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The Kinetics of Murine Hematopoietic Stem Cells In Vivo in Response to Prolonged Increased Mature Blood Cell Production Induced by Granulocyte Colony-Stimulating Factor

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Because of the complexity of appropriate stem cell assays, little information on the in vivo regulation of murine stem cell biology or stemmatopoiesis is available. It is unknown whether and how in vivo the primitive hematopoietic stem cell compartment is affected during a continued increased production of mature blood cells. In this study, we present data showing that prolonged (3 weeks) administration of granulocyte colony-stimulating factor (G-CSF), which is a major regulator of mature granulocyte production, has a substantial impact on both the size and the location of various stem cell subsets pools in mice. We have used the novel cobblestone area forming cell (CAFC) assay to assess the effects of G-CSF on the stem cell compartment (CAFC days 7, 14, 21, and 28). In marrow, in which normally 99% of the total number of stem cells can be found, G-CSF induced a severe depletion of particularly the most primitive stem cells to 5% to 10% of normal values. The response after 7 days of G-CSF treatment was an increased amplification between CAFC day 14 and 7. However, this response occurred at the expense of the number of CAFC day 14. It is likely that the resulting gap of CAFC day 14 cell numbers was subsequently replenished from the more primitive CAFC day 21 and 28 compartments, because these cell numbers remained low during the entire treatment period. In the spleen, the number of stem cells increased, likely caused by a migration from the marrow via the blood, leading to an accumulation in the spleen. The increased number of stem cells in the spleen overcompensated for the loss in the marrow. When total body (marrow and spleen) stem cell numbers were calculated, it appeared that a continued increased production of mature granulocytes resulted in the establishment of a higher, new steady state of the stem cell compartment; most committed stem cells (CAFC day 7) were increased threefold, CAFC day 14 were increased 2.3-fold, CAFC-day 21 were increased 1.8-fold, and the most primitive stem cells evaluated, CAFC day 28, were not different from normal, although now 95% of these cells were located in the spleen. Four weeks after discontinuation of the G-CSF treatment, the stem cell reserve in the spleen had returned to a normal level, whereas stem cell numbers in marrow had recovered to values above normal. This study shows that the primitive stem cell compartment is seriously perturbed during an increased stimulation of the production of mature blood cells. Furthermore, it shows that intricate regulatory feedback loops exist within the stem cell compartment that will enable proper adaptations to stress situations.

GRANULOCYTE colony-stimulating factor (G-CSF) is a prime regulator of in vivo granulopoiesis. This growth factor is now widely administered to patients who recover from chemotherapy or radiotherapy. It has been proven to be able to shorten the neutropenic period in these patients because it can increase the amplification of immature granuloid cells in vivo.[1] The mechanism of this increased amplification can probably be attributed to multiple actions. First, G-CSF may increase the cycling activity of granuloid progenitor cells by shortening their cell cycle time.[2-4] Second, G-CSF may reduce the average transit time of the granuloid compartment.[3,4] Finally, G-CSF may prevent apoptosis of responsive granuloid cells.[5] The exact contribution of each of these parameters in the regulation of in vivo granulocyte production has not yet been assessed. However, whatever the mechanism may be, a prolonged increased granulocyte production will not only affect granuloid cell stages, but also, given the intricate regulatory control processes of in vivo hematopoiesis, will influence other cell compartments. We have previously shown that in vivo G-CSF–stimulated granulopoiesis inhibits erythropoiesis.[6,7] It is conceivable that a prolonged increased production of granulocytes also has an impact on stem cells, because this may indirectly affect the cell flow out of the stem cell compartment. Therefore, it is surprising to see that little is known of the effects of a sustained, enhanced production of mature blood cells on the stem cell compartment. More generally, it is unknown how stem cell proliferation and differentiation, tentatively called stemmatopoiesis, is regulated and what role cytokines play in this process. This lack of knowledge has led to the logical concern as to whether stem cell exhaustion may occur when hematopoietic growth factors are administered to patients.[8] In a murine model it has been shown that G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF), administered during several cycles of cyclophosphamide therapy, significantly reduced marrow transplantation potential, indicating a reduced number of stem cells.[9] Cronkite et al[10] have shown that 1 month after cessation of long-term (128 days) G-CSF treatment, marrow cells were less capable of rescuing lethally irradiated mice. One of the most studied effects of G-CSF on primitive cells is its ability to induce mobilization of stem cells in the blood for stem cell harvesting. These cells have been shown to consist of colony-forming units spleen (CFU-S),[11] but also more primitive, long-term repopulating ability cells (LTRA) are mobilized.[12] It has not yet been determined whether increased peripheral blood stem cells reflect an overproduction of stem cells in marrow and their subsequent release or

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whether the increased frequency in blood is accompanied by a decrease in marrow. In the present study we were interested to see if the murine stem cell compartment would be perturbed at all during a continued increased generation of mature blood cells and, if so, how the primitive hematopoietic cell population would adapt to such a chronic increased production. To determine the characteristics of the murine stem cell compartments, several techniques are available. The classic in vivo stem cell assays (CFU-S,[13] LTRA,[14] self-renewal determination,[15] and marrow repopulating ability [MRA][16]), which often are more qualitative than quantitative, each measure a specific distinct stem cell compartment and are thereby very animal- and time-consuming and labor intensive. Recently, a novel, miniaturized in vitro stem cell assay, the cobbles tone area forming cell (CAFC) assay, has been developed by Ploemacher et al.[17,18] In several reports, the correlation between stem cell subsets as measured by the CAFC assay and the various in vivo stem cell assays has been shown to be very high.[18-22] Because this assay now enables a detailed quantification of all stem cell subsets, we have used this method to assess if and how long-term administration of G-CSF affects the number of cells in bone marrow and spleen and thus in the total animal.

MATERIALS AND METHODS

Mice. In all experiments, female C57Bl/6 mice 12 to 16 weeks old and weighing 20 to 25 g were used. Each data point in the figures was obtained by analyzing individually 3 to 5 mice.

Administration of G-CSF. G-CSF (recombinant human; donated by Amgen, Thousand Oaks, CA) was appropriately diluted and administered by subcutaneously implanted osmotic mini pumps (type Alzet 1007D or 2002; Alza Corp, Palo Alto, CA) to avoid high variation in serum G-CSF levels. To test whether the implanta tion of the pump by itself affected hematologic values, we assessed the effect of a 7- and 14-day implantation of a pump filled with saline. No changes compared to normal, untreated mice were observed (data not shown).

G-CSF was infused at a dose of 2.5 μg/day. This dose results in maximal peripheral blood granulocyte production in our hands. G-CSF was administered for 7, 14, and 21 days.

Preparation of bone marrow and spleen cells. Mice were killed at the times indicated and the spleen and one femur were prepared. Bone marrow cells were collected by flushing the femur three times with 1 mL of α-medium (GIBCO, Grand Island, NY) with a 25-G needle. Spleen cells were obtained by gently pressing the spleen through a stainless steel sieve and collecting the cells in 1 mL of α-medium. Single cells were obtained by repeatedly flushing the spleen cells through a 25-G needle.

Calculating marrow, spleen, and total animal cellularity. Femur and spleen nucleated cell numbers were determined with a Coulter Counter (Coulter, Hialeah, FL). Total marrow cellularity was calculated with the assumption that a femur represents 6% of the total marrow, according to Chervenick et al[23] and Briganti et al.[24] Total animal hematopoietic cell numbers were subsequently calculated, as we have reported before,[6] by adding total marrow cellularity and spleen cellularity. Thus, changes in femur and spleen cellularity are reflected in this calculation.

CAFC assay. The CAFC assay was essentially performed as described by Ploemacher et al.[17,18] This assay is based on a limiting dilution type long-term bone marrow culture. A stromal layer was grown in 96-well microtiter plates (Costar, Cambridge, MA) in Dulbecco's modified Eagle's medium (DMEM; GIBCO), 10% fetal calf serum, 5% horse serum, 10^{-5} mol/L hydrocortison, 3.3 mmol/L L-glutamine, 80 U/mL penicilin, 80 μg/mL streptomycin, 10^{-4} mol/L β-mercaptoethanol, 10 mmol/L HEPES, and 25 mmol/L $NaHCO_3$. Instead of using fresh marrow cells as a source of the stromal layer, we used FMBD-1 cells (a preadipocyte cell line derived from C57Bl/6 mice), which have been reported by Neben et al[25] to result in similar CAFC frequencies as fresh marrow. Furthermore, this cell line is used to determine CAFC frequencies in human marrow cell suspensions.[26] Stromal cell layers were allowed to grow confluently in 14 days. Stromal cell layers were overlaid with bone marrow or spleen cells in 6 dilutions, each dilution threefold apart. For the CAFC assay, the medium was switched to 20% horse serum. Normal cells, G-CSF–marrow cells, and G-CSF–spleen cells were overlayed in 81,000 → 27,000 → 9,000 → 3,000 → 1,000 → 333 cells/well dilution series. Normal spleen cells were overlaid in a 729,000 → 243,000 → 81,000 → 27,000 → 9,000 → 3,000 dilution series. Each dilution was plated 15-fold. Twice a week half of the medium in a well was replaced with fresh medium. To assay the entire stem cell spectrum, the appearance of cobblestone areas (colonies of at least 5 small nonrefractile cells, growing underneath the stromal layer) was evaluated at weekly intervals for 4 weeks. It has been extensively described that the frequency of CAFC day 7 exclusively correlates with CFU-G(E)M/CFU-S-day 7, CAFC day 14 correspond to CFU-S day 12, and CAFC day 28 coincide with cells that have LTRA.[18-22]

The limiting dilution analysis to determine the actual CAFC frequency was performed as described by Ploemacher et al.[17,18] In short, individual wells were scored for the presence or absence of a cobblestone area. The percentage of negative wells as a function of the number of cells per well overlaid was used to calculate the absolute frequency of the various stem cell subsets, using the maximum likelihood solution.[27]

RESULTS

Assessing CAFC frequencies. CAFC frequencies were determined as explained in the Materials and Methods. Table 1 gives an example of the scoring procedure and illustrates how the number of marrow or spleen cells overlaid on the stromal cell layer must span a broad range to cover the frequencies of all CAFC subsets.

Table 1. Illustration of CAFC Day 7 and Day 21 Frequency Estimation in Marrow and Spleen Cell Suspensions of a Mouse Treated With G-CSF for 14 Days

No. of Cells per Well	Marrow Cells Scored at Day 7	Marrow Cells Scored at Day 21	Spleen Cells Scored at Day 7	Spleen Cells Scored at Day 21
81,000	0/15	11/15	0/15	0/15
27,000	0/15	14/15	0/15	1/15
9,000	0/15	15/15	0/15	9/15
3,000	3/15	15/15	0/15	10/15
1,000	7/15	15/15	2/15	14/15
333	11/15	15/15	7/15	15/15
Calculated CAFC frequency/10^5 cells	70.6	0.307	214.3	8.08

This table gives an example of how the frequency of the various CAFC subsets is assessed. For each cell dilution, 15 duplicate wells are scored. For each mouse, 180 wells have to be evaluated weekly (marrow, 6 × 15; + spleen, 6 × 15). Given are the number of negative wells for each dilution for a mouse treated with G-CSF for 14 days. Femur cellularity was 34 × 10^6. Spleen cellularity was 460.3 × 10^6.

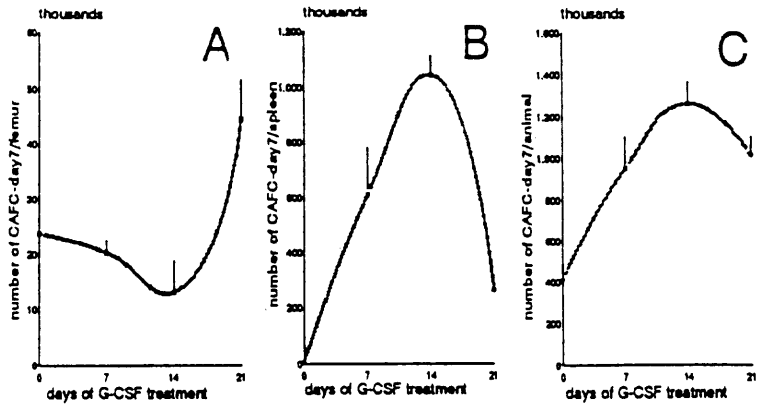

Fig 1. The effect of G-CSF administration on CAFC day 7 in femur (A), spleen (B), and total animal (C). Data are shown as the mean ± 1 SEM.

Effects of G-CSF administration on CAFC day 7. CAFC day 7, the most mature stem cells, behaved oppositely in marrow and spleen. Seven to 14 days of G-CSF administration slightly reduced the number of this cell type in marrow but concomittantly strong increases in the spleen were observed (Fig 1A and B). However, the effects in both marrow and spleen were transient; mice that had been treated for 21 days restored the number of CAFC day 7 in marrow well above normal values, at the expense of splenic CAFC. When total animal CAFC day 7 numbers were calculated, a three-fold increase per animal was found (Fig 1C).

Effects of G-CSF administration on CAFC day 14. Seven and 14 days of G-CSF administration seriously depleted the number of CAFC day 14 cells in marrow (Fig 2A). In the spleen, an accumulation of CAFC day 14 could be shown (Fig 2B). However, continuation of the G-CSF treatment to 21 days again resulted in a reversal of the initial effects. In marrow, CAFC day 14 recovered to normal values at day 21, and in the spleen a decline of cells was found. The time course of total CAFC day 14 numbers differed from that of CAFC day 7 (Fig 2C). After 7 days of G-CSF treatment, a slight decrease of total CAFC day 14 numbers was obtained, but these cells seemed to reach a new steady state at 2.3-fold above normal after 2 and 3 weeks of treatment.

Effects of G-CSF administration on CAFC day 21. In marrow, CAFC day 21 cells were severely reduced at all timepoints (Fig 3A). Toward the end of the treatment, a minor or beginning recovery was observed. In the spleen, strong increases of CAFC day 21 were found (Fig 3B). At 21 days of treatment, no reduction of spleen CAFC day 21 could be shown, which was in contrast to CAFC day 7 and 14. As a consequence, total CAFC day 21 cells were almost exclusively located in the spleen during the treatment. The general pattern of total CAFC day 21 cells was similar to that for CAFC day 14 cells (Fig 3C). However, 3 weeks of G-CSF treatment resulted in a 2.3-fold increase of total CAFC day 14, but total CAFC day 21 cell numbers increased only 1.8-fold.

Effects of G-CSF administration on CAFC day 28. The most primitive stem cells we measured, CAFC day 28, were also continuously severely reduced in marrow (Fig 4A). The loss of marrow CAFC day 28 cell numbers was compensated after 14 and 21 days of G-CSF treatment by splenic increases (Fig 4B). Total CAFC day 28 numbers therefore initially were reduced to 60% of normal, but when the treatment was continued, this decline disappeared and normal cell numbers were restored (Fig 4C).

Effects of G-CSF administration on the ratio between various CAFC subsets in marrow and spleen. Because it was a major aim of this study to determine alterations in the amplification between the distinct stem cell subsets, the ratios of CAFC day 7/14, 14/21, and 21/28 were calculated for marrow and spleen. These ratios give an impression of

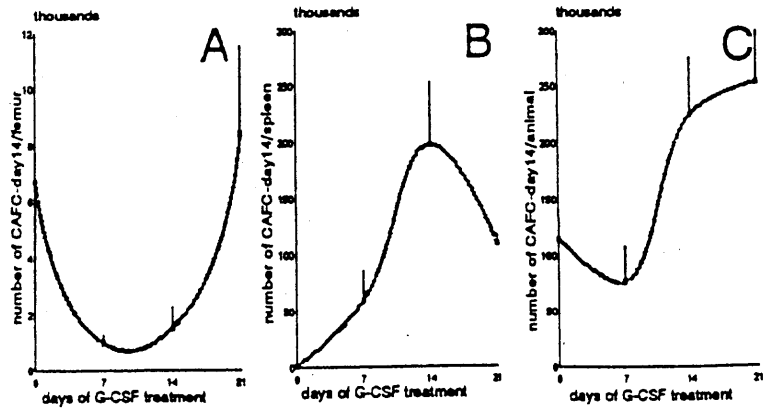

Fig 2. The effect of G-CSF administration on CAFC day 14 in femur (A), spleen (B), and total animal (C). Data are shown as the mean ± 1 SEM.

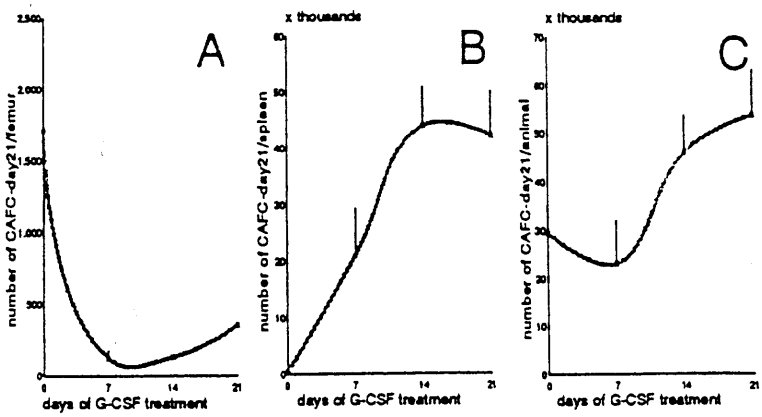

Fig 3. The effect of G-CSF administration on CAFC day 21 in femur (A), spleen (B), and total animal (C). Data are shown as the mean ± 1 SEM.

changes in the amplification between the various cell compartments. Figure 5A and B shows the changes of these ratios in marrow and spleen during 3 weeks of G-CSF treatment. After 7 days, the ratio of CAFC day 7/14 was increased, both in the marrow and in the spleen. However, when the treatment was continued to 21 days, this ratio returned to normal values, but the ratio of CAFC day 14/21 in the marrow was now strongly increased. This phenomenon did not occur in the spleen. The CAFC day 21/28 ratio was hardly affected in the marrow or the spleen.

Behavior of CAFC subsets in marrow and spleen after discontinuation of G-CSF treatment. To determine whether the serious depletion of the earliest marrow stem cells was reversible, the recovery of all CAFC subsets after 14 days of G-CSF treatment was assessed. Figure 6A demonstrates that 4 weeks after G-CSF discontinuation all cell types had indeed recovered. In fact, an overshoot was observed as all subsets reached numbers between 150% to 250% of control values. In the spleen, all subsets returned to normal values at this time point (Fig 6B).

DISCUSSION

It was the aim of these experiments to determine if and how the primitive stem cell compartment would be affected by a continuous increased production of mature granulocytes. Our data provide a general insight into the adaptation of the murine stem cell compartment to a sustained enhanced production of peripheral blood cells. Several major conclusions can be drawn from this study. First, it is evident that administration of G-CSF, which is generally considered to be a lineage-specific, late-acting growth factor, has substantial effects on both the size and the distribution of the primitive hematopoietic stem cell compartment. In marrow, in which normally 99% of total hematopoiesis takes place, G-CSF results in a severe loss of especially the most primitive stem cells. The initial adjustment of the primitive compartment in response to G-CSF is to increase the amplification between CAFC day 7 and 14. The increased total number of CAFC day 7 presumably are required for the enhanced granulocyte production. In marrow, this occurs at the expense of the number of CAFC day 14. However, the decrease of CAFC day 14 numbers in marrow is transient. Replenishment of this compartment, which probably is caused by an increased amplification between CAFC day 14 and 21 (evidenced by the increasing ratio towards the end of the treatment), occurs on its turn at the expense of CAFC day 21 and 28, which remain severely decreased during the treatment period.

The decrease of marrow stem cells can probably be attributed to an increased amplification and concomittant differentiation in more committed cell stages, but also to a massive migration out of the marrow, via the blood, leading to an accumulation in the spleen. Although it has been shown

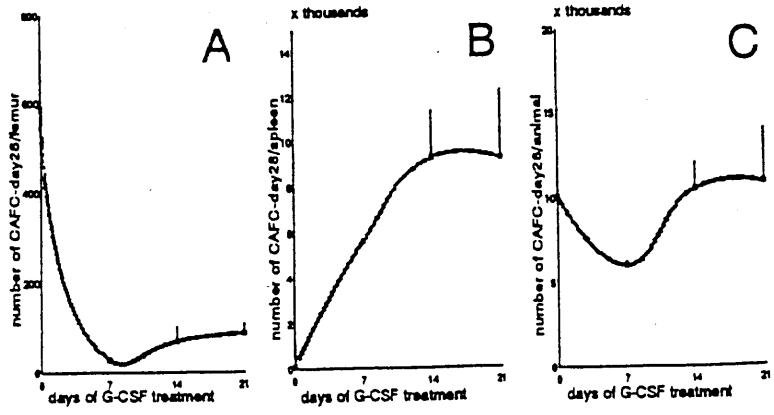

Fig 4. The effect of G-CSF administration on CAFC day 28 in femur (A), spleen (B), and total animal (C). Data are shown as the mean ± 1 SEM.

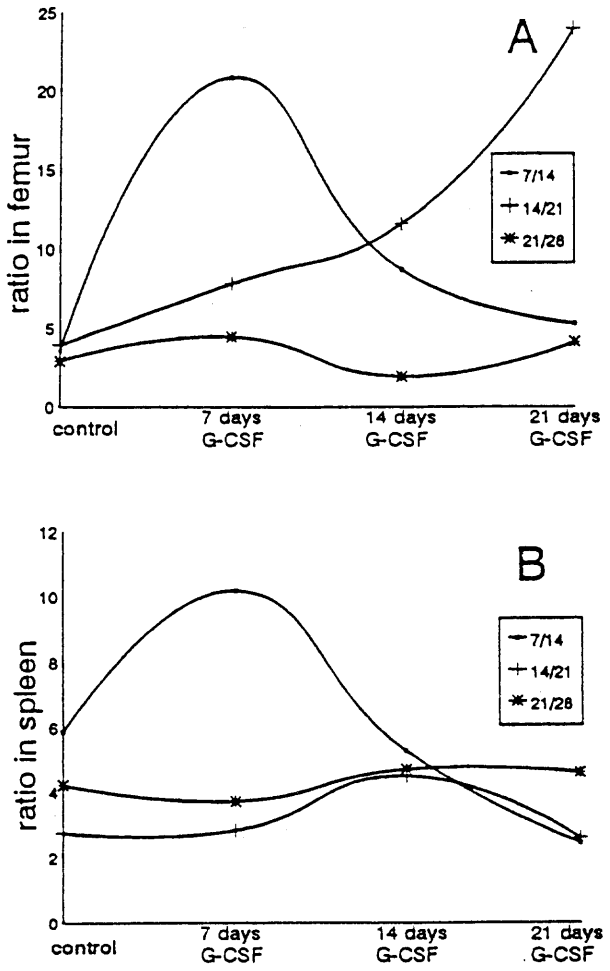

Fig 5. The effect of G-CSF administration on the ratio between the numbers of CAFC day 7 and 14, CAFC day 14 and 21, and CAFC day 21 and 28 in femur (A) and spleen (B).

before that G-CSF and many other agents induce splenic hematopoiesis in mice,[6-28] this has never been properly quantified for the most primitive stem cells. Our data suggest that the mobilization of stem cells by G-CSF is not due to an overproduction of stem cells in marrow, but rather is accompanied by a decreased marrow stem cell reserve. This finding is in agreement with the results of a study reported by Neben et al[21] that show that optimal mobilization induced by cyclophophamide and short-term G-CSF treatment in mice is accompanied by reduced marrow stem cell numbers. In their study, G-CSF alone did not have any impact on marrow stem cells, but it is likely that this discrepancy with our results can be attributed to their short-term (4 days) treatment period. It will be interesting to assess whether the improved mobilization observed by a combination of stem cell factor (SCF) and G-CSF is due to an increased production of primitive cells by SCF and their subsequent release by G-CSF.[29]

Interestingly, our data show that, although G-CSF is continuously administered, splenic stem cell numbers are not constantly increasing. An inverse relationship between the number of stem cells present in marrow and in spleen was observed. CAFC day 7 numbers in marrow increased to values well above normal after 21 days of treatment, which was accompanied by a sharp decline of splenic values. A similar effect was observed for CAFC day 14 numbers. These cells recovered toward normal values in marrow and concomittantly a slight decrease in the level of splenic cells was seen. However, CAFC day 21 and 28 numbers hardly recovered in marrow and here splenic values remained increased. One may only speculate about the origin of the relationship between cells in these two organs and their fate. It is unknown whether backmigration from the spleen to the marrow exists. However, apparently by whatever mechanism, the organism is able to balance the number of stem cells per total animal, irrespective of their location. This finding is reflected in the graphs showing the total number of cells. Although there are major differences between marrow and spleen stem cell content, a prolonged G-CSF treatment gradually resulted in a new steady state of the total stem cell compartment. To meet the increased demand of mature cells, total CAFC day 7 numbers were threefold increased, CAFC day 14 numbers 2.3-fold, CAFC day 21 numbers 1.8-fold, and CAFC day 28 numbers were not different from normal.

We believe that the perturbation of the stem cell compart-

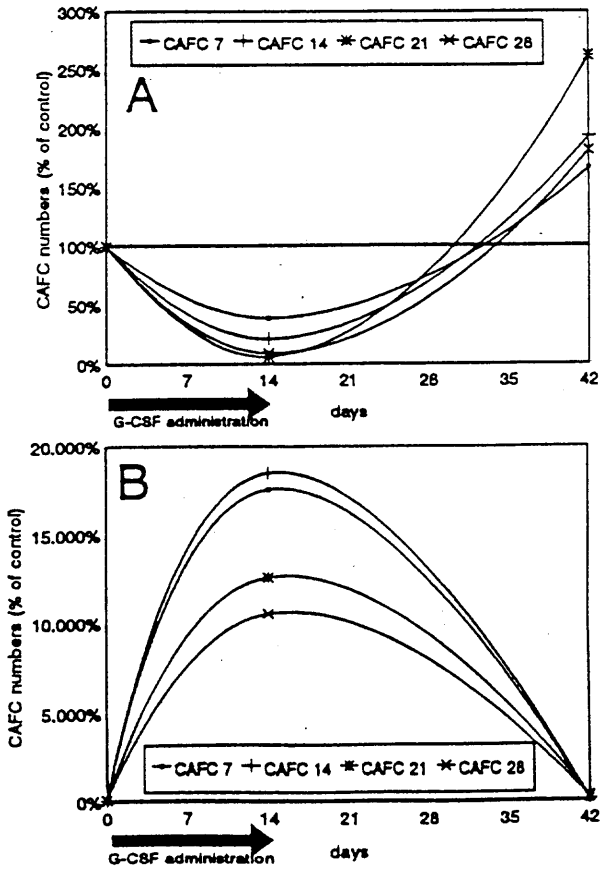

Fig 6. The recovery of CAFC subsets in marrow (A) and spleen (B), after a 14 days of G-CSF administration. Data are shown as the percentage of control.

ment is induced by an indirect effect of G-CSF. In this concept, G-CSF acts on committed granuloid progenitor cells and all observed effects are indirect adaptations of primitive cells to be able to replenish and feed committed compartments. However, another possibility may be that G-CSF, apart from the effects on the granuloid progenitors, also directly stimulates early stem cells. Although G-CSF generally is regarded as a lineage-restricted growth factor, it has been shown that in vitro G-CSF is able to induce cycling activity of quiescent stem cells.[30]

Many topics for further research remain after this study. It will be interesting to determine directly the extent and direction of migration between marrow and spleen via the blood. Furthermore, an even longer administration period or administration of G-CSF to splenec tomized mice may result in additional or different perturbations of the stem cells. In this study, we show that the effects of G-CSF on the stem cell compartment seem to be reversible, although 4 weeks after G-CSF administration stem cell numbers in marrow are still not normalized. It is of interest to assess the nature of this recovery: is replenishment of marrow cells achieved by backmigration from the spleen or is it established by the remaining marrow stem cells? Finally, the question remains as to how these data can be extrapolated to the human situation. Because the CAFC assay has recently been adapted to measure human stem cell subsets,[26] more information on the dynamics of the human primitive compartment can be expected to be published in the near future. This information will be essential to test the safety but also optimize the efficacy of the use of growth factors in the clinic.

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