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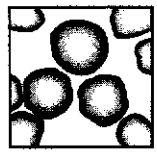

Optimal erythroid cell production during erythropoietin treatment of mice occurs by exploiting the splenic microenvironment

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Abstract. In this study, quantitative effects on erythroid cell production by a prolonged recombinant human erythropoietin (rhEpo) treatment of mice are presented. Epo treatments, given subcutaneously (s.c.) twice per day in doses of 0.5 to 500 U per day, were performed under steady-state production conditions. We found striking differences between the behavior of the different erythroid cell compartments (burst-forming unit erythroid [BFU-E], colony-forming unit erythroid [CFU-E] and erythroid precursors), as well as between the microenvironments of bone marrow and spleen. Whereas the total-body BFU-E was not changed by Epo, a redistribution of BFU-E from marrow to spleen occurred, resulting in decreasing marrow and increasing splenic BFU-E numbers. Splenic BFU-E produced CFU-E as much as 8 times more efficiently than marrow BFU-E at 50 U of Epo. At low Epo doses (to 1 U/day) no difference was found. The CFU-E in the spleen produced erythroblasts at a higher efficiency at all Epo doses (1.5 to 5 times). It seems as if this efficiency was higher at low Epo doses. Because of the migration phenomenon and the excellent microenvironment in the spleen, at the highest Epo concentrations nearly 70% of all erythroid cells reside in the spleen. Even at the highest Epo doses, granuloid cell production was not affected. Similar to the BFU-E, total-body granuloid cells remained constant (despite a shift of granulocyte-macrophage progenitors [CFU-GM]) from marrow to spleen; however, these cells did not flourish in the spleen. Under these conditions, 90% of the granuloid precursors were still localized in the marrow. Erythropoietin did not change the transit time of erythroid cells at high Epo doses.

Key words: Erythropoiesis—Erythropoietin—Microenvironment—Migration—Spleen

Introduction. Hemopoiesis is regulated by a complicated network of interactions between particular growth factors, microenvironments and hemopoietic cells. Erythropoietin is the most prominent regulator of erythropoiesis, mediating the feedback between the mature and immature erythroid cell stages [1].

Although the regulation of erythropoiesis is quite well understood at the cellular level, precise information about the production capacity and the involvement of different hemopoietic sites in vivo is largely lacking.

Knowledge of the Epo dose response is important in our understanding of the dynamics of the system in those circumstances where one wants to predict the behavior of the system after a perturbation. So far attempts to estimate the dose response were rather cumbersome and indirect. They relied on evaluating recovery behavior of erythropoiesis after specific perturbations like bleeding [2,3], hypoxia [4] and hemolytic anemia [5,6]. The availability of rhEpo now permits a more direct approach to the problem, as one can study the system under circumstances of constantly elevated Epo levels. It was the objective of our experiments to determine the dose responses in marrow and spleen under steady-state conditions. Furthermore, we wanted to know if stimulation of the erythroid cell line had consequences for granuloid cell production.

To establish the steady-state conditions, twice-daily injections of rhEpo were used and the response of BFU-E, CFU-E and erythroid precursors was followed over 14 days. From this time course, a time was selected at which dose response evaluations were conducted in subsequent experiments.

Previous model calculations indicated that the dose response should differ between marrow and spleen environments [7]. We therefore simultaneously measured the progenitor cells BFU-E, CFU-E and CFU-GM and the morphologically recognizable granuloid and erythroid precursors (Gran-P, Ery-P) in the marrow, spleen and blood.

Materials and methods

Treatment of mice. Specific-pathogen-free female C57Bl/6 mice (Charles River, Frankfurt, Germany) between 8 and 12 weeks of age and varying in body weight from 20 to 25 g were used. The mice were maintained in a conventional animal room and fed ad libitum with sterilized pelleted food (Hope Farms, Woerden, The Netherlands) and tap water.

rhEpo (generous gift of Boehringer Mannheim GmbH, Almere, The Netherlands) was administered s.c. 2 times daily (0900 and 1700 hours) in an injection volume of 0.1 mL pyrogen-free saline. The administered dose is given as the daily Epo dose in U/mouse/day.

Preparation of femoral bone marrow, spleen and blood cell suspensions. Cell suspensions were prepared as described previously [8] and pooled per group of 3 mice. The hematocrit and reticulocyte counts were determined according to standard procedures. The nucleated blood cells were isolated after centrifugation on Histopaque (Sigma, St. Louis, MO) with a density of 1.085 g/mL. The interphase was removed and washed 3 times with α-medium + 10 mM Hepes, pH 7.2, supplemented with 5% fetal calf serum.

Assay of progenitors. CFU-GM, BFU-E and CFU-E were estimated with the methylcellulose method of Iscove and Sieber [9].

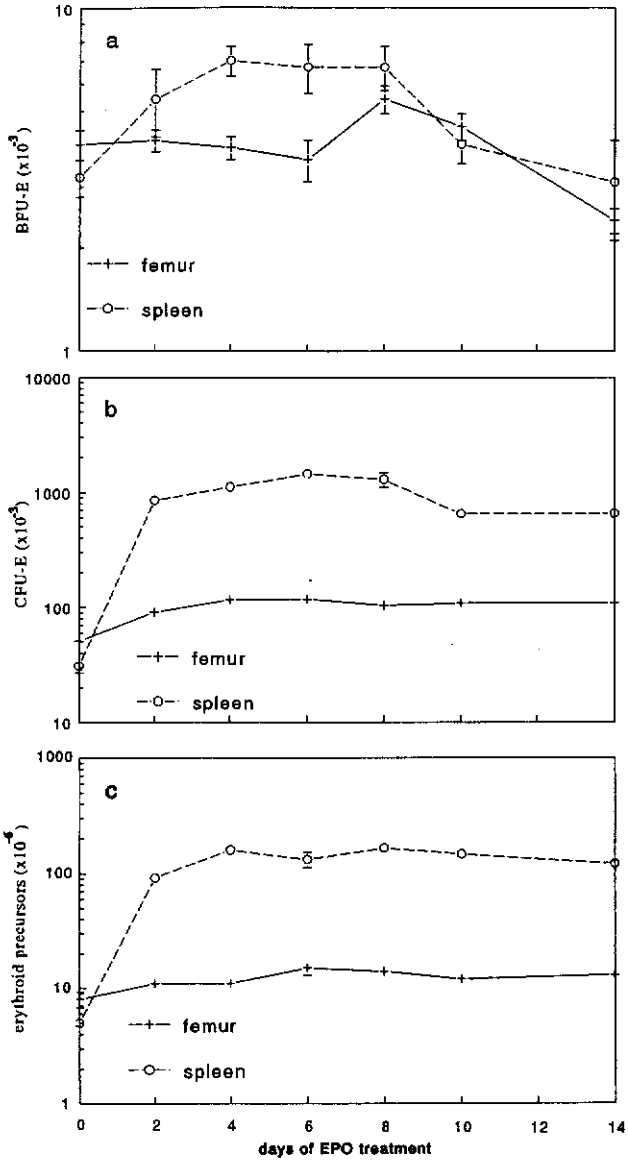

Fig. 1. Response of erythroid cells in the femur and spleen by a 14-day Epo treatment (10 U/day/mouse)
Numbers of BFU-E **(A)**, CFU-E **(B)** and erythroid precursors **(C)** (+ femur, O spleen). (± SEM; n=6)

Calculation of the total body counts. The total body count of a particular cell stage was calculated according to the following equation: total body count = (femoral count x 17) + splenic count, i.e., considering the femur to be approximately 6% of the total bone marrow (the numbers in the peripheral blood were neglected) [10].

Estimation of the number of erythroid and granuloid precursors. The number of morphologically recognizable Ery-P and Gran-P were calculated as percentages after counting 400 cells in duplicate per sample. Percentages were multiplied by total nucleated cell numbers per femur or spleen.

Determination of steady-state conditions for Epo treatment. At regular intervals over a period of 14 days, the femoral and splenic BFU-E, CFU-E and Ery-P contents were determined after administration of a daily Epo dose of 10 U/mouse. When a constant level of a particular cell stage was accomplished, a steady state for this cell stage was assumed.

Dose response of the erythroid and granuloid cells in marrow and spleen. Under steady-state conditions, the BFU-E, CFU-E, Ery-P, CFU-GM and Gran-P were determined at Epo doses of 0.5, 1.0, 2.0, 5.0, 10.0, 50.0, 200.0 and 500.0 U/mouse/day.

Estimate for the transit time of immature erythroid cells. Mice were treated with thiamphenicol (TAP) (Zambon, Milan, Italy) for 3 days via s.c. implantation of a dialysis bag containing 0.5 mL water with 350 mg TAP as described previously [11]. This treatment eliminates all erythroid cells after the BFU-E. After abrogation of the TAP treatment, Epo was administered for 7 days at a daily dose of 5 and 50 U/mouse. The control was injected daily with 2 times 0.1 mL pyrogen-free saline. The peripheral blood reticulocytes were determined for 7 days.

Statistical evaluations. The mean and standard deviation were calculated. A 2-sided Student's *t*-test was used for comparison of two means. The level of significance was set at $p<0.05$.

Results

Steady-state production of erythroid cells during Epo treatment. Epo was administered for 14 days at a daily dose of 10 U per mouse. While the femoral BFU-E counts did not change significantly during 10 days, the splenic counts increased and reached a maximum on day 4 of $7.0 \pm 1.7 \times 10^3$ BFU-E per spleen, which was significantly higher ($p<0.0025$) than the initial counts on day 0 ($2.1 \pm 0.4 \times 10^3$ BFU-E/spleen) (Fig. 1A). Until day 8, a constant level was attained after which a decrease to normal values was observed. At day 14 femoral content was decreased compared to the control value ($p<0.001$).

The CFU-E numbers in both the marrow and spleen demonstrated a more pronounced response compared to the BFU-E numbers (Fig. 1B). While the femoral CFU-E numbers increased to 2 times the control ($p<0.0025$ at day 2 and later), the splenic content overshot the initial value by approximately 50 times. At both hemopoietic sites the maximum value was reached on day 6: $126 \pm 15 \times 10^3$ CFU-E/femur (control $54 \pm 6 \times 10^3$) and $1448 \pm 411 \times 10^3$ CFU-E/spleen (control $31 \pm 13 \times 10^3$). From day 10 on, both the femoral and splenic CFU-E numbers maintained a constant level.

The Ery-P responses in marrow and spleen were similar to those of the CFU-E (Fig. 1C). The Ery-P numbers in the femur were 2 times the initial control value ($p<0.0025$ from day 6 on). The splenic Ery-P numbers reached a maximum on day 4 of 30 times the control, which was less than the increase in CFU-E. From day 6 on, the femoral as well as the splenic Ery-P counts stayed at a constant and significantly elevated level. From these results we have chosen day 10 as appropriate for the steady-state production. We assume that at lower and higher doses of Epo the essentials of the curves are not different. (A pilot study with 50 U gave similar results.)

Epo dose responses of the femoral splenic and total body BFU-E, CFU-E and Ery-P. At daily Epo doses ranging from 0.5 to 500 U per mouse during 10 days, the femoral BFU-E numbers showed a small but significant decrease to 54% of the control at an Epo dose of 2, 5, 50, 200 and 500 U/mouse/day (control: 4.1 ± 1.1; 500 U Epo: 2.2 ± 1.4, $p<0.01$). In the spleen, however, the BFU-E numbers exponentially increased at Epo doses

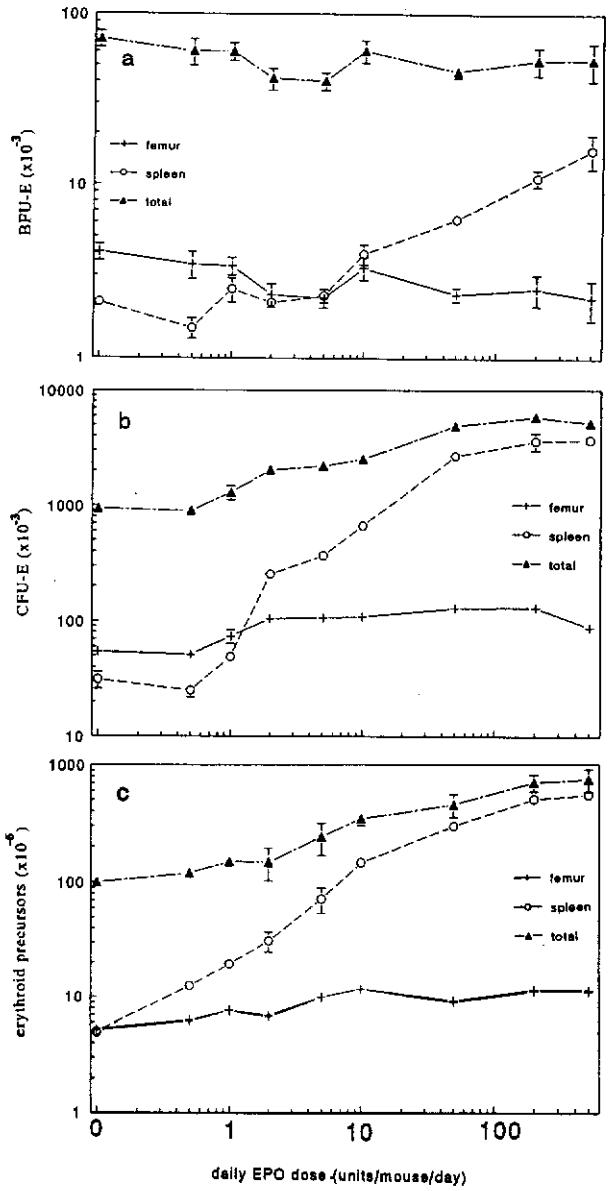

Fig. 2. Dose responses of erythroid cells in the femur and spleen by a 10-day Epo treatment
Numbers of BFU-E (A), CFU-E (B) and erythroid precursors (C) (+ femur, ○ spleen, ▲ total). (± SEM; n =6)

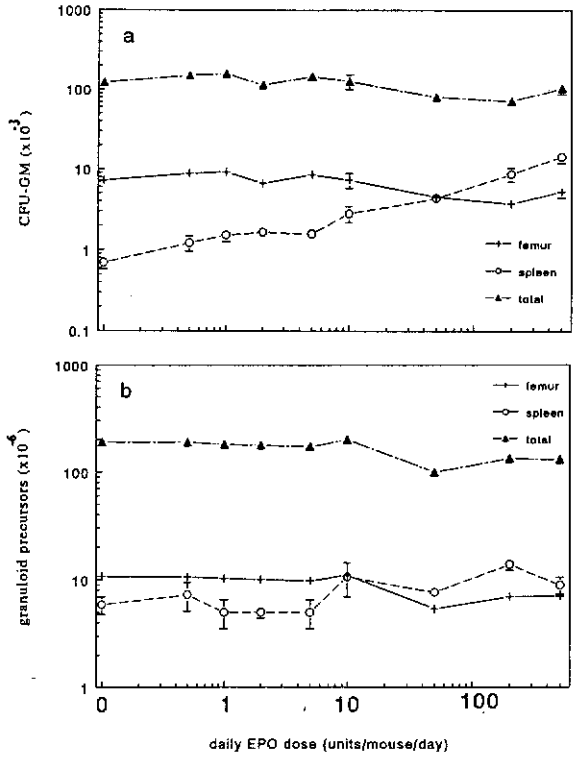

Fig. 3. Effects of Epo administration on granuloid cell stages
Numbers of CFU-GM (A) and granuloid precursors (B) (+ femur, ○ spleen, ▲ total). (± SEM; n=6 [A] n=3 [B])

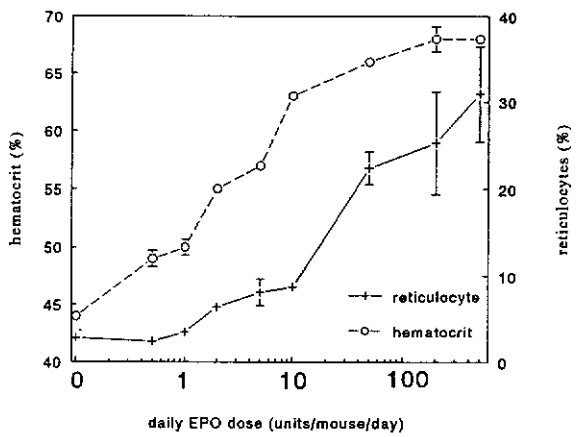

Fig. 4. Dose response of hematocrit (○) and reticulocytes (+) by a 10-day Epo treatment. (± SEM; n=6)

higher than 5 U/mouse/day (control: 2.1 ± 0.4; 500 U Epo 16.0 ± 6.9 $p<0.01$).The total body counts of this cell stage were not significantly affected by different Epo doses (Fig. 2A).

Both the splenic and femoral CFU-E numbers were significantly increased by the Epo treatment. At 2 U/mouse/day the femoral CFU-E reached a maximum response of 2 times the control (105 ± 25x10^3 in comparison with a control of 54 ± 6x10^3 CFU-E/femur, $p<0.005$). Higher levels of Epo had no further effect. The splenic CFU-E increased sharply from 2 U to 50 U/mouse/day and between 50 and 200 U reached a maximum response of 120 times the control (3600 ± 542 vs. 31 ± 13). The total body CFU-E increased maximally to 6 times the control at 200 U.

The femoral and splenic Ery-P numbers increased approximately 2 times and 100 times, respectively.

The total body Ery-P numbers increased 6 times at maximum at an Epo dose of 200 to 500 U/mouse.

Effects of Epo administration on CFU-GM and Gran-P. The splenic, femoral and total body numbers of CFU-GM (Fig. 3A) and Gran-P (Fig. 3B) are given after daily Epo administration

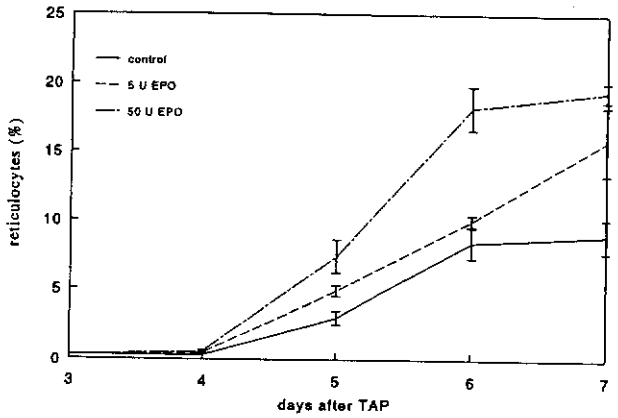

Fig. 5. Epo-dependent reticulocyte response after a TAP treatment (— control, --- 5 U Epo, ····· 50 U Epo). (± SEM; n=6)

for 10 days. The femoral CFU-GM content was not significantly affected up to an Epo dose of 10 U/mouse/day. From then on, CFU-GM demonstrated a significant decrease to approximately 70% of control ($p<0.05$). In contrast, the splenic CFU-GM numbers increased with 5 to 500 U/mouse/day. At a dose of 500 U the splenic CFU-GM numbers were increased 12 times; however, the total body CFU-GM numbers did not change significantly.

The Gran-P numbers behaved similarly. In the marrow a decrease to 65% of the control at high Epo doses and a small increase of the splenic numbers to 2 times the control was observed. The total Gran-P numbers did not change.

Dose response of the hematocrit and peripheral blood reticulocytes. Figure 4 shows the response of the hematocrit and reticulocytes after treatment for 10 days with Epo doses from 0.5 to 500 U/mouse/day. The hematocrit sharply increased above 1 U of Epo, reaching a plateau of 68.1% ± 0.7 at 200 U (control: 44.5% ± 0.4). Up to 10 U of Epo, the reticulocyte counts increased to 8.7% ± 0.4. Between 10 and 500 U, a steeper increase was observed with a maximum value of 30.9% ± 13.5.

Epo-dependent response of peripheral blood reticulocytes after TAP treatment. To investigate if Epo, apart from its stimulatory effect on proliferation, also could influence the cell cycle times of erythroid cells, we used a thiamphenicol treatment to eliminate all erythroid cells more mature than the BFU-E [11]. Differences between the reappearance of peripheral reticulocytes after the treatment can be explained as changes in the transit time of CFU-E and erythroid precursors. Until day 4 after TAP the reticulocyte counts were below the detection level of 0.3% (Fig. 5). After day 4, the reticulocyte counts responded in a dose-related way. On day 6, mice injected with 50 U Epo/day had a reticulocyte response more than 2 times higher than the control and 5 U/day treatments. No time differences in the reappearance of reticulocytes were found between the control and low and high Epo doses.

Discussion

Murine erythroid cell production can occur in the bone marrow and the spleen. The present results show the site dependency of erythroid proliferation related to exogenously administered rhEpo.

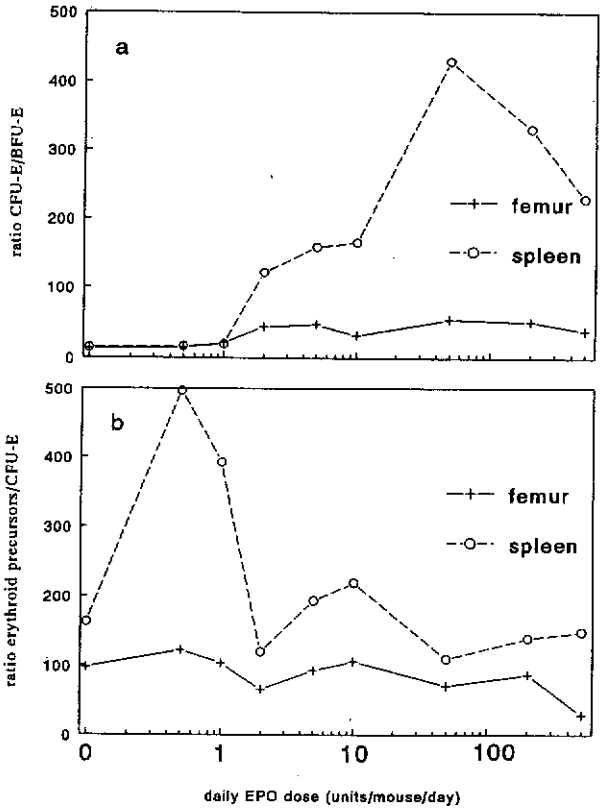

Fig. 6. Epo dose response of erythroid cell ratios CFU-E/BFU-E **(A)**, Ery-P/CFU-E **(B)** (+ femur, ○ spleen).

With an arbitrarily chosen Epo concentration of 10 U per day (about the middle concentration of the used doses), erythroid cell production in marrow and spleen was already maximal on day 4 of the treatment. Despite the fact that the numbers of BFU-E in the spleen and marrow declined somewhat after 8 days, this was not reflected in its offspring (CFU-E and Ery-P) remaining constant between day 4 and 14. For the dose response curves, a 10-day treatment was considered to be sufficient to arrive at steady-state conditions for all concentrations. Possible early oscillations in the production will have been dampened out because the transit times of the sensitive cells are much shorter than 10 days.

We found striking differences between the dose responses of BFU-E on the one hand and CFU-E and erythroid precursors on the other. The latter already increased at 1 U of Epo in the marrow as well as in the spleen, whereas this rise occurred for the BFU-E only in the spleen at an Epo dose 10 times higher. Moreover, the BFU-E decreased in the marrow, whereas the CFU-E and the precursors moderately increased. Furthermore, the splenic BFU-E numbers did not show saturation kinetics, in contrast with those of its descendants.

The total body number of BFU-E did not change in response to Epo, whereas the CFU-E and erythroid precursor numbers rose by a factor of 6. These results can best be explained by a migrational effect of Epo on the BFU-E and a proliferation effect on CFU-E and erythroid precursors. Migration is not restricted to the BFU-E. The CFU-GM, in fact, show a response to Epo similar to that of the BFU-E. Migration was also made likely by the presence of increased numbers of

these progenitor cells in the peripheral blood (not shown). CFU-E were not detectable. A similar pattern for BFU-E, CFU-GM and CFU-E was found previously after phenylhydrazine [12] and thiamphenicol [13] or a granulocyte colony-stimulating factor (G-CSF) treatment [14,15]. Also, the treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) [16] and interleukin-3 (IL-3) [17] induced the appearance of progenitor cells in the peripheral blood. Epo treatment of patients did increase peripheral blood progenitors as well [18]. So migration of early hemopoietic cells is a widely occurring phenomenon in those cases where hemopoiesis is stimulated. The mechanisms of migration remain obscure.

The most striking effect of Epo is exerted on the compartment sizes of the CFU-E and erythroid precursors. In marrow and spleen a dose-dependent increase was found. The marrow CFU-E rose to 2- to 2.5-fold the control value. The spleen response was much higher, to 100-fold at 50 U of Epo. At these Epo concentrations, about 60% of all CFU-E were found in the spleen (normally 3%). The response for Ery-P was similar: a 2-fold increase in the marrow and a 100-fold increase in the spleen. At the highest Epo concentrations, 68% of the total body erythroid precursors resided in the spleen. So in the mouse under these circumstances, the spleen becomes a very important production site. Papayannopoulou et al. [19] came to a similar conclusion after determining the erythroid precursor compartment in marrow and spleen during a short-term (3- to 5-day) treatment with 5 U of human urinary Epo.

A part of the high CFU-E and Ery-P response in the spleen can be attributed to the increased import of BFU-E. On the other hand, it is clear that the output of the BFU-E compartment per BFU-E in the spleen is much higher, so the efficiency of erythroid production is higher. This can be visualized when we replot the data as cell stage ratios (i.e., CFU-E/BFU-E, Ery-P/CFU-E) and compare the splenic and marrow values (Fig. 6). Up to 1 U Epo, no differences were found in the efficiency of CFU-E formation between the marrow and the spleen. Between 1 U and 50 U, the output in the marrow rose by a factor of 2, remaining constant above 2 U Epo. In the spleen, however, this ratio increased to 29 times the control value. So BFU-E in the spleen are generating many more CFU-E than those in the marrow. At the highest Epo concentrations the efficiency seemed to decrease again but was still 15 times control value. It is unknown if a special kind of BFU-E leaves the bone marrow in search of a space with unlimited proliferation possibilities or if the microenvironment of the spleen is more advantageous to erythroid proliferation. This is a subject for further research.

The efficiency of erythroblast production by CFU-E in the marrow remains unaffected at different Epo concentrations. Up to 1 U of Epo, splenic efficiency increased, after which a leveling off to normal values was found (Fig. 6B). Also, for this cell stage the spleen clearly shows a better response than the marrow at each Epo concentration.

Apart from highly increased precursor numbers in marrow and spleen, at high Epo levels proliferative activity is also reflected by the appearance of reticulocytes in the peripheral blood in a dose-dependent way. Simultaneously, the hematocrit representing the red blood cell numbers increased to nearly 70%.

From these results we conclude that murine erythroid production induced by erythropoietin is optimized by a shift in the production sites obtained by a flow of early progenitors from the marrow to the spleen.

Erythropoietin also has an effect on granuloid cells. Similar to the BFU-E, the CFU-GM partly move from the marrow (small decrease) to the spleen (increase above 5 U of Epo). The total numbers remain unaffected. In contrast to the erythroid precursors, which increased in numbers, the total number of granuloid precursors did not change. An increase in the spleen compensates for a small decrease in the marrow. Even at the highest Epo concentration, we did not find inhibitory effects (competition) on granulopoiesis, which takes place primarily in the marrow (more than 90% measured as granuloid precursors). This is in contrast to our findings that a treatment of G-CSF inhibits marrow erythropoiesis [14,15]. The increased production of marrow granulopoiesis obviously leads to removal of erythropoiesis which escapes to the spleen. The increased marrow erythropoiesis with Epo does not extinguish granulopoiesis in the marrow, however.

Further interpretation of the proliferative effects of Epo would require information on changes in cell cycle times, maturation times and the definitions of cell compartments. Our results to this end show that the overall transit time of CFU-E and Ery-P plus marrow reticulocyte maturation is not influenced by Epo. After a bone marrow depression by thiamphenicol, the peripheral blood reticulocytes do appear simultaneously at high or low Epo.

The present study clearly indicates that the spleen is a favorable microenvironment for erythropoiesis. An implication of this observation is the physiological significance of migration of immature hemopoietic cells. This may be a way to support rapid cell production more efficiently. In this light one may also interpret enhanced BFU-E and spleen colony-forming units (CFU-S) blood levels found after multiple acute bleeding or hemolytic anemia [20,21].

Another implication is that the differentiation program of a particular cell, especially with respect to the dose-response characteristics, depends upon the microenvironment. It would be worthwhile to examine whether it is a general in vivo phenomenon that hemopoietic cells stimulated by a growth factor can show different quantitative behaviors in different microenvironments. The molecular mechanism by which a particular cell type can perform additional mitoses remains to be clarified. Does an enhancing factor produced by the microenvironment or an adaptation of the intrinsic properties of the cell—for example, by expression of high affinity receptors [22]—play a role? Or does erythropoiesis under normal in vivo conditions (low Epo) occur inefficiently, leading to premature death of progenitor cells (apoptosis), which could be prevented by high concentrations of Epo [23]? These possibilities are currently under investigation.

Acknowledgments

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