Separation of the Erythropoietin-responsive Progenitors BFU-E and CFU-E in Mouse Bone Marrow by Unit Gravity Sedimentation

By D. S. Heath, A. A. Axelrad, D. L. McLeod, and M. M. Shreeve

The sedimentation velocity profiles of the entities in mouse bone marrow responsible for erythropoietic burst formation (BFU-E) and for erythrocytic colony formation (CFU-E) have been studied under conditions designed to determine whether the values observed are real or result from cell interactions occurring during culture of the fractions. Bone marrow cells of adult C3Hf/Bi mice were subjected to unit gravity sedimentation in a bovine serum albumin gradient, and fractions were assayed in plasma culture. Because it was found that cell concentration affected the efficiency of erythropoietic burst formation in culture, aliquots were plated at two different cell concentrations, as well as at a fixed proportion of each fraction. The modal sedimentation velocity of the BFU-E population averaged 3.9 mm/hr and that of the CFU-E population, 6.4 mm/hr; both were found to be independent of cell concentration or method of dividing the fractions. Cells from fractions of different sedimentation velocity were mixed with one another or with unfractionated cells. No significant inhibition or stimulation of erythropoietic burst formation was seen. We concluded that the observed values represented the true modal sedimentation velocities of BFU-E and CFU-E in normal mice. To determine whether a change in the physiologic state of the animals affected the sedimentation velocities of BFU-E or CFU-E, marrow cells from mice hypertransfused with red cells were compared with those from controls. The modal sedimentation velocity of BFU-E was unaffected by hypertransfusion, nor was there any change in the number of BFU-E under these conditions. The number of CFU-E was substantially reduced without a significant change in modal sedimentation velocity.

In mammalian hemopoietic cell cultures containing erythropoietin (Epo), colonies develop which are composed of hemoglobin-synthesizing cells that mature into non-nucleated red cells. These, referred to as erythrocytic colonies, are composed of up to 65 benzidine-staining cells. In plasma cultures initiated with a low dose of Epo and seeded with bone marrow cells from adult C3H mice, the colonies regularly reach peak numbers after about 2 days and subsequently disappear. If cultures are initiated with a high dose of Epo and then continually fed with the hormone, crops of new colonies arise after about 6 days. These colonies exist in isolated groups; the groups themselves are relatively few in number and Poisson distributed in the cultures. The colonies within the groups are less discrete than the 2-day erythrocytic colonies, and they are composed of cells that vary over a larger range of...
size and apparent hemoglobin content. We have called such large groups of colonies "erythropoietic bursts."³

The entities responsible for the formation of erythropoietic bursts appeared to behave differently from those that gave rise to erythrocytic colonies. Thus, the number of erythropoietic bursts produced by marrow cells taken from hypertransfused mice was at least as great as that produced by cells from control mice, while the number of erythrocytic colonies produced by marrow cells from hypertransfused donors was substantially lower than normal. Moreover, in fractions of bone marrow cells separated by unit gravity sedimentation, the peak of erythropoietic burst-forming activity was clearly separable from the peak of erythrocytic colony-forming activity. We therefore concluded that the erythropoietic burst is derived from an entity distinct from the CFU-E,¹² which is responsible for erythrocytic colony formation and proposed for this new entity the term "erythropoietic burst-forming unit that responds to Epo" or BFU-E.³

In the present contribution, we provide further data on the physical separation of BFU-E from CFU-E in murine bone marrow cell suspensions. Evidence is presented from fraction-mixing experiments that the observed sedimentation velocity profiles are the true profiles of these progenitors and do not result from spurious effects of stimulator or suppressor cells in bone marrow. We further show that, while hypertransfusion with red cells reduces the number of CFU-E, it has no effect on either the sedimentation velocity or number of BFU-E in murine marrow.

MATERIALS AND METHODS

Mice

Eight- to ten-week-old male C3Hf/BiUt mice (descended from C3Hf/BioCi), bred and raised in the Division of Laboratory Animal Science, University of Toronto, were used in all experiments.

Hypertransfusion

Mice were hypertransfused by the method of Jacobson et al.⁴ Injections of 0.5 ml of washed, packed red blood cells from syngeneic mice were administered on each of days 0, 1, 2, and 6.

Preparation of Cell Suspensions

For all unit gravity sedimentation experiments, a pool of femoral bone marrow cells collected from five to seven mice was pooled, and a single cell suspension was prepared as in McLeod et al.⁵ After centrifugation, the cells were resuspended in supplemented HMEM containing 0.33% bovine serum albumin (BSA) at a concentration of 4 × 10⁶ nucleated cells/ml.

Unit Gravity Sedimentation

The method used was essentially that of Miller and Phillips.⁵ The apparatus consisted of a cylindrical chamber (diameter, 11.5 cm) with a conical base. One per cent and 2% solutions of BSA were prepared in nonsterile supplemented HMEM by dissolving powdered BSA (Sigma, Step V) in the medium, and sterilized by filtration through a 0.45-µ Millipore filter. The chamber was loaded with 20 ml of cell suspension (6 × 10⁶ cells total) and sedimentation was carried out at 4°C for 3.5 hr. The chamber was then drained at a rate of 15 ml/min. The first 100 ml of medium (i.e., volume filling the conical base of the chamber) was discarded. The remaining contents of the chamber were collected in 15-ml fractions, and maintained at 4°C until they were cultured. Samples of each fraction were taken for total and nucleated cell counts (Coulter Electronics of Canada Ltd., Mississauga, Ont.).
Each fraction was centrifuged at 160 g for 10 min just prior to being cultured, and care was taken to remove all excess medium from the resulting cell pellet. In most experiments, cells were resuspended at $5 \times 10^6$ nucleated cells/ml, using the appropriate volume of collection medium, and cultured for CFU-E and for BFU-E, as described below. In some experiments, however, all cell fractions or pools of fractions were resuspended in a constant volume of medium; thus each culture contained a constant portion of a fraction.

**Cultivation of Erythrocytic Colonies**

The method of McLeod et al. was used. Cells were cultured at a concentration of $2.5 \times 10^6$ nucleated cells/0.1 ml culture in medium containing 0.25 U/ml of Epo (Connaught, Step III) for 2 days.

**Cultivation of Erythropoietic Bursts**

Cells were cultured at a concentration of $5 \times 10^5$ nucleated cells/ml in medium of similar composition as used for erythrocytic colonies, except that the concentration of Epo was increased to 1.0 U/ml.

In the initial experiments, the culture medium containing cells was distributed in 0.1-ml aliquots (containing 0.1 U Epo/0.1 ml culture) into the wells of a microtitration tray (yielding 17-18 microcultures). The cultures were placed in large petri dishes containing a smaller dish of water, and were incubated at 37°C in a humidified atmosphere containing 5% CO$_2$ in air for 7-9 days. Cultures were fed by the addition of 0.01 ml of medium NCTC-109 containing 0.1 U Epo to the surface of each clot at 24, 48, 72, and 96 hr.

In the later experiments the culture medium containing cells was distributed in 0.5-ml aliquots into larger wells from a titration tray (Disposo-Trays, Linbro Chemical Co., Inc., New Haven, Conn.). The cultures were incubated as above and fed by the addition of 0.05 ml of medium NCTC-109 containing 0.5 U Epo at 24 and 48 hr. At 72 and 96 hr, the cultures were fed with medium NCTC-109 only (0.05 ml/well).

**Fixation and Staining**

After the appropriate time of incubation, clots were turned out onto slides and fixed with 5% glutaraldehyde in 0.01 M phosphate buffer (pH 7.0-7.2). The slides were stained, after being washed and dried, with benzidine-peroxide reagents and counterstained with Harris' hematoxylin as described in McLeod et al. 2

**Scoring Colonies and Bursts**

The entire plasma clot was scored for colonies or bursts. All groups of eight or more benzidine-positive nucleated cells were counted as erythrocytic colonies. Aggregates appearing as isolated groups of small colonies or as single large colonies composed of 50 or more nucleated cells, at least some of which were benzidine-positive, were scored as erythropoietic bursts. For each experimental point, 10-12 of the 0.1-ml cultures or 4-5 of the 0.5-ml cultures were counted.

**RESULTS**

**Appearance of Erythropoietic Bursts**

Mouse bone marrow cells were plated at $5 \times 10^6$ nucleated cells/ml in plasma culture medium containing Epo at 1 U/ml, and subsequently fed with additional Epo. Initially, large numbers of scattered discrete colonies of 8-40 benzidine-positive cells formed in the cultures, but by 7 days the majority of these colonies had lysed and new groupings of cells appeared. Each consisted of either an isolated group of small colonies or a single, irregularly shaped large mass of cells (Fig. 1) and contained up to 1000 cells or more. We have considered both as representing erythropoietic bursts because morphologically
Fig. 1. Photomicrographs of plasma cultures showing the appearance of erythropoietic bursts. The cultures were harvested at 7 days and fixed and stained with benzidine-peroxide, hematoxylin. (A) Three erythropoietic bursts are visible. The burst on the left is composed of groups of small colonies; the one in the upper right is more compact. (B) A single erythropoietic burst at higher magnification. Benzidine-positive cells were in the minority in this burst.
intermediate forms were not uncommon in the cultures and because we could not distinguish between the unit gravity sedimentation profiles of the entities in bone marrow that gave rise to the single large cell masses and those that produced the large groups of small colonies. Bursts contained some cells with no demonstrable hemoglobin, and often a majority of the cells in the bursts were of this apparently less mature type. No bursts formed if cultures were set up without Epo; very few bursts developed if cultures were not fed with additional Epo.
Table 1. Summary of Modal Sedimentation Velocities of CFU-E and BFU-E in Various Experiments

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Experimental Conditions</th>
<th>Modal Sedimentation Velocities (mm/hr)</th>
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<td>RBC</td>
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<tr>
<td>39</td>
<td>Standard</td>
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<tr>
<td>40</td>
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<td>41</td>
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<tr>
<td>45</td>
<td>Standard</td>
<td>2.0</td>
</tr>
<tr>
<td>49</td>
<td>Mice hypertransfused</td>
<td>2.2</td>
</tr>
<tr>
<td>59</td>
<td>Standard</td>
<td>2.2</td>
</tr>
<tr>
<td>75</td>
<td>Standard</td>
<td>2.1</td>
</tr>
<tr>
<td>78</td>
<td>Standard</td>
<td>2.1</td>
</tr>
<tr>
<td>79</td>
<td>Lower than standard cell concentration used for erythropoietic burst culture</td>
<td>2.1</td>
</tr>
<tr>
<td>80</td>
<td>Constant portion of each fraction cultured, variable cell concentration</td>
<td>2.2</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>1.8–2.2</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>2.06</td>
</tr>
</tbody>
</table>

Effect of Variation in Number of Cells Plated on Number of Erythropoietic Bursts

The relation between the number of cells plated and the number of bursts that subsequently developed was nonlinear. Few or no bursts formed at cell concentrations less than $10^5$ cells/0.5 ml culture, and a fall-off in burst-forming efficiency occurred when cells were plated at concentrations above $5 \times 10^5$ cells/0.5 ml culture. A cell concentration of $2.5 \times 10^5$ nucleated cells/0.5 ml culture was found to produce the most consistently high efficiencies of burst formation, and therefore this concentration was used to assay marrow cell fractions for their ability to form bursts.

Unit Gravity Sedimentation of Bone Marrow Cell Suspensions

Bone marrow cells were subjected to unit gravity sedimentation, and cells in each of the fractions were assayed for their ability to produce erythrocytic colonies at 2 days and erythropoietic bursts at 7–9 days. The results are shown in Fig. 2 and Table 1. The red blood cell peak was sharply defined; its modal sedimentation velocity ranged between 1.8 and 2.2 mm/hr in different experiments.

The majority of erythrocytic colonies developed in cultures of cells with high sedimentation velocities; the sedimentation profile for erythrocytic colony formation had a broad peak, with a modal sedimentation velocity that ranged between 5.7 and 7.0 mm/hr in different experiments. In contrast, the majority of erythropoietic bursts developed in cultures of more slowly sedimenting cells; the sedimentation profile of these cells had a sharp peak, with a modal sedimentation velocity that ranged between 3.7 and 4.1 mm/hr in different experiments and a small shoulder that sloped downward toward higher sedimentation ve-
BFU-E AND CFU-E

Table 2. Effect of Plating Sedimentation Velocity Fractions at Different Cell Concentrations: Experiment No. 78

<table>
<thead>
<tr>
<th>No. of Cells Plated</th>
<th>No. of Erythropoietic Bursts/0.5 ml Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fractions 1–8</td>
</tr>
<tr>
<td>$2.5 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>$1.25 \times 10^5$</td>
<td>$12.4 \pm 2.1$</td>
</tr>
</tbody>
</table>

...This shoulder in the BFU-E profile corresponded with a small peak in the CFU-E profile.

Effect of Plating Sedimentation Velocity Fractions at Different Cell Concentrations

It was a consistent finding that no or very few bursts could be cultured from the cells of the high sedimentation velocity fractions of the gradient. A pool of the rapidly sedimenting fractions 1–8 (previously shown to have little or no burst-forming activity) and the slowly sedimenting fraction 13 (with the majority of the burst-forming activity) were separately plated at the routine cell concentration of $2.5 \times 10^5$ cells/0.5 ml culture, and at half that cell concentration. The numbers of erythropoietic bursts produced by each were then determined. From the results in Table 2, it can be seen that the pool of rapidly sedimenting fractions 1–8 gave rise to no bursts when plated at the cell concentration of $2.5 \times 10^5$ cells/0.5 ml culture. However, when the cell suspension was diluted to $1.25 \times 10^5$ cells/0.5 ml culture, bursts developed. Some inhibitory influence thus seemed to exist in the cell suspension that contained the rapidly sedimenting cells.

The cells of fractions 1–8, although plated at the same concentration as those in fraction 13, proliferated in the cultures to reach a higher cell density. Most of these cells, which formed sheets or colonies, did not stain with benzidine. Concomitantly with the growth of these cells, there was a noticeable drop in the pH of the cultures (phenol red indicator). This did not occur when the cells of fractions 1–8 were plated at the lower cell concentration or in fraction 13 at either cell concentration. In the slowly sedimenting fraction 13, reduction in the cell concentration did not result in an increase in the number of erythropoietic bursts that were produced. The question of whether the inhibitory effect of high cell concentrations is due to a lowering of the pH in the cultures of the high sedimentation velocity fractions or to other factors is currently under investigation in this laboratory.

We next determined the sedimentation velocities of cells capable of forming erythropoietic bursts under two sets of assay conditions. In one of these (Exp. No. 79), all fractions were plated at a fixed low cell concentration ($1.25 \times 10^5$ cells/0.5 ml culture). In the other (Exp. No. 80), each fraction or pool of fractions was resuspended for culture in a constant volume of medium; cell concentrations thus varied from culture to culture. In those fractions that contained cells of high sedimentation velocity, the total cell counts were low, and so only low cell concentrations were plated. The results are shown in Table 1. The modal sedimentation velocities for burst-forming activity were similar in the two experiments, and they were also in the same range as those found in...
earlier experiments. Thus, the modal sedimentation velocities obtained for the entities responsible for erythropoietic burst formation remained the same, even when the fractions were assayed at low cell concentration where the inhibitory effect was eliminated. The sedimentation profiles obtained at low cell concentrations differed slightly from those in which the fractions were assayed at higher cell concentrations, in that at low cell concentrations some burst-forming activity could be detected in the higher sedimentation velocity fractions.

**Effect of Mixing Fractions Obtained by Sedimentation of Marrow Cells at Unit Gravity**

Two experiments (Nos. 83 and 84) were performed in which bone marrow cells were fractionated by unit gravity sedimentation in the usual way, and three different cell suspensions were prepared: (A) a pool of rapidly sedimenting cells from fractions 1-8, which were expected to possess little or no burst-forming activity; (B) a pool of more slowly sedimenting cells from fractions 13 and 14, expected to include the peak erythropoietic burst-forming activity; (C) unfractionated cells from the same original bone marrow cell suspension kept at 4°C during the sedimentation procedure. In Exp. 83, cells of suspension A were plated at 3.1-12.5 \times 10^6 \text{ cells/0.5 ml culture}, and cells of suspensions B and C were plated at 6.25-25.0 \times 10^6 \text{ cells/0.5 ml culture}. The cultures were fed and scored for erythropoietic bursts at day 8. As control, a sample of the same bone marrow cells was left in a tube to sediment at 4°C in the same medium and was then mixed and assayed at the same time as the sedimentation fractions (up to 10 hr).

The data obtained in this experiment provided a measure of the surviving number of CFU-E in marrow which had been loaded into the separation chamber: \(2.4 \times 10^6 \text{ CFU-E in } 3.5 \times 10^6 \text{ nucleated cells}\). In the pooled sedimentation fractions that included the peak CFU-E concentration, there were \(7.2 \times 10^5 \text{ CFU-E in } 2.2 \times 10^6 \text{ nucleated cells}\). Thus a CFU-E enrichment of approximately 4.7-fold was realized by unit gravity sedimentation. The total number of CFU-E recovered, calculated by adding together the numbers from all the fractions, was \(1.4 \times 10^6 \text{ CFU-E in } 3.2 \times 10^7 \text{ nucleated cells}\); this represents a CFU-E recovery of 57\%. In a separate experiment, it was shown that the loss of CFU-E from the original marrow at 4°C without sedimentation was in the order of 50\%.

The total number of BFU-E loaded into the separation chamber was \(5.2 \times 10^5 \text{ in } 3.5 \times 10^6 \text{ nucleated cells}\). In the BFU-E peak fraction, there were \(1.3 \times 10^5 \text{ BFU-E in } 2.9 \times 10^6 \text{ nucleated cells}\). The total number of BFU-E recovered, calculated by adding together the numbers from all the fractions, was \(4.2 \times 10^5 \text{ in } 3.2 \times 10^7 \text{ nucleated cells}\). This would represent a threefold enrichment and an 81\% recovery of BFU-E, except for the fact that the number of erythropoietic bursts was not proportional to the number of cells plated; thus, no reliable figure for either enrichment or recovery of BFU-E could be obtained from these data.

The results in Fig. 3 show that the efficiency of erythropoietic burst formation was low at low cell concentrations in all three suspensions, and increased
Fig. 3. Titrations of fractionated and unfractionated bone marrow cell suspensions for their ability to form erythropoietic bursts. Suspension A—a pool of fractions 1–8, expected to contain little erythropoietic burst-forming activity and including most CFU-E responsible for erythrocytic colony formation. Suspension B—a pool of those fractions expected to contain the major part of the erythropoietic burst-forming activity. Suspension C—unfractionated cell suspension.

with increasing concentration of the slowly sedimenting cells (B) and of the unfractionated cells (C). An inhibitory effect of higher cell concentration was again seen in the cultures of rapidly sedimenting cells (A). Suspension B, which contained the majority of erythropoietic burst-forming activity, yielded a titration curve similar in shape to that of the whole unfractionated cell suspension C. This suggested that no inhibitory cells separable by unit gravity sedimentation were affecting the expression of erythropoietic burst-forming capacity in the unfractionated suspension.

Cells from each of the suspensions were plated either alone or mixed together in the cultures, and the effect on the number of erythropoietic bursts was determined. Results are shown in Table 3, where the observed numbers of erythropoietic bursts produced by the mixtures are compared with the numbers expected if the cells in each pool had acted independently and additively. Note that in all instances the mixtures gave higher numbers of bursts than expected if the numbers produced by each suspension alone were additive. In one experiment, heavily irradiated bone marrow cells (5000 rads $^{137}$Cs $\gamma$-rays) from the unfractionated suspension C were added to cultures of either suspension A or suspension B. A mild augmentation of burst formation was again observed. However, when cells from suspension B alone and from suspension C alone were cultured at twice the usual concentration (i.e., $5 \times 10^5$/ml), the number of bursts which subsequently formed also more than doubled, as was already noted in the titrations depicted in Fig. 3. It was thus evident that, while the efficiencies of erythropoietic burst formation were cell concentration-
Table 3. Effect of Mixing Cells of Different Sedimentation Velocities on Erythropoietic Burst Formation

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Rapidly Sedimenting (1-8)</th>
<th>Slowly Sedimenting (13, 14)</th>
<th>Unfractionated</th>
<th>Mixture</th>
<th>No. of Cells Plated per Culture</th>
<th>No. of Erythropoietic Bursts per Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>83</td>
<td>$6.2 \times 10^6$</td>
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<td>$2.5 \times 10^5$</td>
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<td>$6.2 \times 10^4$</td>
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<td>$6.2 \times 10^4$</td>
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<td>$6.2 \times 10^4$</td>
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<tr>
<td>84</td>
<td>$6.2 \times 10^4$</td>
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<td>$6.2 \times 10^4$</td>
<td>$1.25 \times 10^5$</td>
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dependent, the effects of cell concentration were nonspecific in the sense that cells at increased concentration facilitated erythropoietic burst formation, irrespective of their source.

Effect of Hypertransfusion

We have previously shown that hypertransfusion of mice with red blood cells reduces the number of CFU-E but leaves the number of BFU-E of marrow unaltered. To determine whether hypertransfusion affected the sedimentation velocity of the BFU-E of marrow, bone marrow cells from hypertransfused mice were sedimented at unit gravity, and fractions were assayed for their content of BFU-E and CFU-E. The control was a pool of marrow from normal mice treated in the same way. As seen in Fig. 4, the modal sedimentation velocities of CFU-E and BFU-E from the marrow of hypertransfused mice were similar to those of CFU-E and BFU-E from normal marrow. The total number of CFU-E was substantially reduced as compared to normal. The profile for BFU-E from hypertransfused mice was superimposable upon the BFU-E profile from normal mice. Although this comparison was made between two independent separations done on different days, the absolute numbers of BFU-E per fraction were found to be similar. Thus neither the sedimentation velocity nor the number of marrow BFU-E was affected by hypertransfusion.

DISCUSSION

The erythrocytic colony-forming activity of C3H/Bi mouse bone marrow sedimented with a modal velocity ranging from 5.7 to 7.0 mm/hr, while the
Fig. 4. A comparison of the sedimentation profiles of CFU-E and BFU-E from normal (A) and hypertransfused (B) mice. Solid line: colonies per fraction assayed at 2.5 x 10⁶ cells/0.1 ml culture. Broken line: erythropoietic bursts formed per fraction assayed at 5.0 x 10⁶ cells/0.1 ml.

The modal sedimentation velocity of erythropoietic burst-forming activity lay between 3.7 and 4.1 mm/hr. This difference suggested that two distinctly different entities, separable on the basis of size, were responsible for the two activities. However, before this conclusion could be drawn with confidence, a number of other explanations for the observed profiles had to be considered.

Worton et al.⁶ have pointed out that whenever a population of cells is fractionated, the spectrum of cells in each fraction may become different, and this could affect the efficiency of assays based on colony formation. Cells that stimulate or inhibit colony formation might be concentrated in particular fractions, and thus interfere with determination of the true profiles of those entities that were actually giving rise to the colonies. This factor could be especially relevant to the assay of erythropoietic bursts from fractionated populations of bone marrow cells because the number of bursts was not directly proportional to the number of cells plated. An inhibitory effect on burst formation was, in fact, demonstrated in our cultures from fractions containing cells of higher sedimentation velocity. When the concentration of these cells was reduced in the cul-
tures, however, the inhibitory effect disappeared. Under these conditions, the modal sedimentation velocity for burst-forming activity was found to be the same as in earlier experiments in which this precaution had not been taken.3

Similarly, if a population of stimulatory cells were concentrated in certain fractions, the sedimentation profile for burst-forming activity could actually represent the profile of the stimulator cell. When the fractions containing cells with low modal sedimentation velocity (about 3.9 mm/hr) were mixed in culture with unfractionated cells or with cells of high sedimentation velocity, a stimulatory effect was, in fact, noted. However, a similar stimulatory effect was also observed when low concentrations of cells of high sedimentation velocity were mixed with unfractionated cells. Therefore, it could not be concluded that a stimulatory cell population was concentrated in the fractions that supported maximum burst formation. It would appear that the stimulation resulted from a nonspecific, perhaps protective, effect of the presence of greater numbers of cells in the cultures. This view was supported by the observation that efficiency of burst formation was greater at high than at low cell concentrations in titrations of both the fractionated and unfractionated cell populations, and that the addition of heavily irradiated cells had a mild stimulatory effect on burst formation. These findings strongly indicated that the observed sedimentation profile for erythropoietic burst-forming activity represented the true distribution of a burst-forming entity in the gradient and was not merely an artifact of the experimental system.

The term “erythropoietic burst-forming unit that responds to Epo,” or “BFU-E,” is proposed for this entity.3 The term could be taken to mean that the BFU-E is the cell (or cells) that, when plated in culture, replicates and differentiates to create an erythropoietic burst. However, another possibility would be that the formation of a burst might result from an interaction between BFU-E and CFU-E which induces a sustained self-replication of the CFU-E. Three facts argue against this. First, in most experiments the number of bursts cultured from the BFU-E peak fraction exceeded the number of colonies that could be cultured from the same fraction. Second, if the BFU-E stimulated the CFU-E, one would expect that cells from the BFU-E peak fraction would have the greatest effect in stimulating burst formation when added to unfractionated cells because the unfractionated cell suspension contained higher numbers of CFU-E than did cells from the BFU-E peak fraction. However, no specific stimulatory action on burst formation by cells from the BFU-E peak fractions could be demonstrated. There appeared to be as much “stimulation” by the more rapidly sedimenting cells when they were mixed with unfractionated cells. Finally, it should be noted that if the BFU-E were merely a stimulator of CFU-E one would expect the efficiency of burst formation to decrease in cultures of bone marrow cells from hypertransfused animals, because, as discussed below, fewer CFU-E would be available for stimulation. The results of the present and previous3 experiments have shown that this did not occur. We conclude that the BFU-E is itself the entity which gives rise to the mass of progeny cells that we recognize in culture as an erythropoietic burst.

Recent work in other laboratories has confirmed and extended our observations. Iscove and Sieber,7 using methylcellulose cultures, have shown that with a single initial dose of Epo at high concentrations (3 U/ml), burst-like colonies
BFU-E and CFU-E developed in relatively large numbers and reached macroscopic dimensions (up to \(10^6\) cells) at 10 days of culture. With the same culture system, Wagemaker et al.\(^8\) have found distinct differences between the kinetics of the response of CFU-E and of BFU-E in mice exposed to intermittent hypoxia.

The increase in efficiency of erythropoietic burst formation with increasing marrow cell number seen in our experiments, as well as in those of Iscove and Sieber, raises the question of whether or not interaction between two cells is required for the expression of burst-forming capacity. However, recent work in our laboratory\(^9\) indicates that with improved culture conditions, burst numbers become proportional to marrow cell numbers, making such a hypothesis unnecessary. This finding, of course, does not imply that erythropoietic burst formation is necessarily clonal; more direct experiments will be required to determine whether or not that is the case.

A small peak was repeatedly seen in the sedimentation profile of CFU-E which corresponded to a shoulder or small peak in the BFU-E profile at around 5 mm/hr (Figs. 2 and 4). This finding raises the possibility that there exists in mouse marrow a minority cell population capable of carrying out either of two functions—erythrocytic colony formation at 2 days or erythropoietic burst formation at 8 days, depending on the concentration of Epo in the medium. Alternatively, a minority component of the CFU-E population could have a sedimentation velocity similar to that of a minority component of the BFU-E population, without the two being identical to one another. Further work will be necessary to distinguish between these possibilities.

It has been repeatedly suggested in the past that Epo has more than one target for its action in the mammalian hemopoietic system. McCool et al.\(^{10}\) used sedimentation at unit gravity to separate two cell populations (fractions I and II) from adult rat marrow. Cells of fraction I, with sedimentation velocity about 6.6 mm/hr, were strongly stimulated by Epo to produce heme-synthesizing progeny. From their sensitivity to vinblastine, these cells were considered to be actively proliferating. Cells of fraction II, with sedimentation velocities of 1.3-3.9 mm/hr, were believed to be nonproliferating; their hemoglobin synthesis was at most only slightly stimulated by Epo. The latter must thus have represented rather mature erythroid cells. These features suggested that cells of fraction I in the rat may be comparable with CFU-E. However, no cells comparable with BFU-E were detected in that study.

With a bovine serum albumin density gradient, Clissold\(^11\) recently separated two classes of Epo-responsive cells in anemic rabbit marrow depleted of hemoglobin-containing cells by immune lysis. Cells of the two classes differed with respect to their size and the time course of their response to Epo in vitro, which was measured in terms of \(^{59}\)Fe incorporation into heme. However, only one of the two classes was detected in similarly treated normal rabbit marrow, making it difficult to relate her results to those in the present work.

Stohlman et al.,\(^12\) on the basis of experiments in vivo, proposed a model of erythropoiesis in which Epo acts not only on morphologically unrecognizable committed erythroid cells (previously shown to differentiate into morphologically recognizable proerythroblasts under its influence\(^13\)), but also on the differentiated erythroid cells from proerythroblasts to early polychromatophilic erythroblasts (to increase their rate of hemoglobin synthesis). A similar model
has been used to account for differences in drug sensitivity observed between
the responses to Epo measured at 24 ("early") and at 48 ("late") hr after ad-
ministration of the hormone, and to explain the greater intensity of response
to Epo when administered in fractionated doses, as opposed to a single dose.

However, the notion that there may be more than one class of cells within the
compartment of Epo-responsive, committed, but morphologically unrecogniz-
able erythroid elements of mammalian marrow seems to have originated with
Reissmann and his co-workers. They showed that if a single dose of busul-
fan was given to hypertransfused mice erythropoiesis was totally inhibited for
several days but afterward returned. Regeneration of the capacity for respons-
siveness to Epo was itself found to be Epo responsive. Relatively large doses of
Epo were required for this effect, and the magnitude of the recovery was related
to the dose of Epo administered. It was concluded from these experiments that
Epo had two actions within the (morphologically unrecognizable) Epo-respon-
se cell compartment: stimulation of proliferation of committed erythroid stem
cells and stimulation of transformation of these stem cells into proerythro-
blasts. An age distribution of the committed erythroid stem cells was postulated
to account for the two responses. Thus the same agent, Epo, which through dif-
ferratiation increased the outflow of cells from the Epo-responsive cell com-
partment, also appeared to provide the signal for increased proliferation of
their progenitors within this compartment.

In all these instances, the properties of Epo-responsive cells had to be in-
ferred from studies on the behavior of the differentiated descendants of these
cells, and not on the Epo-responsive cells themselves. Furthermore, they de-
pended on measurements of radioiron incorporation into newly formed red
cells, an assay method sensitive to changes at the population level, rather than
at the cellular level.

The present data, obtained by direct assay at the cellular level in vitro, pro-
vide further evidence in favor of the concept of two different Epo-responsive
cell populations within the compartment of committed but morphologically unrecognizable erythroid cells of mammalian bone marrow. The data show that
these entities have distinctive physical characteristics, the BFU-E having a
lower modal sedimentation velocity (and therefore smaller diameter) than the
CFU-E. They also have distinctive physiologic characteristics, the BFU-E re-
quiring a higher concentration of Epo for erythropoietic burst formation than
the CFU-E for erythrocytic colony formation. The BFU-E also has several
properties that are compatible with its being the progenitor of the CFU-E.
(1) BFU-E has the capacity to produce more numerous progeny over a longer
period of time in culture than CFU-E. (2) Each erythropoietic burst is com-
posed of groups of colonies which morphologically resemble erythrocytic col-
onies that arise from CFU-E. This situation is what would be expected if each
BFU-E produced a number of CFU-E. (3) Although precise estimates of fre-
cuency await the development of a more reliable quantitative assay method for
BFU-E, there appear to be many more CFU-E than BFU-E in the normal mar-
row and spleen of the mouse. (4) Hypertransfusion markedly reduces the num-
ber of CFU-E, in agreement with previous findings, but has no effect on either
the number or modal sedimentation velocity of BFU-E. Finally, we have shown
that, when marrow cells from hypertransfused mice are grown under conditions of high Epo concentration which would favor the production of erythropoietic bursts, new CFU-E arise in the cultures; these could be demonstrated by the capacity of the trypsinized cultures to produce erythrocytic colonies at low Epo concentration in secondary culture. Thus BFU-E could remain available in the absence of Epo to give rise to CFU-E when later exposed to Epo, either in vivo or in vitro.

In conclusion, on the basis of the available data, we believe that what has in the past been termed the (morphologically unrecognizable) “erythropoietin-responsive cell” compartment of mammalian hemopoietic tissue may be considered to be composed of at least two Epo-responsive subcompartments, each of which can now be assayed separately in vitro. In this model, the CFU-E would correspond to the immediate precursor of proerythroblasts, as has been suggested by Gregory et al. The BFU-E would represent its Epo-responsive progenitor, the committed erythroid stem cell. While the evidence in favor of this hypothesis is strong, direct experiments will be necessary to establish if the BFU-E and CFU-E stand in progenitor-progeny relationship to one another, i.e., whether the BFU-E spontaneously or under the influence of Epo gives rise to a clone of CFU-E, and if the BFU-E is capable of self-renewal.

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REFERENCES


Separation of the erythropoietin-responsive progenitors BFU-E and CFU-E in mouse bone marrow by unit gravity sedimentation

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