

Effects of filgrastim on granulopoietic cells of mice pretreated with methotrexate

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ABSTRACT: We have evaluated the effect of filgrastim on proliferation and differentiation activity of granulopoietic cells in mice pretreated with methotrexate. Filgrastim was injected daily, from day 8 to 28 after cytotoxic agent administration. The granulopoiesis changes were measured by assessment of GM-CFU cells content, marrow and spleen granuloid cells pool as well as circulating neutrophils. In MTX pretreated mice, bone marrow GM-CFU oscillating values were higher than normal values, but these changes were not followed by high proliferative activity in granuloid precursor cell compartment. After MTX treatment, filgrastim administration was unable to stimulate marrow granulopoiesis as observed in normal mice. In the spleen, MTX led to dramatic changes in the proliferative activity of GM-CFU cells, but did not result in spleen granuloid cell changes. However, filgrastim treatment induced a spleen granuloid amplification, similar to the changes observed in circulating neutrophils values. We suggest that these findings can be explained by inhibition of differentiation of marrow GM-CFU cells into the more mature granulopoietic cells and/or by an inhibited proliferative activity of marrow granuloid cells. They can be also explained in terms of an unfavorable marrow microenvironment for granulopoiesis, contrary to a supportive spleen microenvironment.

Introduction

The folate antagonist methotrexate (MTX) is widely used in cancer chemotherapy and is also used to treat a variety of nonmalignant conditions. This drug is employed in the treatment of bladder carcinoma (Hsu *et al.*, 2001), trophoblastic neoplasm (Bower *et al.*, 1998), head and neck cancer (Lin *et al.*, 1999) and breast cancer (Colleoni *et al.*, 2002). It has also become an important therapy in the treatment of severe psoriasis (Kuijpers

and van de Kerkhof, 2000), and rheumatoid arthritis (Papadopoulos *et al.*, 2002). However, the use of MTX for antineoplastic treatment is limited by its toxic effects on rapidly proliferating tissues, particularly the bone marrow.

Previous studies have indicated that high doses of MTX appears to affect cells at relatively late stages of hematopoiesis (Vogler *et al.*, 1973) inducing a reversible developmental arrest in the progenitor cell population (Stromhaug *et al.*, 1995; Stromhaug and Warren, 2000). In addition, a declination in the number of mononuclear cells reflecting a significant depletion of nonclonogenic precursor cells (Blau *et al.*, 1996), was observed in mice treated with a single injection of MTX.

On the other hand, the use of hemopoietic growth factor such as filgrastim (recombinant human granulocyte colony – stimulating factor (rhG-CSF),

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shows clinical usefulness in the treatment of neutropenias associated with the use of chemotherapeutic agent (Iqbal *et al.*, 2000; Edelman *et al.*, 2000). Filgrastim stimulates granulopoiesis with certain side effects on migration of early progenitors cells from bone marrow to spleen (Barrios *et al.*, 2000; Bungart *et al.*, 1990).

However, no systematic accounts have been reported on the effects of filgrastim on the growth rate of the granulocyte cells surviving methotrexate treatment.

In the present study, we have examined the *in vivo* granulopoietic cells recovery after administration of filgrastim in mice pretreated with methotrexate. We measured the granulocyte – macrophage colony forming unit (GM-CFU) cells correlating these changes with those in the femoral and splenic granulocyte pools and circulating neutrophils.

Material and Methods

Mice

Adult female Swiss CF1 mice between 25 to 30 g (3 months of age) were used for the experiments. They were maintained in a conventional environment with food pellets and water *ad libitum*, according to institutional guidelines. Mice were examined daily for signs of illness.

Methotrexate (MTX) treatment

Methotrexate was purchased from Lederle Laboratories (Argentina). MTX 25 mg/m² (8.5 mg/kg) was dissolved in sterile water and administered by intraperitoneal injection to all mice on days 0 and 7.

Filgrastim treatment

Filgrastim, recombinant human granulocyte colony-stimulating factor (rhG-CSF), was purchased from Roche Laboratories (Argentina). Half of the mice pretreated with methotrexate were injected with Filgrastim (30 µg/kg/day) administered subcutaneously, on days 8 to 28, starting 24 h after the last dose of MTX. Control mice were injected with equivalent volumes of PBS. The filgrastim doses used in mice are usually higher than those in human, at least in part due to limited cross-species reactivity (Shimamura *et al.*, 1987).

At days 0, 4, 8, 12, 16, 20, 24 and 28, 6-7 filgrastim treated mice and 6-7 control mice treated with PBS were sacrificed and the experiment done.

Cell numbers

Cells were counted using a Neubauer hemocytometer. Blood (50 µL) was collected from

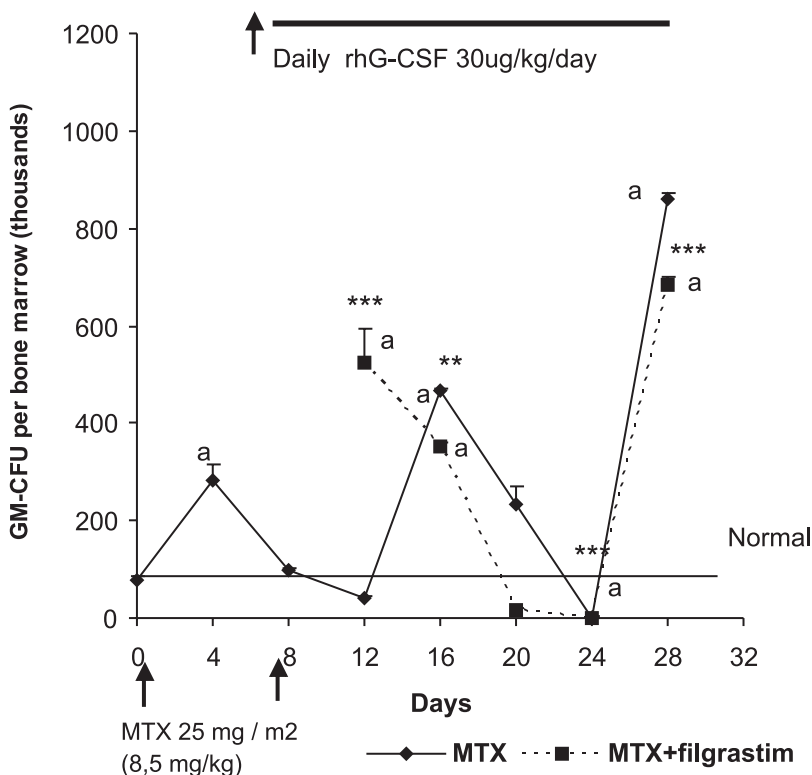


FIGURE 1. Changes in marrow granulocyte - macrophage colony forming unit cell numbers ($\times 10^{-3}$). GM-CFU cell numbers were determined in pooled femurs from 3 mice per experiment. Data represent Mean \pm SEM. Where number differs significantly from MTX + filgrastim treated groups (\blacksquare) from MTX treatment alone (\blacklozenge), it is indicated (***) $p < 0.001$ and (**) $p < 0.01$. And where MTX + filgrastim or MTX treatment alone differs from normal values it is indicated (a): $p < 0.001$.

cardiac puncture and nucleated cells were suspended immediately with diluting fluid. From a further small sample, blood films were prepared, stained with May Grünwald - Giemsa, and a differential count was made. After blood sampling, mice were humanely killed, and femora and spleen were removed. Marrow cells were flushed from femora and suspended by being passed several times through a hypodermic needle. Bone marrow cell numbers were calculated under the assumption that one femur contains 6% of total marrow (Bogss, 1984). Spleens were homogenized in glass-teflon tissue grinders until single cell suspension was obtained. Granuloid precursor cells are referred as morphological recognizable nucleated myeloid precursor cells: myeloblast through segmented neutrophils.

Assay for progenitor cells

Splenic and femoral granulocyte-machrophage colony forming units (GM-CFU) cells were assayed by the methylcellulose method of Iscove and Sieber (Iscove and Sieber, 1975). GM-CFU culture was supplemented with 20% pokeweed mitogen-stimulated murine spleen conditioned medium (Burgess *et al.*, 1980). Bone marrow and spleen cells from methotrexate treated mice were plated respectively at 5×10^4 and 2×10^5 cells /ml

and after 8 days of incubation at 37°C in a humidified atmosphere with 5% CO₂, cultures were scored for colonies. Colonies greater than 50 cells were scored and colony forming cells were calculated per spleen or per bone marrow.

Statistics

Statistical significant differences between groups were assessed by Student's test. Multiple group comparisons were performed using analysis of variance (ANOVA). Filgrastim treated mice were compared with mice pretreated with the cytotoxic agent injected with PBS and with normal mice. All data are expressed as means \pm SEM.

Results

Effects of filgrastim administration on bone marrow and spleen GM-CFU cell values of methotrexate pretreated mice

Figure 1 illustrates the behavior of bone marrow GM-CFU cell numbers in MTX and MTX + filgrastim experiments. MTX shows a modest effect killing

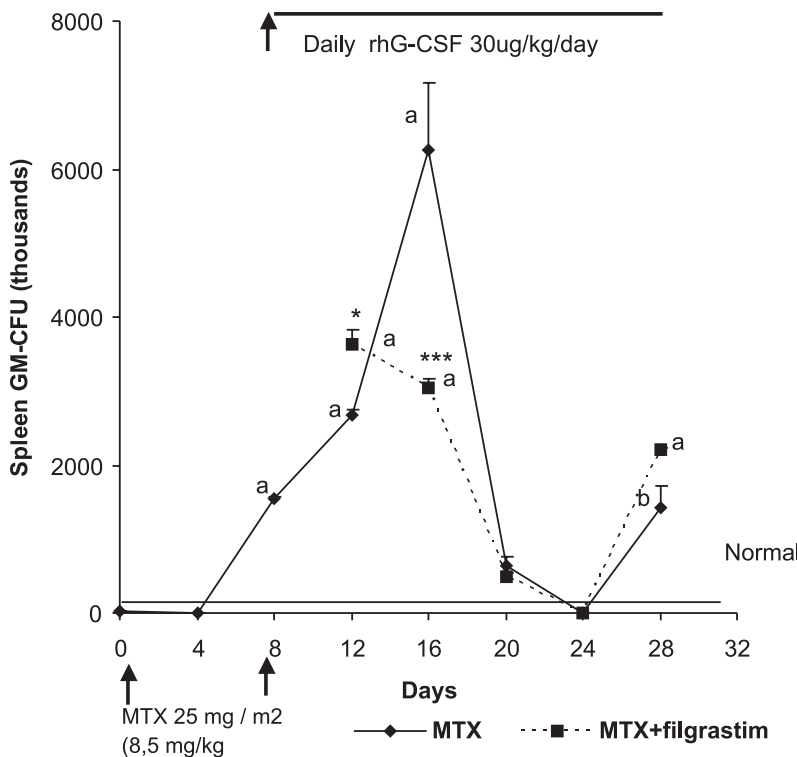


FIGURE 2. Changes in spleen granulocyte - macrophage colony forming unit cell numbers ($\times 10^{-3}$). GM-CFU cell numbers were determined in pooled spleens from 3 mice per experiment. Data represent Mean \pm SEM. Where number differs significantly in MTX + filgrastim treated groups (■) from MTX treatment alone (◆), it is indicated (***) $p < 0.001$. And where MTX + filgrastim or MTX treatment alone differs from normal values it is indicated (a): $p < 0.001$ and (b): $p > 0.01$.

marrow GM-CFUs (except on day 24). The surviving fractions of these hemopoietic progenitor cells were able to induce overshooting which allowed GM-CFU contents to reach values higher than normal. In control groups (MTX), after the first methotrexate dose on day 0, GM-CFU stage cell was approximately 3.5 times higher than normal control values on day 4 and returned to normal values on day 8. When MTX treatment was repeated on day 7, changes in GM-CFU progenitor cell values demonstrated a biphasic pattern. After a nadir on day 12, the GM-CFU cell numbers increased 6-fold on day 16, and a second nadir seen on day 24 was followed by an increase of nearly a 11-fold on day 28. When filgrastim was administered to MTX pretreated mice, the pattern of hemopoietic growth factor treated mice was similar to control mice. In marrow of filgrastim treated mice, the number of GM-CFU cells, except on day 12, was lower than the cytotoxic agent administration without filgrastim. Decreased numbers of GM-CFU observed along the experiment, in both groups (MTX and MTX + filgrastim), are compatible with cell migration from the marrow to the spleen, as previously observed when a cytotoxic agent was administered alone or in combination with a cytokine (Goris *et al.*, 1990; Morrison *et al.*, 1997).

The splenic GM-CFU progenitor cell numbers in both, methotrexate (MTX) and cytotoxic agent plus

filgrastim treated mice (MTX + filgrastim), exhibited dramatic changes when compared with normal mice values (Fig. 2). In control animals injected with methotrexate on days 0 and 7, after a fall to undetectable levels on day 4, a quick recovery took place followed by an high increase of splenic GM-CFU cell numbers from day 8 to 16. The maximum values, reached on day 16 were 200 times higher than the control values. Then, splenic GM-CFU cells level had a severe drop between days 20 – 24 and after that, they reached a level above normal values on day 28. After the addition of filgrastim to control methotrexate treated mice (starting on day 8) the recovery followed a broadly similar pattern to mice treated with cytotoxic agent alone, except on day 16 when the GM-CFU cell values of MTX plus filgrastim group were lower compared with those in mice treated with MTX.

Effects of filgrastim on bone marrow and spleen granulocytic precursor cells population values in mice previously exposed to methotrexate treatment

Effects of MTX and of MTX plus filgrastim on bone marrow granulocyte precursor cell values are shown in figure 3. In contrast to the effects on marrow GM-CFUs, no significant increase of marrow granuloid cells was seen throughout the experiment, when compared with

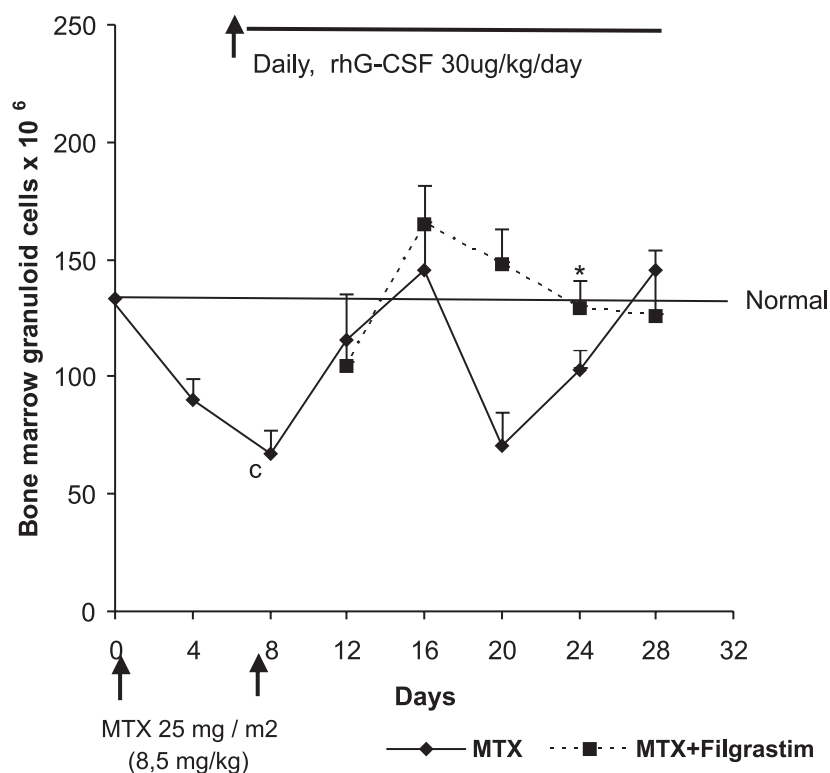


FIGURE 3. Changes in bone marrow granuloid cell numbers $\times 10^6$. All mice were injected ip with MTX (methotrexate) 25 mg/m² (8,5 mg/kg) on days 0 and 7. Filgrastim was administered from day 8 to 28 at a dose of 30 μ g/kg/day. Control mice were given vehicle alone. Data represent Mean \pm SEM. Where number differs significantly in MTX + filgrastim treated groups (■) from MTX treatment alone (◆), it is indicated (*) $p < 0.05$. And where MTX treatment alone differs from normal values it is indicated (c): $p < 0.05$.

normal values. The injection of methotrexate on days 0 and 7 resulted in a 51% decrease of femoral granuloid cell values on day 8 ($p < 0.05$) followed by a return to normal values between days 12-16. After that, a transient no significant reduction in the femoral granuloid compartment was seen before a new recovery took place. Administration of filgrastim, starting on day 8, prevented the drop in femoral granuloid cells found on day 20 in mice treated with MTX alone. There were not stimulating effects of the hemopoietic growth factor along the period of observation, except a transient increase of granuloid precursor cells per femur noted on day 24 in filgrastim treated mice ($p < 0.05$), when compared with control values. These results suggest a decrease in the proliferation capacity of bone marrow myeloid precursors in mice pretreated with methotrexate.

The effects of two doses of methotrexate treatment alone or followed by filgrastim administration on spleen granuloid precursor cells values are shown in figure 4.

While methotrexate treatment alone had little or no effect on granulocytic population content, daily administration of filgrastim starting on day 8 (24 h after the last dose of MTX), had an important stimulatory

effect on splenic granuloid precursor values which increased between 5 to 11-fold from days 16 to 28. This finding suggests that splenic tissue of mice treated with MTX and filgrastim acquired a supportive microenvironment for granulopoiesis.

However, when we calculated (under the assumption that one femur contains 6% of total marrow) the bone marrow and spleen contribution to the total granuloid precursors numbers, we observed that the spleen was a minor granulopoietic site if compared with the bone marrow (Table 1).

Circulating neutrophil counts in mice previously exposed to methotrexate. Effect of filgrastim administration

In mice receiving MTX, numbers of circulating neutrophils showed no significant changes (Fig. 5). However, daily filgrastim administrations to methotrexate-pretreated mice were unable to maintain significant increased levels of peripheral neutrophils as observed in normal mice, and only increased significantly neutrophil count between days 24 – 28 ($p < 0.05$). The splenic granulopoiesis seems to have contributed to this increase in neutrophil values.

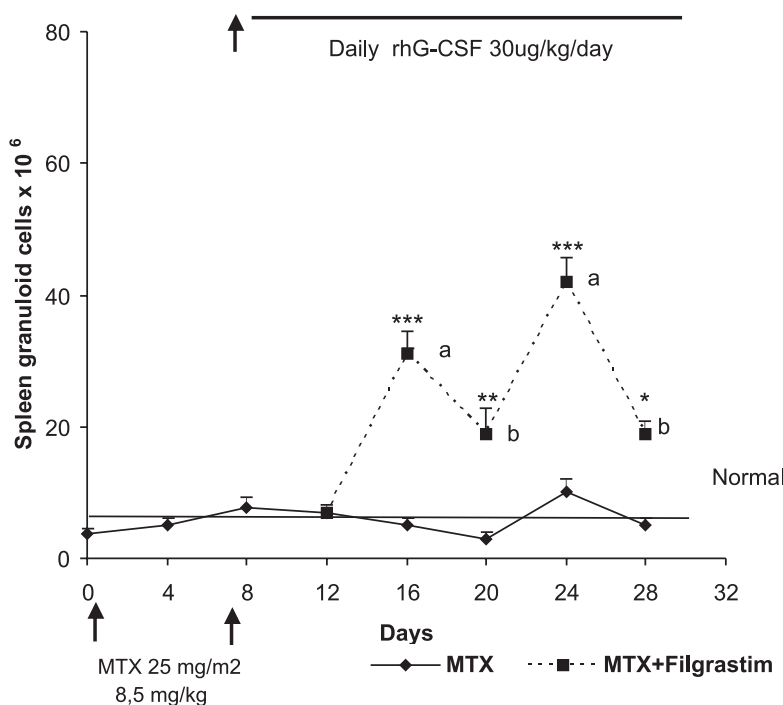


FIGURE 4. Changes in spleen granuloid cell numbers x 10⁶. All mice were injected ip with MTX (methotrexate) 25 mg/m² (8,5 mg/kg on days 0 and 7. Filgrastim was administered from day 8 to 28 at a dose of 30 μg/kg/day. Control mice were given vehicle alone. Data represent Mean ± SEM. Where number differs significantly in MTX + filgrastim treated groups (■) from MTX treatment alone (◆), it is indicated (***) $p < 0.001$; (**) $p < 0.01$ and (*) $p < 0.05$. And where MTX + filgrastim differs from normal values it is indicated (a): $p < 0.001$; (b): $p > 0.01$.

Discussion

The *in vivo* effect of filgrastim administration on granulopoietic cells proliferative activity was evaluated in mice pretreated with MTX. Data obtained in the present study confirm the previous observation that MTX administration shows adverse effects at relatively late stages of granulopoietic development (Pannacciulli *et al.*, 1982; Bogliolo *et al.*, 1988; Stromhaug and Warren, 2000). Under physiological conditions, an increase in bone marrow or spleen GM-CFU population is followed by a differentiation into granulocyte precursor cells, with a concomitant increase in circulating neutrophils. In addition, the bulk of the granuloid production occurs in the marrow. In MTX treated mice the granulopoiesis is perturbed. In our study the hemopoietic tissues such as bone marrow and spleen showed differential behavior as regards the changes in granuloid cell values. In bone marrow of MTX pretreated mice, GM-CFU progenitor cells reached oscillating values higher than normal ones indicating a high proliferative activity while granuloid precursor cells have shown a reduction of values

followed by a return to normal ones. Still, high proliferative activity was not observed. On the other hand, spleen GM-CFU cells values showed alternate phases of over – and undershooting in mice treated with MTX alone, but the increased splenic GM-CFUs values did not result in an increased compartment of granuloid precursors in this hemopoietic organ.

The total recovery of granulopoiesis from MTX-induced damage was not reached after 28 days of cytotoxic drug treatment and a new steady state was not yet seen at the end of our experiments. Administration of filgrastim did not modify greatly the MTX changes induced in murine femoral or spleen GM-CFU cells stage. However, there is a remarkable difference between marrow and spleen as regards the amplification within the granuloid precursor cells. Filgrastim did not result in an increase of marrow granuloid cell number, when compared with MTX pretreated mice, as observed in normal mice (Barrios *et al.*, 1998). This finding can be explained by an inhibited differentiation of GM-CFU into the more mature granulopoietic precursor cells or by an inhibited amplification of marrow granuloid cells

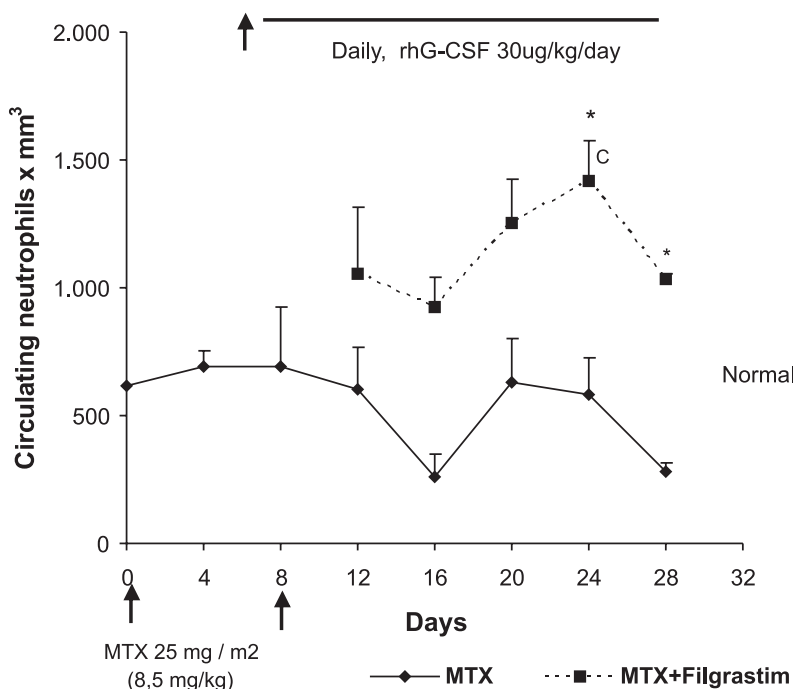


FIGURE 5. Changes in circulating neutrophil level $\times \text{mm}^3$. All mice were injected ip with MTX (methotrexate) 25 mg/m² (8,5 mg/kg) on days 0 and 7. Filgrastim was administered from day 8 to 28 at a dose of 30 $\mu\text{g}/\text{kg}/\text{day}$. Control mice were given PBS. Data represent Mean \pm SEM. Where number differs significantly in MTX + filgrastim treated groups (■) from MTX treatment alone (◆), it is indicated (*) $p < 0.05$; and where it differs from normal values, it is indicated: (c) $p < 0.05$.

TABLE 1.

Bone marrow and spleen contribution to granuloid recovery following filgrastim therapy in mice pretreated with methotrexate (MTX)

Day	Treatment	Marrow granuloid cells x 10 ⁶	Spleen granuloid cells x 10 ⁶
0	None	130.9 ± 10.1	5.0 ± 1.8
4	MTX	90.8 ± 10.3	5.1 ± 1.0
8	MTX	67.2 ± 8.5 c	7.8 ± 2.1
12	MTX	116.3 ± 19.3	7.0 ± 1.4
	MTX + filgrastim	105.3 ± 14.6	7.5 ± 0.8
16	MTX	145.2 ± 18.8	5.8 ± 0.8
	MTX + filgrastim	165.5 ± 11.4	31.3 ± 4.5***a
20	MTX	71.5 ± 12.2	3.5 ± 0.5
	MTX + filgrastim	148.8 ± 14.7	19.3 ± 5.2**b
24	MTX	103.0 ± 7.4	10.3 ± 2.4
	MTX + filgrastim	129.6 ± 16.4*	42.8 ± 7.2***a
28	MTX	145.1 ± 7.4	5.0 ± 1.1
	MTX + filgrastim	126.0 ± 19.8	19.5 ± 2.4*c

Values shown are Mean ± SEM. All mice were injected intraperitoneally with MTX 25 mg/m² (8.5 mg/kg) on days 0 and 7. MTX + filgrastim groups were injected with filgrastim (30 mg/kg) from day 8 to 28. Control MTX groups were given PBS.

*** (p > 0.001), ** (p < 0.01), * (p < 0.05) compared with control MTX group
a: (p < 0.001), b (p < 0.01), c (p < 0.05) compared with normal values (day 0)

of MTX pretreated mice. Furthermore, an unfavorable marrow MTX - induced microenvironment could be present.

On the other hand, in mice receiving filgrastim after MTX treatment, the spleen microenvironment becomes more supportive for granulopoiesis, and filgrastim enhanced the spleen granuloid cell values of MTX pretreated mice. These observations could be explained by a progenitor cell migration from the marrow to the spleen (a minor hemopoietic site in normal mice if compared with the bone marrow) induced by the chemotherapy treatment (Goris *et al.*, 1990; Morrison *et al.*, 1997). The splenic granuloid amplification stimulated by filgrastim administration paralleled the changes in circulating neutrophils values of mice

injected with filgrastim. However, in total granuloid cells production, the marrow was the most important hemopoietic site.

In summary, this study provides evidence that MTX treatment toxicity appears affecting late stages of bone marrow granulopoiesis, and the lack of filgrastim stimulating effect on marrow granuloid precursor cell compartment is consistent with the evidence presented above.

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