GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Effect of Immobilized Granulocyte Colony-Stimulating Factor on Hemopoietic Precursors of Various Classes during Cytostatic-Induced Myelosuppression

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Experiments were performed on the model of cytostatic myelosuppression induced by cyclophosphamide. We compared the effect of immobilized granulocyte CSF (the preparation was created in Russia) and reference standard preparation of granulocyte CSF on the development of neutrophilic leukopenia and hemopoietic precursors of various classes. It was found that preparations of granulocyte CSF decreased the duration and degree of peripheral blood neutropenia. The granulocytopoiesis-stimulating effect was related to stimulation of multipotent hemopoietic precursors, granulocyte-erythroid-macrophage-megakaryocyte precursors, and granulocyte precursors. Induction of division and maturation of multipotent hemopoietic precursors, granulocyte-erythroid-macrophage-megakaryocyte precursors, and granulocyte precursors and recovery of cellularity of the granulocytic hemopoietic stem after administration of immobilized granulocyte CSF were observed at later terms compared to treatment with the reference preparation of granulocyte CSF.

Key Words: *immobilized granulocyte colony-stimulating factor; multipotent hemopoietic precursor cell; precursor cells of the granulocyte-erythroid-macrophage-megakaryocyte, granulocyte-macrophage, and granulocyte types; cyclophosphamide*

Much progress in the therapy for leukopenia and anemia of different genesis was achieved after the discovery and preparation of recombinant forms of specific hemopoiesis-stimulating agents (CSF, IL, and erythropoietin). The advantages of these drugs are extremely high activity and selectivity for certain hemopoietic stems [1,13,15]. However, the therapeutic action of growth factors is often accompanied by musculoskeletal pain, splenomegaly, allergic reactions, and other adverse effects [4]. The use of these drugs in medical practice is also limited by rapid elimination [10].

Much attention is paid to polyethylene oxide-conjugated medicinal agents, because immobilization of molecules on low-molecular-weight carriers helps to preserve their activity and to reduce side effects of the drugs. For example, pegylated granulocyte CSF (G-CSF, Neulasta) decreases the duration of neutropenia and mobilizes hemopoietic precursor cells from the bone marrow to the peripheral blood [5,6]. This is accompanied by a significant increase in the time

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of drug circulation in blood plasma. Therefore, these preparations hold much promise for the use in medical practice. However, the indications for treatment with pharmaceutical agents (*e.g.*, blood-stimulating drugs) can be formulated only after the evaluation of fine mechanisms for their action.

Here we compared the effect of immobilized granulocyte CSF (IMG-CSF; Scientific Futures Management, Russia) and reference preparation of granulocyte CSF on hemopoietic precursor cells of various classes under conditions of cyclophosphamide (CP) treatment.

MATERIALS AND METHODS

Experiments were performed on 540 male 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 single intraperitoneal injection of alkylating agent CP (one-third of the maximum tolerated dose, 83 mg/kg). All mice of the treatment groups received subcutaneous injections of non-glycosylated human G-CSF Filgrastim (neupogen, Hoffman-La Roche Ltd.) and IMG-CSF (Scientific Futures Management) in a dose of 100 μg/kg for 5 days after administration of CP. Control mice received an equivalent volume of physiological saline (0.2 ml) under similar conditions (cytostatic control). The intact control group consisted of intact animals.

The number of neutrophilic leukocytes in the peripheral blood was measured on days 1-12 after cytostatic treatment [2]. The animals were euthanized by cervical dislocation under ether anesthesia. The number of granulocyte-erythroid-macrophage-megakaryocyte colonies (CFU-GEMM) was evaluated in the bone marrow. These colonies consisted of 4 types of hemopoietic cells (erythrokaryocytes, granulocytes, macrophages, and megakaryocytes). Granulocyte associations (CFU-G) were counted in the bone marrow [2,14].

The concentration of bone marrow CFU with non-differentiated cells (CFU-N, more than 1500 blast cells) was estimated by the method of limiting dilutions [2,14]. After culturing, CFU-N were collected, suspended to single nucleated cells (pipetting), and washed from methylcellulose. The cells were put in 35-mm Petri dishes with 2 ml medium, incubated for 12 days under standard conditions, and passaged (3 times). The number of colonies was evaluated after each passage. A cytomorphological study was performed. The effect of G-CSF preparations on differentiation of CFU-N was studied. The aggregates from treated animals obtained after the 3rd passage were suspended to individual cells, the concentration was brought to 10^3 cells per ml with culture medium for the growth of granulocyte-macrophage colonies (CFU-GM) and CFU-G, the suspension was transferred to 96-well flat-bottom plates (150 μl per well), and the number of CFU-GM and CFU-G was evaluated after 7 days [2].

The results were analyzed by standard methods of variation statistics. The significance of differences was evaluated by parametric Student's *t* test or nonparametric Mann–Whitney *U* test. Exact Fisher test was used to analyze the rated data. The frequency of CFU-N was estimated by the standard linear method of Poisson distribution.

RESULTS

IMG-CSF decreased the duration and degree of peripheral blood neutropenia during CP-induced myelosuppression (Fig. 1, *d*). The stimulatory effect of this agent was also observed during regeneration of hemopoiesis, which was seen from the development of neutrophilic leukocytosis on days 7 and 10 of the experiment. Comparison of the stimulatory effects of IMG-CSF and neupogen on granulocytopoiesis showed that pegylated cytokine had a more moderate effect without overshoot (day 7).

IMG-CSF accelerated recovery of granulocytic hemopoietic stem under conditions of CP-induced suppression. Published data show that the active substance is hardly released from the conjugate of polyethylene glycol-cytokine [3], which explains slow increase in the number of peripheral blood neutrophils after treatment with IMG-CSF. Smoother stimulation of granulocytopoiesis by pegylated cytokine is an advantage of the pegylated cytokine over neupogen. Long-term administration of neupogen can cause side effects (syndrome of acute respiratory failure, lung injury, splenomegaly, in rare cases with spleen rupture) [4,12], inhibition of thrombocytic hemopoietic stem, and development of acute myeloleukemia (subtype M1) [9].

The pegylated preparation of G-CSF (Neulasta) promotes the release of hemopoietic precursors into circulation [5,6]. It is accompanied by mobilization of S-phase $CD34^+$ cells. They are characterized by different expression of genes for early hemopoiesis, erythroid differentiation, and late stages of myeloid maturation [5]. Our experiments showed that neupogen and IMG-CSF have a strong effect on functional activity of primitive hemopoietic precursors in the bone marrow of cytostatic-treated animals. For example, the reference preparation of cytokine stimulated the release of CFU-N on days 3 and 4 after CP injection and did not affect colony formation during the period of stimulation (day 2; Fig. 1, *a*). A linear dependence

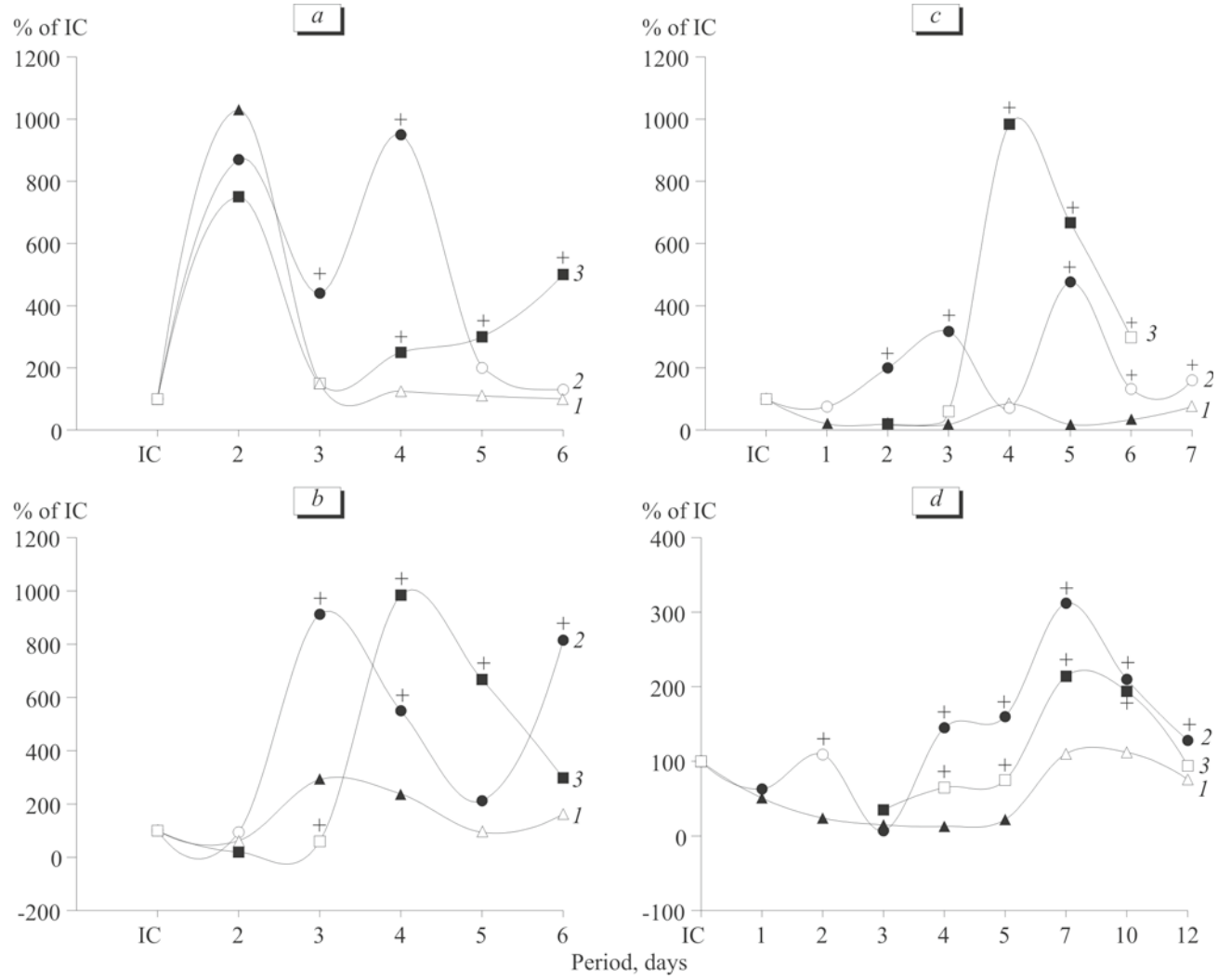

Fig. 1. Effect of G-CSF preparations on the content of CFU-N (*a*), CFU-GEMM (*b*), and CFU-G in the bone marrow (*c*) and number of neutrophilic granulocytes in the peripheral blood (*d*) from CP-treated CBA/CaLac mice. Filled symbols: significant differences of the test parameter from the intact control group (IC; $p<0.05$); $^{+}p<0.05$, differences of mice receiving neupogen (*2*) and IMG-CSF (*3*) after treatment with CP from the cytostatic control (physiological saline, *1*; $p<0.05$).

was revealed between the intensity of this process and number of myelokaryocytes in a well, which attested to high proliferative activity of CFU-N (Table 1). Moreover, neupogen accelerated differentiation of multipotent hemopoietic precursors into granulocyte precursor cells (days 3 and 6; Fig. 2, *b*). At the same time, CFU-GM formation was significantly suppressed in the culture of non-differentiated cells of tertiary colonies (days 2 and 4; Fig. 2, *a*).

In contrast to the reference preparation, IMG-CSF increased the concentration of CFU containing non-differentiated cells starting from day 4; this parameter attained the maximum by day 6 (Fig. 1, *a*). The release of CFU-N did not depend on the concentration of nucleated cells in culture (low correlation coefficient). It can be suggested that pegylated cytokine increases proliferative activity of some multipotent hemopoietic precursors. IMG-CSF stimulated the maturation of CFU-N: we observed an increase in the count of CFU-GM (days 3, 5, and 6) and CFU-G (days 5 and 6) in the culture of non-differentiated cells (Fig. 2).

The cells present in colonies from animals of the cytostatic control group and treatment groups are characterized by long-term repopulation (more than 4 passages). The secondary, tertiary, and quaternary colonies practically did not differ from the primary culture in size (variations by not more than 10%) and morphology. These colonies consisted of not only non-differentiated cells, but also of aggregates consisting of erythroblasts, granulocytes, and mononuclear phagocytic cells. These CFU were probably formed from hemopoietic stem cells. The number of nucleated cells in CFU-N did not differ in the treatment group and cytostatic control group (neupogen group, by 15%; IMG-CSF group, by 20%). These data suggest that G-CSF preparations mobilize hemopoietic

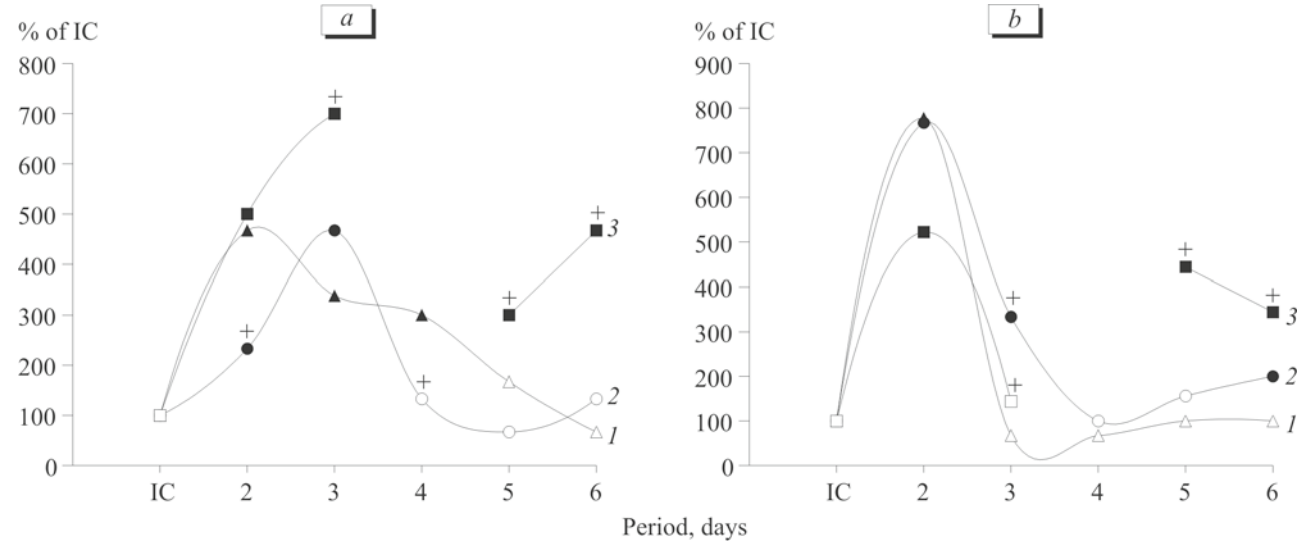

Fig. 2. Effect of G-CSF preparations on the growth of CFU-GM (*a*) and CFU-G (*b*) in tertiary cultures of multipotent hemopoietic precursors from CP-treated CBA/CaLac mice. Ordinate: amount of CFU in the methylcellulose medium. Here and in Fig. 3: filled symbols, significant differences of the test parameter from the intact control group (IC; $p<0.05$); $^{+}p<0.05$, differences of the parameter in groups of neupogen (*2*) and IMG-CSF (*3*) from the cytostatic control (physiological saline, *1*; $p<0.05$).

stem cells, whose proliferative activity is below the control level (without preparations).

Our results extend the knowledge of the properties of polyethylene oxide-conjugated medicinal agents. Immobilization of G-CSF on carriers not only contributes to mobilization of immature precursor cells to the blood, but also induces their proliferation and differentiation into more mature hemopoietic precursors (CFU-GM and CFU-G).

During cytostatic stress, the preparations of G-CSF had a stimulatory effect on mature precursor cells. Parenteral administration of neupogen significantly increased the growth of CFU-GEMM (days 3, 4, and 6) and CFU-G (days 2, 3, and 5; Fig. 1, *b*, *c*). A stimulatory effect of IMG-CSF on the formation of CFU-GEMM and CFU-G was observed in the later period than that of the reference cytokine preparation (days 4, 5, and 6 after CP treatment). The degree of colony formation progressively decreased with increasing neutrophilic granulocyte count in the peripheral blood. Both, G-CSF and pegylated cytokine increased the ClFU-G/CFU-G ratio (on days 2-6 and 4-5, respectively; Fig. 3).

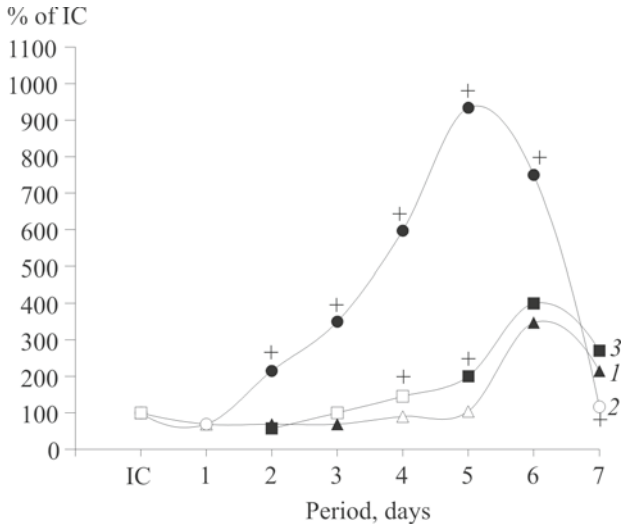

Fig. 3. Effect of G-CSF preparations on maturity index of CFU-G in CP-treated CBA/CaLac mice. Ordinate: ClFU-G/CFU-G ratio.

The size of mixed colonies in mice receiving neupogen (40-70%, $p<0.05$) and IMG-CSF (by not more than 20%) surpassed that in animals of the cytostatic control group. Cytomorphological study of individual colonies showed that they contain not only blast forms of granulocytes and promyelocytes (as in the cytostatic control group without treatment), but also metamyelocytes and stab granulocytes. Cytokine preparations stimulated not only proliferation, but also differentiation of precursor cells (*e.g.*, CFU-GEMM).

Thus, IMG-CSF induces division and maturation of CFU-GEMM and CFU-G under conditions of CP treatment. In experiments with neupogen, induction was observed during myelosuppression and recovery of white blood cells. By contrast, these changes in pegylated cytokine-treated animals accompany an increase in the number of neutrophilic granulocytes.

According to the modern notions, G-CSF is a linearly restricted regulator of neutrophil production. This agent is potent in stimulating terminal stage of neutrophil development [1,10]. The cytokine also plays a role in activation of resting primitive precursor cells and therefore can be assigned to the group

TABLE 1. Correlation Coefficient (r) between the Concentration of Multipotent Hemopoietic Precursors in the Bone Marrow and Content of CFU-N in a Well under Conditions of Cytostatic Myelosuppression (Significance of Correlation Coefficient at r=0.7; p<0.05)

Days	Physiological saline	Neupogen	IMG-CSF
2	0.88; p<0.05	0.95; p<0.05	0.29
3	0.88; p<0.05	0.98; p<0.01	0.16
4	0.96; p<0.01	0.98; p<0.01	0.37
5	0.23	0.52	0.42
6	0.31	0.22	0.51

Note. Intact control, r=0.78; p<0.05.

of earliest growth factors including also cell growth factor, FLT 3 ligand (fms-like tyrosine kinase receptor-3), IL-6, IL-11, and IL-12 [1]. Stimulation of division and maturation of CFU-N, CFU-GEMM, and CFU-G by the growth factor during cytostatic myelosuppression observed in our experiments agrees with published data. However, we believe that the direct stimulatory effect of G-CSF on multipotent hemopoietic precursors should be confirmed in *in vitro* experiments. The effect of this cytokine is probably related to the influence on early hemopoietins stimulating transition of primitive hemopoietic precursors in the bone marrow from G_0 phase to the stage of proliferation followed by multistage differentiation of precursor cells into specialized blood cells [1]. It should be emphasized that G-CSF can synergistically interact with GM-CSF and IL-3 (intermediate-acting lineage-nonspecific factors), thus stimulating the formation of CFU-GM, megakaryocyte colonies, and mixed colonies [11].

Conjugation of GM-CSF with polyethylene glycol increased the molecular weight of this compound and decreased the specific weight of active molecule at the same molecular weight with the reference preparation [10]. These changes modulate the neutrophil response. As differentiated from G-CSF (half-life period 3-4 h [10]), the pegylated preparation is slowly eliminated by the kidneys. It contributes to gradual accumulation of the agent. Published data show that 80% of the total clearance of G-CSF are provided by cytokine receptors [8]. The increase in blood neutrophil count is followed by a sharp decrease in cytokine concentration. IMG-CSF impairs the regulatory mechanism of the clearance, which probably results from saturation of neutrophil receptors at high doses of pegylated G-CSF [6]. This determines more pronounced response of precursor cells to IMG-CSF compared to that observed after treatment with neupogen.

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