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Role of Stromal and Thy 1,2$^+$ Cells in the Mechanisms of Action of Immobilized Granulocyte Colony-Stimulating Factor during Cytostatic-Induced Myelosuppression

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The effects of immobilized granulocyte colony-stimulating factor (mediated by cells of the hemopoiesis-inducing microenvironment) on hemopoietic precursors of various classes were studied on the model of cytostatic-induced myelosuppression (administration of cyclophosphamide). The action of this preparation was compared with that of the standard preparation of granulocyte colony-stimulating factor. Thy 1,2$^+$ cells potentiated the effects of immobilized and standard granulocyte colony-stimulating factors on granulocyte-erythroid-macrophage-megakaryocyte precursors. Stromal cells were shown to potentiate the influence of these agents on granulocyte precursors. Induction of proliferation of precursor cells by the immobilized factor mediated by cells of the hemopoiesis-inducing microenvironment persisted for a longer period compared to that induced by the standard product.

Key Words: *immobilized granulocyte colony-stimulating factor; granulocyte-erythroid-macrophage-megakaryocyte precursors, granulocyte precursors, and fibroblast precursors; Thy 1,2$^+$ cells; stromal cells; cyclophosphamide*

Products of granulocyte colony-stimulating factor (G-CSF) are used in clinical practice for prevention of neutropenia after cytostatic therapy. This factor via binding to specific high-affinity receptors (or intermediate-affinity receptors at high concentration of the cytokine in blood and tissues) stimulates proliferation and induces differentiation of granulocyte-macrophage and granulocyte precursors [1,2,6].

Direct activation of committed hemopoietic precursors is not the only mechanism, which mediates granulocytopoiesis-stimulating effects of G-CSF. For example, T lymphocytes regulating proliferation and differentiation of hemopoietic precursors [2] also carry surface receptors for G-CSF [6,8,13]. Hence, the effect of G-CSF on hemopoietic precursors can be mediated by cells of the hemopoiesis-inducing microenvironment (HIM).

The disadvantages of recombinant cytokine preparations are low efficiency of these agents (under conditions of short-term treatment) and development of serious complications (after long-term treatment). The method for immobilization of molecules on low-molecular-weight carriers allows us to prolong the action of drugs (due to a longer period of circulation

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in blood serum) and to prevent undesirable effects [4,7,10].

Here we studied the role of stromal cells and Thy $1,2^+$ cells in the effect of products from immobilized G-CSF (IG-CSF) compared to standard G-CSF (SG-CSF) under conditions of cytostatic-induced myelosuppression.

MATERIALS AND METHODS

Experiments were performed on 230 CBA/CaLac mice (class I conventional strain) aging 2-2.5 months and obtained from the nursery of the Institute of Pharmacology.

Cytostatic myelosuppression was induced by an intraperitoneal injection of alkylating agent cyclophosphamide in a single dose 83 mg/kg. Some mice of the treatment groups received subcutaneous injection of human non-glycosylated SG-CSF preparation filgrastim (Neupogen, Hoffman-La Roche Ltd., 100 μg/kg) and IG-CSF preparation (Scientific Futures Management Company, 100 μg/kg) for 5 days. IG-CSF is non-glycosylated G-CSF, whose molecules undergo conjugation on polyethylene glycol by the accelerated electron beam (wide energy range, 1-5 MeV) and γ-radiation [14]. Control animals received an equivalent volume of the solvent (0.2 ml 5% glucose) under similar conditions (cytostatic control). Baseline parameters were measured in intact animals (intact control).

The minimal effective titer of monoclonal antibodies (MCA) anti-Thy 1,2 (clone 5A-8 CL 8600, Cedarlane, 1:100) for the cytotoxic reaction and complement cytotoxicity were evaluated in experiments with thymocytes from intact CBA/CaLac mice. The suspension of bone marrow cells was purified from erythrocytes and divided into the adherent and nonadherent fractions (plastic adhesion) to study the role of Thy $1,2^+$ cells in the regulation of colony formation. The concentration of nonadherent karyocytes was brought to 2×10^6 cells/ml using RPMI 1640 medium with 10 mM HEPES. MCA anti-Thy 1,2 (10 μl/ml cell suspension) were added to a part of this solution (50%). The control and treated suspensions of cells were maintained at 4°C for 45 min. Both samples were mixed 10:1 with rabbit serum (complement source) and placed in a thermostat (37°C). The control and treated cells were twice washed by centrifugation. The concentration of these cells was brought to 2×10^5 nucleated cells/ml with the culture medium containing 79% RPMI 1640, 1% methylcellulose, 20% FBS, 280 mg/ml L-glutamine, 4 μM 2-mercaptoethanol, and 50 mg/liter gentamicin (Sigma). A part of the semiviscous cell suspension (0.5 ml) was placed in 24-well plates above the washed layer (5×10^5 adherent myelokaryocytes from mice of the corresponding group). SG-CSF (4 ng/ml) or IG-CSF (4 ng/ml) was added to another part of the suspension. The mixture (0.5 ml) was placed in 24-well plates. Depending on the culture system, incubation was performed for 12 days (granulocyte-erythroid-macrophage-megakaryocyte colonies, CFU-GEMM) or 7 days (granulocyte colonies, CFU-G). Incubation was conducted in a CO_2 incubator at 37°C, 5% CO_2, and 100% humidity. The number of newly formed CFU-GEMM and CFU-G was estimated by the end of incubation [3]. Additionally, the growth of fibroblast colonies (CFU-F) in culture of adherent myelokaryocytes, intensity of fibroblast-like cell monolayer formation, and effect of this monolayer on the growth of CFU-GEMM and CFU-G were studied [3].

The significance of differences was evaluated by parametric Student's *t* test or nonparametric Wilcoxon–Mann–Whitney *U* test. Exact Fisher test was used to analyze the rated data.

RESULTS

Functional activity of Thy $1,2^+$ cells and stromal cells was studied after cyclophosphamide injection. Adherent myelokaryocytes produced a strong stimulatory effect on the growth of CFU-GEMM (days 2 and 3) and CFU-G (days 2-6) during cytostatic-induced myelosuppression (Fig. 1, *a*, *d*). The increased release of hemopoietic colonies was associated with stimulation of CFU-F formation (by 350-170%, $p<0.01$ compared to the intact control) and intensive formation of fibroblast-like cell monolayer. These data indicate that administration of the alkylating agent stimulates the formation of fibroblasts from stromal precursors. It should be emphasized that *de novo* formation of fibroblastoid hemopoietic islets in the bone marrow is observed on day 3 after cytostatic treatment [5]. The maturation of granulocyte precursors into mature cells occurs in these structures [4].

At the same time, nonadherent myelokaryocytes carrying surface antigen Thy $1,2^+$ selectively increased the number of CFU-GEMM (days 3 and 6), but had no effect on the formation of CFU-G (Fig. 2, *a*, *d*).

After administration of the alkylating agent, fibroblast-like (stromal) cells have a stimulatory effect on CFU-G. This effect is related to an increase in the pool of stromal precursors and improvement of hemopoietic cell function. The effect of Thy $1,2^+$ cells is mainly directed toward CFU-GEMM.

G-CSF preparations had different effect on the cyclophosphamide-induced inhibition of hemopoietic precursors. SG-CSF had a stimulatory effect on the formation of CFU-GEMM in the culture of nonadherent bone marrow cells, while and IG-CSF promoted the release of CFU-G, respectively (Fig. 3).

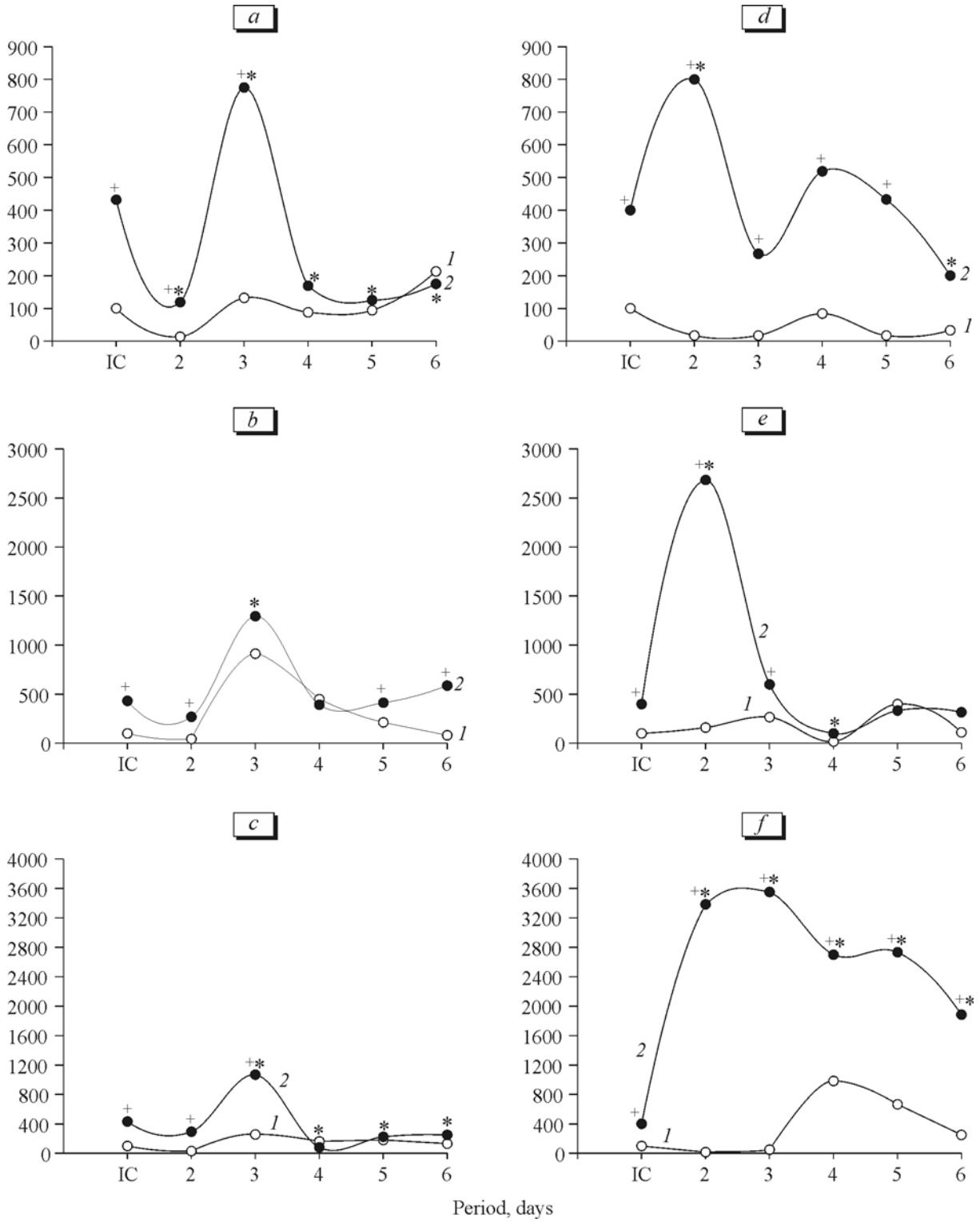

Fig 1. Effect of adherent myelokaryocytes on the G-CSF-induced stimulation of growth of CFU-GEMM (*a*, *b*, *c*) and CFU-G (*d*, *e*, *f*) in the culture of nonadherent bone marrow cells from cyclophosphamide-receiving CBA/CaLac mice. Administration of the solvent (*a*, *d*), SG-CSF (*b*, *e*), and IG-CSF (*c*, *f*). Here and in Figs. 2 and 3: ordinate, number of precursor cells in the bone marrow, % of the intact control (IC, 100%). Group without (*1*) and with adherent myelokaryocytes (*2*). $p<0.05$: *compared to the intact control; +compared to *1*.

Specific effect of IG-CSF on hemopoietic precursors is probably associated with new biophysical properties of the pegylated cytokine. A carrier polyethylene glycol probably modifies the interaction of G-CSF with membrane-expressed CD114 and/or soluble G-CSF receptor (produced by mature myeloid cells) [9].

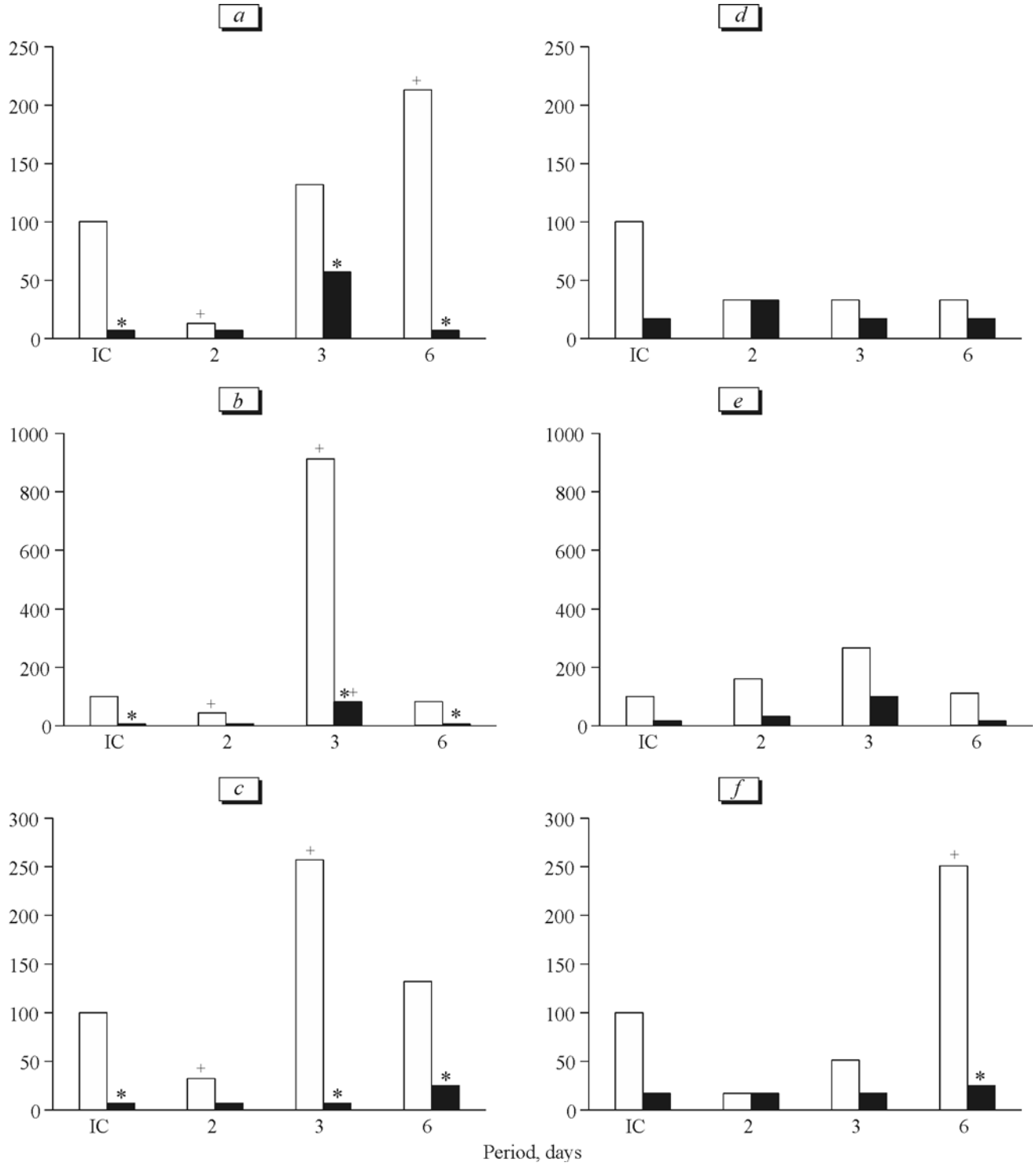

Fig 2. Effect of Thy 1,2⁺ cells on the G-CSF-induced stimulation of growth of CFU-GEMM (*a*, *b*, *c*) and CFU-G (*d*, *e*, *f*) in the culture of nonadherent bone marrow cells from cyclophosphamide-receiving CBA/CaLac mice. Administration of the solvent (*a*, *d*), SG-CSF (*b*, *e*), and IG-CSF (*c*, *f*). Light bars, group with Thy 1,2⁺ cells; dark bars; group without Thy 1,2⁺ cells. $p<0.05$: *compared to light bars; ⁺compared to the intact control.

HIM cells provide rapid recovery of hemopoietic cells after administration of G-CSF preparations under conditions of cytostatic treatment. For example, the sublayer of stromal cells potentiated the G-CSF-mediated growth of granulocyte colonies (days 2 and 3; Fig. 3, *b*, *d*). Changes in CFU-GEMM were insignificant. After treatment with IG-CSF, adherent HIM cells had a stimulatory effect

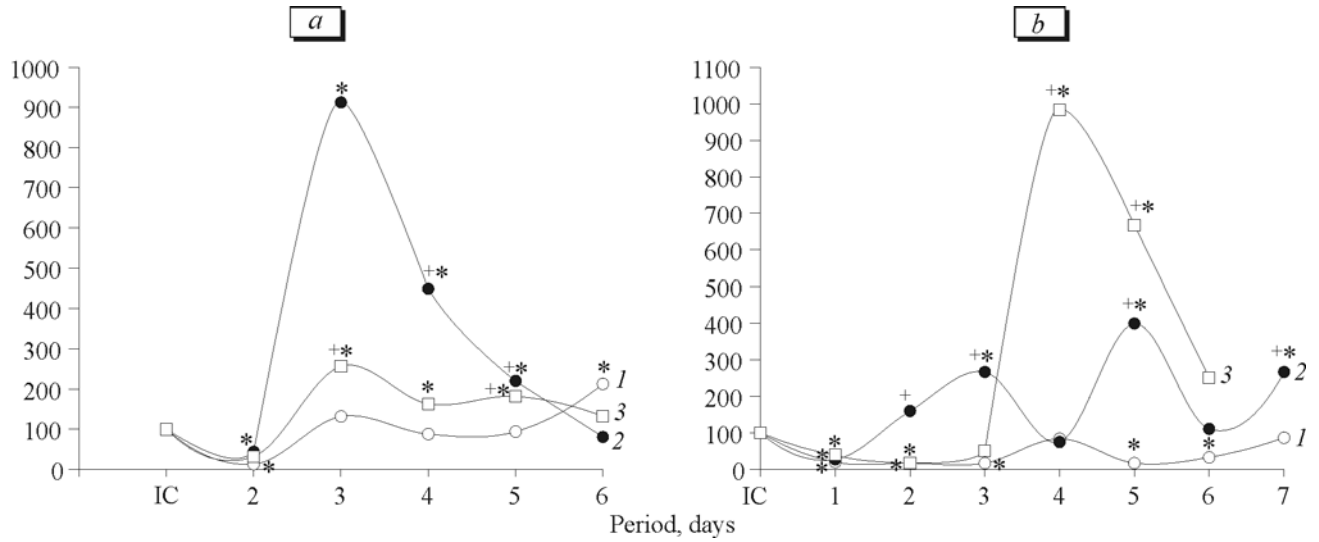

Fig. 3. Effect of G-CSF products on the growth of CFU-GEMM (*a*) and CFU-G (*b*) in the culture of nonadherent bone marrow cells from cyclophosphamide-receiving CBA/CaLac mice. Cytostatic control (physiological saline, *1*); after treatment with cyclophosphamide and Neupogen (*2*); after treatment with cyclophosphamide and IG-CSF (*3*). $p<0.05$: *compared to the intact control; +compared to *1*.

on CFU-GEMM (days 2 and 3) and CFU-G (days 2-6; Fig. 3, *c*, *e*).

Experiments with MCA showed that Thy $1,2^+$ cells significantly increase the division rate of CFU-GEMM, but not of CFU-G (IG-CSF group and SG-CSF group; Fig. 2, *b*, *c*).

Our results indicate that the stimulatory effect of SG-CSF and IG-CSF on CFU-GEMM under conditions of cyclophosphamide treatment is mediated by the T lymphocyte-dependent mechanisms. The influence of CFU-G was shown to depend on functional activity of stromal cells.

$CD4^+$ and $CD8^+$ lymphocytes [13] and T cells that are restricted by histocompatibility classes I and II [8] can express G-CSF receptors. We showed that course treatment with G-CSF significantly increases the number of CFU-F in the culture of adherent bone marrow cells ($6.88\pm0.52\times10^5$ *vs*. $2.38\pm0.18\times10^5$ cells in the cytostatic control, day 3). The number of intact CFU-F was $0.88\pm0.13\times10^5$. It cannot be excluded that the indirect effect of G-CSF products on hemopoietic precursors is realized through specific receptors on HIM cells (T lymphocytes and fibroblasts).

The effects of IG-CSF and SG-CSF on CFU-G are mainly realized through stromal cells. It is associated with the fact that intercellular contacts play the major role in the mechanisms of proliferation and differentiation of mature precursors for granulocytopoiesis during cytostatic-induced myelosuppression [2]. The lower concentration of adhesion molecules on mobilized multipotent hemopoietic precursors determines a major role of Thy $1,2^+$ cells in the stimulatory effect of study products.

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