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Effects of G-CSF on Erythropoiesis[a]

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INTRODUCTION

Hemopoietic cell growth is governed by a complex network of interdependent cytokines and growth factors. Positive as well as negative signals are available to the system for finely tuning a steady-state cell production. The main positive regulator for erythropoiesis is erythropoietin, the level of which is indirectly controlled by the oxygen tension of the blood, and which is produced in the kidneys. Granulopoiesis is greatly enhanced by granulocyte colony-stimulating factor (G-CSF), a growth factor that can be produced by several cells of the microenvironment of the hemopoietic organs by external signals.[1] Both processes are fed by common ancestors of the pluripotent stem cells. It is largely unknown how and when regulatory signals act on cells from this pool to differentiate into one of the specific lineages. It can be envisaged that a big demand for granulopoiesis or erythropoiesis (after chemo- or radiotherapy) could give problems for the timely and sufficient flow of cells into the progenitor compartments of the opposite lineage. Competition between the lineages for a common predecessor has been suggested from *in vitro*[2] and *in vivo*[3] experiments. Support for such a competition might be derived from the *in vivo* side effects of G-CSF administration on erythropoiesis.[4–7] To get more insight into the mechanism of the negative effects of G-CSF on erythropoiesis, we have administered the recombinant human growth factors EPO and G-CSF to normal and splenectomized mice alone and in combination and measured the erythroid response during 1–3 weeks.

MATERIAL AND METHODS

Animal Treatment

Most experiments were performed with C57B1 mice, 8–12 weeks of age. The mice were treated with growth factors by subcutaneous injection twice daily. Recombinant human G-CSF was a gift from AMGEN (Thousand Oaks, CA, USA). Recombinant human erythropoietin was a gift from Boehringer Mannheim

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(Almere, The Netherlands). The mice were kept in a conventional animal room and fed ad libitum. Splenectomy was carried out six months before the experiments were performed.

Assay of Progenitors and Stem Cells

This was done as previously described.[5] In short, bone marrow cells were obtained by flushing the femora with α-medium (GIBCO, UK) plus 10 mM HEPES, pH 7.2. Spleen cells were suspended in α-medium by disrupting the spleen through a metal sieve. Nucleated blood cells were isolated after centrifugation on a Histopaque layer (1.083 g/mL, Sigma). The interphase was removed and washed with α-medium plus 5% fetal calf serum. Erythrocytes were lysed in 1 mL 0.157 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2. Remaining nucleated cells were isolated by centrifugation. These cells were cultured in a methylcellulose medium for the assay of CFU-GM, BFU-E, and CFU-E. CFU-GM and BFU-E cultures were supplied with 2 U EPO/mL and pokeweed mitogen–stimulated spleen cell–conditioned medium. CFU-E cultures were supplied with 500 mU EPO. CFU-S-d8 assays were performed with the Till and McCulloch technique.[8]

Total Body Cell Number

To get an impression about the marrow and splenic contribution to a certain cell compartment, the total cell numbers were calculated, assuming that the femur contained 1/17 of the total marrow and neglecting the number in the peripheral blood. Each experimental point was obtained from three treated mice, of which the marrow and spleen cells were pooled. The experiments were performed 3–5 times.

RESULTS

Effects of G-CSF Hemopoiesis

The effects of a G-CSF administration (150 μg/kg/day) were followed for 20 days with respect to (A) granulopoiesis, (B) migration of cells, and (C) erythropoiesis.

A: The total number of granuloid progenitors (CFU-GM) continuously increased to 250% (FIG. 1a). This increment was due to the spleen contribution; marrow CFU-GM numbers did not change. The granuloid precursors (morphologically recognizable cells) increased in both compartments, leading to a comparable total rise to 250% (FIG. 1b). As a result, the number of neutrophilic granulocytes in the peripheral blood increased to 10-fold the normal values (FIG. 2). The effects of this G-CSF treatment on the CFU-S-d8 were qualitatively and quantitatively different. The marrow numbers decreased to 20% of initial values, splenic numbers increased, and total numbers showed a transient decrease (FIG. 3).

B: The G-CSF treatment caused a time-dependent high presence of early hemopoietic cells in the peripheral blood. CFU-S numbers increased nearly 20-fold, the BFU-E 27-fold, and the CFU-GM even 90-fold (FIG. 4). The presence of high numbers in blood and the simultaneous changes in the bone marrow

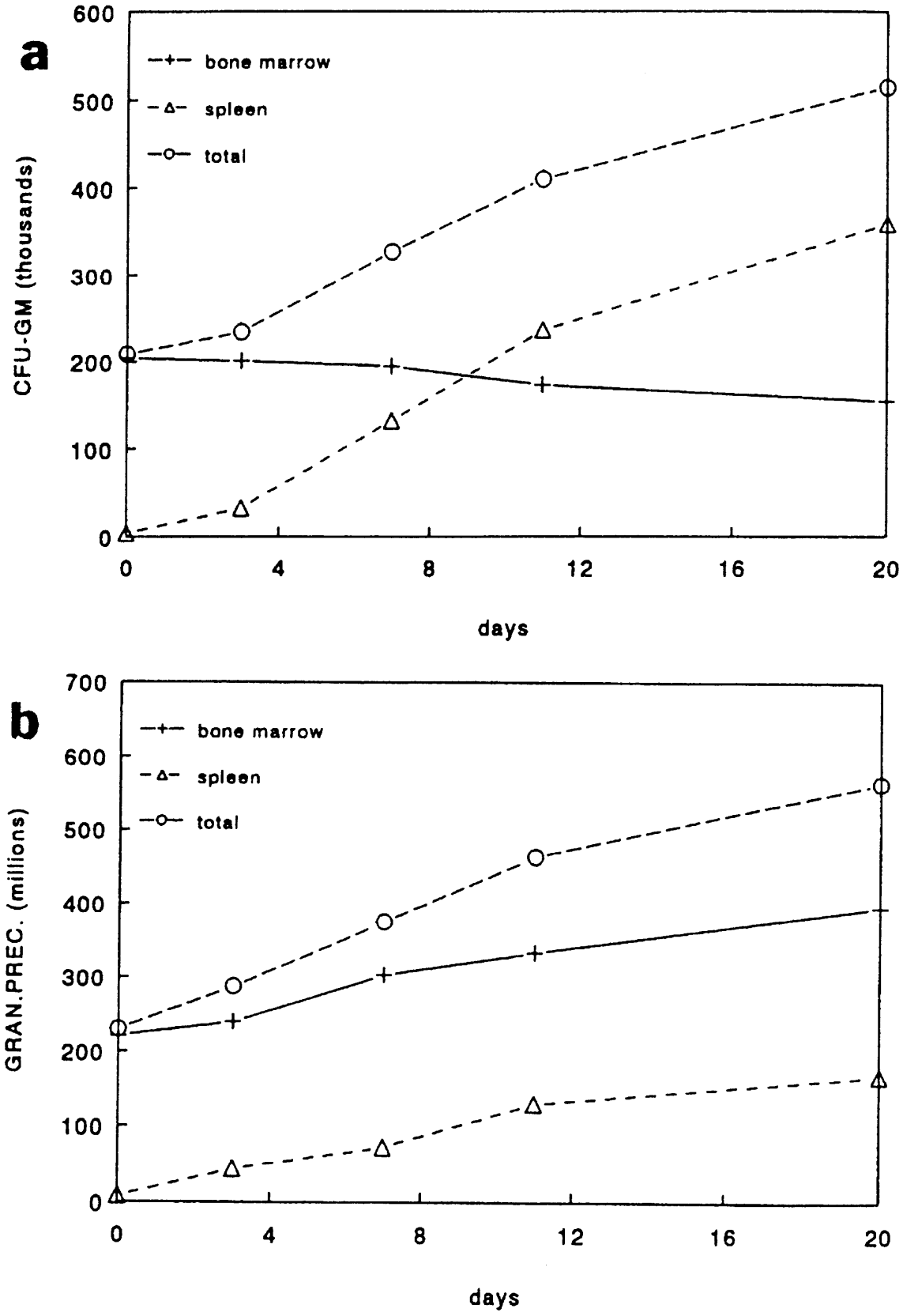

FIGURE 1. Granuloid effects (CFU-GM: **a**; granuloid precursors: **b**) of a G-CSF treatment of mice.

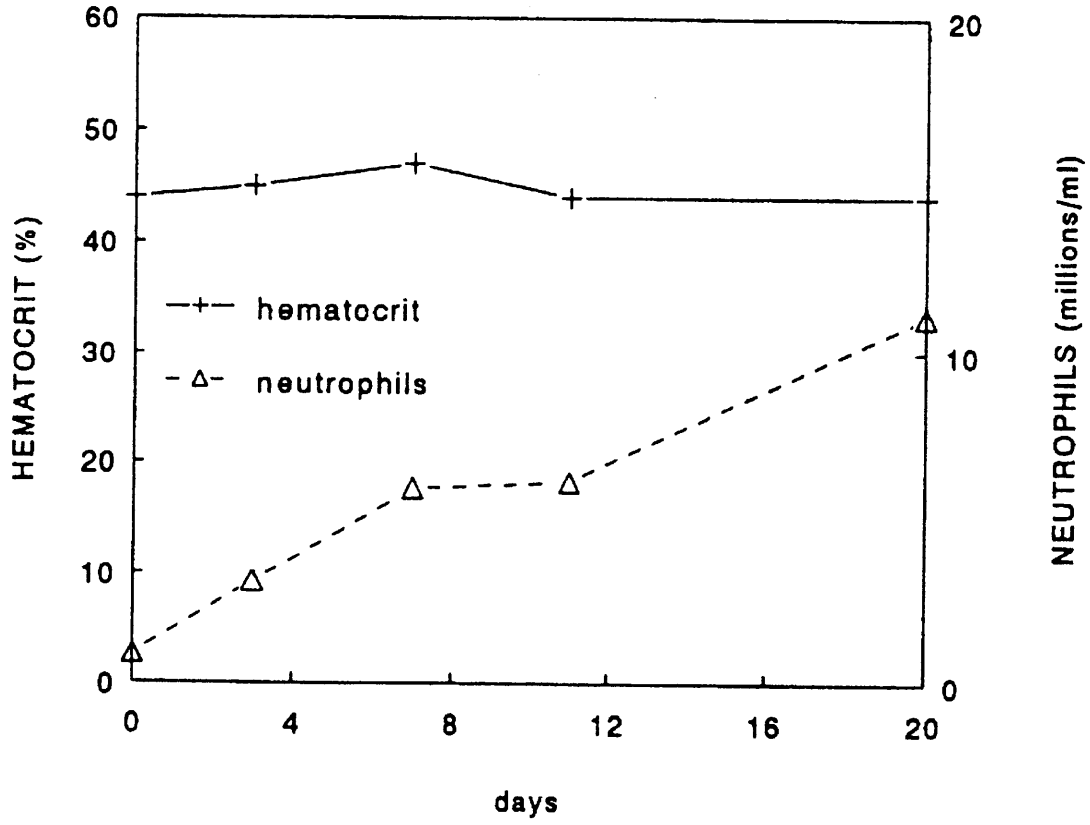

FIGURE 2. Peripheral blood changes during a G-CSF treatment.

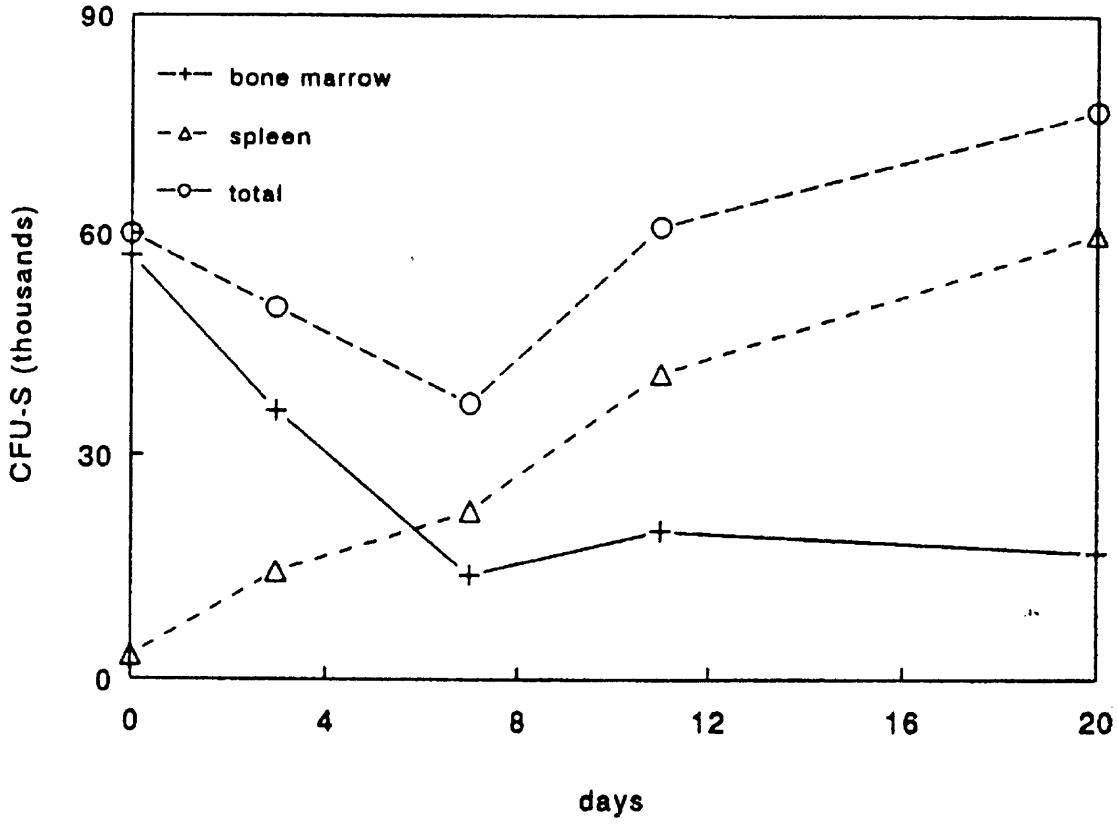

FIGURE 3. Effect of G-CSF on CFU-S-d8.

(generally a decrease) and spleen (increase) are usually considered to be a migrational event between bone marrow and spleen.

C: After a lag phase of seven days, the total numbers of the erythroid progenitor BFU-E increased to 180% of control values after 20 d (FIG. 5a). Again, marrow numbers decreased to 20% and splenic values increased (30-fold). The total numbers of late erythroid progenitors, CFU-E, sharply decreased to 20% after three days of treatment. Within three weeks, the numbers increased again to subnormal levels. This inhibitory effect is totally exerted in the marrow, where CFU-E's practically disappeared after seven days. Splenic numbers rose to 20 times the initial values (FIG. 5b). The net effect of these inhibitory and stimulatory events on erythropoiesis did not cause a change in the hematocrit (FIG. 2).

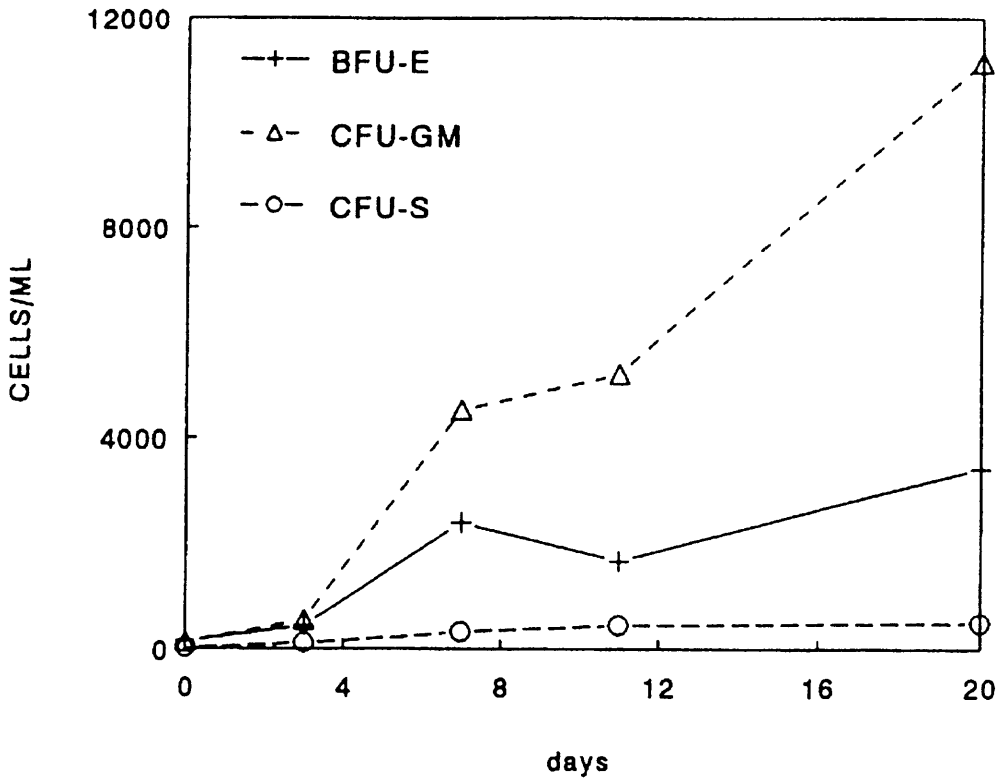

FIGURE 4. Numbers of peripheral blood progenitors and CFU-S during a G-CSF treatment (day 0 values: ○, 30; +, 147; △, 140).

The Amelioration of G-CSF–Induced Inhibitory Effects by EPO

In the next set of experiments, G-CSF (150 μg/kg/d) and EPO (50 U/mouse/d) were simultaneously administered for 10 days to normal mice. Because the effects were most pronounced in the CFU-E compartment, only these are given in comparison with an EPO or G-CSF treatment alone (FIG. 6 and FIG. 5b). The increase in marrow CFU-E to 400% on day 2 by an EPO treatment alone remained constant after 2 days (FIG. 6a). The same increase was also found in the combination treatment, but after day 2 a decrease to 20% of normal values was observed,

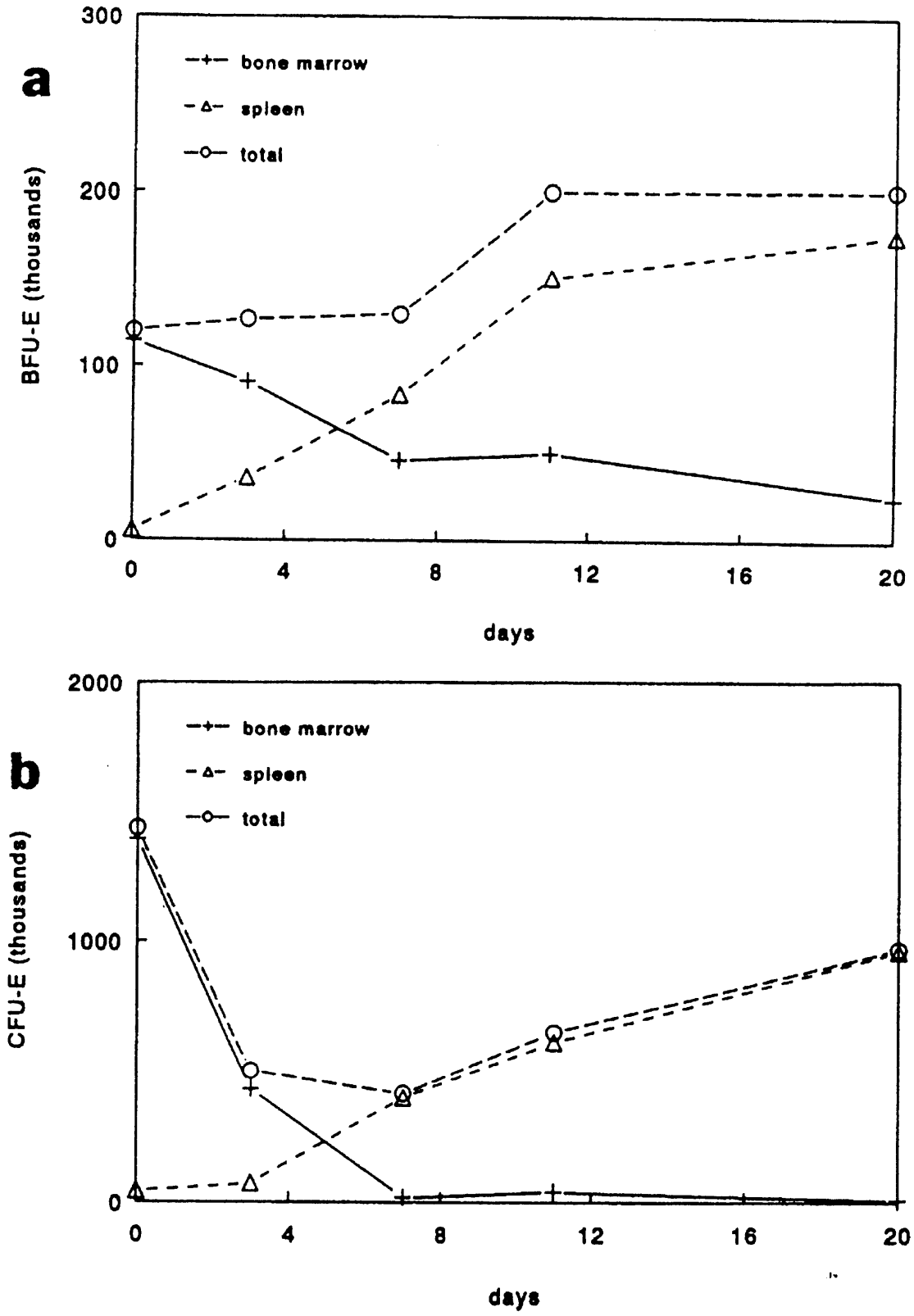

FIGURE 5. Erythroid effects (BFU-E: **a** and CFU-E: **b**) during G-CSF administration.

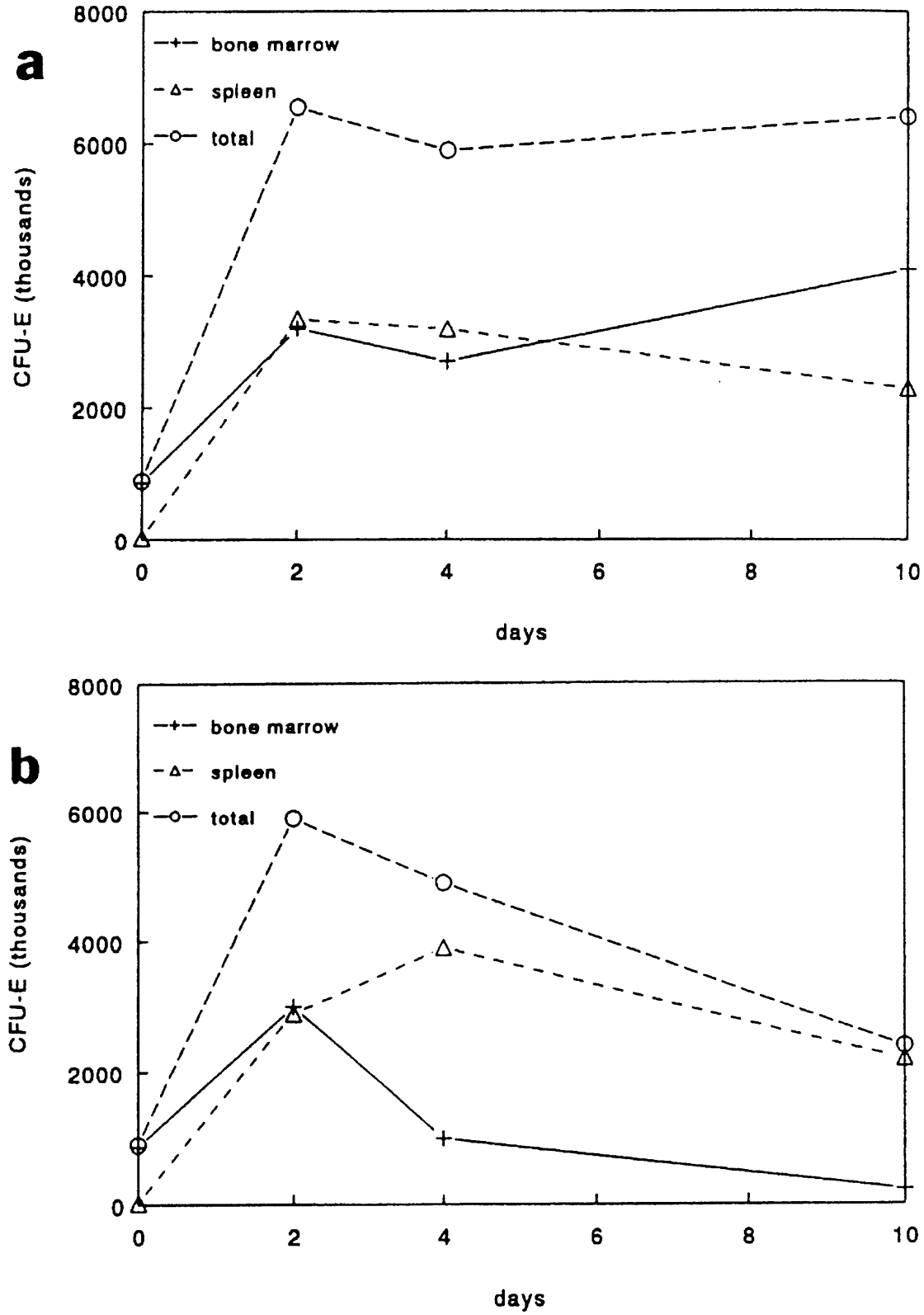

FIGURE 6. Effects of a 10-day EPO (50 U/d) **(a)** or G-CSF (150 μg/kg/d) and EPO **(b)** treatment on CFU-E numbers in the marrow and spleen.

which remained considerably above values of G-CSF alone (FIG. 6b and 5b). The abundant response of CFU-E numbers to EPO in the spleen was similar in the combined treatment (≈150-fold). Over the period of 10 days, the total body response of CFU-E remained above normal values. The increases in reticulocyte numbers and hematocrit were similar for EPO, and EPO plus G-CSF treatments (FIG. 7).

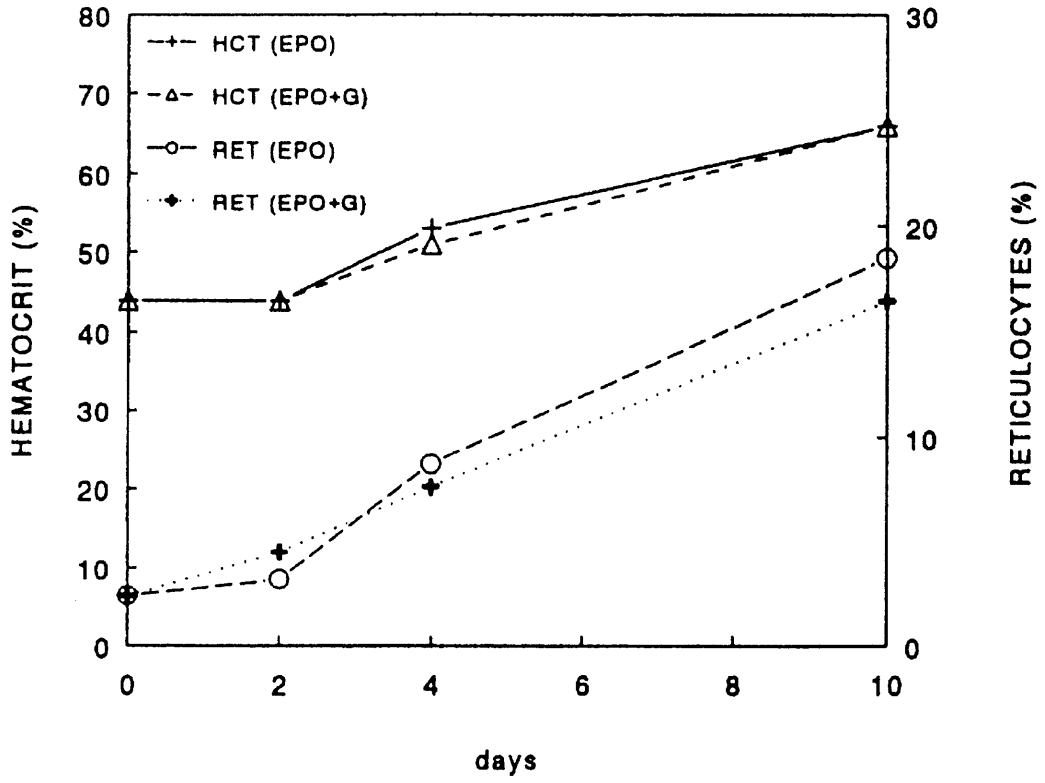

FIGURE 7. Changes in the hematocrit (HCT) and reticulocytes (RET) during a 10-day EPO (or G-CSF + EPO) treatment.

The Effect of Splenectomy on Growth Factor–induced Changes on Erythropoiesis

The inhibitory effects of G-CSF on femoral erythropoiesis of normal mice were largely compensated for by increased splenic production. We wanted to investigate to what extent the removal of the spleen could improve the negative effects in the marrow. G-CSF (doses 10, 25, and 100 μg/kg/day) and EPO (doses 0.5, 5, and 50 U/kg/day) were administered in all combinations to splenectomized mice for seven days. FIGURE 8 shows the effects on CFU-E numbers after seven days. Whereas EPO dose-dependently increased the number of CFU-E above values in normal mice, G-CSF dose-dependently decreased stimulated CFU-E formation to levels even below those of normal mice (cf. FIG. 6).

Hematocrit values decreased through G-CSF, from 45 to 41 percent. This light anemic condition can be normalized with high EPO (FIG. 9).

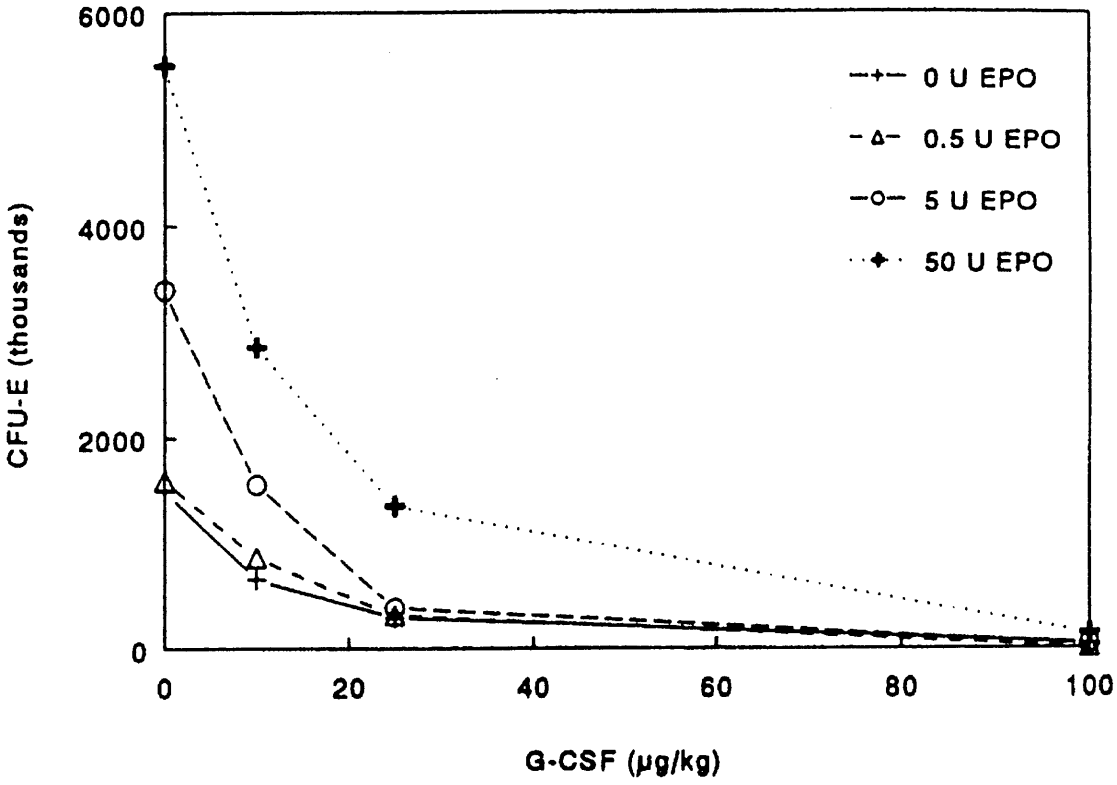

FIGURE 8. Changes in marrow CFU-E numbers of splenectomized mice after a 7-day (EPO + G-CSF) treatment.

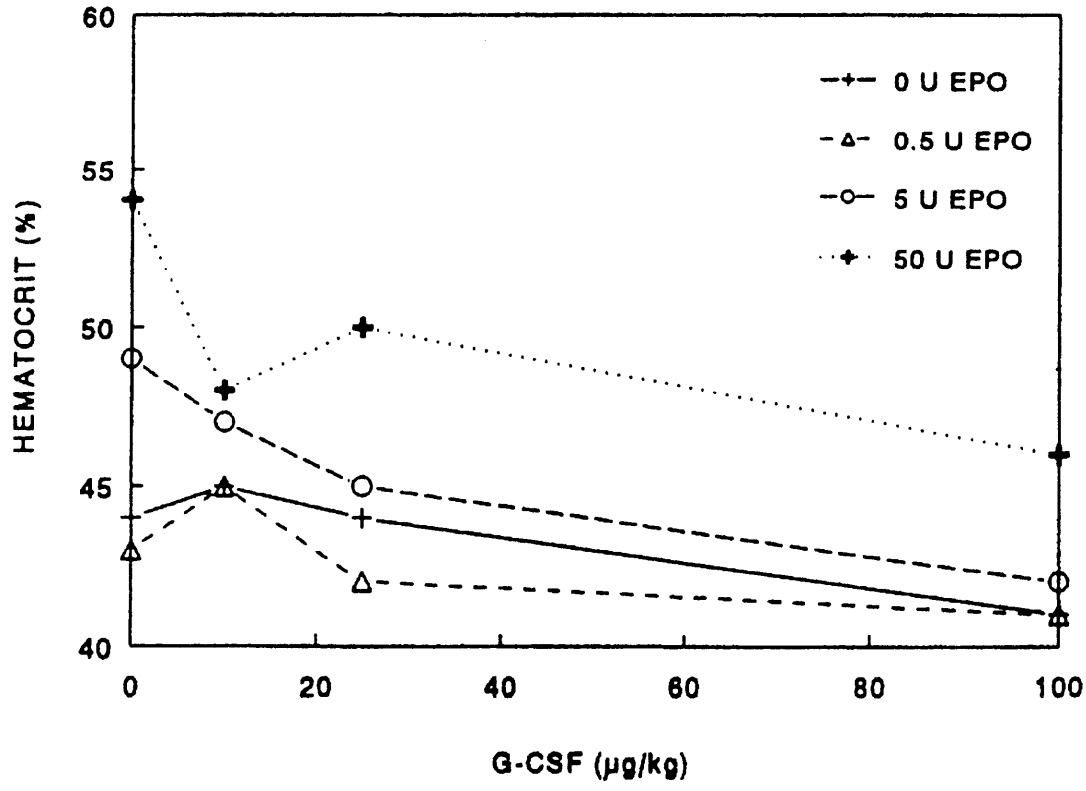

FIGURE 9. Changes in the hematocrit of splenectomized mice after a 7-day (EPO + G-CSF) treatment.

Efficiency of CFU-E Production

Because the CFU-E is the descendant of the BFU-E, the ratio of CFU-E/BFU-E can be considered as an indicator for the production of numbers of CFU-E per BFU-E. We compared this ratio of bone marrow and spleen cells from mice after different treatments with growth factors (TABLE 1). Normal marrow and spleen ratios being similar increased 4- and 30-fold, respectively, during an EPO treatment. A G-CSF treatment severely reduced the ratio in marrow (to 3%) and to a lesser extent in the spleen (to 40%). In splenectomized mice, the marrow efficiency increased to 260% of that in normal mice (12 → 31). In EPO-treated splenectomized mice, the ratio rose to 600% (50 → 300). These values approximate the splenic values of normal EPO-treated mice. G-CSF dose-dependently decreased this value (300 → 13).

TABLE 1. Efficiency of CFU-E Production[a]

Treatment	Organ	CFU-E/BFU-E
None	bone marrow	12
None	spleen	12
[b]EPO (10 d, 50 U)	bone marrow	50
EPO (10 d, 50 U)	spleen	360
[c]G-CSF (16 d, 150 μg)	bone marrow	0.4
G-CSF (7 d, 150 μg)	spleen	5
Splenectomy	bone marrow	31
Splenectomy, EPO 7 d, 50 U	bone marrow	300
Splenectomy, G-CSF 7 d, 150 μg	bone marrow	4
Splenectomy, EPO 50 U + G-CSF 10 μg	bone marrow	55
Splenectomy, EPO 25 μg	bone marrow	24
Splenectomy, EPO 100 μg	bone marrow	13

[a] Growth factor in amount per day per mouse [b] or per kilogram.[c]

EPO Sensitivity of Different Marrow and Spleen CFU-Es

The different behavior of marrow and spleen erythropoiesis of G-CSF–treated mice led us to examine the sensitivity of CFU-Es from different sources to EPO *in vitro*. FIGURE 10 shows a highly sensitive CFU-E from normal bone marrow (R_{50} (half maximal response) = 50 mU). G-CSF drastically increased this value to 200 mU: the CFU-E were much less sensitive. Splenic values were in between (R_{50} = 100 mU), but no difference could be detected between CFU-E from normal and G-CSF–treated mice.

DISCUSSION

As we and others[3–7] have shown previously, G-CSF has multiple effects on hemopoiesis. Besides the positive actions on granulopoiesis, a migration of early progenitors was induced from the marrow to the spleen, which had consequences

for the production of erythroid cells and granuloid cells. The inhibitory effects on erythropoiesis and specifically on the CFU-E formation could be compensated for by increased splenic erythroid activity through migration of BFU-E (and CFU-S). No anemia developed. This inhibition in marrow erythropoiesis could only be partly overcome by simultaneous EPO administration. The rise in hematocrit by EPO was not affected by G-CSF. When the spleen as an erythroid production site was missing, a mild anemia occurred, which could be prevented by a high dose of EPO, at least for seven days. Longer treatments would also possibly lead to anemia. So only under this extreme situation where hemopoiesis was completely confined to the marrow, erythroid inhibition became apparent in the peripheral blood. It is very unlikely that the inhibitory effects could be attributed

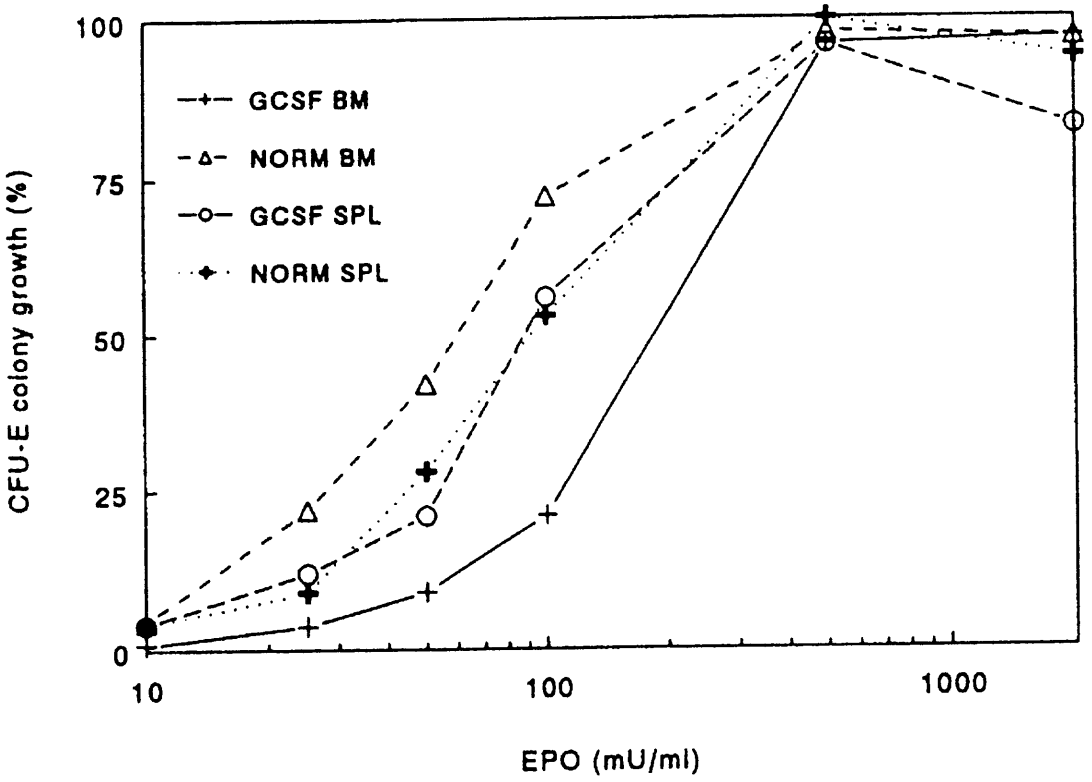

FIGURE 10. EPO-dose response of CFU-E from bone marrow and spleen of normal and G-CSF–treated mice.

to a competition for stem cells between granulopoiesis and erythropoiesis. Besides a total body increase in granuloid progenitor numbers during a G-CSF treatment, also BFU-E numbers increased, whereas the CFU-S numbers hardly changed. Furthermore the decrease in BFU-E numbers in the marrow was similar to that of the CFU-S (the ratio of BFU/CFU-S did not change). This should not occur when the number of CFU-S would have been rate limiting. Humans (AIDS patients) treated simultaneously with EPO and G-CSF recovered from anemia and neutropenia, indicating no competition at an early common stem-cell level.[9] Probably the G-CSF treatment triggers more CFU-S into cycle, so that total numbers of CFU-GM as well as those of the BFU-E can rise. From our experiments, it is clear that the BFU-E–CFU-E transition is somehow affected by a G-CSF treatment. The

ratio CFU-E/BFU-E could be greatly influenced by the growth factors, being high in the spleen after an EPO treatment and low in the marrow after G-CSF. Splenic values in the marrow were only observed after splenectomy during an EPO treatment. Only under these conditions was granulopoiesis also partly inhibited (granuloid precursors and peripheral neutrophils; results not shown here). The CFU-E/BFU-E ratios appeared to be quite different in marrow and spleen, indicating a much more efficient erythropoiesis in the spleen than in the marrow. Differences between marrow and spleen erythropoiesis also were observed *in vitro* in an EPO sensitivity assay. Whereas splenic CFU-E from normal or G-CSF–treated mice had similar sensitivities, marrow CFU-E from G-CSF–treated mice had a much lower sensitivity than those from normal mice. There is no absolute correspondence between the ratios CFU-E/BFU-E (advocated as efficiency of production) and the CFU-E sensitivities to EPO. Normal marrow CFU-E have a high sensitivity, but the ratio CFU-E/BFU-E is normal. Marrow CFU-E from G-CSF–treated mice have a low sensitivity and a very low ratio. The decrease in EPO sensitivity possibly can be explained by a change in EPO receptor properties or numbers. Other growth factors (GM-CSF) have been shown to be able to induce a down-regulation of EPO receptors in a cell line.[10] G-CSF could change receptor numbers or affinity of M-CSF (decrease) or IL-1 (increase) in murine bone marrow cells.[11] The shift in the EPO sensitivity has a remarkable resemblance to the sensitivity curves recently described by Nakamura *et al.*[12] for EPO-sensitive cell lines. Two different forms of EPO receptors, the full-length form with a high sensitivity and a truncated form with a low sensitivity, were responsible for this shift. If those results would be extrapolated to ours, it would imply that in G-CSF marrow only the truncated form would be present and that in normal marrow mostly (only?) the full-length EPO receptor would be present. It remains mysterious why such an effect would only become manifest in the marrow and not (or to a much lesser extent) in the spleen. Furthermore, the effect seems to be restricted to the *in vivo* situation because G-CSF (100 ng/mL) added to *in vitro* cultures of CFU-E or BFU-E did not appear to be inhibitory to colony growth (own observations). Another explanation for inhibitory effects on erythropoiesis could be that G-CSF induced inhibitory factors, which only acted locally and had a different composition in the marrow and the spleen (microenvironmental differences).

The different possibilities for the explanation of the multiple effects of G-CSF on erythropoiesis remains subject for further research.

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DISCUSSION OF THE PAPER

FU-KUEN LIN (*Amgen, Thousand Oaks, CA*): What kind of G-CSF are you using? Human?

WILLEM NIJHOF (*State University of Groningen, Groningen, The Netherlands*): Yes.

LIN: It is known that human G-CSF has very little activity in mice. That is why you have to use such a high dose. I'm wondering if you are actually generating antibody against the G-CSF, which will have an effect on your results. The other question is, What are your culture conditions for your CFU-E?

NIJHOF: You say that there may be antibody formation against the G-CSF, but we find granulopoiesis is not inhibited. In fact, it is increased. So if there had been a lack of natural G-CSF by this antibody formation, I would have expected a decrease in granulopoiesis, and not of erythropoiesis. The observed results are not in agreement with antibody formation.

LIN: Have you looked at this over a long time period?

NIJHOF: No; 20 days was the maximum.

CHAIRMAN BERNHARD KUBANEK (*University of Ulm, Ulm, Germany*): Dr. Nijhof, in the splenectomized animals, you showed that there was a decrease of the hematocrit in 20 days, which was very moderate. I wonder how your reticulocyte counts were in these animals, because if you don't have any CFU-E, the transit time from BFU-E to the reticulocyte has to be shortened, if you get such a slight decrease of the hematocrits. This is puzzling in this model.

NIJHOF: In these mice, the period was shorter than 20 days, actually 7 or 10 days, and, as you saw, the hematocrit went down slightly, from 45 to 41. All the other immature cells, for instance erythroid precursors, went down as well.

CHAIRMAN KUBANEK: So you had a decreased absolute reticulocyte count?

NIJHOF: Right.

IVAN N. RICH (*University of Ulm, Ulm, Germany*): Willem, even though you got a decrease in GM in the bone marrow, what was the effect on the progeny of the GM? Did myeloid cells increase in the bone marrow? If you have an increase of myeloid cells in the bone marrow, you have a decrease of both BFU-E and CFU-E in the bone marrow. So couldn't these myeloid precursors be pushing out the BFU-E and CFU-E? After all, there isn't much room in the marrow.

NIJHOF: I don't think so; I think that the migration phenomenon is not primarily dependent on space but is a more active process. Every stimulating disturbance in an organism gives you such a migrational wave and is totally independent of how many cells are there. The progenitors are the only migrating cells. They make up 0.1–0.2% of the marrow cells; by leaving they are not creating space. The large increase (about 75%) in cell numbers occurs in the myeloid compartment, but these cells do not migrate.

W. DAVID HANKINS (*The Johns Hopkins University School of Medicine, Baltimore, MD*): I probably didn't catch something, and maybe Mark Koury will want to comment, but a few years ago when I was at Vanderbilt, we were trying to

stimulate target cells for SFFV, and Mark came up with a scheme that the best way to get target cells was to treat the animals with LPS. During those experiments, he noted something that we were calling migration of intermediate BFU-E from the marrow to the spleen. I don't know whether there is such a thing as simple migration, but could your decrease in the BFU-E be due to inhibition in the marrow rather than migration?

NIJHOF: No. Inhibition of BFU-E in the marrow is not compatible with increased numbers in blood and spleen. Migration of BFU-E from bone marrow via the blood to the spleen is. Nevertheless we find that 20% of the BFU-E remain in the marrow, whereas all CFU-E disappear. So the point is, I think that somehow the transition between BFU-E and CFU-E is inhibited. A lack of space as an explanation for the observed migration phenomenon is unlikely, as I explained before. The space concept would have been attractive, if the decrease only had occurred in the most abundant erythroid cell compartment, the erythroblasts.

HANKINS: I'll make one other quick comment regarding what I learned about LPS. One of the first things that happens after LPS administration is a substantial increase in serum G-CSF. Also, Makao Ogawa showed that G-CSF was one of a few cytokines that would put stem cells into cycle. Do you have any thoughts on what, if anything, G-CSF has to do with your observation?

NIJHOF: We didn't measure thymidine kills and things like that, so I cannot answer your question exactly. But I think that the CFU-S are triggered into cycle, producing more CFU-GM and BFU-E, and that continues.

CHRISTIAN BREYMANN (*University Hospital, Zurich, Switzerland*): In treating an anemic cancer patient with G-CSF, will you suppress his erythropoiesis?

NIJHOF: I have asked many clinicians if a human being is able to switch from bone marrow to spleen erythropoiesis. If I could extrapolate my results to the human being, assuming that splenic erythropoiesis is also possible, then I would say there would be no harm at all. As I showed with the mice, an inhibition was only observed in the peripheral blood when the mice had been splenectomized and when both processes had to be confined to the marrow. Only then did we see this decrease in hematocrit, and not in the other situation, where splenic erythropoiesis can completely take over the inhibition in the marrow.

BREYMANN: But the cancer patient shouldn't be anemic if the spleen takes over?

NIJHOF: That is what I don't dare to say, but what I would like to find out from the clinicians. If you find an enlarged spleen, does it always mean that there has been an increase in erythropoiesis?

CHAIRMAN KUBANEK: It is very difficult to study, because either you do it with tracer studies or you can look at myeloid precursors in peripheral blood. In fact, we did this after bone marrow transplantation, and we had some indication that there is extramedullary hemopoiesis under stress conditions. It is minute, however, and not very important, certainly not as important as in a mouse, because a mouse has its hemopoiesis in a very confined space. They have no place to expand, whereas humans do have space to expand. I think this might be one explanation for some of the findings, and some of the difference between humans and mice.

MARK J. KOURY (*Vanderbilt University, Nashville, TN*): I wanted to ask about the EPO-responsive curves and the culture conditions. It seems to me that because CFU-E are grown among other cells from the bone marrow you have effects of the products of those cells rather than effects that are intrinsic within the cell. It may be a different sensitivity, but only under the conditions of the culture.

NIJHOF: Of course, you are completely right. The possibility of microenvironmental differences between marrow and spleen requires attention.