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Experimental Hematology

Migration of Stem Cells and Progenitors between Marrow and Spleen Following Thiamphenicol Treatment of Mice

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Abstract. Recovery of hemopoiesis was studied after a 3-day treatment with the antibiotic thiamphenicol (TAP). A contrasting behavior of the spleen colony-forming units (CFU-S), granulocyte–macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E), and erythroid colony-forming units (CFU-E) numbers in the bone marrow versus those in the spleen was found. Whereas the cell numbers reached nadirs in the marrow, they peaked 30 to 100-fold above control values in the spleen on day 4. Simultaneously the number of CFU-S, BFU-E, and CFU-GM, but not of CFU-E, increased drastically in the peripheral blood. The tritiated thymidine kill of the splenic CFU-S was too small to explain the endogenous splenic production of these cells. A quantitative analysis further revealed that an effective erythropoiesis was established in the spleen. As a consequence, the first part of a reticulocytosis was mainly due to the splenic contribution, whereas the second part predominantly originated from a delayed marrow erythropoiesis. In contrast, the CFU-GM of the spleen did not effectively differentiate into granuloid precursors. The bulk of the granuloid production occurred in the marrow. The best explanation for these results is a net migration of CFU-S, BFU-E, and CFU-GM from the marrow to the spleen during early recovery, and a back-migration of CFU-GM to the marrow later in the recovery phase. These observations indicate a link between migration of hemopoietic cells and their differentiation at the two hemopoietic sites.

Key words: Hemopoietic stem cells — Hemopoiesis — Migration — Thiamphenicol

Hemopoietic stress is often accompanied by expansion of hemopoiesis to extramedullar sites. The relationship, however, between medullar and extramedullar, i.e., splenic, hemopoiesis is hardly understood.

Between bone marrow and spleen, marked differences in the regenerative behavior of immature hemopoietic cells (spleen colony-forming units [CFU-S], granulocyte–macrophage colony-forming units [CFU-GM], erythroid burst-forming units [BFU-E], and erythroid colony-forming units [CFU-E]) have been reported during recovery of hemopoiesis after hemopoietic stress induced by phenylhydrazine [1–3], treatment with bacterial compounds [4–6], and the administration of hemopoietic growth factors [7–9]. Under these circumstances migration of marrow cells to the spleen was considered to be at least partly responsible for these differences. Recently, a new manipulation was described to change the ratio of marrow to spleen hemopoiesis drastically. The antibiotic thiamphenicol (TAP) appeared to induce an antagonistic behavior of the BFU-E, CFU-E, and CFU-GM in bone marrow and spleen [10]. These observations were suggestive for migration from the bone marrow to the spleen.

In the present study, we focused attention on the quantification of the cell pools involved in generating the differences between bone marrow and spleen. This was an attempt to elucidate whether differences between both hemopoietic sites are due to an exchange, i.e., migration, of hemopoietic cells between bone marrow and spleen, and if so, whether these cells undergo a differentiation and amplification process in the new environment.

Therefore, we followed the recovery of stem cells (CFU-S, day 8) and progenitors (CFU-GM, BFU-E, and CFU-E) in the bone marrow, peripheral blood, and spleen of mice after treatment with TAP. In contrast to former studies [10, 11], the present study was performed without bleeding to circumvent interference of severe anemic stress with recovery.

In a previous report [12], we extensively examined the effects on hemopoiesis during a 4-day TAP treatment, indicating that TAP was an effective suppressor of erythropoiesis and to a lesser extent of granulopoiesis [12]. Here we report on the recovery after termination of TAP application.

Materials and methods

Treatment schedule. Specific-pathogen-free female C57bl/6 mice (Charles River, FRG) between 8 and 12 weeks of age were treated with TAP (Inpharzam S.A., Zambon Group, Cadempino, Switzerland) for 3 days via a s.c. implanted dialysis bag containing 0.5 ml water with 350 mg TAP as described previously [10]. Recovery was studied starting just after removal of the dialysis bag; this was considered day 0 after TAP treatment.

Preparation of femoral bone marrow and spleen cell suspensions. Cell suspensions were prepared as described previously [12] and pooled per group of three mice. The concentration of nucleated cells in the bone marrow and spleen cell suspensions was determined using a Coulter counter.

Peripheral blood cells. Blood of groups of three mice, which were bled from the orbital plexus under ether anesthesia, was pooled in heparinized (50 U heparin per ml blood) tubes. The hematocrit, white blood cell (WBC), and reticulocyte counts were determined

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Table 1. Effect of TAP on different hemopoietic cell stages

Cell stage	Femoral content (× 10^3 cells per femur)[a]			Splenic content (× 10^3 cells per spleen)[a]		
	C[b]	Day 0[c]	Percent[d]	C[b]	Day 0[c]	Percent[d]
CFU-S	5.8 ± 0.4	3.4 ± 0.2	59	3.1 ± 0.2	1.8 ± 0.1	58
CFU-GM	20.6 ± 1.4	45.7 ± 3.5	222	3.9 ± 0.4	9.2 ± 0.8	236
Gran P[e]	11.2 ± 0.4	7.9 ± 0.4	71	5.5 ± 0.4	1.8 ± 0.8	33
BFU-E	4.6 ± 0.5	7.5 ± 0.8	163	4.7 ± 0.6	6.1 ± 0.9	130
CFU-E	53 ± 3	<1	<2	31 ± 5	<2	<6
Ery P[e]	5.2 ± 0.5	0.3 ± 0.1	6	10.9 ± 0.8	4.0 ± 0.5	37

[a] Mean femoral and splenic content of hemopoietic cells ± SEM of three separate experiments.
[b] The untreated control (C).
[c] After 3 days of TAP treatment (day 0).
[d] The content on day 0 as percentage of the control.
[e] The granuloid precursor (Gran P) and erythroid precursor (Ery P) counts as presented × 10^3.

according to standard procedures. The peripheral blood cells were layered on Ficoll–Isopaque (Sigma and Nyegaard, Norway, respectively) with a density of 1.085 g/ml, and the nucleated cells were isolated after centrifugation [13]. The cells were washed three times with α-medium plus 10 mM HEPES, pH 7.2, supplemented with 5% fetal calf serum.

Spleen colony assay. The number of CFU-S was determined according to the method of Till and McCulloch [14] as described previously [12]. The tritiated thymidine ([^{3}H]TdR) kill of these cells was performed according to Becker et al. [15] and was described in detail previously [12].

Culture of progenitors. CFU-GM, BFU-E, and CFU-E were assayed with the methycellulose method of Iscove and Sieber [16] as described previously [12].

Yield of CFU-E from the peripheral blood after density gradient centrifugation. Blood with a high number of CFU-E was artificially prepared by mixing spleen cell suspensions, highly enriched for CFU-Es (post-TAP plus bleeding, day 4 [17]) with a peripheral blood sample of untreated controls (10^6 nucleated spleen cells per ml peripheral blood). This mixture was separated by centrifugation on Ficoll–Isopaque as described above. The yield of CFU-E after centrifugation was calculated as percentage of an incubation control of the spleen cell suspension.

Calculation of the total body cell numbers and the splenic fraction of hemopoietic cells. The total cell number of a particular cell stage was calculated by adding the numbers in the marrow ($17 \times C_{fem}$), the spleen (C_{spl}), and the peripheral blood (1.5 ml, C_{bl}). The fraction of cells in the spleen can be obtained by the formula: $C_{spl/tot} = C_{spl}/(17 \times C_{fem} + C_{spl} + C_{bl})$ [18]. The contribution of the spleen to the reticulocyte numbers is calculated by multiplying the $C_{spl/tot}$ values for erythroid precursors of the previous day with the peripheral blood reticulocyte values, which were corrected for differences in the hematocrit and expressed as percent of control. (We assumed that the blood volume remained constant.)

Statistical evaluations. The mean and standard error of the mean (SEM) were calculated. A two-tailed Student's *t*-test was used for comparison of two means. The level of significance was set at $p = 0.05$.

Results

Effect of a 3-day TAP treatment on hemopoietic cells

After a 3-day TAP treatment, i.e., at day 0 of the recovery phase, comparable changes in the pool size of hemopoietic cells in the femoral bone marrow and the spleen were observed (Table 1). The morphologically recognizable erythroid precursors and in particular the late erythroid progenitor, CFU-E, were found severely suppressed, whereas the granuloid precursors showed a more moderate reduction. A decline of the CFU-S numbers to approximately 60% of the control accompanied a significant increase of the pool size of the early progenitors (BFU-E and CFU-GM). The CFU-GM was substantially elevated to approximately 220% in the femur and to 240% in the spleen. The BFU-E numbers increased to a lesser extent, i.e., to 160% and 130%, in the femur and the spleen, respectively.

Recovery of the femoral and splenic CFU-S, CFU-GM, BFU-E, and CFU-E after the TAP treatment

These cell stages demonstrated a biphasic recovery pattern in the femoral bone marrow (Fig. 1A). After a first rebound from days 0 to 3, a nadir from days 4 to 6 occurred, followed by a second recovery until day 10. The CFU-S and CFU-GM reached the minimum value on day 4, the BFU-E on day 5, and the CFU-E on day 6. The nadir of these hemopoietic cell stages was significantly lower than the control value. The early progenitors, i.e., CFU-GM and BFU-E, exceeded the control value on days 8 and 10, respectively. On day 14 all stages returned to control levels.

The splenic content of the hemopoietic cell stages exhibited a monophasic recovery with a simultaneous peak for all cell stages on day 4 (Fig. 1B). The maximum value on day 4 for the CFU-S and BFU-E was approximately 30 times higher than normal, and the CFU-GM and CFU-E were even 100 times higher than normal control values. On day 14 these cell numbers, except for the CFU-E, had decreased to a level that was still significantly higher than the control value.

[^{3}H]TdR kill of the femoral and splenic CFU-S

Just after TAP treatment (day 0), the kill of both the femoral and splenic CFU-S was significantly elevated with respect to the control (from 16% ± 3% to 38% ± 2% and from 3% ± 1% to 17% ± 2%, respectively; Fig. 2). In general, until day 10 the femoral and splenic CFU-S exhibited a constantly elevated kill, with the exception of day 4 in the marrow. On day 14 the kill returned to the control level. The kill of the splenic CFU-S varied between 10% ± 2% (day 2) and 17%

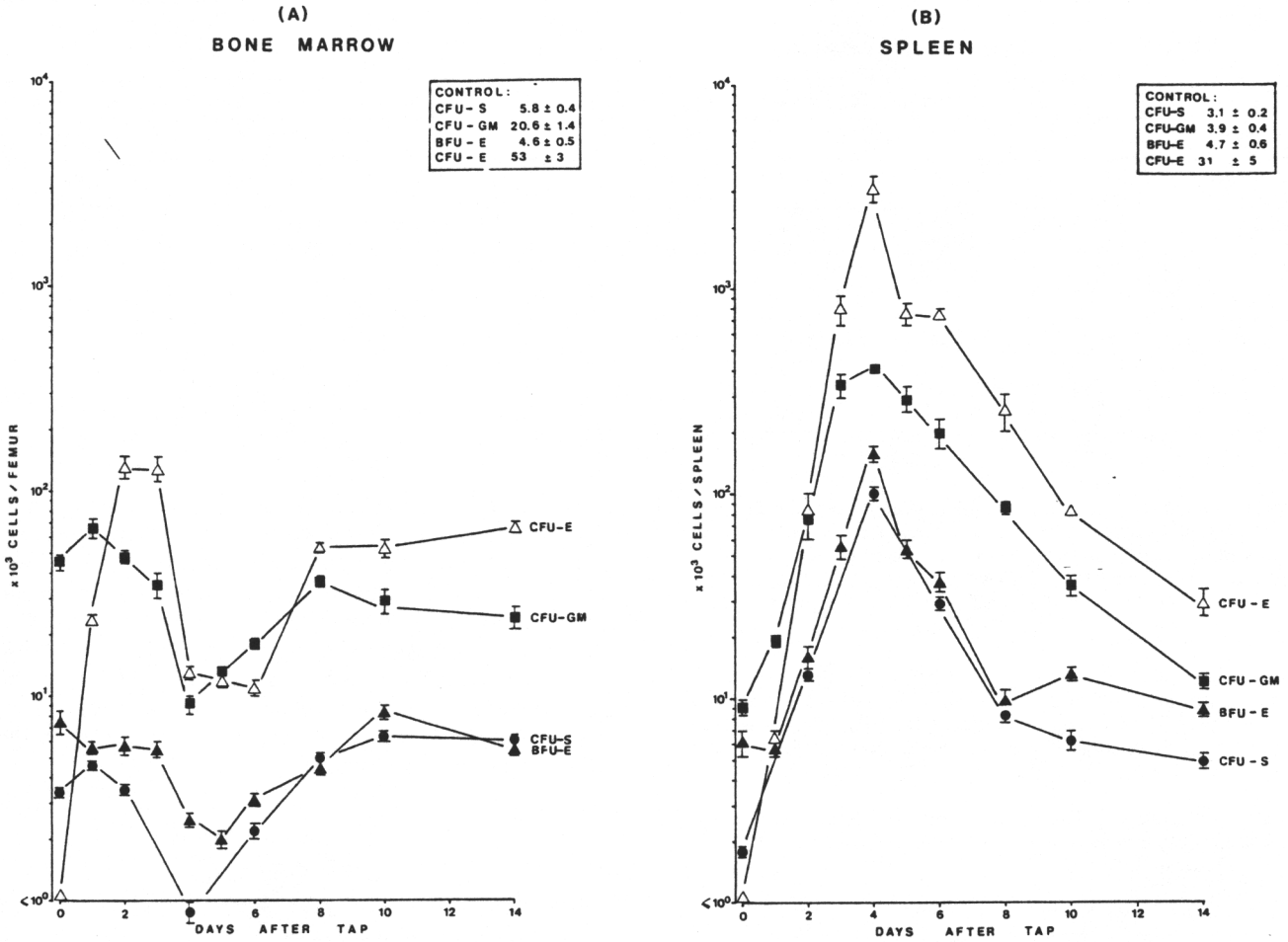

Fig. 1. Recovery of femoral and splenic CFU-S, CFU-GM, BFU-E, and CFU-E. The mean femoral (A) and splenic (B) CFU-S (●—●), CFU-GM (■—■), BFU-E (▲—▲), and CFU-E (△—△) numbers ± SEM of three separate experiments are given. *Insets:* untreated control cell numbers in the femur and the spleen.

± 2% (day 0), and it was significantly higher than the control value on days 0, 4, 8, and 10. At the end of the observation period (day 14), the kill of the splenic CFU-S returned to the control level.

Peripheral blood levels of CFU-S, CFU-GM, BFU-E, and CFU-E

Shortly after the TAP treatment, the numbers of CFU-S, CFU-GM, and BFU-E in the peripheral blood drastically increased (Fig. 3). The maximum values, reached on day 4, were 10–50 times the control values. After 8 days the CFU-S and BFU-E returned to normal values, whereas the CFU-GM numbers were still significantly increased. During the observation period no CFU-E could be detected in the peripheral blood (detection limits 100 CFU-E per ml blood).

Yield of CFU-E after density gradient centrifugation

To test the validity of the Ficoll–Isopaque (density 1.085 g/ml) isolation procedure for CFU-E, splenic cell suspensions highly enriched for CFU-E were added to the peripheral blood of controls and centrifuged on the density gradient. The yield of CFU-E from the blood was similar to that of the splenic cell suspension, separated on the gradient (i.e., 74% ± 5% vs 86% ± 7%), and it was slightly higher than that of the nucleated cells (72% ± 4% vs 59% ± 8%; Table 2), proving that CFU-E were not inactivated during the isolation procedure.

Recovery of the femoral and splenic precursors

Starting from approximately 70% of the control value, the femoral granuloid precursors peaked on day 5 at about 175% of control (Fig. 4A). Between days 10 and 14 a gradual decrease, to the control value, was observed. A steady recovery was observed for the erythroid precursors in the marrow (with an indication of a temporary plateau) reaching the control level at day 10. The splenic granuloid precursors exhibited a very rapid increase, peaking on day 3 at a level of seven times the control. They returned to the control value between day 10 and 14 (Fig. 4B). The erythroid precursors started an even more pronounced increase on day 2 and peaked on day 5 at a level of 18 times the control. The splenic

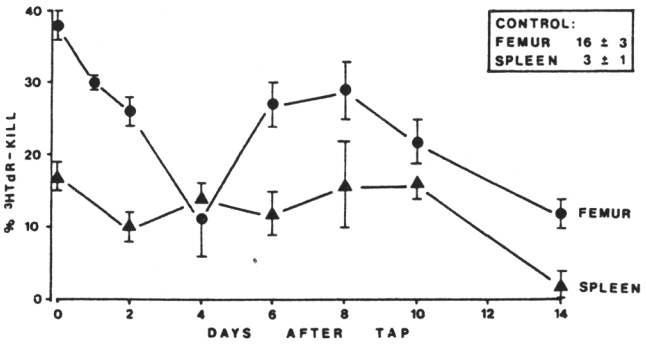

Fig. 2. [³H]TdR kill of the femoral and splenic CFU-S. The mean ($n = 3$) value ± SE of the femoral (●—●) and splenic (▲—▲) CFU-S is presented. *Inset:* control value of the femoral and splenic [³H]TdR kill.

content of erythroid precursors also returned to the control value between day 10 and 14.

Recovery of the hematocrit, reticulocyte, and WBC counts

After an initial drop to 70% of the control value, the WBC peaked on day 5, overshooting the control by 55%. The WBC returned to the control level on day 8 (Fig. 5).

The reticulocyte counts stayed at a very low level from day 0 to 3. Subsequently a reticulocytosis occurred, peaking

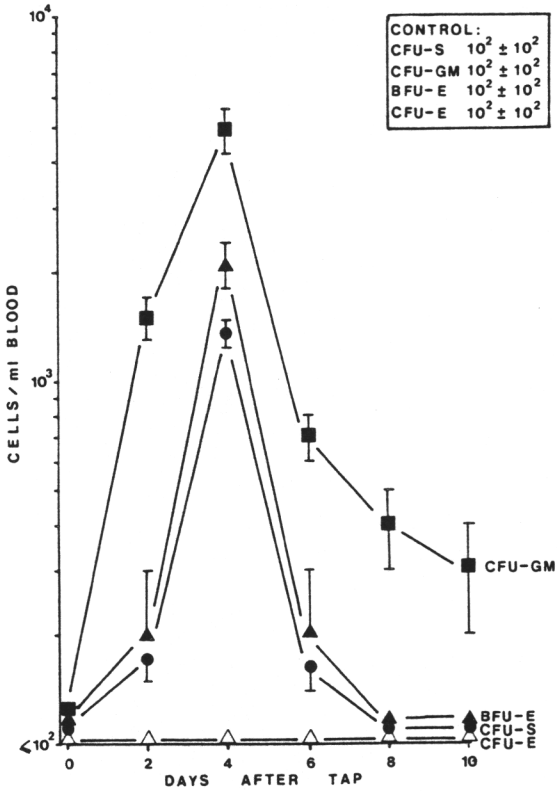

Fig. 3. Peripheral blood levels of the CFU-S, CFU-GM, BFU-E, and CFU-E. The mean blood counts ± SEM per ml peripheral blood are given. *Inset:* the control levels. For *symbols* see *Figure 1.*

Table 2. Yield of nucleated cells and CFU-E after density gradient centrifugation

Specimen	Yield (percent)	
	Nucleated cells	CFU-E
Peripheral blood plus 10^6 spleen cells per ml	72 ± 4	74 ± 5
Spleen cell suspension	59 ± 8	86 ± 7

at 7.0% ± 0.7% on day 6, remaining high until day 10, and returning to the control level on day 14. The hematocrit continued to decrease from day 0 on and reached a minimum of 36.5% ± 0.6% (82% of the control) on day 4. From this point on, the recovery of the hematocrit started and attained the control level on day 10.

Total hemopoietic production and splenic contribution

On the basis of the assumption that the femur represents 6% ($1/17$) of the total bone marrow and that the total hemopoietic cell number is the sum of the cells found in the bone marrow, spleen, and peripheral blood, the total cell numbers and the splenic fraction of the total were calculated and expressed as percent of control or percent of the actual total, respectively (Fig. 6). Figure 6A shows that the CFU-E peak preceded the erythroid precursors peak by 1 day. Figure 6B demonstrates an early increase of the CFU-GM that was followed by a regnerative wave of granuloid precursors several days later.

The splenic fraction of progenitors and precursors (Fig. 6C and D) shows that on day 4 after TAP practically the total CFU-E and CFU-GM pools were present in the spleen. Regarding the CFU-E, the spleen contained 90% of all CFU-E in the animals (control: 3%), whereas the CFU-GM fraction rose from 1% to 70%. Remarkably, the splenic CFU-GM fraction was accompanied only by a small fraction of granuloid precursors (10% on day 4). In contrast, 80% of the erythroid precursors were present in the spleen on day 5.

Discussion

The present study demonstrates an antagonistic behavior of immature hemopoietic cells (CFU-S, CFU-GM, BFU-E, and CFU-E) in the bone marrow and spleen after TAP-induced hemopoietic suppression. The minimum in the femur and maximum in the spleen during recovery of these cell stages occurred simultaneously. The course of the numbers in the peripheral blood coincided with that in the spleen. The levels of these cells found in the blood were extreme. On day 4 after TAP the blood contained 10–50 times as many immature cells as in the control. The total amount of CFU-S found in the blood on day 4 was equivalent to at least one normal femur content. The blood levels of the CFU-S and BFU-E were similar to or even higher than the levels reported after administration of bacterial compounds [4, 5] and phenylhydrazine injection [2]. These findings strongly indicate cell migration from the marrow to the spleen.

A further support for migration is the fact that the increase of the splenic CFU-S content from day 0 to day 4 cannot be

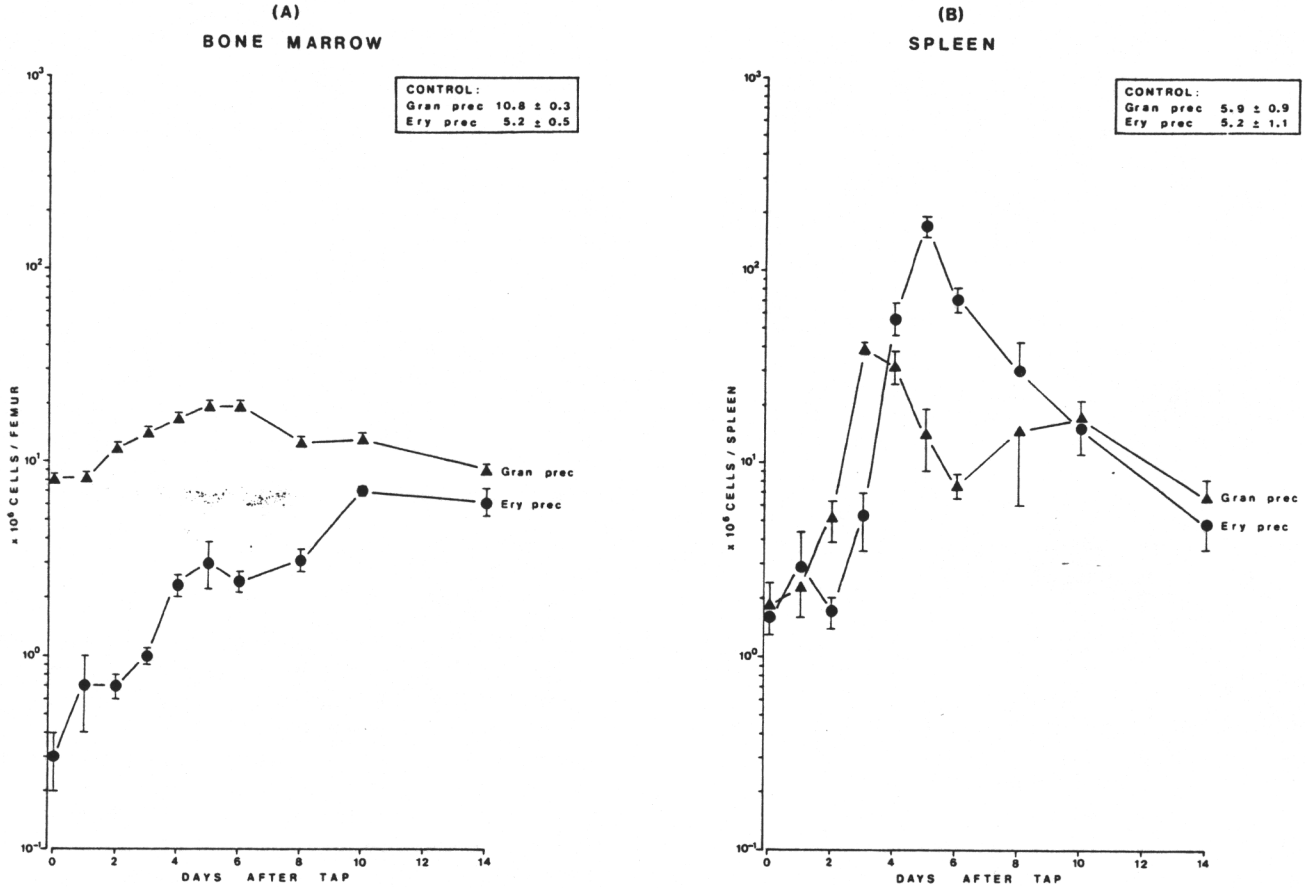

Fig. 4. Recovery of the granuloid and erythroid precursors (morphologically recognizable immature cells) in the femoral bone marrow (**A**) and spleen (**B**). After counting of 400–800 nucleated cells in May-Grünwald–Giemsa-stained cytospin preparations, the femoral and splenic numbers of morphologically recognizable precursors were determined. The mean (n = 3) femoral (**A**) and splenic (**B**) numbers of granuloid (▲—▲) and erythroid (●—●) precursors ± SEM are presented. *Inset:* control values.

explained only by endogenous, i.e., splenic, proliferation. The [^{3}H]TdR kill data show that only half of the CFU-S are in cycle at maximum. (Time of S-phase is approximately half of the cell cycle, and the maximal number in S-phase is 20%–25%.) Assuming a maximum self-renewal probability of 0.65 and a cell cycle time of 8 h, one can calculate that the endogenous CFU-S can at maximum increase sixfold within a 4-day period:

$$S = S_0 \exp[(2p - 1) \cdot a/T \cdot t]$$

where S = the pool size of the CFU-S, p = self-renewal probability, a = fraction in cell cycle, T = cell cycle time, and t = total time of observation [19]. However, a 50-fold increase was found. Thus, migration of the CFU-S from the bone marrow to the spleen (until day 4) appears to contribute substantially to the course of the splenic CFU-S during this period. Similar arguments hold for splenic BFU-E and CFU-GM, whose increase cannot be explained solely on the basis of enhanced differentiation rates of CFU-S. Thus the splenic increase of CFU-S, CFU-GM, and BFU-E appears to be predominantly due to migration from the bone marrow. The increment of the splenic CFU-E should most likely be ascribed to differentiation of BFU-E, because the CFU-E could not be found in the peripheral blood, although the detection procedure was fairly sensitive. However, we cannot rule out the

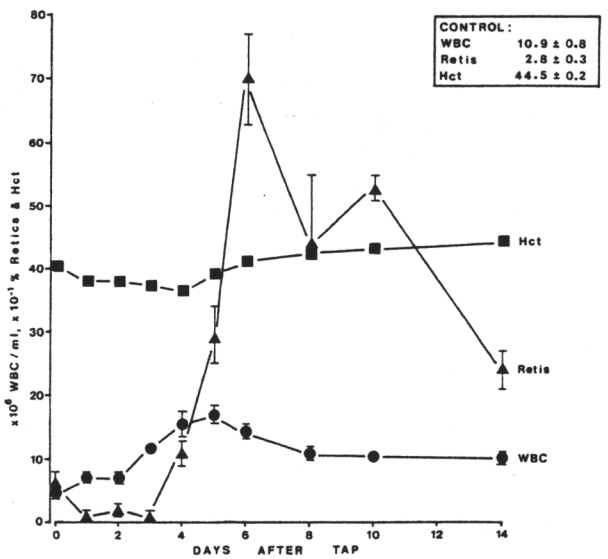

Fig. 5. The WBC (*WBC*), reticulocyte counts (*Reti*) and hematocrit (*Hct*). The mean (n = 3) WBC (●—●), Reti counts (▲—▲), and Hct (■—■) ± SEM are given.

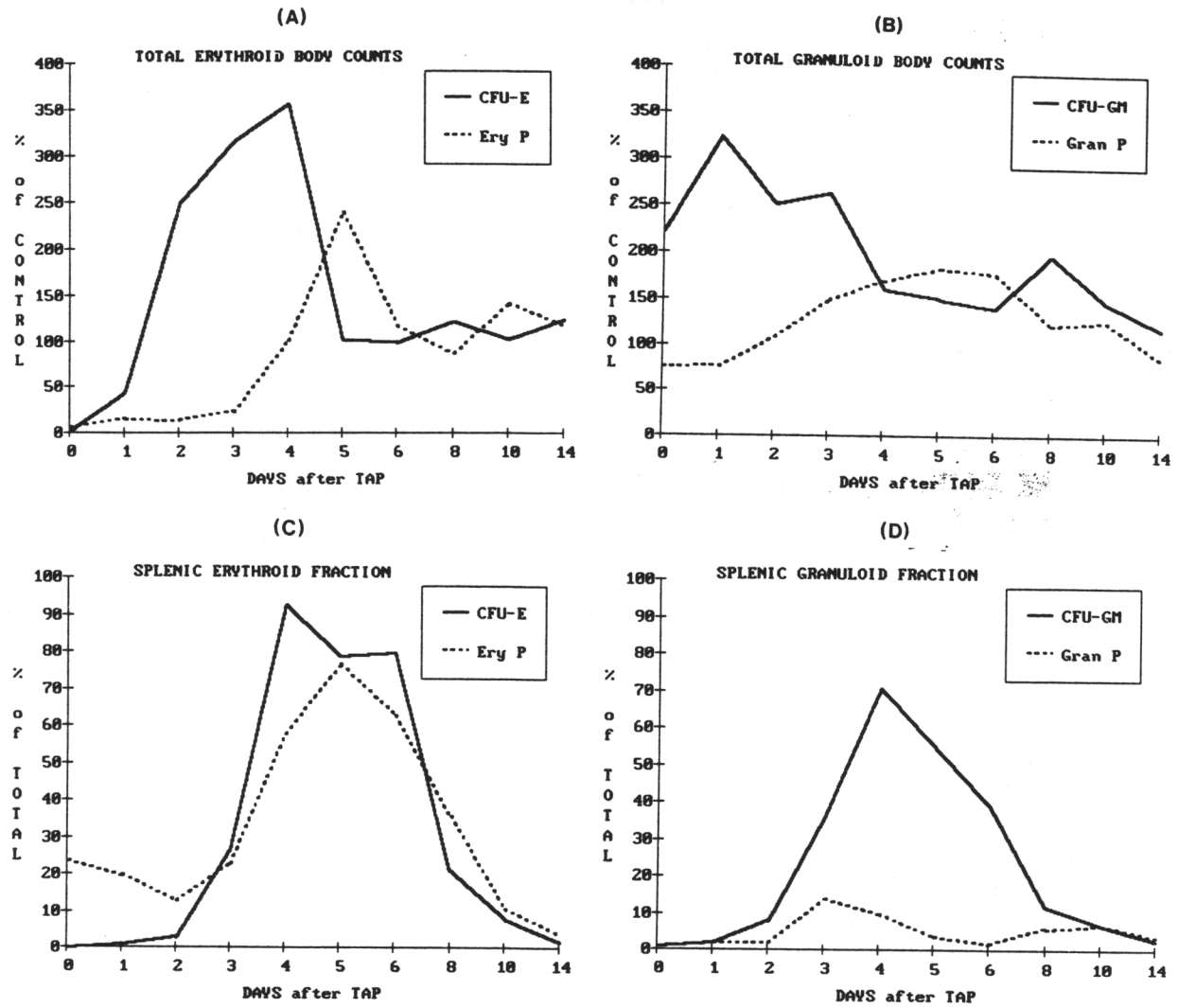

Fig. 6. Total body counts and splenic fraction of erythroid and granuloid cell stages. The mean total body counts of CFU-E (——) and erythroid precursors (Ery P, ·····) (**A**), CFU-GM (——) and granuloid precursors (Gran P, ·····) (**B**) are presented. In (**C**) and (**D**) the mean splenic fraction of the erythroid and granuloid cell stages is given, respectively. Both parameters were calculated according to the equations as given in the *Materials and methods*.

possibility that the CFU-E might have an extremely high exchange rate, which could lead to blood values below detection limits. On the other hand, the discrepancy in migration behavior between the BFU-E and the CFU-E supports the observation of other authors [2]. In addition, the decrease of femoral CFU-E between days 3 and 4 can be fully explained by differentiation into femoral erythroblasts. Within 24 h 3.4 cell divisions can occur (cycle time of CFU-E and erythroblasts is 7 h [20]). This would lead to a multiplication of 10.6. The actual increase in erythroblast numbers (1.3×10^6) was 11.4 times the decrease in CFU-E (114,000). These close values rule out migration of CFU-E.

To investigate to what extent the cells, localized in the marrow and spleen, effectively differentiated into the new environment and contributed to mature blood cells, we have calculated the hemopoietic production and the splenic contribution to total hemopoiesis for the relevant cell stages [18].

The small splenic fraction of granuloid precursors in comparison with the total cell numbers (Fig. 6B) is an indication that in spite of the excessive presence of the CFU-GM in the spleen, this progenitor does not effectively give rise to granuloid precursors in this particular organ. This means most probably that the CFU-GM migrate back from the spleen to the bone marrow without losing the ability to differentiate there. In addition, the peripheral blood level of the CFU-GM remained elevated until the end of the observation period; this supports the idea of a bidirectional traffic of the CFU-GM. Maturation of the CFU-GM seems to take place predominantly in the bone marrow. This contrasts with erythropoiesis. Figure 6A and C show that all CFU-E present in the spleen differentiate to become erythroid precursors. There is no evidence for back-migration of erythropoietic cells from the spleen to the marrow.

One can extend these calculations to establish the contribution of the spleen and bone marrow to the erythroid and granuloid recovery. Figure 7A shows the reticulocyte counts replotted from Figure 5 with different shadings indicating the presumptive origin of the cells. A transit time of 1 day is

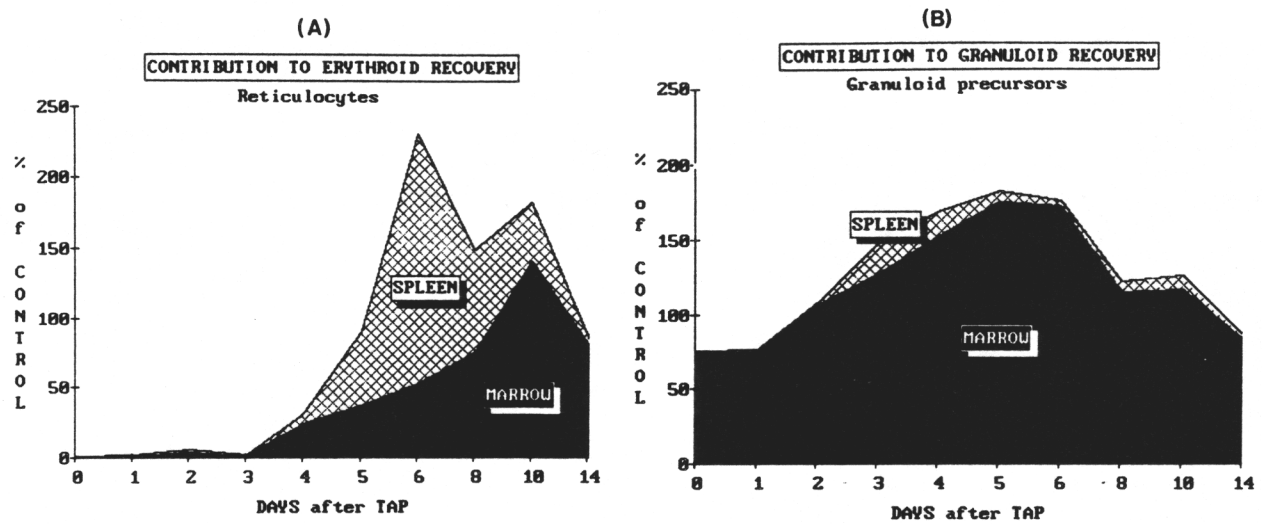

Fig. 7. The contribution of the spleen and marrow to erythroid (**A**) and granuloid (**B**) recovery. In the *area under the curves* of the reticulocytes (**A**) and total granuloid precursors (**B**), the contribution of the spleen and marrow is indicated as calculated on basis of the erythroid and granuloid precursors at both hemopoietic sites.

assumed between erythroid precursors and reticulocytes (Figs. 5 and 6C). The peak of the reticulocytes on day 6 predominantly originates from splenic erythropoiesis, whereas the peak on day 10 stems from the bone marrow. In contrast, the splenic contribution to granuloid recovery measured as granuloid precursor numbers from Figure 6B and D is very small (Fig. 7B).

The physiological significance of the exchange of immature cells remains to be clarified. Migration from the bone marrow to the spleen already takes place before the control level of these cells is reached in the marrow. Therefore, physical limitations of the bone cavity during recovery, as suggested in a former study [21], seems not to be a valid reason for migration.

In previous studies [12, 22, 23] we demonstrated the involvement of the intermediate cell stages (CFU-E, erythroid, and granuloid precursors) in the regulation of the stem cell (CFU-S, day 8). The differentiation and proliferation ([^{3}H]TdR kill) of the CFU-S appeared to depend upon the level of the intermediate cell stages. The present study confirms this concept with respect to the cycling activity of the femoral CFU-S. During the first 3–4 days of marrow recovery, the erythroid cells increased sharply. During this period, cycling activity of CFU-S decreased. From days 4–6 CFU-E numbers declined again; this decline was accompanied by another increase of CFU-S cycling. After day 8, when CFU-E and erythroid precursor numbers returned to normal values, the cycling activity of CFU-S also decreased to its normal level.

In contrast, the cycling activity of splenic CFU-S remained more or less constant, above normal, despite excessive changes in CFU-E and precursor numbers. This might suggest that splenic CFU-S are less responsive to regulatory signals. On the other hand, the cycling activity of splenic CFU-S was consistently below the marrow activity. Bearing in mind the continuous import of highly cycling cells from the marrow, this observation indicates the presence of inhibitory signals acting on the newly imported cells. Such an inhibitory activity in the spleen was previously reported under similar circumstances [24]. The present data confirmed that major differences exist between marrow and spleen microenvironments. Despite these differences the migration phenomena did not disturb a fast overall recovery. This indicates an elaborate interaction between local (microenvironmental) and systemic (humoral) regulation and the processes involved in the control of cell migration.

A detailed examination of these phenomena within the frame of a mathematical model of hemopoiesis discriminating bone marrow and spleen is presently being undertaken.

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