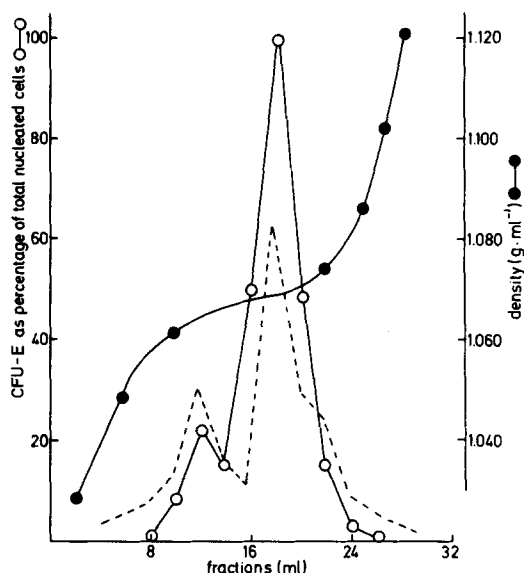


FIGURE 4 Percentage of CFU-E in fractions collected after a Percoll density gradient centrifugation. (---) Total nucleated cells (arbitrary units).

and 0.09% CFU-GM were present. The enrichment of the CFU-E preparation was two- to three-fold by this procedure.

### The Morphology of the CFU-E

On May-Grünwald-Giemsa-stained smears, CFU-E very much resembled early erythroblasts. The diameter of the cells as measured on the photographs was  $16 \pm 0.3 \mu\text{m}$  ( $\pm$  SEM,  $n = 67$ ). The large nucleus with irregular chromatin was surrounded by very basophilic cytoplasm. Fig. 5a shows some cells from a 100% CFU-E preparation with a density of 1.070 g/ml. Fig. 5b shows cells isolated from the gradient at 1.065 g/ml. This fraction contained 66% CFU-E. Cells in both fractions had a similar appearance. Many cells in the light density fraction were in mitosis (not quantitated).

Electron microscopy revealed more structural characteristics (Fig. 6). Many large mitochondria were found together in one area of the cell or closely aligned along the nucleus. Sections through the center of the cell contained  $29 \pm 1.6$  ( $\pm$  SEM,  $n = 33$ ) mitochondria. The cytoplasm was packed with ribosomes. A few membranous structures could be observed, possible remnants of nuclear membranes or endoplasmic reticulum. A Golgi apparatus was present. The nucleus contained a large nucleolus and had only a small proportion of condensed chromatin. The nucleus could have deep indentations. The diameter of the CFU-E as measured on the micrographs was  $8.5 \pm 0.1 \mu\text{m}$  ( $\pm$  SEM,  $n = 127$ ). The cells from the minor peak showed similar features (Fig. 6d). Also electron-microscopically, many cells were caught in mitosis. For comparison we show an erythroblast obtained from spleens of mice recovered after 6 d of a TAP-treatment (Fig. 6d). These spleens (>500 mg) contained only small amounts of CFU-E (1%) and were largely erythroid (90%) (polychromatic and orthochromatic

TABLE V  
Cell Recovery after a Percoll Gradient Centrifugation

|                       | % ± SEM |        |
|-----------------------|---------|--------|
| Total nucleated cells | 77 ± 4  | n = 15 |
| CFU-E                 | 100 ± 9 | n = 13 |
| BFU-E                 | 59 ± 10 | n = 8  |
| CFU-GM                | 89 ± 10 | n = 4  |

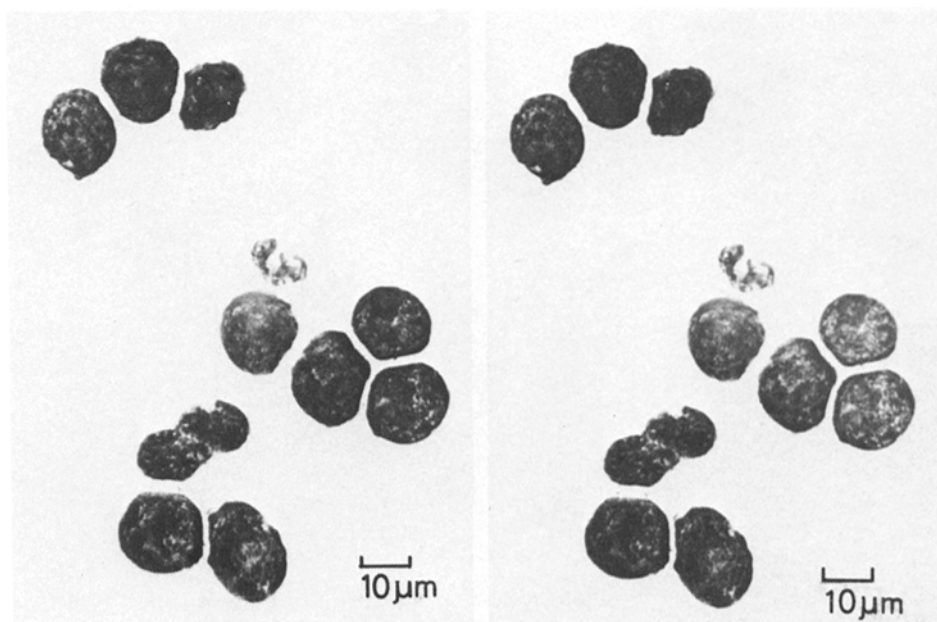


FIGURE 5 (a) May-Grünwald Giemsa-stained smear of cells with a density of 1.070 g/ml. (b) May-Grünwald Giemsa-stained smear of cells with a density of 1.065 g/ml.

erythroblasts). The erythroblast had much less mitochondria ( $11 \pm 0.9$ ,  $n = 27$ ). The nucleus consisted largely of condensed chromatin and the nucleolus was absent or small. On electron micrographs this cell could be easily distinguished from a CFU-E.

#### *Growth Characteristics of the CFU-E*

The CFU-E were very actively dividing cells. Within a few hours after plating with 0.5 U EPO, cell doublings were observed (Fig. 7). Doublet formation was maximal 7 h after plating. Practically no single cells were present anymore. The numbers then decreased and the formation of four-cell clusters

increased. The maximal number was reached 14 h after plating. Simultaneously with the decrease in the four-cell clusters, the number of eight-cell colonies increased to a maximum at 22 h. From these growth curves an average cycle time of 7 h could be derived (peak distances). The counted numbers of two- and four-cell clusters were less than the number of eight-cell colonies because the clusters were not yet settled in one focal plane and some were missed in scoring. After 24 h, sometimes colonies of 32 cells were observed. The cell clusters formed up to 19 h were still benzidine-negative. From 19 to 24 h, the colonies became moderately positive. Only after 48 h did the cells show a bright brown-red appearance after benzidine

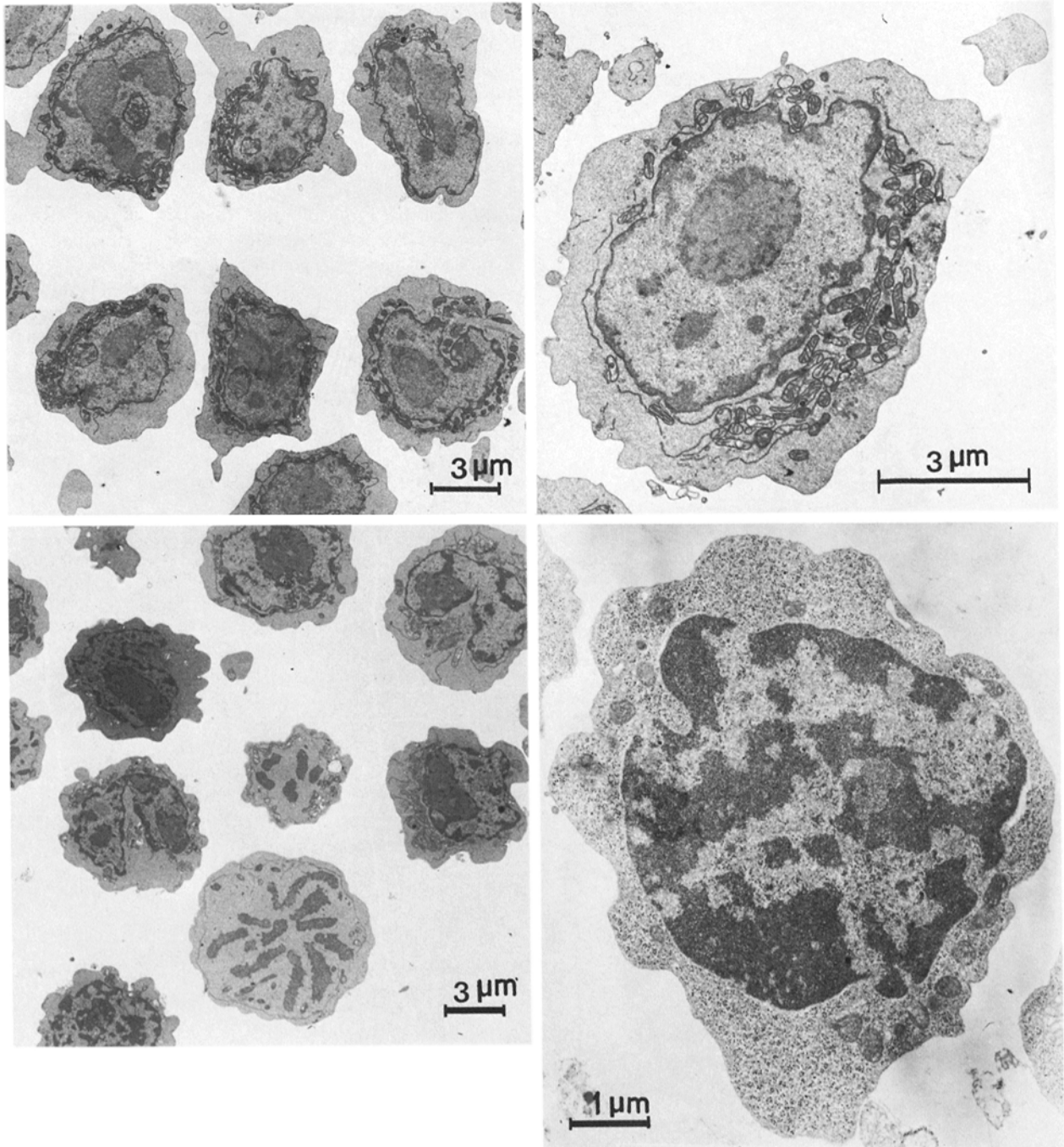


FIGURE 6 Transmission electron micrographs of several CFU-E of 1.070 g/ml (a), close-up of a CFU-E (b), cells with a density of 1.065 g/ml (c), and a late erythroblast (d).

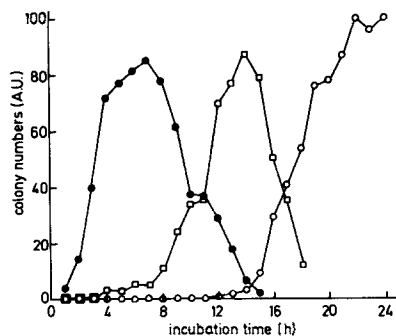


FIGURE 7 Proliferation of CFU-E progeny observed during 24 h with 0.5 U EPO (A.U. = arbitrary units), (●) two cells; (□) four cells; (○) 8-32 cells.

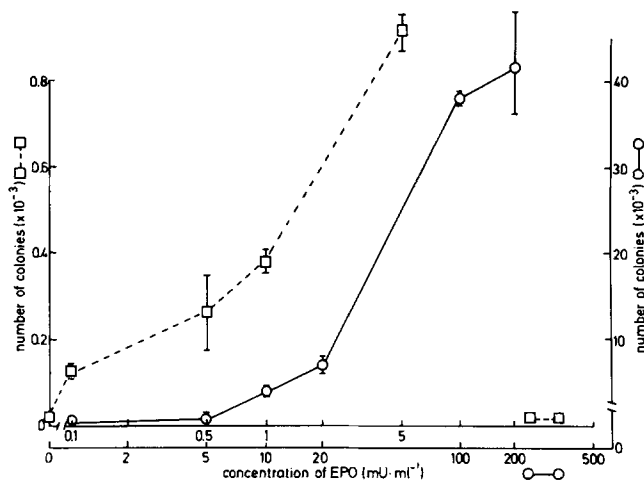


FIGURE 8 Dose-response relationship between EPO and the formation of colonies (□) low range; (○) high range concentration  $\pm$  SD.

staining. Benzidine-detectable hemoglobin accumulation began coincident with eight-cell colony appearance.

The CFU-E were very sensitive to EPO (Fig. 8). An increase in the numbers of colonies could be found after the addition of 0.5 mU EPO. The plateau value was found at 100 mU. When no EPO was added to the cultures, the cells still were able to synthesize DNA and proliferate (Fig. 9). Up to 8 h, no difference was observed with the cultures to which EPO was added. At 14 h the DNA synthesis had decreased by 50%, but was still significant. This coincided with the production of four-cell clusters (qualitatively checked). Only a few eight-cell colonies were found when no EPO was added. Most of them happened to be benzidine-negative and had a degenerating appearance.

## DISCUSSION

To elucidate the mechanism of hormone action and the consequent differentiation of primitive cells, it is essential that the target cells be highly purified. The above experiments show that we succeeded in isolating a target cell for erythropoietin, the erythroid precursor cell: CFU-E. The most important step in the isolation procedure was the pretreatment of the animal. The animal was made very anemic by bleeding together with treatment with the drug thiamphenicol. The high erythropoietin content, a consequence of the treatment (15, 16), caused a rapid differentiation of CFU-E into erythrocytes without further proliferation and differentiation of the CFU-E precursors, CFU-S (colony-forming-unit-spleen, pluripotent stem cell) and

BFU-E (8). Erythropoietic tissues (marrow-spleen) became void of cells that ultimately could disturb the isolation of progenitor cells. When the inhibiting drug was removed the system became highly activated and a wave of erythropoiesis could be observed in the marrow and spleen. Splens from animals 4 d after thiamphenicol-treatment were excellent sources of large numbers of CFU-E. Up to 400 mg, splens still had low amounts of late erythroblasts. Larger splens could have considerable amounts of CFU-E, but contaminating erythroblasts became more and more a problem in further purification steps. The enrichment of CFU-E by this pretreatment compared with the numbers in normal marrow was 100-fold in splens between 250 and 400 mg. We also showed that this method could be used for obtaining large amounts of the hemopoietic progenitors BFU-E and CFU-GM.

The importance of the elutriation procedure was the removal of the erythrocytes, lymphocytes, and the majority of the BFU-E and CFU-GM. A three- to five-fold enrichment in CFU-E concentration was obtained, leading to a 30-50% CFU-E cell suspension. On a Percoll density gradient the progenitor cell concentration could be further enhanced to practical purity (80-100% seeding efficiency). By means of these three simple steps,  $\sim 25 \cdot 10^6$  CFU-E could be obtained from two mouse splens within 2 h. A part of the failure with other techniques may be due to inactivation of the CFU-E by the long isolation procedure (17). The CFU-E resembled morphologically very much the early erythroblasts. On simple May Grünwald-Giemsa-stained smears, they were undistinguishable from basophilic erythroblasts. Electron microscopy, however, showed distinct morphological features.

The high numbers of large mitochondria, the high ratio of condensed chromatin and the large nucleolus in the nucleus were striking in the CFU-E. This cell also was easily distinguishable from the pluripotent stem cell described by Van Bekkum et al. (5). Elutriation data lead to a calculation of a cell diameter between 9 and 11  $\mu\text{m}$  for the CFU-E.

The smaller diameters (8.5  $\mu\text{m}$ ) in EM or larger ones (16  $\mu\text{m}$ ) on smears were probably due to shrinkage or spreading, respectively. We did not try to purify the CFU-E from the remaining elutriation fractions. The properties of these CFU-E may be different from those of the bulk of the CFU-E. It is possible that cells in these fractions represent CFU-E in a different phase of the cell cycle (small cells in fraction 1:G<sub>1</sub>

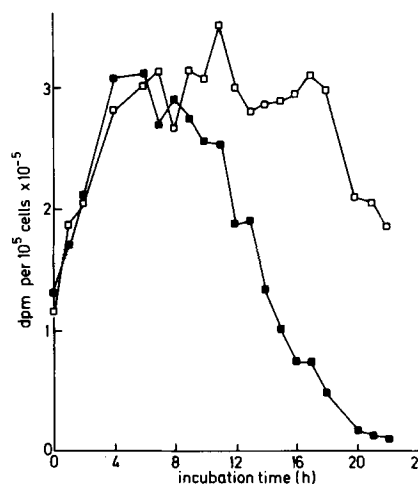


FIGURE 9 Synthesis of DNA in CFU-E cultured in vitro during 24 h. (□) With EPO; (■) Without EPO.

phase; large cells in fraction III:G<sub>2</sub>-M phase). Further research is necessary to investigate this possibility.

The high resolution of the Percoll gradient enables us to separate the CFU-E into two subpopulations with a buoyant density of 1.065 and 1.070 g/ml, the latter being the most constant and with the highest seeding efficiency. The nature of these different cells is currently under research.

The diameters of the active spleen CFU-E are in close agreement with those of Wagemaker et al. (18) who used normal marrow CFU-E separated on a 1 g gradient and a continuous BSA gradient. Depending on the cell cycling state, they determined diameters between 8.3 and 10.5  $\mu$ m. The experiments of Nicola et al. (17) also suggest that a CFU-E is a large basophilic cell, based on stained smears. The buoyant density profile of normal marrow CFU-E showed a single peak at 1.077 g/ml (18). Our results with highly active spleen CFU-E show a bimodal density curve with cells banding at 1.065 and 1.070 g/ml. Our density values are comparable with those of Johnson et al. (19) who used fetal livers as a source of progenitor cells and found CFU-E with a density of 1.062 g/ml and 1.070 g/ml. The variability in the numbers of cells banding at 1.065 and 1.070 g/ml is very intriguing and is currently under investigation. We have indications that cells in these fractions are in a different phase of the cell cycle.

Functionally, the isolated CFU-E were very active cells showing a high generative capacity. Within 24 h, up to 5 cell divisions could occur. The average cycle time of these cells in vitro was 7 h, as was derived from the in vitro growth curves (Fig. 7).

The first two cell divisions in vitro were independent of EPO addition, as was shown by the incorporation of [<sup>3</sup>H]thymidine into DNA, and as checked microscopically. The high in vivo concentration of EPO (16) may have triggered the cells during 7–14 h in vitro.

The progeny of the CFU-E did not synthesize hemoglobin during the first 19 h in culture. The majority of the four-cell clusters then had developed into eight-cell colonies. From then on, the production of hemoglobin started so that cell differentiation had arrived at the polychromatic erythroblast stage. It is tempting to state that the doublet formation represents the differentiation of CFU-E into proerythroblasts. Differentiation of the latter into basophilic erythroblasts is expressed by the formation of the four-cell clusters. After one more cell division, polychromatic erythroblasts are formed (first 5 h during the

formation of the eight-cell-colony peak), which can give another cell division and/or start with the production of hemoglobin. This sequence of events would enable us to study exactly every single differentiation step of the CFU-E up to the orthochromatic erythroblasts stage after 48 h.

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