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Hemopoiesis during Thiamphenicol Treatment. I. Stimulation of Stem Cells during Eradication of Intermediate Cell Stages

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Abstract. Continuous treatment of C57bl/6 mice for 4 days with the cytostatic antibiotic thiamphenicol revealed a dual response of hemopoietic cells. On one hand, morphologically recognizable erythroid precursors and late progenitors (erythroid colony-forming units; CFU-E) and, to a lesser extent, granuloid precursors were found substantially reduced. On the other hand, early granuloid (granulocyte–macrophage colony-forming units; CFU-GM) and erythroid (erythroid burst-forming units; BFU-E) progenitors increased on day 3 to 220%–240% and 120%–130% of the control value, respectively. This was accompanied by a decline of the initial spleen colony-forming units (CFU-S) (day 8) pool size to approximately 60%. These patterns were similar in the bone marrow and the spleen. In addition, the tritiated thymidine kill of femoral and splenic CFU-S rose significantly ($p < 0.05$) from 16% ± 3% to 38% ± 2% and from 3% ± 1% to 17% ± 2%, respectively. A sudden decline of peripheral reticulocytes between days 2 and 3 from 2.8% ± 0.3% to 0.6% ± 0.2% was observed, whereas the hematocrit gradually decreased from day 1 to day 4 from 45.2% ± 0.1% to 39.3% ± 0.3%. The white blood cells were not affected. From these results we conclude that stem cells were stimulated as a consequence of the suppression of the intermediate cell stages. As analyzed in the accompanying paper, this confirms a prediction stated by a quantitative theoretical concept of in vivo stem cell regulation.

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

The ability to balance blood cell formation after various perturbations is a characteristic property of hemopoiesis. The regulation of this process is very complex and only partly elucidated. In order to identify the essential rules governing hemopoietic regulation a mathematical model has been developed [1–4]. It was predicted by the model that a selective reduction of intermediate erythroid and granuloid cell stages should lead to an activation of stem cells. This regulatory mechanism is predominantly based upon indirect evidence [2, 4]. To test the model predictions the antibiotic thiamphenicol (TAP) is an interesting drug. It has been shown to affect the intermediate hemopoietic cell stages, whereas stem cells and early progenitors are relatively refractory [5–7]. These experiments, however, were performed under anemic stress by bleeding, which caused a high erythropoietin response. This will certainly interfere with the regulatory phenomena. Therefore, in the present study the short-term effects of TAP on hemopoietic cells in bone marrow as well as spleen were studied without bleeding. This experimental design enabled us to get a better insight in the effects of TAP and to test qualitatively the role of intermediate cell stages in the regulation of stem cells as hypothesized in the model of Wichmann and Loeffler [2]. In this report we present the experimental findings, and in the accompanying paper we compare the data with the model predictions and discuss the implications for the model [8].

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Materials and methods

Treatment of animals. Specific pathogen-free female C57bl/6 mice (Charles River, FRG) between 8 and 12 weeks of age (20–25 g) were used. The mice were maintained in a conventional animal room and fed ad libitum with pelleted food (Hope Farms, The Netherlands) and tap water. TAP (Inpharzam, S.A., Zambon Group, Cadempino, Switzerland) was administered s.c. via a dialysis bag as described previously [7].

Cell suspensions. Bone marrow cell suspensions were obtained by flushing one femur with 1 ml α-medium (GIBCO, UK) plus 10 mM HEPES, pH 7.2. Single-cell suspensions were made by repeated flushing through a 25-gauge needle. Spleens were pressed through a stainless steel sieve (100 mesh) and suspended in α-medium through 20- and 25-gauge needles, respectively. Bone marrow and spleen cell suspensions of groups of three mice were pooled. The femoral and splenic nucleated cells were counted in a Coulter counter.

Peripheral blood cells. Blood of groups of three mice that were bled from the orbital plexus was pooled in heparinized (50 U heparin per ml blood) tubes. The hematocrit (HCt), WBC, and reticulocyte counts were determined according to standard procedures. The leukocytes were isolated after centrifugation on a discontinuous gradient of Ficoll–Isopaque (Sigma and Nyegaard, Norway, respectively) with a density of 1.085 g/ml [9]. The interphase was removed and washed three times with α-medium plus 10 mM HEPES, pH 7.2, supplemented with 5% fetal calf serum.

Marrow, spleen, and blood cell differentials. On May-Grünwald–Giemsa-stained cytospin preparations the precursors were classified as pro (El), basophilic (E2), polychromatic (E3), and orthochromatic erythroblasts (E4), myeloblasts (G1), promyelocytes (G2), myelocytes (G3), banded and segmented granulocytes (G4), and lympho-

Table 1. Femoral nucleated cell and precursor content during TAP treatment

Days of TAP	Nucleated cells	Granuloid precursors G1–4	Erythroid precursors E1	Erythroid precursors E2	Erythroid precursors E3 + E4
0	22.9 ± 0.2	11.2 ± 0.4	0.4 ± 0.2	1.6 ± 0.2	3.2 ± 0.4
1	19.3 ± 0.1	7.4 ± 0.3	0.2 ± 0.2	1.4 ± 0.4	3.5 ± 0.1
2	14.1 ± 0.3	7.5 ± 0.4	<0.1	<0.1	0.7 ± 0.1
3	12.8 ± 0.8	7.9 ± 0.4	<0.1	<0.1	0.3 ± 0.1
4	11.0 ± 0.4	7.7 ± 0.3	<0.1	<0.1	<0.1

Mean femoral content (× 10^6 cells) ± SEM of three separate experiments.

Table 2. Splenic nucleated cell and precursor content during TAP treatment

Days of TAP	Nucleated cells	Granuloid precursors G1–4	Erythroid precursors E1	Erythroid precursors E2	Erythroid precursors E3 + E4
0	129 ± 2	5.5 ± 0.4	<0.1	0.4 ± 0.2	9.9 ± 1.3
1	118 ± 2	5.2 ± 0.6	<0.1	1.0 ± 0.4	8.2 ± 1.2
2	113 ± 3	5.6 ± 1.1	<0.1	0.3 ± 0.1	8.1 ± 1.2
3	108 ± 7	1.8 ± 0.8	<0.1	<0.1	4.0 ± 0.5
4	100 ± 3	2.0 ± 0.4	<0.1	<0.1	5.4 ± 0.8

Mean splenic content (× 10^6 cells) ± SEM of three separate experiments.

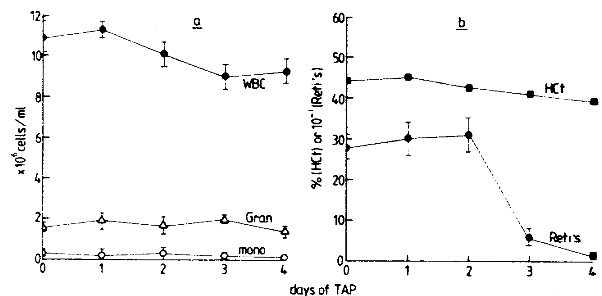

Fig. 1. (a) The mean ± SEM of the WBC (*WBC*), granulocyte (*Gran*), and monocyte (*mono*) counts of three separate experiments is given. (b) Mean percentage ± SEM of the hematocrit (*HCt*) and reticulocyte (*Reti's*) counts.

cytes according to standard criteria by counting 400–800 nucleated cells in duplicate. Blood cell preparations were classified as lymphocytes, granulocytes, and monocytes by counting 100 leukocytes.

Progenitor cell assay. Granulocyte–macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E), and erythroid colony-forming units (CFU-E) were estimated with the methylcellulose method of Iscove and Sieber [10]. One-ml suspensions of 0.2–5 × 10^5 nucleated femoral bone marrow cells or 2–5 × 10^5 nucleated spleen cells were plated in duplicate. Cultures for BFU-E and CFU-E were supplemented with 1.0 U and 0.25 U erythropoietin (Terry Fox Laboratory, Vancouver), respectively. An optimal amount of pokeweed mitogen-stimulated spleen cell-conditioned medium [11] was added to the CFU-GM and BFU-E cultures.

Spleen colony assay. The number of spleen colony-forming units (CFU-S) was determined according to Till and McCulloch [12]. Into seven to ten recipient mice, which had received 9 Gy (dose rate 0.5 Gy/min) 24 h previously, donor cells were injected via the orbital sinusoids. After 8 days the spleens were fixed in Tellyesnicky's solution and the colonies were counted.

Tritiated thymidine ([^{3}H]TdR) kill. The [^{3}H]TdR kill of stem cells and progenitors was determined according to Becker et al. [13]. Briefly, cell suspensions of 10–20 × 10^6 nucleated cells per ml were incubated with 0.74 MBq [^{3}H]TdR (spec. act. 814 MBq/μmol; Amersham, UK) per ml for 20 min at 37°C in a water-saturated atmosphere with 5% CO_2. After incubation the cells were washed three times with α-medium plus 10 mM HEPES, pH 7.2, supplemented with 5% fetal calf serum. The [^{3}H]TdR kill was expressed as percentage of the incubated control.

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

The effects of continuously administered TAP to C57bl/6 mice during a 4-day period were determined on the peripheral blood cells, granuloid and erythroid precursors, progenitors (CFU-GM, BFU-E, and CFU-E), and stem cells (day-8 CFU-S) in the spleen and the bone marrow.

Peripheral blood cells. Over the 4-day period the WBC counts tended to decrease but the difference with the control value was not significant (Fig. 1a). The granulocyte and monocyte counts did not change. The hematocrit significantly decreased from 44.5% ± 0.2% to 42.3% ± 0.1% on day 2, down to 39.3% ± 0.3% on day 4 (Fig. 1b). The reticulocyte counts did not significantly change during the first 2 days of treatment. A significant decline from 3.1% ± 0.4% to 0.6% ± 0.2% occurred between days 2 and 3. After 4 days a reduction to 0.1% ± 0.1% was observed (Fig. 1b).

Femoral and splenic nucleated cells. The femoral nucleated cell content was reduced to approximately 50% within 4 days of TAP treatment (Table 1). Because of morphological abnormalities (vacuolization), the classification of the granuloid precursors was hampered. Therefore, they were combined into one group (G1–4). After 1 day of treatment the femoral granuloid precursors were found significantly diminished to about 70% (Table 1). Thereafter their number remained constant. In contrast, the erythroid precursors vanished completely (below the detection level) on the 4th day. The decrease was most pronounced between days 1 and 2. After 4 days of TAP treatment the splenic nucleated cell content decreased by approximately 20% (Table 2). In the spleen a significant repression of granuloid precursors paralleled a decline of basophilic (E2) and poly- and orthochromatic erythroblasts (E3 + E4). The major changes occurred between days 2 and 3. The proerythroblasts (E1) were below the detection level throughout the 4-day period.

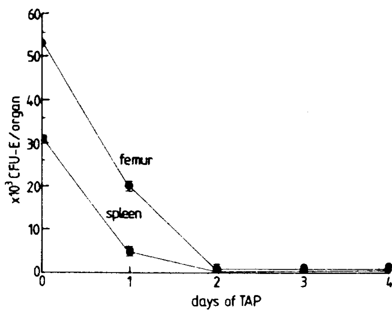

Fig. 2. Course of the femoral (●——●) and splenic (■——■) CFU-E content expressed as the mean ± SEM of three separate experiments.

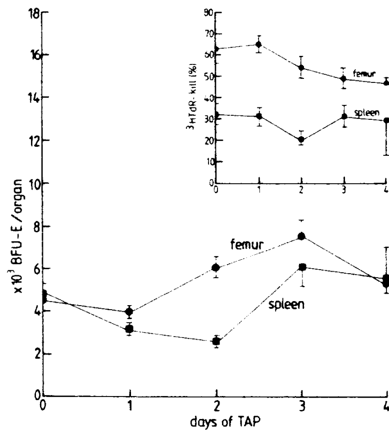

Fig. 3. The mean femoral (●——●) and splenic (■——■) BFU-E content of three separate experiments. *Inset:* mean [³H]TdR kill ± SE (n = 3).

Late erythroid progenitors (CFU-E). TAP treatment resulted in a rapid significant decline of both the femoral and splenic CFU-E content (Fig. 2). A reduction to 20% and 40% in the spleen and femur, respectively, was observed already after 1 day of treatment. Two days of treatment were sufficient to suppress the CFU-E content below the detection level (approximately 2×10^3 CFU-E per organ).

Early erythroid progenitors (BFU-E). After 1 day of delay in the femur and a temporary decline in the spleen until day 2 a significant elevation of the BFU-E content was observed on days 3 and 4 (Fig. 3). The [³H]TdR kill of the BFU-E of control animals was twice as high in the femur as in the spleen (60% vs 30%). The kill of the BFU-E remained constant in the femur and the spleen during 3 days of TAP treatment.

Early myeloid progenitors (CFU-GM). The femoral and splenic CFU-GM showed a continuous increase after a delay of 1 day (Fig. 4). On day 4 the femoral and splenic content was approximately two to three times the control value. The [³H]TdR kill for the CFU-GM in the femur was about twice as high as in the spleen (45% vs 19%). The kill did not change significantly during 4 days.

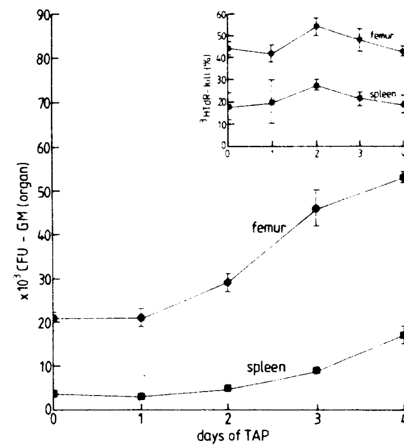

Fig. 4. The mean femoral (●——●) and splenic (■——■) CFU-GM content ± SEM of three separate experiments. *Inset:* mean [³H]TdR kill ± SE (n = 3).

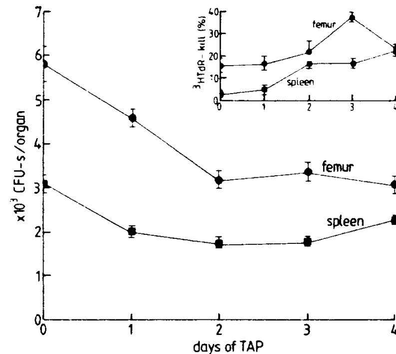

Fig. 5. Course of the femoral (●——●) and splenic (■——■) CFU-S content ± SEM of three separate experiments. *Inset:* mean [³H]TdR kill ± SE (n = 3).

Stem cells (day-8 CFU-S). Within the first 2 days of TAP treatment the CFU-S content decreased to approximately 60% of the control in the femur as well as in the spleen. The difference from control was already statistically significant on day 1. In the subsequent days the content remained below 60% of the control (Fig. 5). A difference in the femoral and splenic [³H]TdR kill was observed in control animals (16% ± 3% vs 3% ± 1%, respectively). Starting from the low control values a significant increase was found on day 3 in the bone marrow to 38% ± 2% ($p < 0.05$) and in the spleen to 17% ± 2% ($p < 0.05$). On day 4 the [³H]TdR kill in the bone marrow and the spleen was approximately the same (22% vs 24%).

Discussion

The present study shows a remarkable dual response of hemopoietic cells during TAP treatment in mice. On one hand, in the bone marrow as well as in the spleen, the erythroid progenitors CFU-E and the precursors E1–E4 were found to be severely repressed, whereas immature granuloid cells G1–G4 were less affected. On the other hand, however, a reduced

pool size and an increased proliferative state of the CFU-S accompanied elevated levels of the early progenitors BFU-E and CFU-GM.

The repressive effects of TAP and the TAP analogue chloramphenicol (CAP) on hemopoiesis, especially the preferential susceptibility of erythroid precursors, has been described previously [14, 15]. The course of the peripheral blood cell numbers, i.e., the reduction of the reticulocytes after day 2 and the decline of the hematocrit from day 1 on, in relation to the fate of the precursors, does not unequivocally show a simultaneous or sequential disappearance of the erythroid cell stages during TAP. Therefore, it remains unclear at this stage whether TAP evokes an overall inhibition of the proliferation of immature erythroid cells, or an inhibition of the differentiation of the BFU-E to CFU-E, or whether both processes are affected simultaneously.

The effects on granulopoiesis were less pronounced than on erythropoiesis. The data on the femoral and splenic granuloid precursors showed a decline that was not reflected in the peripheral blood neutrophils within 4 days. Among the granuloid cells we observed cells with morphological abnormalities (vacuoles, enlargement). This is already an indication of a toxic effect. Also, the fact that the total nucleated cell count in the femur was reduced by 50%, half of which could be accounted for by erythroid cells, shows that TAP does not exclusively affect erythropoiesis.

In contrast to the effects on the more mature hemopoietic cells, TAP apparently induced a stimulatory effect on the CFU-S both in the bone marrow and the spleen. A decrease of the CFU-S pool size coincided with an increase of the pool size of the CFU-GM and BFU-E, whereas the proliferative state of the CFU-S was significantly elevated on days 2 and 3 in the spleen and bone marrow, respectively. It has been reported that increased cycling of stem cells will reduce their seeding efficiency in the spleen [16]. If not corrected for, this would lead to a decrease of CFU-S numbers. It is very unlikely that this could be an explanation for the observed data, because a substantial decline in numbers (day-1 spleen, day-2 marrow) corresponds with an unchanged proliferative state. Former studies suggested an inhibition of the proliferation of the CFU-S and BFU-E during TAP treatment [6, 8], whereas others, in concert with the present study, demonstrated an increase of the femoral culture colony-forming unit (CFU-C) pool during CAP treatment [17]. The discrepancy between the former and the present study may be due to differences in the serum levels of this drug attained during treatment. At high serum levels cytoplasmic protein and DNA synthesis might have been inhibited in addition to mitochondrial protein synthesis. This may explain the nonproliferative state of immature cells during TAP in that study [6, 8]. In vitro experiments, however, did not support this hypothesis [18].

Concerning the mode of action of TAP, it has been shown that this drug inhibits mitochondrial protein synthesis [6]. Because the role of the mitochondrion and the biogenesis of mitochondria in cellular proliferation and differentiation have not been clarified in detail, it is difficult to explain the susceptibility of a particular cell to this drug. Even more, the accessibility to the mitochondria, i.e., the permeability of the cytoplasmic membrane for this drug, is unknown. Therefore, the relative refractoriness of stem cells and early progenitors remains unexplained. The high mitochondrial content of the CFU-E [19] may provide an indication for the extreme susceptibility of this cell stage to this drug, particularly because the transition of the BFU-E to CFU-E would necessitate an extensive biogenesis, i.e., production of mitochondria.

Whereas the inhibitory effects of TAP and CAP are overwhelming in the literature, a few reports describe stimulatory effects in vivo on granulocyte production in a case of neutropenia [20] as well as in vitro on CFU-GM numbers by CAP [21]. Whatever the mechanism might be, TAP had no stimulatory effects in these cases. A CAP-induced accumulation of CFU-S and CFU-C in mice has been ascribed to an enhanced proliferation and a maturation arrest of these cell stages [17]. Our study, however, shows a stimulation of the proliferation and differentiation of the CFU-S, whereas the cycling of early progenitors was unaffected. The stimulatory phenomena in stem cells and progenitors make a direct toxic action by TAP on CFU-S (decreased numbers) very unlikely. We therefore conclude that the depletion of intermediate cell stages (in particular erythroid) plays a role in the stimulation of stem cells. Evidence for the involvement of erythroid precursors in the regulation of the hemopoietic stem cell has also been presented after eradication of erythroid precursors by ^{55}Fe-treatment [22]. The present results are a qualitative support of the hypothesized regulatory events between intermediate cell stages and stem cells in the model of Wichmann and Loeffler [2]. A detailed model analysis of these experimental results is described in the accompanying paper, where the data are compared with the predictions and, moreover, the relationship of the TAP experiment to other experiments involving manipulations of intermediate cell stage is discussed [8].

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