Effect of Transplantation of Peripheral Blood Mononuclears Obtained Using Granulocytic Colony-Stimulating Factor and Hyaluronidase on Regeneration of Hemopoietic Tissue during Myelosuppression

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The mechanisms of hemopoiesis recovery after transplantation of peripheral blood mononuclears obtained using granulocytic CSF and granulocytic CSF in combination with hyaluronidase were studied on the model of cytostatic myelosuppression. It was found that regeneration of the hemopoietic tissue resulted from an increase in the pool of erythroid and granulomonocytic precursors and in their functional activity. The increase in the count of fibroblast precursors in the bone marrow, higher production of hemopoietins by adherent myelokaryocytes, and an increase in the level of humoral factors in the serum were detected after injection of the transplants. A higher therapeutic efficiency of cell material obtained after combined use of granulocytic CSF and hyaluronidase was shown.

Key Words: *progenitor cells; transplantation; granulocytic colony-stimulating factor; hyaluronidase; myelosuppression*

Cell therapy is now widely used in clinical oncology for the treatment of hematological complications [10]. The peripheral blood mononuclear fraction, enriched by progenitor elements mobilized from the bone marrow, is one of the materials most often used for transplantation. Stem cell release into circulating blood is often stimulated by granulocytic CSF (G-CSF) [6,10]. On the other hand, it was experimentally shown that the count of precursor cells among mononuclears stimulated with G-CSF can be increased by additional treatment with hyaluronidase [2]. This enzyme involved in hyaluronic acid metabolism can essentially modulate the status of extracellular matrix [4,11] and glycocalyx of cells and their receptors [9]. Therefore, the capacity of progenitor elements treated with hyaluronidase to realization of their growth potential *in vivo* after their transplantation attracts special interest.

We studied therapeutic activity of cell material (peripheral blood mononuclears) obtained using G-CSF and hyaluronidase on the model of cytostatic myelosuppression.

MATERIALS AND METHODS

The study was carried out on 2-month-old male CBA/CaLac mice (n=250; 18-20 g; first-category conventional inbred animals from Breeding Center of Institute of Pharmacology). Cell material for transplantation was collected from mice injected with: 1) G-CSF alone; 2) G-CSF in parallel with hyaluronidase.

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Granulocytic CSF (Neupogen, Hoffman-la Roche) was injected subcutaneously (125 μg/kg/day) for 3 days, hyaluronidase (Lidase, Microgen) was injected intraperitoneally in a daily dose of 20 arb. units for 2 days. Blood cells were collected on day 4 after the beginning of injections. The peripheral blood mononuclear fraction was collected using Histo-paque-1077 (Sigma) [3]. The cell material was diluted in a medium containing 90% RPMI-1640 (Sigma), 10% inactivated FCS (Sigma), and 40 U/ml heparin (Biochemie) to a concentration of 15×10^6 cell/ml.

Myelosuppression was induced by a single intraperitoneal injection of $^1/_2$ of maximum tolerated dose of 5-fluorouracyl (Ebewe Farma GmbH; 114 mg/kg). One day after cytostatic treatment, the animals received a single intravenous injection of syngeneic cell material obtained beforehand by one of the two methods in a dose of 1.5×10^6 cell/mouse. Controls received an equivalent volume (100 μl) of nutrient medium used for cell dilution. On days 5, 7, and 12 after the cytostatic injection, parameters of the peripheral blood and bone marrow hemopoiesis were determined using an ABACUS automated hematological analyzer (Diatron) and standard hematological tests [5]. The content of erythroid, granulomonocytic, and fibroblast CFU in the hemopoietic tissue and proliferative activity and rate of hemopoietic precursor maturation were evaluated by culture methods [3]. The production of erythropoietic and colony-stimulating activities by individual fractions of the hemopoiesis-inducing microenvironment (HIM) and serum content of humoral factors were studied [3].

The results were processed by variation methods of statistics using Student's *t* test and nonparametric Mann—Whitney *U* test.

RESULTS

Injection of 5-fluorouracyl decreased cell count in the peripheral blood (Table 1). These changes reflected the bone marrow hemopoiesis processes. A sharp drop in the counts of bone marrow immature and mature neutrophilic granulocytes and erythrokaryocytes was observed (Fig. 1). On day 5 of the experiments their counts decreased to 6.7, 0.3, and 6.2% of basal levels, respectively. The reaction of the pool of committed precursor cells was characterized by a decrease in the count and rate of division of erythroid CFU on days 5 and 7 and of granulomonocytic CFU on day 5 of the experiment (Fig. 2). These changes were observed despite more intense production of growth factors by HIM cells and increased serum erythropoietic activity (Fig. 3), and were presumably caused by disorders in the hemopoietic precursor cooperation with the microenvironment elements [1]. On the other hand, the

TABLE 1. Parameters of Peripheral Blood in CBA/CaLac Mice Injected with 5-Fluorouracyl (Group 1) and during Cell Therapy for Myelosuppression with Mononuclears from Mice Injected with G-CSF (Group 2) and G-CSF+Hyaluronidase (Group 3) ($X\pm m$)

Day of study; group		Reticulocytes, ‰	Erythrocytes, 10^{12}/liter	Total leukocyte count, 10^9/liter	Neutrophils, 10^9/liter		Monocytes, 10^9/liter	Eosinophils, 10^9/liter	Lymphocytes, 10^9/liter	Platelets, 10^9/liter
					stab	segmented				
Basal level		5.29±0.18	11.59±0.40	7.50±0.29	0.09±0.03	1.63±0.12	0.11±0.03	0.11±0.03	5.56±0.34	680.14±16.78
Day 5	1	2.90±0.14*	9.38±0.17*	3.11±0.26*	0±0*	0.03±0.01*	0±0*	0.03±0.01*	3.06±0.25*	329.86±37.10*
	2	4.43±0.20*+	9.84±0.51*	3.64±0.41*	0.02±0.01*+	0.02±0.02*	0.01±0.01*	0.06±0.02	3.45±0.43*	368.33±21.4*
	3	4.57±0.20*	10.51±0.38	4.21±0.20*	0.04±0.01	0.06±0.01*	0±0*	0.03±0.01*	4.09±0.18*	536.0±49.0×
Day 7	1	3.00±0.31*	8.84±0.15*	3.50±0.25*	0.03±0.01*	0.16±0.04*	0±0*	0.02±0.01*	3.30±0.25*	452.71±54.20*
	2	6.14±0.10*+	9.55±0.51*	5.07±0.19*+	0.09±0.02+	0.6±0.1*+	0.01±0.01*	0.07±0.01+	4.30±0.18*+	871.43±67.50*+
	3	7.14±0.26*×	9.69±0.24*+	5.93±0.20*+×	0.14±0.01+×	0.67±0.04*+	0.01±0.01*	0.06±0.01+	5.05±0.19+×	935.1±83.5*+
Day 12	1	7.57±0.75*	7.38±0.30*	8.57±0.46	0.17±0.04	1.96±0.029	0.04±0.02	0.12±0.03	6.29±0.40	940.14±89.10*
	2	6.57±0.80	8.16±0.09*+	10.60±0.44*+	0.31±0.08*	2.83±0.27*+	0.06±0.03	0.22±0.04	7.25±0.19*+	1359.4±181.0*
	3	6.71±1.08	9.09±0.35*+×	10.80±0.38*+	0.38±0.06*+	3.68±0.36*+×	0.05±0.03	0.24±0.05	6.46±0.54	1143.8±109.4*

Note. $p<0.05$ compared to: *intact control, +cytostatic control, ×between groups 2 and 3.

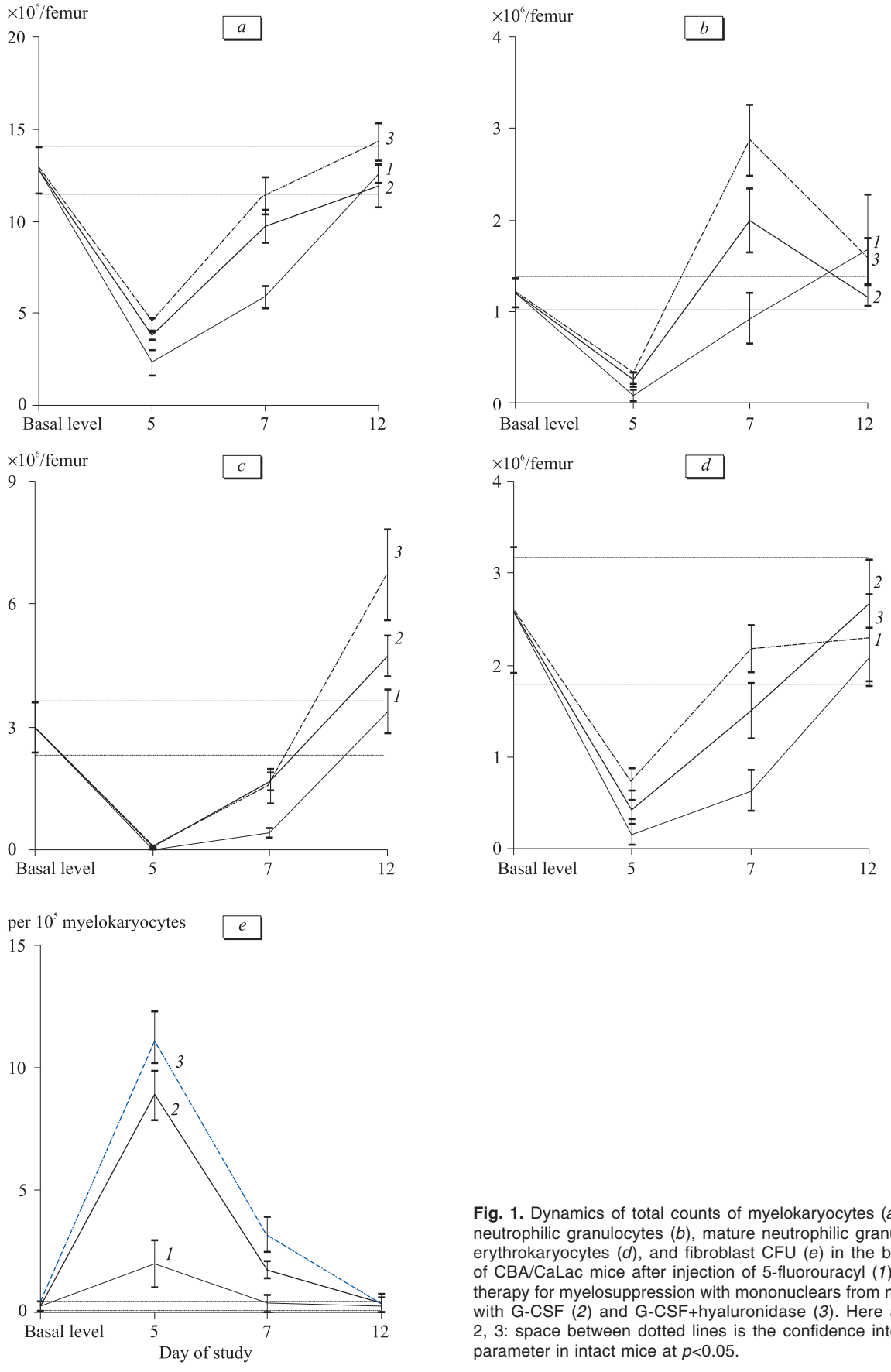

Fig. 1. Dynamics of total counts of myelokaryocytes (*a*), immature neutrophilic granulocytes (*b*), mature neutrophilic granulocytes (*c*), erythrokaryocytes (*d*), and fibroblast CFU (*e*) in the bone marrow of CBA/CaLac mice after injection of 5-fluorouracyl (*1*), during cell therapy for myelosuppression with mononuclears from mice injected with G-CSF (*2*) and G-CSF+hyaluronidase (*3*). Here and in Figs. 2, 3: space between dotted lines is the confidence interval for the parameter in intact mice at $p<0.05$.

increase in the count of fibroblast CFU in the bone marrow observed on day 5 indicated activation of recovery of hemopoietic tissue stroma (Fig. 1, *e*). On the whole, the results of the experiment completely coincided with our previous data on the hemotropic effect of 5-fluorouracyl [1].

Study of the effects of cell therapy on hemopoiesis processes showed that injection of peripheral blood

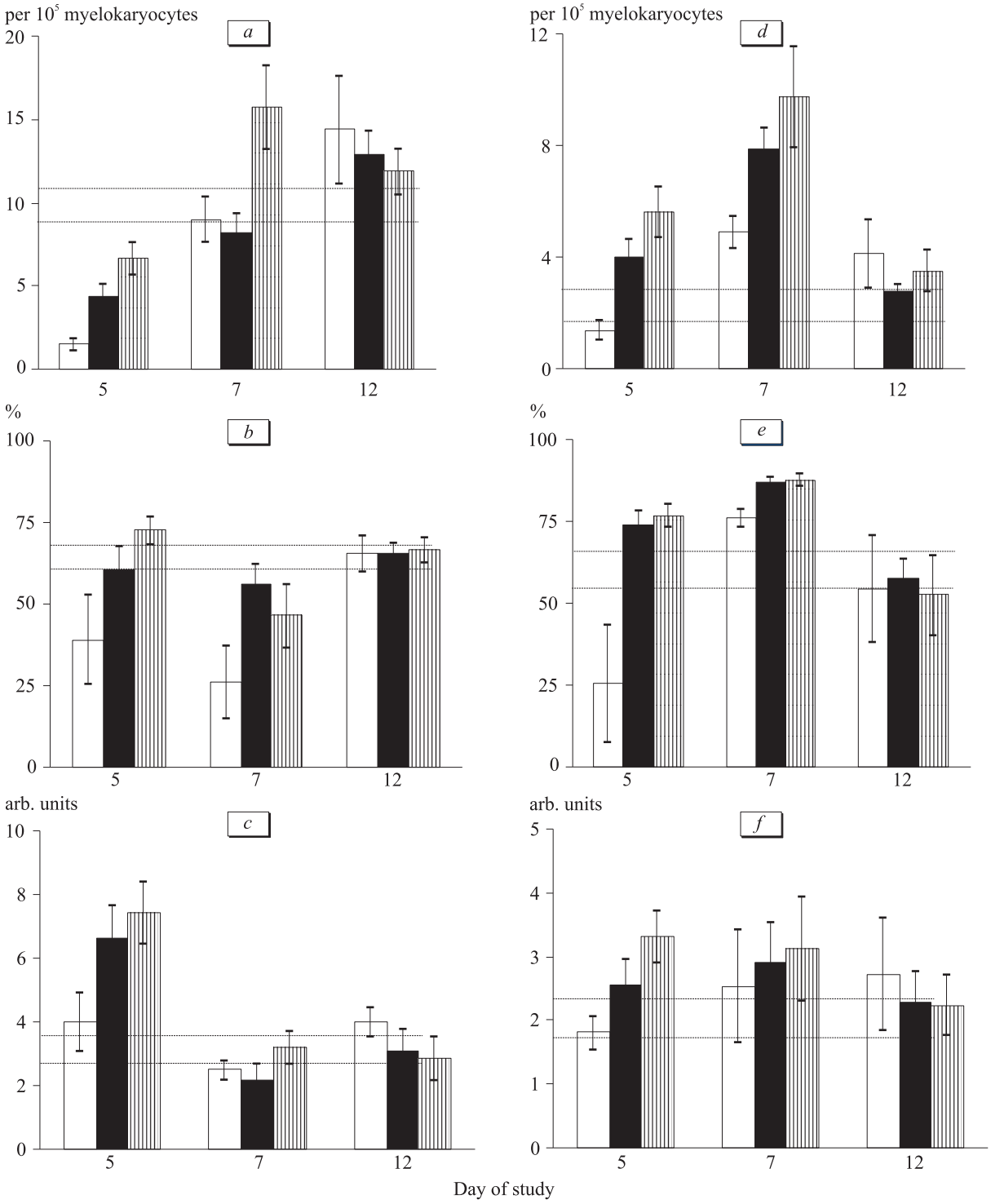

Fig. 2. Dynamics of hemopoietic precursor counts (*a*, *d*), their percentage in the mitotic cycle S phase (*b*, *e*), and the rate of maturation (*c*, *f*) in the bone marrow of CBA/CaLac mice injected with 5-fluorouracyl (light bars), during cell therapy for myelosuppression with mononuclears obtained from mice treated with G-CSF (dark bars) and G-CSF+hyaluronidase (vertically hatched bars). *a-c*: erythroid CFU; *d-f*: granulomonocytic CFU.

mononuclears after myelosuppression induction significantly stimulated regeneration of the hemopoietic tissue. The counts of peripheral blood of reticulocytes, erythrocytes, and total counts of leukocytes, stab and segmented neutrophils, monocytes, and platelets significantly increased in both groups virtually during all periods of the study. However, more significant changes were observed in mice injected with cell material obtained after combined treatment with G-CSF and hyaluronidase (Table 1). Significant shifts in the

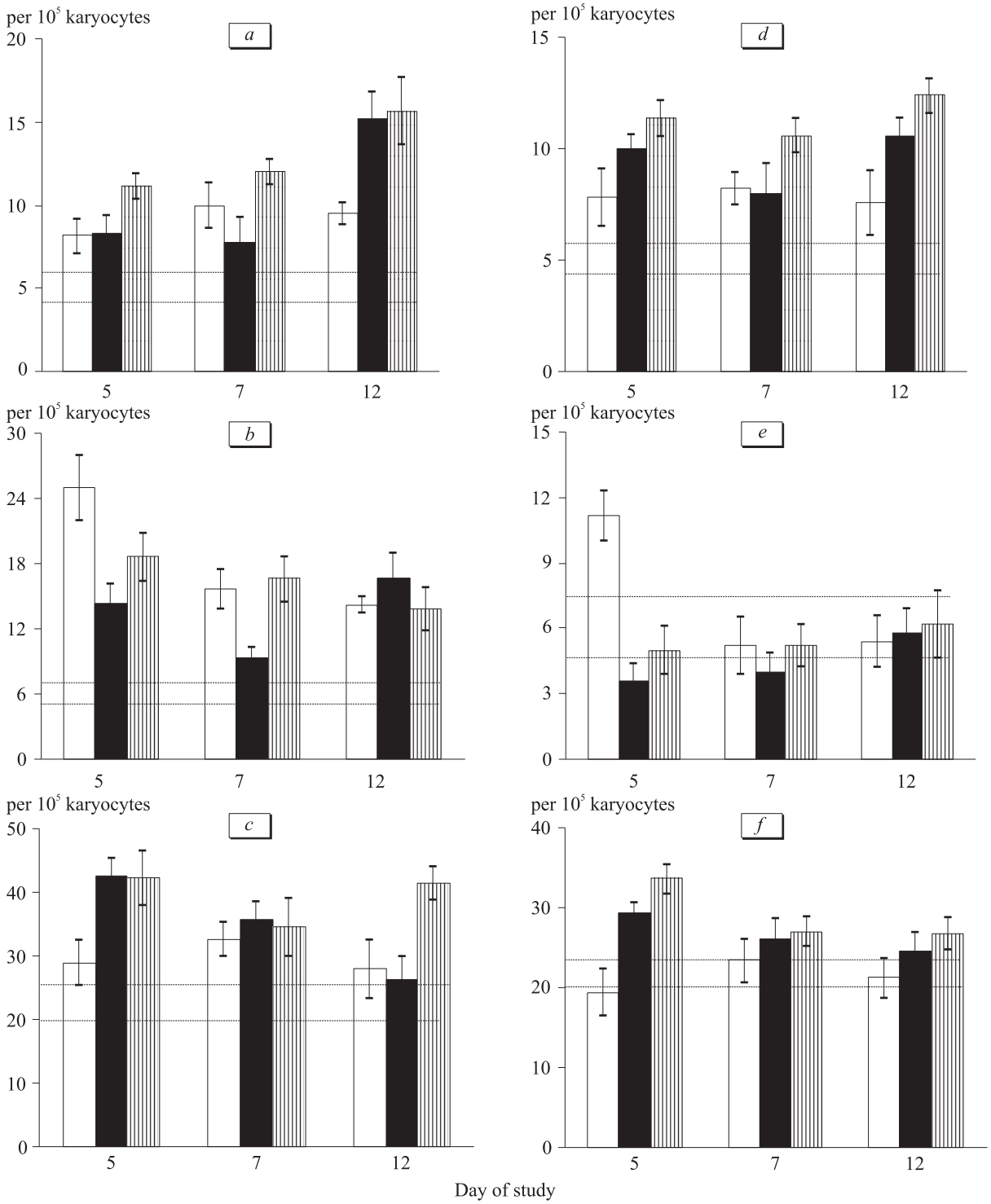

Fig. 3. Changes in the levels of erythropoietic (*a*-*c*) and colony-stimulating (*d*-*f*) activities of conditioned media of serum adherent (*a*, *d*) and nonadherent myelokaryocytes (*b*, *e*), of serum (*c*, *f*) from CBA/CaLac mice after injection with 5-fluorouracyl (light bars), during cell therapy for myelosuppression with mononuclears from mice injected with G-CSF (dark bars) and G-CSF+hyaluronidase (verically hatched bars).

bone marrow hemopoiesis were observed. Cell therapy led to a significant increase in the counts of immature and mature neutrophilic granulocytes and erythrokaryocytes in the hemopoietic tissue. The counts of these cells were significantly higher in mice receiving nuclears from animals treated with both preparations. The maximum difference between the parameters in animals of the experimental groups was 44.2, 41.8, and 45.3% for immature (day 7), mature (day 12) neutrophilic granulocytes and erythrokaryocytes (day 5), respectively (Fig. 1).

Study of the mechanisms of stimulation of hemopoietic tissue regeneration during cell therapy revealed the key role of the increase in the bone marrow counts of erythroid and granulomonocytic precursor cells, increase in their proliferative activity, and rate of maturation (Fig. 2). More significant changes were in all cases observed in mice receiving the material obtained using hyaluronidase. For example, the counts of erythroid and granulomonocytic CFU in this group on day 7 were higher than after transplantation of cells obtained from animals injected with G-CSF alone by 90.9 and 61.5%, respectively. In addition, cell transplantation increased the count of fibroblast CFU in the bone marrow, also more pronounced after transplantation of material from mice treated with both agents (Fig. 1, *e*). The detected changes in the pool of fibroblast CFU indicate more rapid recovery of the HIM stromal compartment, which is particularly important in myelosuppression caused by injection of antimetabolites damaging the HIM elements [1].

Study of the role of humoral factors in the regeneration of the hemopoietic tissue after cell transplantation detected the following phenomena. A significant increase in the production of erythropoietic and colony-stimulating activities by adherent bone marrow mononuclears was noted, reaching the maximum after injection of the material from mice treated with G-CSF and hyaluronidase for mobilization of stem cells (Fig. 3). In parallel, production of hemopoiesis-active substances by nonadherent myelokaryocytes (mainly lymphoid elements [3]) was reduced, presumably because of the lymphotropic and immunosuppressive [7,8] effects of cell therapy, caused by the presence of mesenchymal precursors in the transplants [2]. Cell therapy was associated with an increase in serum erythropoietic and colony-stimulating activities, presumably due to a direct increase in the content of elements capable of producing humoral growth factors. On the other hand, the increase in the level of erythropoietic activity during delayed period after injection of mononuclears from animals treated with G-CSF and hyaluronidase could result from recovery of the functions of the structures (kidneys and liver) responsible for erythropoietin formation. It is known that 5-fluorouracyl damages mature cells, which leads to disorders in the production of biologically full-value erythropoietin in the presence of hyperproduction of its inactive forms [12].

Hence, regeneration of the hemopoietic tissue in myelosuppression after injection of peripheral blood mononuclears enriched with stem cells [2,10] is accelerated due to increased pool of hemopoietic precursors, recovery of HIM structure and function, and elevated hemopoietin content in the serum. Cell material obtained after mobilization of the bone marrow progenitor elements [2] by combined G-CSF and hyaluronidase treatment exhibited much higher therapeutic activity.

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