Three Stages of Erythropoietic Progenitor Cell Differentiation Distinguished by a Number of Physical and Biologic Properties

By Connie J. Gregory and Allen C. Eaves

Previous studies have shown that erythroid precursors at sequential stages of differentiation along the red cell pathway can be distinguished by differences in the size and maturation kinetics of the colonies to which they give rise in vitro. Using criteria based on these two parameters, it is thus possible to identify three distinct erythroid progenitor cell populations in the mouse, known as day 8 BFU-E, day 3 BFU-E, and CFU-E. These cell types have now been shown to differ in a number of other respects, including progenitor cell size, sensitivity to cycle-active agents, response to plethora, and effects of the W/W' genotype. In addition, a comparison of the differences found between day 8 BFU-E and day 3 BFU-E on one hand and those distinguishing day 3 BFU-E and CFU-E on the other provides support for the view that early erythropoietic cell differentiation involves a series of changes that take place long before competence to synthesize hemoglobin becomes manifest.

CURRENT CONCEPTS of the early stages of hemopoietic cell differentiation in man are largely based on experiments performed in mice using clonal assays that detect primitive progenitor cell types. Many of the more recent in vitro colony assay procedures have been successfully adapted for use with human hemopoietic cells, so that the progenitors of each of the various colony types obtained can now be studied directly in man. However, definition of the interrelationships between different types of colony-forming cells in vitro of human origin continues to rely on preliminary findings in mice, where controlled manipulations in vivo as well as in vitro are possible. The present experiments were undertaken as part of such a study to characterize different stages of erythropoietic cell differentiation in the mouse, with a view to future investigations of the role of equivalent cell types in human disease.

When semisolid cultures containing relatively high concentrations of erythropoietin (Ep) and either mouse or human marrow cells are examined at various times after plating, a spectrum of different-sized colonies containing variable numbers of erythroblast clusters becomes apparent during the first 3 wk. These different-sized colonies are detected not simultaneously but sequentially, starting with single, isolated erythroblast clusters, the smallest type of erythroid colony usually recognized. In larger erythropoietic colonies it appears that erythroblast cluster formation represents a terminal phase of erythropoietic colony growth and therefore occurs later, depending on the proliferative ca-
pacity of the original progenitor cell. Moreover, the mature red cells ultimately produced do not survive well in the culture medium, so that very few of the earliest colonies formed contribute to the erythroid colony counts obtained at later times.

We recently showed by analysis of spleen colony contents that in the mouse the different-sized erythroid colonies seen after increasing incubation periods are derived from progenitor cell types at sequential stages of differentiation along the erythropoietic pathway. The three classes of erythropoietic progenitors defined have been termed CFU-E (colony-forming units-erythroid, progenitors of single or paired clusters seen after 2 days), day 3 BFU-E (burst-forming units-erythroid, progenitors of early bursts containing four or more clusters and seen after 3–4 days), and day 8 BFU-E (progenitors of late bursts containing four or more clusters and seen after 8–10 days). These three classes of erythropoietic progenitors have also been found to exhibit progressive changes in the concentration of Ep required in vitro for their detection. Thus maximum numbers of CFU-E derived colonies are obtained with as little as 0.03 U Ep/ml culture medium, whereas early bursts derived from the next most primitive progenitors detectable in vitro (day 3 BFU-E) require 50–100 times more Ep for maximum expression under otherwise identical conditions.

In the present studies a number of additional properties and responses of these various erythropoietic precursor populations in the mouse were investigated. We found that day 8 BFU-E, day 3 BFU-E, and CFU-E in normal marrow could be physically separated from one another and that each of these populations were affected to different extents in the adult anemic W/W<sup>+</sup> mouse, although in contrast no effect on day 8 BFU-E, day 3 BFU-E, or CFU-E was demonstrable in the normal adult f/f mouse. Following red blood cell transfusion, which causes a significant reduction in CFU-E numbers, the number of day 3 BFU-E remained unchanged, as found previously for day 8 BFU-E. On the other hand, day 3 BFU-E more closely resembled CFU-E than day 8 BFU-E in their cycling characteristics.

**MATERIALS AND METHODS**

*Mice*

Except where specified otherwise, the mouse strain used in these experiments was an F<sub>1</sub> hybrid of C57BL/6B × C3H/HeB (abbreviated as B6C3F<sub>1</sub>) obtained from the barrier stock of Biobreeding Laboratories, Ottawa. All mutant mice and their +/+ controls were purchased from the Jackson Laboratory, Bar Harbor, Maine. Mice of genotype W/W<sup>+</sup> and their congenic +/+ littermates were derived from crosses between WB/RE-W/+ and C57BL/6J-W/+ parents. Mutant FL/1Re-/+ L<sup>o</sup>/L<sup>o</sup> mice and their nearly congenic controls, FL/4Re-/+ L<sup>o</sup>/L<sup>o</sup>, were also used in some studies. In all experiments with B6C3F<sub>1</sub> hybrids and for most experiments with mutants 2–4-mo-old mice of either sex were used, although no consistent differences were seen in mice up to 7 mo of age.

*Assays*

A detailed description of the methods, source of culture reagents, and criteria used for scoring colonies and bursts derived from CFU-E, day 3 BFU-E, and day 8 BFU-E has been given previously. Briefly, suspensions of femoral marrow or spleen cells were prepared in 2% fetal calf serum (FCS) in Alpha medium unless specified otherwise and then plated in replicate 1-ml cultures in 35-mm Petri dishes and incubated in a humidified atmosphere at 37°C. The final culture mixture contained 0.4% methylcellulose, 30% FCS, 1% bovine serum albumin (BSA) (except in
some CFU-E assays after it was found that the addition of BSA was unnecessary for this cell type), $10^{-4} M$ 2-mercaptoethanol, cells, and Ep.

Optimal Ep concentrations of 0.05 U/ml were used for routine CFU-E determinations, and colonies derived from CFU-E were scored on the second day of incubation. A concentration of 2.5 U Ep/ml, which was optimal for day 3 BFU-E and suboptimal for day 8 BFU-E, was used routinely for both types of BFU-E measurements; the same cultures were then simply scored twice, first for early bursts on the third or fourth day after plating and then again for late bursts 8–10 days after plating. Under these conditions the plating efficiency of CFU-E, day 3 BFU-E, and day 8 BFU-E had previously been shown to be constant for the range of cell concentrations used in these studies.

**Cell Separation Experiments**

Cells were separated by velocity sedimentation using a 11.2-cm-diam glass sedimentation chamber (Staput apparatus from O.H. John, Montreal). Twenty milliliters 3%, FCS containing the marrow cells ($8 \times 10^7$ total nucleated cell content) were run into the chamber, followed by a 600-ml gradient of 15–30% FCS. The cells were allowed to sediment at 4°C for $-3^{1/2}$–5 hr at unit gravity. The first 100 ml was discarded and then the remainder collected in 13-ml fractions. The cells in each fraction were then concentrated by centrifugation (250 g for 20 mm) and resuspension in 3 ml. Nucleated and total cell counts were performed using a hemocytometer. CFU-E and BFU-E assays were carried out as described above.

**$^3$H-Thymidine Suicide Experiments**

In each experiment four tubes of $8 \times 10^6$ freshly suspended marrow cells in 2 ml Alpha medium without nucleosides or serum were used. Tube 1 contained cells and medium alone. Tubes 2 and 3 also contained 20 and 100 $\mu$Ci/ml of tritiated thymidine (methyl-$^3$H-TdR, 19 Ci/mM, Radiochemical Centre, Amersham-Searle, England), respectively. The fourth tube contained cells in medium plus 20 $\mu$Ci/ml $^3$H-TdR as well as 400 $\mu$g unlabeled TdR.

Following incubation at 37°C for 20 min, 10 ml 2%, FCS containing 200 $\mu$g unlabeled TdR/ml was added to each tube, and the cells were then centrifuged at 250 g for 20 min at 4°C. The cell pellets were resuspended and rewashed in 10 ml 2%, FCS containing 40 $\mu$g TdR/ml. The cells in each tube were finally resuspended in 2 ml of the same medium and plated. Recovery of all progenitor cell types in tubes 1 and 4 after incubation and washing by comparison to cells held on ice was 80%–100%. Percentage suicide values were calculated from the ratio of the number of CFU-E, day 3 BFU-E, or day 8 BFU-E detected in groups 2 and 3 relative to the numbers of these found in groups 1 and 4.

**Vinblastine Experiments**

Groups of B6C3F1 mice were injected intravenously with graded doses of vinblastine sulfate (Velbe, Eli Lilly, Toronto) just previously dissolved and diluted in pyrogen-free 0.9% saline, such that each mouse received a total volume of 0.5 ml. Mice were killed 20–22 hr later, and marrow cell suspensions were prepared and assayed immediately.

**Red Blood Cell Transfusions**

Transfused mice were given three injections of 1 ml packed washed B6C3F1 red cells in saline or Hank’s balanced saline solution. Mice with hematocrits of 65% or more 6 days later were considered plethoric.

**RESULTS**

The results of two types of experiments are reported here. The first part of this study describes a number of properties that serve to distinguish CFU-E, day 3 BFU-E, and day 8 BFU-E found in the marrow of normal mice. In the second part the differential effects of a number of perturbations in vivo on the population size of each of these progenitor cell compartments are reported.
Velocity Sedimentation Studies

To determine whether or not CFU-E and day 3 and day 8 BFU-E could be physically distinguished, normal mouse marrow cells were separated using the velocity sedimentation technique of Miller and Phillips. Initially fractions were collected after 3½ hr of sedimentation. Subsequently experiments were run for 5 hr, since it was found that the longer sedimentation period gave a better resolution of the two BFU-E profiles and allowed a clear separation of both of these cell types from the large, broad peak of the faster-sedimenting CFU-E population. A typical profile for a 5-hr run is shown in Fig. 1.

Table 1 summarizes the results of all experiments performed in terms of the different modal sedimentation rates obtained for each of the three cell types assayed. In addition, possible effects of the Ep concentration used for these measurements were investigated in one experiment (No. 5 of Table 1) in two different ways. In this experiment CFU-E–derived colony counts were performed on day 2 in the cultures containing 2.5 U Ep/ml as well as in the routine CFU-E assay cultures containing 0.05 U Ep/ml. The number of CFU-E detected in each fraction was always equal or less for the higher Ep concentration, as we have always found for normal marrow, and no evidence of a shift in the general shape of the CFU-E profile was seen.

To ensure that the suboptimal Ep conditions used to obtain day 8 BFU-E profiles yielded a sedimentation velocity representative of this population, cells from appropriate fractions of experiment No. 5 were also plated in cultures containing 10 U Ep/ml. In comparison to the cultures containing 2.5 U Ep/ml,
In each experiment a single pool of marrow cells was used. Per cent suicide values were obtained as described in Materials and Methods.

Table 1. Differential Sedimentation Velocities of Murine Progenitors of Erythroid Colonies and Early and Late Bursts

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Modal Sedimentation Velocity (mm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU-E</td>
</tr>
<tr>
<td>1</td>
<td>7.1*</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
</tr>
<tr>
<td>3</td>
<td>7.9</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>6.2 (5.8-6.6)*</td>
</tr>
<tr>
<td>6</td>
<td>6.9 (6.3-7.5)*</td>
</tr>
<tr>
<td>Mean ± 1 SEM</td>
<td>7.0 ± 0.3</td>
</tr>
</tbody>
</table>

* A flat-topped broad CFU-E peak extending from 6.1 to 8.2 mm/hr was obtained in this experiment.
† In these experiments CFU-E appeared to sediment as two peaks, with the modal velocities indicated in parentheses.

In this experiment day 8 BFU-E were also assayed in cultures containing 10 U Ep/ml, in which case the modal sedimentation velocity obtained was 3.7 mm/hr.

The number of late bursts detected at the higher Ep concentration was regularly increased by a factor of 1.5-2.0. However, as indicated in Table 1 the modal sedimentation velocity remained the same.

The sedimentation velocities shown in Table 1 for CFU-E and for day 8 BFU-E are similar to those originally described by Axelrad et al. using plasma clot cultures and confirmed subsequently by others. The present studies show that early bursts are derived from a population of cells intermediate in size between the day 8 BFU-E from which they derive and the CFU-E to which they give rise.

Experiments With Cycle Active Agents

It has previously been reported that CFU-E in normal mouse marrow represent an actively cycling population, in contrast to the progenitors of late bursts. It was therefore of interest to examine the cycling state of day 3 BFU-E, since these are known to occupy a position in the erythropoietic line intermediate between day 8 BFU-E and CFU-E. This study was done in two different ways. First, suicide values were obtained for CFU-E and day 3 and day 8 BFU-E from normal mouse marrow following a 20-min exposure in vitro of the cells to high specific activity tritiated thymidine (for details see Materials and Methods). The results of such experiments are presented in Table 2. It can be seen that the suicide values for CFU-E and day 8 BFU-E are high and low,
respectively, as previously reported. The value obtained for day 3 BFU-E is intermediate but suggests that in normal mice most day 3 BFU-E, like CFU-E, are actively cycling. The greater suicide rate exhibited by CFU-E presumably reflects a relatively shorter $G_1 + G_2 + M$ characteristic of this more differentiated cell type.

Similar results were obtained with the second cycle active agent used, vinblastine sulfate. In these experiments groups of mice were injected with graded doses of drug and killed for marrow CFU-E and BFU-E determinations 20–22 hr later. Figure 2 shows the three different dose-response curves obtained. Although greater interexperimental variation was encountered in these studies in vivo by comparison to the thymidine suicide experiments performed in vitro during the same time period, the vinblastine data are useful because they provide a more sensitive measure of the minor proportion of cells in a predominantly cycling population that did not cycle during the period of drug exposure. Thus the data in Fig. 2 suggest that more than 99.5% of CFU-E were killed under conditions that reduced the day 3 BFU-E population by only 95%.

Studies of the Effect of Transfusion-induced Plethora

The results of the above experiments are consistent with the previously advanced hypothesis that the assay for early bursts in vitro and the 3-day $^{59}$Fe incorporation assay for Ep-responsive cells in vivo detect the same or overlapping cell populations, since both have now been shown to be actively cycling. Further support for this hypothesis was obtained when the effect of transfusion-induced plethora on day 3 BFU-E numbers was investigated. Parallel groups of normal and transfused mice, selected as plethonic 6 days later on the basis of their increased hematocrits at that time, were killed and assayed individually for marrow CFU-E and day 3 and day 8 BFU-E numbers. The pooled results from two such experiments are given in Table 3. As shown previously this procedure causes a significant although not total reduction in CFU-E numbers. In contrast, day 3 BFU-E numbers, like day 8 BFU-E numbers, were found to remain unchanged or were slightly increased.
PROPERTIES OF BFU-E AND CFU-E

Table 3. Differential Effect of Hypertransfusion-induced Plethora on the Number of Murine Progenitors of Erythroid Colonies and Early and Late Bursts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Mice</th>
<th>Plethoric Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit</td>
<td>50.5 ± 0.7</td>
<td>71.8 ± 1.7</td>
</tr>
<tr>
<td>CFU-E/femur Day 3</td>
<td>48,900 ± 6900</td>
<td>13,100 ± 5700</td>
</tr>
<tr>
<td>BFU-E/femur Day 3</td>
<td>3,610 ± 230</td>
<td>4,200 ± 360</td>
</tr>
<tr>
<td>Day 8 BFU-E/femur</td>
<td>3,500 ± 280</td>
<td>4,620 ± 360</td>
</tr>
</tbody>
</table>

Values shown represent the mean ± 1 SEM of the combined results from a total of 11 normal mice and 9 plethoric mice assayed individually in two separate experiments.

Studies in W/W" and f/f Mutant Mice

Previous studies have shown that a defect in CFU-E production following Ep injection in vivo is seen in the adult anemic W/W" mouse, although this defect is presumably not a direct result of the anemia characteristic of this mouse, since the same phenomenon is observed in W/W" mice rendered plethoric. Similar findings have also been reported using 59Fe incorporation as an endpoint of Ep responsiveness in vivo. In contrast, a significant defect in this response has not been found in adult mice of genotype f/f, although evidence of defective erythropoiesis in erythroid precursors active in the early phases of major hemopoietic regeneration has been well documented in f/f mice. Since day 8 and day 3 BFU-E are known to represent sequential stages of erythroid precursor development starting close to CFU-S and extending close to CFU-E, it was also of interest to examine the behavior and numbers of these two cell types in W/W" and f/f mice.

Time-course studies of burst formation in cultures containing 2.5 U Ep and marrow or spleen cells from either genotype yielded a pattern similar to that previously described and seen also in simultaneous cultures prepared with cells from the appropriate +/+ controls. Early bursts were seen by day 3 in all cultures, and no difference was apparent in the spectrum of burst sizes en-

Table 4. Population Size Measurements of Various Types of Erythropoietic Precursors in W/W" and +/+ Littermate Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CFU-E</th>
<th>Day 3 BFU-E</th>
<th>Day 8 BFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per femur</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>62,300 (34,800-100,000)</td>
<td>3,260 (960-6,860)</td>
<td>5,650 (1,560-10,100)</td>
</tr>
<tr>
<td>W/W&quot;</td>
<td>37,100 (19,500-74,600)</td>
<td>2,160 (980-3,330)</td>
<td>2,080 (910-3,050)</td>
</tr>
<tr>
<td>+/+ × 100%</td>
<td>59.6%</td>
<td>66.3%</td>
<td>36.8%</td>
</tr>
<tr>
<td>Per spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>22,300 (1,200-178,000)</td>
<td>4,720 (200-46,500)</td>
<td>10,100 (1,500-37,900)</td>
</tr>
<tr>
<td>W/W&quot;</td>
<td>2,980 (600-14,800)</td>
<td>540 (150-1,600)</td>
<td>2,120 (750-9,410)</td>
</tr>
<tr>
<td>+/+ × 100%</td>
<td>13.4%</td>
<td>11.4%</td>
<td>21.0%</td>
</tr>
</tbody>
</table>

Values shown are the geometric means of data obtained from six experiments in which 11 or 12 mice of each genotype were assayed individually and 9 mice of each genotype were assayed three at a time. Numbers shown in parentheses are the highest and lowest values encountered in each set of measurements. Geometric means were used to accommodate the range of values obtained with spleen. Arithmetic and geometric means gave similar results for marrow.
countered throughout the following week of incubation. Similarly, variation of the concentration of Ep present yielded Ep dose-response curves similar for mutant and +/+ cells, although the Ep requirement of each progenitor cell class differed as previously described.12

However, when the population size of CFU-E, day 3 BFU-E, and day 8 BFU-E in each of these genotypes was measured, consistent differences were found between $W/W$ mice and their +/+ littermates (Table 4). This difference was not the case for mice of genotype $f/f$, where similar results were obtained for $f/f$ mice and their +/+ controls (Table 5). Mice of genotype $W/W^{*}$ showed decreased numbers of erythropoietic progenitors of all types. In the marrow this reduction was greater for day 8 BFU-E ($73\%$) than for both day 3 BFU-E ($34\%$) and CFU-E ($40\%$). Population size measurements for spleen showed much greater intermouse variation; however, in this organ in $W/W^{*}$ mice erythroid progenitors seemed to be even more depressed and all three cell types assayed appeared approximately equally affected ($80\% - 90\%$ reduction).

### DISCUSSION

Many studies have suggested that the differentiation of hemoglobin-synthesizing cells from the most primitive class of committed erythroid precursors is a multistep process. In the mouse, at least three stages of this early phase of erythropoietic cell differentiation can be identified by colony assays in vitro, where the time of colony maturation and ultimate colony size serve to define different progenitor cell classes.24,25 These are known as day 8 BFU-E, day 3 BFU-E, and CFU-E, ranked in order of increasing maturation. As cells move

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Exp. No.</th>
<th>Per femur</th>
<th>Day 3 BFU-E</th>
<th>Day 8 BFU-E</th>
<th>Per spleen</th>
<th>Day 3 BFU-E</th>
<th>Day 8 BFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>$+/+$</td>
<td>1</td>
<td>—</td>
<td>1,440</td>
<td>2,620</td>
<td>1</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>2</td>
<td>13,800</td>
<td>1,440</td>
<td>2,620</td>
<td>2</td>
<td>18,400</td>
<td>3,910</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3,940</td>
<td>2,690</td>
<td>4,070</td>
<td>3</td>
<td>3,910</td>
<td>2,990</td>
</tr>
<tr>
<td>Mean</td>
<td>13,800</td>
<td>2,690</td>
<td>4,070</td>
<td></td>
<td>18,400</td>
<td>2,990</td>
<td>3,450</td>
</tr>
<tr>
<td>$f/f$</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>1,800</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18,400</td>
<td>1,900</td>
<td>5,560</td>
<td>2</td>
<td>18,400</td>
<td>3,910</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>—</td>
<td>3,910</td>
<td>2,990</td>
<td>3</td>
<td>—</td>
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<tr>
<td>Mean</td>
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<td>2,990</td>
<td>3,450</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

In each experiment parallel assays were performed on cells pooled from three mice of each genotype matched for age and sex.
through each of these early compartments, they exhibit a progressive decrease in proliferative capacity and a concomitant increase in Ep sensitivity in vitro.

The studies reported here were undertaken to obtain additional information about changes in vivo that accompany (1) the differentiation of day 3 BFU-E from day 8 BFU-E and (2) the subsequent differentiation of CFU-E from day 3 BFU-E.

Little is known about the dependence of these differentiation steps on cell cycle-related events. The present results show that in normal marrow the proportion of cells killed following short-term exposure to ³H-TdR or vinblastine is small for day 8 BFU-E, higher for day 3 BFU-E (50%, kill for ³H-TdR), and even higher for CFU-E (Table 2, Fig. 2). The modal cell volumes of these cell types in normal marrow (Fig. 1, Table 1) also show systematic changes corresponding to their differentiated states. It is well known that cell volume is related to cycle state, and hence even for an otherwise homogeneous population the relative distribution of cells in different phases of the cell cycle will affect the sedimentation profile obtained. At least some of the differences observed between day 8 and day 3 BFU-E and CFU-E sedimentation profiles could be explained in this way. For example, it might even be argued that the formation of bursts in culture within 3 days rather than 1 wk represents a change in the response of cells plated at opposite ends of the same cell cycle. This hypothesis appears unlikely, however, since ³H-TdR suicide values similar to those of normal day 3 BFU-E have been obtained for day 8 BFU-E found in regenerating hemopoietic tissue, and under these conditions the ratio of day 3 to day 8 BFU-E numbers remains approximately the same as in normal marrow.

An additional consideration in the interpretation of the present cycling and sedimentation profile data is the possibility that the duration of S (the DNA synthetic phase) relative to the total cell cycle may be substantially different for day 8 and day 3 BFU-E in normal marrow. From the preliminary experiments reported here it is unfortunately not possible to distinguish between such variations in the length of S and variations in the proportion of normal day 8 and day 3 BFU-E that are “in cycle.” It is possible, however, that the cell volume differences observed between these two populations are at least in part associated with differences in their differentiated states, a phenomenon seen more dramatically when day 3 BFU-E and CFU-E are compared than when day 8 and day 3 BFU-E are compared. It is interesting that a similar correlation between progenitor cell size, proliferative capacity, and responsiveness to trophic stimuli has been found for cells forming granulopoietic colonies in vitro, suggesting that changes in cell volume may be a general feature of early hemopoietic differentiation events.

The lack of effect of plethora and hence reduced circulating Ep levels on the maintenance of day 8 and day 3 BFU-E populations in vivo (Table 3) suggests that physiologic concentrations of Ep may not play a significant role in regulating the flow of cells out of the day 8 BFU-E compartment and into the day 3 BFU-E population. In man such a role could be ascribed to leukocyte-derived factors required in addition to Ep for burst formation by human BFU-E. Although to date evidence of a similar phenomenon in the murine system has not
been reported, it is tempting to speculate that a similar type of non-Ep factor also exists in this species. The restriction of action of such a factor to the most primitive erythropoietic cell types (BFU-E or their equivalent in man\textsuperscript{3}) would then provide an explanation for both the lack of effect of plethora on day 3 BFU-E (Table 3) and the dramatic increase in Ep sensitivity that accompanies the differentiation of CFU-E from day 3 BFU-E in mice.\textsuperscript{2}

The studies with $W/W^t$ mice provide further documentation of differences between day 3 and day 8 BFU-E (Table 4). They also reinforce the concept that the genetically determined anemia in $W/W^t$ mice is due primarily to alterations in the behavior of primitive hemopoietic precursors\textsuperscript{23} and suggest that the defective response of normal or plethoric $W/W^t$ mice to Ep in vivo\textsuperscript{12,13} may be explained by the greatly reduced numbers of all erythropoietic progenitor cell types found in the $W/W^t$ spleen, since their intrinsic Ep responsiveness appears to be normal. Further application of assays in vitro to studies of genetically determined hemopoietic abnormalities should continue to contribute to an understanding of the primary lesions involved. Similarly, the definition of equivalent erythroid precursor cell classes in man should also be useful in the future investigation of human hemopoietic disorders.

**ACKNOWLEDGMENT**

We are grateful to M. Heppner, C. Smith, and S. Thomas for expert technical assistance and to Dr. K. Humphries for the HP-67 program used to calculate sedimentation velocities.

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