Mechanisms of Hemostimulating Effect of Granulocytic Colony-Stimulating Factor during Cytostatic Treatment

E. D. Gol'dberg, A. M. Dygai, V. V. Zhdanov, G. N. Krivopalova, V. I. Masycheva, N. M. Pustoshilova, N. S. Pushkareva, E. V. Simanina, and S. A. Sinichkina

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Original Russian-made preparation of human recombinant granulocytic colony-stimulating factor possesses *in vitro* activity similar to that of standard preparation, activates *in vivo* production of humoral factors by adherent cells of the hemopoiesis-inducing microenvironment, and stimulates hemopoiesis in the bone marrow at various levels.

Key Words: *granulocytic colony-stimulating factor; granulocytopoiesis; microenvironment*

The main disadvantage of contemporary antineoplastic drugs is their toxic effect on actively proliferating cell systems. Drastic inhibition of leukocyte production during chemotherapy is sometimes the main indication for discontinuation of the treatment in oncological patients despite pronounced cancerolytic effect [1,6].

Therefore, elaboration of new drugs and rational use of known methods of correcting changes in the blood induced by cytostatic drugs are of particular importance [2].

Natural bioactive compounds, regulators of hemopoiesis, in particular, hemopoietic growth factors, are used for the treatment of hemodepressions [7,10]. The effect of human recombinant granulocytic colony-stimulating factor (G-CSF) on hemopoietic cells and regulatory apparatus of the blood system is now intensively studied and effective methods of pharmacological correction of hypoplastic state of the blood system are elaborated.

The purpose of the present study was to elucidate specific activity and mechanisms of hemostimulatory effect of original Russian-made G-CSF preparation on a model of myelosupression caused by cyclophosphamide.

Institute of Pharmacology, Tomsk Research Center, Siberian Division of the Russian Academy of Medical Science; Engineering-and-Technological Institute of Bioactive Compounds, "Vector" Research-and-Production Association, Berdsk.

MATERIALS AND METHODS

The experiments were carried out with 70 CBA/CaLac male mice aged 2-2.5 months (collection of Research Laboratory of Experimental Biomedical Modeling, Tomsk Research Center). Control and experimental mice received single intraperitoneal injection of cyclophosphamide (Biokhimik, Saransk) in a maximum permissible dose of 250 mg/kg. On days 1-5 after cyclophosphamide treatment experimental mice were injected subcutaneously with 125 mg/kg human recombinant G-CSF (Vector), control mice received the same volume of vehicle with 2% rheopolyglucin. Initial parameters were obtained during examination of intact mice.

Human recombinant G-CSF was isolated from recombinant SG200-50/pGGF8 *E. coli* strain. Semisynthetic human G-CSF gene was constructed at M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry [4]. The expression plasmid GGF8 contained a sequence of promoters of *E. coli* tryptophan operon and synthetic translation enhancer (Vector). Competent SG200-50 *E. coli* cells transformed by GGF8 plasmid were cultured in 1 liter LB medium [5] supplemented with 0.075 mg/ml ampicillin for 16 h at 30°C on a shaker at 120 rpm. The cells were centrifuged and G-CSF was isolated immediatelly or after storage at -20°C.

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For isolation of G-CSF the cells were resuspended in buffer A (10 mM Tris-HCl, pH 8.0) containing 2-mercaptoethanol. After centrifugation the pellet was washed with buffer A and dissolved in 8 M urea. Further purification of G-CSF was performed by gel filtration of protein extract on G-25 Sephadex (Pharmacia), chromatography on CM cellulose (Serva) and gel filtration on G-75 Sephadex (Pharmacia). The obtained protein (0.2-0.8 mg/ml) was dialyzed against 10 mM potassium phosphate buffer (pH 7.2) containing 0.15 M NaCl. The solution was sterilized by filtration through a membrane filter (0.22 μ pore size) and stored at -20°C.

The yield was 2-3 mg G-CSF from 10 g of biomass with 95-98% purity confirmed by high-performance liquid chromatography (HPLC) and electrophoresis. G-CSF preparation contained no lipopolysaccharide or DNA impurities.

Bovine serum albumin (67 kD), ovalbumin (45 kD), myoglobin (17.8 kD), and lysozyme (14.4 kD) were used as molecular weight markers for protein electrophoresis under denaturating conditions [8].

The content of G-CSF was measured on a Chromoscan 200 device (Joyce Loebl) and protein concentration was determined as described elsewhere [9,11]. HPLC was performed on a Pro RPC TM column (Pharmacia); mobile phase consisted of 0.1% trifluoroacetic acid — acetonitrile in 0.1% trifluoroacetic acid, gradient elution.

The animals were killed on days 4-6, 8 and 10 after cyclophosphamide injection by cervical dislocation under ether narcosis. Quantitative and qualitative content of periferal blood was estimated routinely and the total number of myelokaryocytes in the bone marrow was determined. The content of committed precursor cells of erythropoiesis (CFU-E) and granulomonocytopoesis (CFU-GM) in the bone marrow was determined by *in vitro* cloning in methylcellulose [3]. Colony-stimulating and erythropoietic activities in conditioned media of adherent and nonadherent cells of hemopoiesis-inducing microenvironment were tested on intact murine myelokaryocytes in semisolid culture medium [3].

Erythrocyte-free suspension of nonadherent bone marrow cells from intact CBA mice in semisolid methylcellulose medium was used as an *in vitro* test system for determination of specific activity of G-CSF. The G-CSF samples (Russian-made G-CSF and standard preparation approved by WHO) were added to the culture medium at varying concentrations (5-100 ng/ml). The cells were incubated for 7 days at 100% humidity and 3.5% CO_2. Aggregates containing more than 50 hemopoietic cells were regarded as colonies. Individual colonies were examined under an inverted phase contrast microscope.

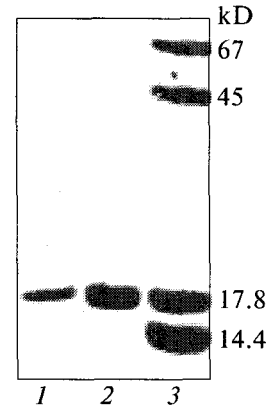

Fig. 1. Electrophoresis of recombinant human granulocyte colony-stimulating factor in 15% polyacrylamide gel under denaturating conditions at 10 (*1*) and 40 (*2*) μg load. *3* — marker proteins.

The results were processed statistically using Student's *t* and nonparametric Wilcoxon—Mann—Witney tests.

RESULTS

The test G-CSF showed a clear-cut dose-dependent effect in the *in vitro* system. Granulocyte colonies (80%) predominated in culture, while the content of granulocyte-macrophage and macrophage colonies was 10% each. Colony-stimulating activity of the examined preparation was comparable with that of standard (WHO approved) drug (Table 1).

Experiments on mice showed that G-CSF significantly increased the total number of myelocytes (up to 300%) in comparison with the control (cyclophosphamide) on days 4 and 8 due to more than 2-fold increase in the number of bone marrow neutrophils (day 8) and partly, lymphoid cells (days 5 and 8; Fig. 2). On the whole, the changes in the peripheral blood corresponded to the dynamics of bone marrow parameters: the total number of leukocytes increased significantly from day 5 to 8 due to an increase in the number of neutrophilic granulocytes to 385% on day

TABLE 1. *In Vitro* Colony-Stimulating Activity of Human Recombinant G-CSF (Number of Colonies per 10^5 Intact Nonadherent Myelokaryocytes of CBA mice)

Concentration in culture, ng/ml	G-CSF	
	standard (WHO)	test
0	1.75	1.75
5	3.00	2.50
10	3.75*	4.50*
20	4.00*	6.75*

Note. *$p<0.05$ compared to background activity (concentration=0).

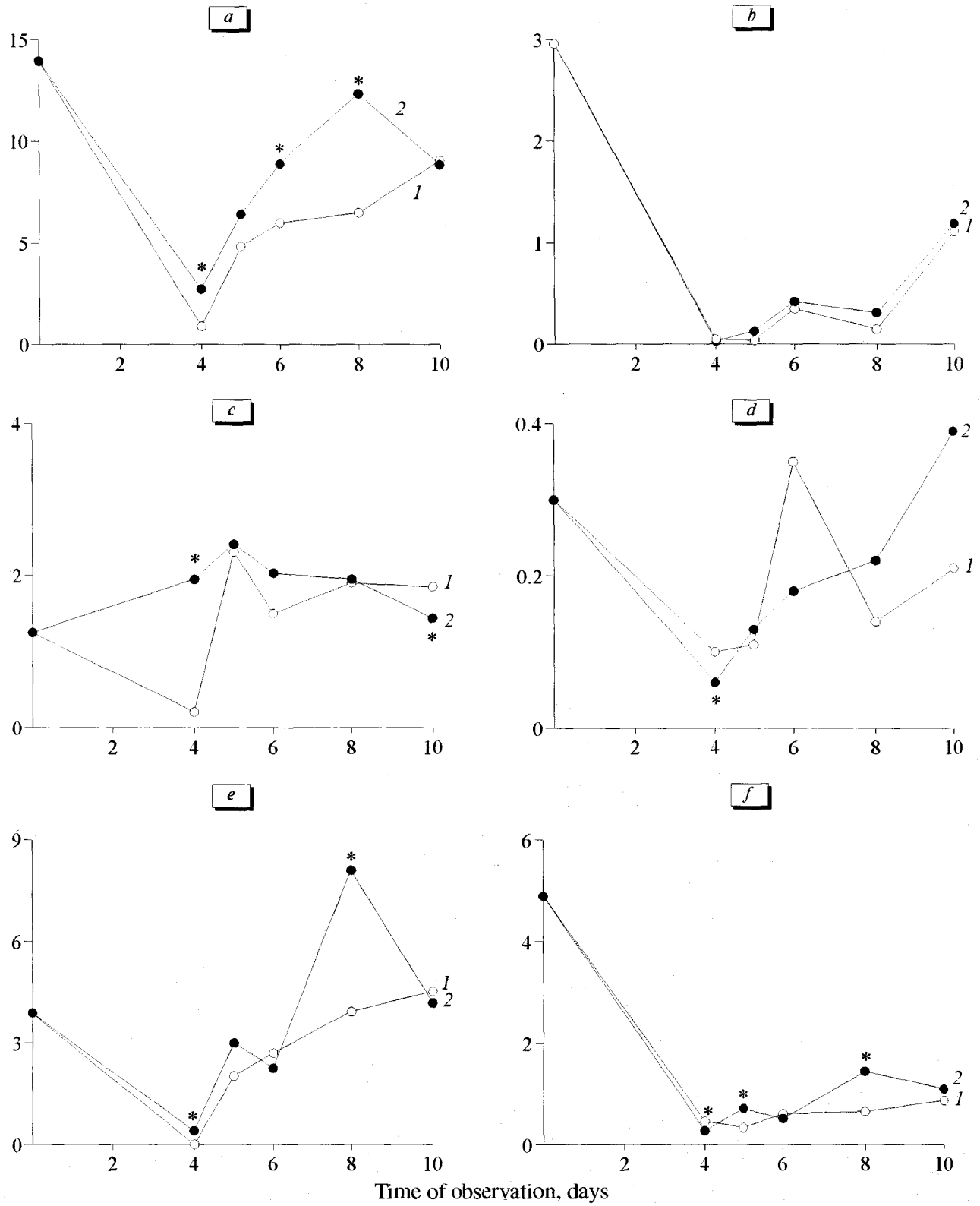

Fig. 2. Total number of myelokaryocytes (*a*), erythroid cells (*b*), immature neutrophilic leukocytes (*c*), monocytes (*d*), mature neutrophilic leukocytes (*e*), and lymphoid cells (*f*) in the bone marrow of CBA mice after injection of cyclophosphamide alone (*1*) or in combination with granulocytic colony-stimulating factor (*2*). Ordinate: cell number, $\times 10^6$/femur. Here and in Fig. 3 and 4: *$p<0.05$ compared to cyclophosphamide.

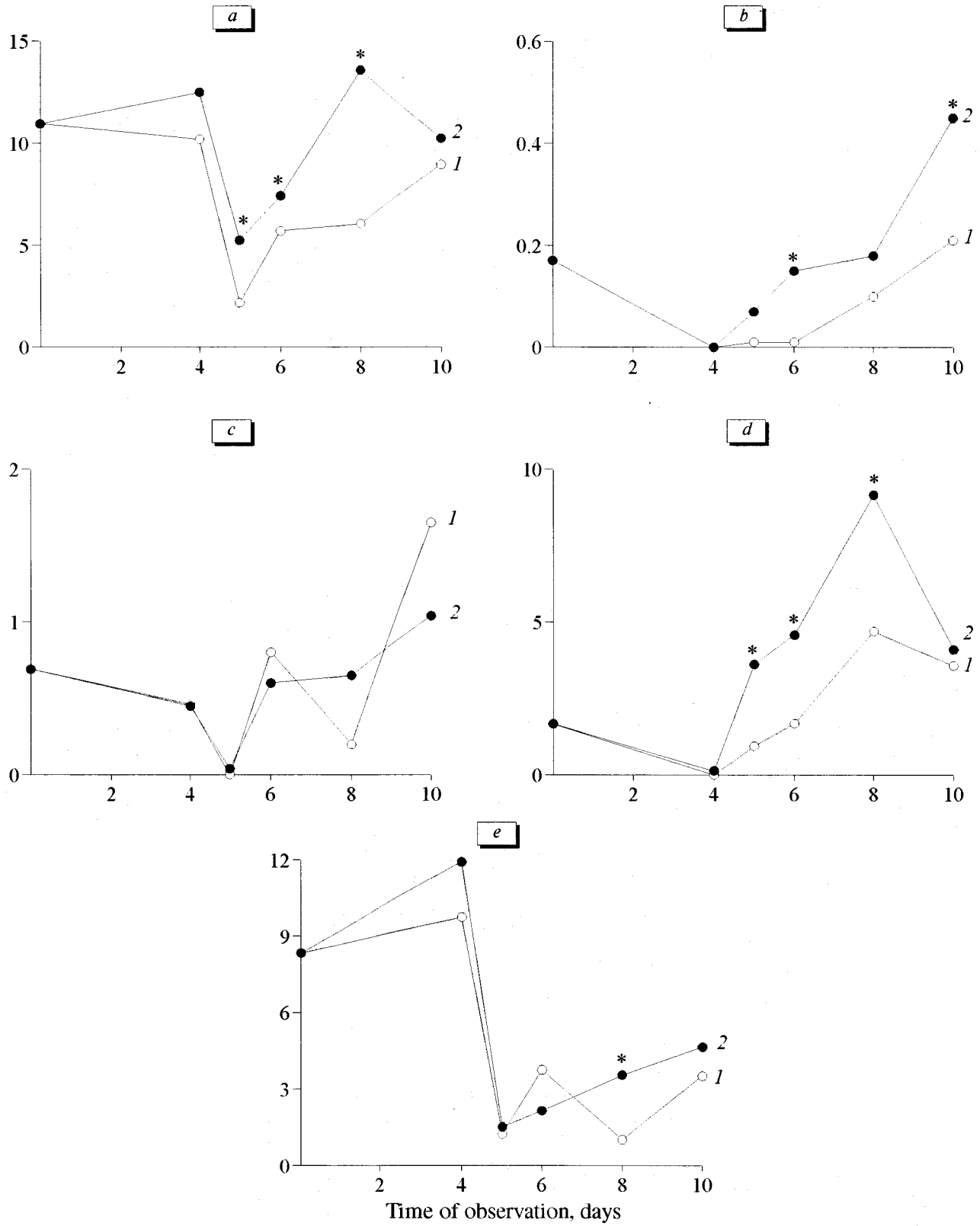

Fig. 3. Total number of leukocytes (*a*), band (*b*) and segmented (*c*) neutrophils, monocytes (*d*), and lymphocytes (*e*) in peripheral blood of CBA mice after treatment with cyclophosphamide alone (*1*) or in combination with granulocytic colony-stimulating factor (*2*). Ordinate: cell number, $\times 10^9$/liter.

TABLE 2. Content of Hemopoietic Precursor Cells (per 10^5 Nonadherent Myelokaryocytes) in the Bone Marrow of CBA Mice after Injection of Cyclophosphamide Alone or in Combination with Recombinant G-CSF ($X \pm m$)

Time of observation, days	CFU-E		CFU-GM	
	cyclophosphamide	cyclophosphamide+ G-CSF	cyclophosphamide	cyclophosphamide+ G-CSF
Before injection	6.83±0.70	6.83±0.70	4.00±0.45	4.00±0.45
4	7.50±0.43	5.00±0.26*+	4.50±0.43	9.83±0.31*+
5	13.33±0.67*	9.60±0.51*+	14.50±0.76*	19.17±1.01*+
6	12.80±1.02*	5.60±0.51+	9.50±0.76*	8.25±0.85*
8	15.00±0.86*	5.33±0.49+	7.00±0.37*	4.50±0.43+
10	9.00±1.18	4.60±0.51*+	4.17±0.48	5.00±0.68

Note. $p<0.05$: *compared to the initial level, +compared to the control (cyclophosphamide).

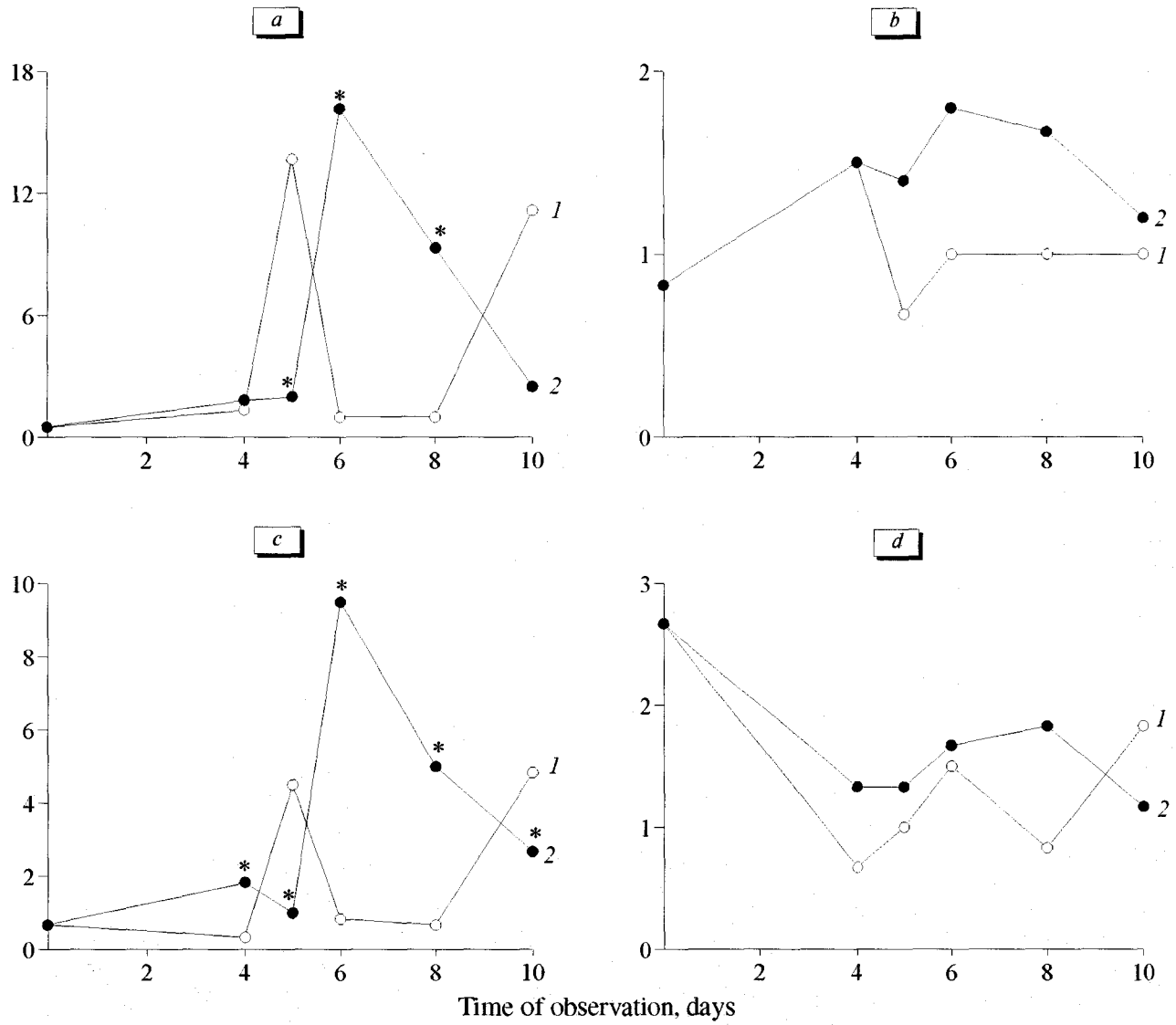

Fig. 4. Dynamics of erythropoietic (*a, b*) and colony-stimulating (*c, d*) activities of media conditioned by adherent (*a, c*) and nonadherent (*b, d*) cells of bone marrow of CBA mice after treatment with cyclophosphamide alone (*1*) or in combination with granulocytic colony-stimulating factor (*2*). Ordinate: number of colonies per 10^5 intact myelokaryocytes.

5 after the combined cyclophosphamide and G-CSF treatment (Fig. 3).

No hemopoietin-induced stimulation of erythropoiesis was observed throughout the experimental period. At the same time, the content of CFU-GM in hemopoietic tissue increased on days 4 and 5 after treatment in experimental mice compared to the control (Table 2).

Recombinant G-CSF did not change secretory activity of nonadherent cells of the bone marrow microenvironment, but enhanced colony-stimulating and erythropoietic activities of adherent myelokaryocytes 1 day after their maximum activities were observed in the control mice (Fig. 4).

Thus, original G-CSF demonstrated high hemostimulating activity in modeled myelosuppression caused by single injection of cyclophosphamide. The effect of G-CSF was limited to stimulation of granulocyte and lymphocyte production. Reconstitution of hemopoiesis after combined treatment with cyclophosphamide and G-CSF was primarily determined by enhanced functional activity of the adherent microenvironmental fraction, which promoted maturation of hemopoietic precursor cells.

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