EXPERIMENTAL BIOLOGY

Delayed Effects of Long-Term Administration of Granulocyte Colony-Stimulating Factor to Mice

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We studied the effects of chronic administration of granulocyte colony-stimulating factor in nonmobilizing doses to mice. Over 18 months of the study, 55% animals of the treatment group died of unknown cause, blood diseases and tumors were found in 20% mice, and in 5% animals pathological changes were absent. Control mice had no diseases (normal values of total and differential leukocyte count). The diagnoses made over the first 7 months mainly included myeloproliferative diseases. Solid tumors were found at later terms. Suppurative inflammation at the site of injection was observed in all mice after 3-month treatment with granulocyte colony-stimulating factor. Our results indicate that chronic administration of granulocyte colony-stimulating factor in low doses leads to the development of etiologically different tumors and sharply reduced animal life span. The use of granulocyte colony-stimulating factor during allogeneic transplantation of hemopoietic stem cells can be hazardous for donors.

Key Words: *granulocyte colony-stimulating factor; hemopoietic stem cells; myeloproliferation; tumor transformation*

The cytokine granulocyte colony-stimulating factor (G-CSF) is extensively used in clinical practice for the therapy of severe neutropenias and infections, stimulation of tumor cell proliferation with subsequent chemotherapy, and mobilization of hemopoietic stem cells (HSC) from the bone marrow to the peripheral circulation.

A large-scale clinical trial showed that chronic administration of G-CSF (more than 12 years) in the therapy of patients with familiar (inherited) neutropenia often causes myelodysplastic syndrome and acute myeloid leukemia [10]. Several injections of G-CSF to mobilize HSC were shown to affect the donor organism. For example, administration of G-CSF in daily doses of 5-10 μg/kg for 3-5 days is accompanied by the appearance of tetraploid myeloid cells in the peripheral blood of donors. The development of neutropenia in donors is probably related of apheresis [1,7]. Some patients exhibit transient splenomegaly [5], pain sensation in the abdomen and bones, arthritis, arterial thrombosis, and anaphylaxis [7].

In experiments on mice we studied delayed effects of chronic treatment with G-CSF. The doses of G-CSF in mice were similar to those used to mobilize HSC in humans. G-CSF concentration in blood plasma from healthy donors is 25.0±19.7 pg/ml. The mobilizing dose of G-CSF is 5-10 μg/kg (350-700 μg per 70 kg). Total blood volume in the human organism is 6 liters. G-CSF concentration in

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blood plasma during mobilization is 4000-fold higher compared to the basal level. Blood volume in mice is 1.2 ml. Body weight of mice is 20-30 g. The concentration of G-CSF in blood plasma of mice is similar to that in humans. Administration of G-CSF in a dose of 25 μg/kg to mice is followed by an increase in its concentration by 20,000 times.

The effectiveness of HSC mobilization depends on the dose and time of treatment with G-CSF. G-CSF in a dose of 5-10 μg/kg is administered to accelerate the recovery of neutrophils after chemotherapy (7-14-day treatment) and to mobilize HSC in humans (4-6-day treatment) [6]. The mobilizing dose of G-CSF in mice is much higher than in humans (200-300 μg/kg for 5-17 days). The number of precursor cells of different maturity increases in the peripheral blood (by tens times) [2,6]. No effective mobilization is observed after administration of G-CSF in a dose of 25 μg/kg. Under these conditions the number of peripheral blood precursor cells (spleen colony-forming units, CFU-S) increases only by 4 times. However, HSC count in the bone marrow decreases by 1.5-2.0 times [2].

Here we studied the delayed effects of long-term treatment with G-CSF.

MATERIALS AND METHODS

Experiments were performed on female CBF_1 (CBA×C57Bl/6) and DBF_1 mice (DBA2×C57Bl/6) aging 5 months and weighing 24-26 g. These hybrid mice have different resistance to spontaneous tumorigenesis. The experimental group consisted of 20 treated mice (10 mice of each genotype) and 10 control animals.

G-CSF (Neupogen 48 Mio U, F. Hoffman-La Roche) was dissolved in physiological dose with 0.1% bovine serum albumin (Sigma). The solution of G-CSF (0.2 ml) was administered subcutaneously in doses of 25 (4 days, pulse treatment) and 5 μg kg (20 days, chronic treatment). Hence, the total monthly dose of G-CSF was similar in both treatment courses. The peripheral blood was sampled from animals with pulse (before and 1 day after each course of injections) and chronic treatment (once a month). Leukocyte count and leukogram were estimated. The course of injections was repeated monthly for 6 months. The state of animals was monitored over 12 months. The number of peripheral blood leukocytes and differential leukocyte count were evaluated once a month.

Histological samples from sick animals were treated and studied routinely. The samples were fixed in Bouin's fluid. Paraffin sections were stained with hematoxylin and eosin or with Giemsa solution (Romanovsky method). The results were analyzed by Student's *t* test.

RESULTS

G-CSF significantly decreased mouse life span (Fig. 1). Over 18 months of the study, 22 animals of the treatment group (55%) died of unknown cause. Blood diseases and tumors were found in 8 mice (20%). Control mice had no diseases. These animals were characterized by normal leukocyte count and hemogram.

Pulse treatment with G-CSF in low dose had little effect on leukocyte count in the peripheral blood of mice (Fig. 2, *a*). The number of leukocytes and percentage of granulocytes in mice after chronic administration of G-CSF were higher than in controls and animals receiving pulse treatment (Fig. 2, *b*). Reticulocyte count in most mice increased after pulse treatment with G-CSF. These data show that the number of peripheral blood leukocytes depends on the route of treatment with G-CSF (at the same dose of test substance). Leukocyte count in control animals varied within the normal limits (6-14×10^9/liter).

Tumorigenesis was preceded by a decrease in the number of peripheral blood leukocytes in mice of the G-CSF pulse treatment group (compared to the control, Table 1). Among all sick animals, the exception was the DBF_1-8 mouse. Postmortem examination showed that severe cachexia in this mouse was caused by liver tapeworms.

Two mice (CBF_1-4 and CBF_1-9) died during the 4th course of pulse treatment. The number of

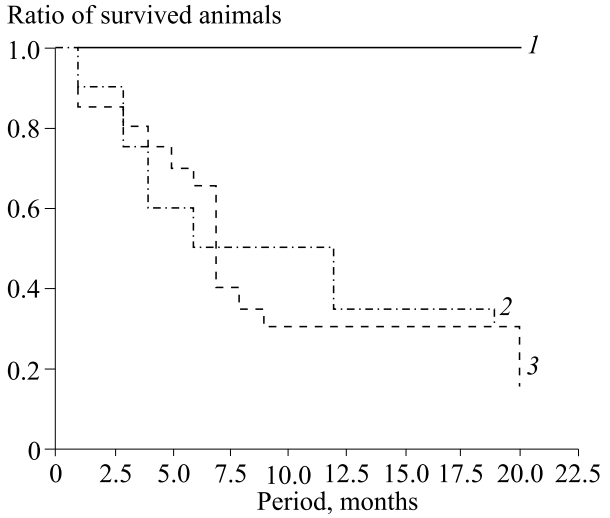

Fig. 1. Overall survival of mice. Control (*1*); chronic treatment with G-CSF (*2*); and pulse treatment with G-CSF (*3*). Abscissa: duration of experiment, months. Ordinate: ratio of survived animals (% of the total number of animals).

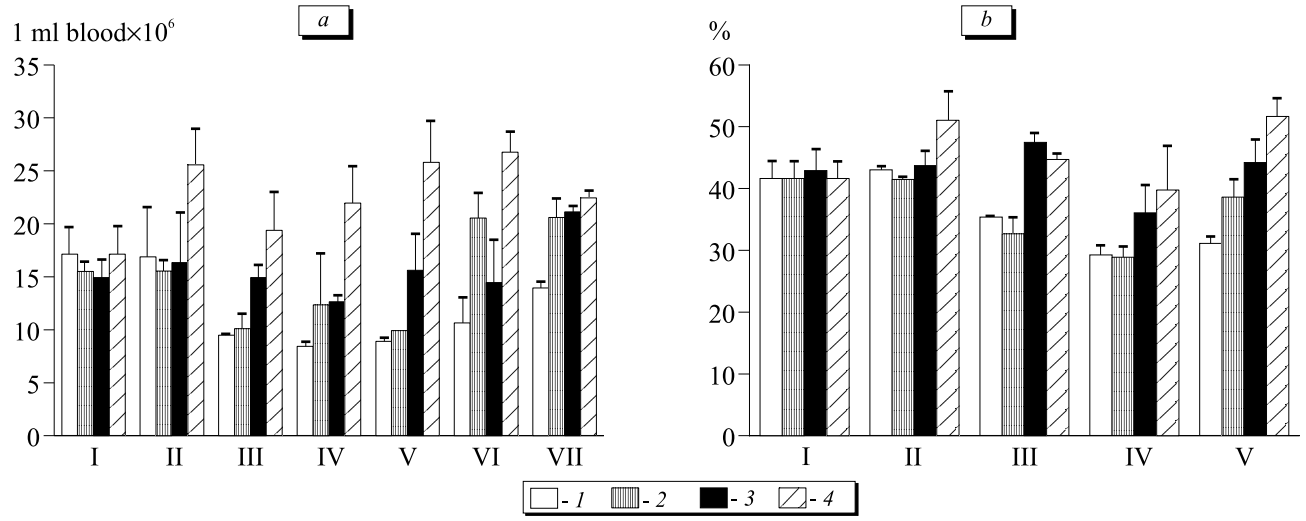

Fig. 2. Leukocyte count (*a*) and percentage of granulocytes (*b*) in the peripheral blood of G-CSF-receiving mice. Control (*1*); pulse treatment, before administration (*2*); pulse treatment, after administration (*3*); and chronic treatment (*4*).

leukocytes and percentage of granulocytes in both mice increased after the 3rd course of injections. The cause of death was not determined. By the 4th course, leukocyte count in CBF_1-5 was lower than in other animals of the control and treatment group. However, leukocyte count in this mouse sharply increased before the 5th course (from 5.2×10^9 to 2.05×10^{10}/liter). The percentage of mature granu-

TABLE 1. Peripheral Blood Leukocyte Count in Diseased and Died Mice after Pulse Treatment with G-CSF (as Compared to Animals of the Control Group)

Genetic strain, number of mouse	Time of disease detection or death, months after the start of treatment	Most probable diagnosis	Leukocyte count during or before death, 10^9/liter	Leukocyte count in control mice, 10^9/liter
CBF_1-4	4	Unknown	4.8	8.05±1.50
CBF_1-9	4	Unknown	8	
CBF_1-5	4	Myeloproliferative disease with signs of histiocytic sarcoma	20.5	
CBF_1-8	5	Unknown	19.4	13.0±3.9
CBF_1-7	7.5	Undifferentiated myeloid leukemia	4.4	13.30±2.06
CBF_1-3	8-18	Unknown		11.1±2.7
CBF_1-10	8-18	Unknown		
CBF_1-1	Alive for 20 months			11.1±2.7
CBF_1-2	Alive for 20 months			
CBF_1-5	Alive for 20 months			
DBF_1-4	1.5	Unknown	17.4	9.40±2.16
DBF_1-9	1.5	Unknown	13.5	
DBF_1-1	6	Cachexia	10.9	13±2
DBF_1-3	6	Cachexia	6.8	
DBF_1-5	6	Cachexia	9.2	
DBF_1-6	6	Cachexia	10.8	
DBF_1-7	8-18	Unknown	17.5	22.3±9.9
DBF_1-2	18	Thyroid hyperplasia	13.6	
DBF_1-8	18	Helminthic invasion	63.5	
DBF_1-10	18	Cachexia	12.9	

locytes was 72.5%. The mouse was euthanized due to these changes in differential leukocyte count. The diagnosis of myeloproliferative disease with signs of histiocytic sarcoma was made after histological examination of samples (classification of mouse leukemia [3]).

Leukocyte count in the peripheral blood from CBF_1-7 of the pulse-treatment group increased 1.5 months after the 6th course of G-CSF (3.92×10^{10}/liter). This parameter decreased after 3 days (4.4×10^9/liter), and the mouse died. The diagnosis of undifferentiated myeloid leukemia was made after histological examination of samples (classification of mouse leukemia [3]).

Three mice of the G-CSF pulse treatment group were euthanized due to severe cachexia 18 months after the start of the study. Severe hyperplasia of the thyroid gland was found in one of these animals. No deterioration was observed in other mice.

After chronic administration of G-CSF, leukocyte count in all animals was much higher than in controls (Fig. 2, *b*). Suppurative inflammation of the injection site was found in all animals 3 months after the start of study. The observed effect was not associated with aseptic failure, since control animals did not exhibit these changes. Progressive partial facial alopecia was of the same type in most animals (Fig. 3).

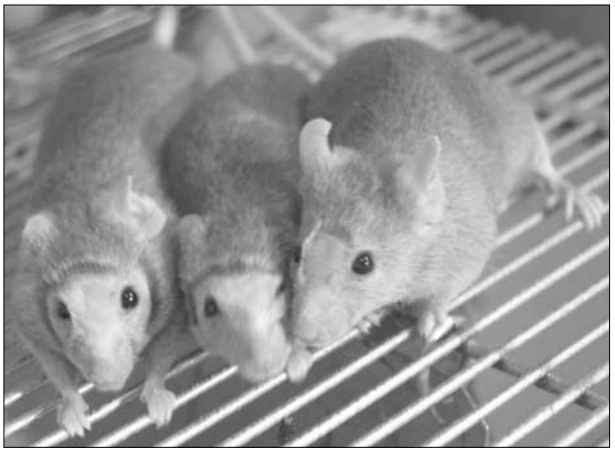

Fig. 3. Mice in chronic administration of G-CSF (5 months after the start of treatment).

TABLE 2. Peripheral Blood Leukocyte Count in Diseased and Died Mice after Chronic Treatment with G-CSF (as Compared to Animals of the Control Group)

Genetic strain, number of mouse	Time of disease detection or death, months after the start of treatment	Most probable diagnosis	Leukocyte count during or before death, 10^9/liter	Leukocyte count in control mice, 10^9/liter
CBF_1-9	1	Liver enlargement		12.15±2.50
CBF_1-10	1	Unknown		
CBF_1-3	2	Cachexia, forelimb necrosis	18.1	9.40±2.16
CBF_1-4	2	Cachexia, forelimb necrosis	24	
CBF_1-8	3	Abscess		8.0±1.5
CBF_1-1	5	Cachexia		13.0±3.9
CBF_1-5	6	Cachexia, splenic myelofibrosis		3±2
CBF_1-2	18	Pyogenic abscess of the parathyroid salivary gland	41.6	22.3±9.9
CBF_1-6	18	Tumor in the femoral region (angiosarcoma)	8.7	
CBF_1-7	18	Cachexia, splenic hyperplasia	6.9	
DBF_1-10	1	Liver enlargement	13.4	12.15±2.50
DBF_1-5	3	Unknown		8.0±1.5
DBF_1-8	4	Liver enlargement		8.05±1.50
DBF_1-3	8-18	Unknown	29.6	22.3±9.9
DBF_1-6	18	Spleen hyperplasia	16.4	22.3±9.9
DBF_1-1	Alive for 20 months		22.3	22.3±9.9
DBF_1-2	Alive for 20 months		20.3	
DBF_1-4	Alive for 20 months		27.5	
DBF_1-7	Alive for 20 months		27.6	
DBF_1-9	Alive for 20 months		19.2	

Signs of cachexia in 2 mice (CBF_1-3 and CBF_1-4) were observed 2 months after the start of chronic treatment with G-CSF. One of the forelimbs in each mouse was necrotized. The animals were euthanized. The cause of deterioration was undetermined. CBF_1-1 was euthanized due to severe cachexia after 5 months of chronic treatment with G-CSF. Signs of myelofibrosis were revealed during histological examination of the spleen.

DBF_1-6 was euthanized due to large tumor in the femoral region 18 months after the start of chronic treatment with G-CSF. Tumor cells did not invade the peritoneum. The tumor was encapsulated and contained a considerable number of vessels and small inclusions of connective tissue cells. The diagnosis of angiosarcoma was made after histological examination. Pyogenic abscess of the parathyroid salivary gland and sharp increase in the number of peripheral blood leukocytes (4.16×10^{10}/liter) were found in CBF_1-2. Myeloid hyperplasia of the spleen (megakaryocytosis) was revealed in both animals. Two animals of this group were characterized by cachexia. Peripheral blood leukocyte count decreased to 8.7×10^9 (CBF_1-6) and 6.9×10^9/liter (CBF_1-7). The percentage of peripheral blood monocytes increased in both mice. Histological examination revealed fatty degeneration of the liver and bone marrow in CBF_1-6. Table 2 illustrates the premorbid dynamics of leukocyte count.

Hence, most animals died of different causes after 18-month administration of G-CSF in a low dose. However, the mortality rate of control animals corresponded to the mean life span of these hybrids.

Our results indicate that chronic administration of G-CSF in very low doses is followed by the development of tumors and diseases of different etiology and sharp decrease in the life span of animals. The use of G-CSF during allogeneic transplantation of HSC can be hazardous for the donor. Anemia and thrombocytopenia in donors were usually attributed to leukopheresis. Experiments on animals showed that pulse treatment and, particularly, chronic administration of G-CSF are followed by reticulocytosis and partial alopecia and contributes to the development of infections. The permanent increase in leukocyte count after chronic administration of G-CSF cannot be explained by pyogenic abscess of the injection site. These changes persisted after the disappearance of pyogenic abscess. No correlation was found between leukocyte count and toxicity in humans. However, leukocytosis in primates was accompanied by infiltration of the brain parenchyma with neutrophils (instruction for Neupogen). The rabbits with this disorder were characterized by pulmonary hemorrhage, microvascular occlusion, and focal pneumonia [9]. Acute pulmonary toxicity in 1 donor was observed after 4-day treatment with G-CSF [11]. Granulocyte infiltration was revealed in the liver and lungs of mice [8]. Acute myeloid leukemia (M1 according to the FAB classification) in 1 donor of HSC developed 1 month after mobilization (Japan) [4]. A large-scale trial with donors is required to evaluate the relationship between acute myeloid leukemia and G-CSF administration. In our experiments, most animals died over the first 10-12 months of treatment. The majority of blood tumors were myeloid. Solid tumors were revealed in the later period.

Since no diseases were found in control mice, we can conclude that these disorders are induced by repeated treatment with G-CSF. The mechanism of G-CSF-induced tumorigenesis requires further investigations. The understanding of this mechanism will elucidate the mechanisms of normal regulation of hemopoiesis and pathogenesis of leukemias.

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