Pharmacological Properties of Granulocytic Colony-Stimulating Factor Pegylated Using Electron Beam Synthesis Nanotechnologies

A. M. Dygai[1], G. N. Zyuz'kov[1], V. V. Zhdanov[1], P. G. Madonov[2], E. V. Udut[1], L. A. Miroshnichenko[1], T. Yu. Khrichkova[1], E. V. Simanina[1], L. A. Stavrova[1], A. V. Artamonov[2], A. A. Bekarev[2], D. N. Kinsht[2], A. V. Chaikovskiy[1], T. S. Markova[1], and R. V. Gurto[1]

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Granulocytic CSF pegylated using electron-beam synthesis nanotechnology exhibits pronounced granulomonocytopoiesis-stimulating and SC-mobilizing activity. More potent stimulation of committed precursors against the background of less pronounced activation of polypotent hemopoietic cells is a peculiarity of hemostimulating action of pegylated using electron-beam synthesis nanotechnology granulocytic CSF in comparison with its non-modified analog. The mobilizing effect of pegylated using electron-beam synthesis nanotechnology granulocytic CSF on early progenitor elements surpasses that of non-conjugated cytokine.

Key Words: *pegylated granulocytic colony-stimulating factor; nanotechnologies; hemopoiesis; progenitor cells; mobilization; regenerative medicine*

Detailed study of the mechanisms of hemopoiesis allowed creation of new pharmacological substances exhibiting hemostimulating properties on the basis of analogs of endogenous hemopoiesis regulators [2,7]. Preparations of recombinant human granulocytic CSF (G-CSF) are widely used in clinical practice for correction of granulocytopoiesis disorders. There are non-conjugated and chemically pegylated forms of this factor [7,15]. However, practical use of non-modified G-CSF is largely limited due to its toxicity and high incidence of side effects and complications [13]. At the same time, pegylated G-CSF (pegfilgrastim) is a practically nontoxic compound [15]. Moreover, this preparation is characterized by high physical stability, solubility, half-elimination time, and relatively low susceptibility to proteolytic enzymes. However, the technology of chemical synthesis of pegfilgrastim is a multi-stage and complex process involving highly toxic substances requiring various methods of purification. At the same time, nanotechnology of electron-beam (radiation) synthesis also makes it possible to create pharmacological preparations, *e.g.* pegylated protein compounds [6,8,9], and is free from above-described drawbacks.

Here we studied the pharmacological effects of pegylated using electron-beam synthesis nanotechnology G-CSF (PG-G-CSF) the mechanisms underlying these effects.

MATERIALS AND METHODS

Experiments were carried out on 2-month-old male CBA/CaLac mice (n=689), conventional certified mouse strain obtained from the nursery of Institute of

[1]Institute of Pharmacology, Siberian Division of the Russian Academy of Medical Sciences, Tomsk; [2]Scientific Future Management Company, Novosibirsk, Russia. ***Address for correspondence:*** mmu@pharm.tsu.ru. A. M. Dygai

Pharmacology, Siberian Division of Russian Academy of Medical Sciences. Hemostimulating activity of the test preparation was studied on the model of cytostatic myelosuppression induced by intraperitoneal injection of cyclophosphamide (Verofarm) in the maximum tolerated dose 250 mg/kg. Mobilization of progenitor cells was studied on intact mice. PG-G-CSF (Research Institute of Pharmacology, Siberian Division of Russian Academy of Medical Sciences in cooperation with Scientific Future Management Company) was injected subcutaneously in a dose of 100 μg/kg/day for 5 days and *per os* for 10 days. Immobilization of non-glycosylated G-CSF at the low-molecular-weight polyethylene glycol was carried out using the nanotechnology of electron-beam synthesis employing a directed flow of accelerated electrons [8,9,11]. Neupogen (Hoffman-La Roche Ltd) was used as the reference preparation; it was injected subcutaneously in a dose of 100 mg/kg/day for 5 days.

For evaluation of hemostimulating activity after parenteral and peroral administration of the test preparations we determined parameters of peripheral blood and bone marrow hemopoiesis and content of granulocyte-erythroid-macrophage-megakaryocytic (GEMM), granulomonocytic (GM), granulocytic (G), and fibroblast (F) CFU in BM and production of colony-stimulating activity (CSA) by individual fractions of hemopoiesis-inducing microenvironment (HIM) after subcutaneous injection of the preparations [5]. For evaluation of mobilizing properties of the preparation in both administration routes, the counts of CFU-GM, CFU-E, CFU-F, and MSC in the peripheral blood were determined [4,6].

The data were processed by methods of variation statistics using Student's *t* test and nonparametric Mann–Whitney *U* test. The incidence of MSC in BM and peripheral blood was evaluated using generalized linear model for Poisson distribution [14].

RESULTS

Cyclophosphamide treatment reduced the content of mature and immature neutrophilic granulocytes in BM

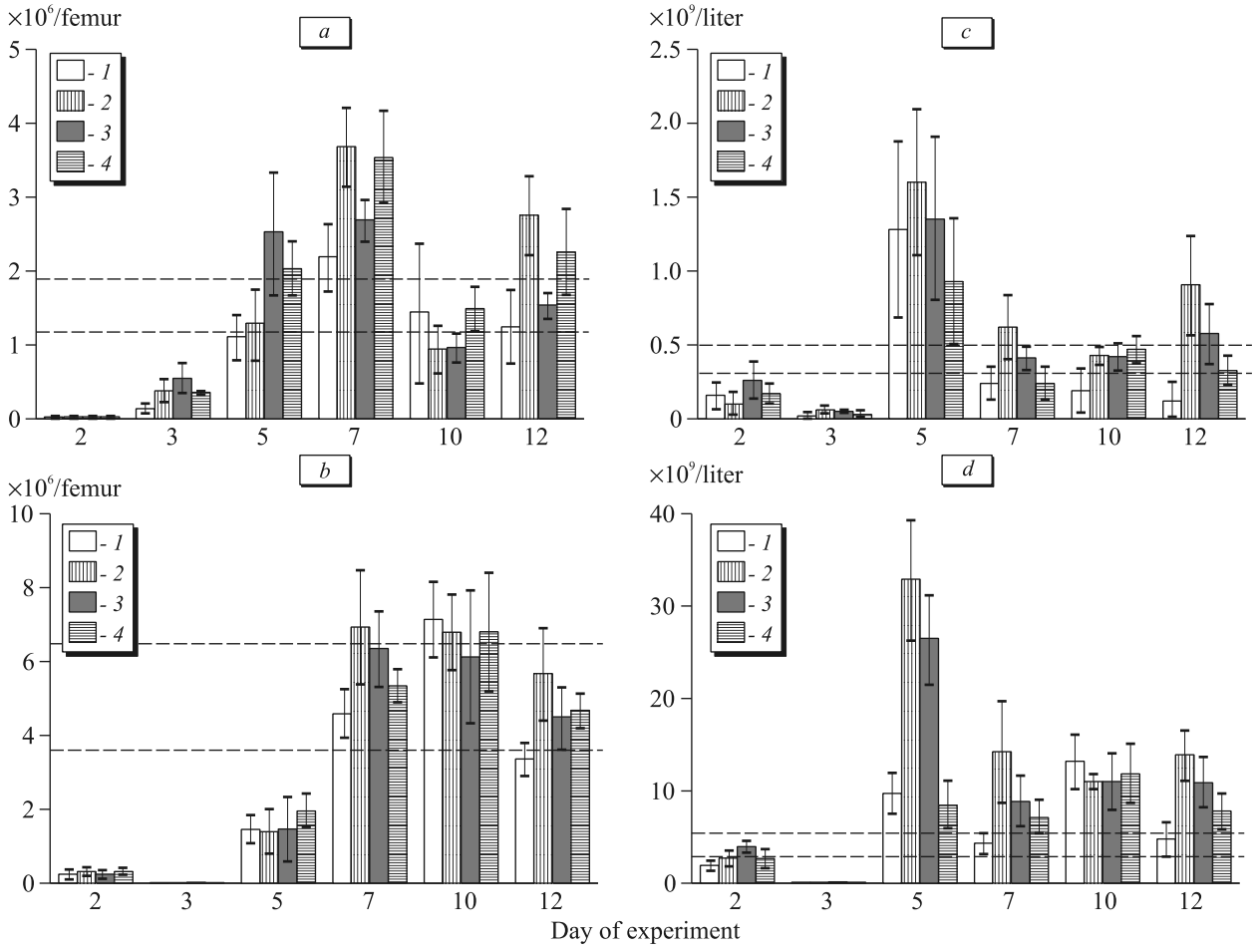

Fig. 1. Content of immature (*a*) and mature (*b*) neutrophilic granulocytes in BM, stab (*c*) and segmented neutrophils (*d*) in the peripheral blood of CBA/CaLac mice after administration of cyclophosphamide (*1*) and non-modified G-CSF (*2*), PG-G-CSF subcutaneously (*3*), and PG-G-CSF *per os* (*4*) against the background of modeled myelosuppression. Here and in Fig. 2, 3: confidence intervals at $p<0.05$. Area between dotted lines shows confidence interval for the test parameter in intact mice at $p<0.05$.

and the number of segmented neutrophils in the peripheral blood (Fig. 1). At the same time we observed considerable activation of compensatory processes in the hemopoietic tissue manifesting in increased functional activity of granulomonocytic (CFU-GM) and polypotent hemopoietic elements (CFU-GEMM) in BM (Fig 2).

Administration of G-CSF preparations led to stimulation of granulocytopoiesis. The number of neutrophilic granulocytes in the hemopoietic tissue increased in all experimental groups (including groups with peroral administration of PG-G-CSF, Fig. 1, *a*, *b*) The most pronounced changes were found in the count of immature granulocytes in groups with parenteral administration of both non-conjugated CSF and PG-G-CSF. These changes in the hemopoiesis were accompanied by appropriate increase in peripheral blood neutrophil count (Fig. 1, *c, d*).

The reactions of the pools of progenitor cells of different classes to parenteral administration of G-CSF differed significantly. PG-G-CSF markedly stimulated committed progenitor blood cells (Fig. 2, *b, c*). The number of CFU-GM increased on days 2 and 3 of the experiment (to 176 and 138% of the control), CFU-G content was elevated practically through the observation period (the peak was observed on day 5 and constituted 2000% of control). Changes in the content of polypotent hemopoietic stem cells were substantially less pronounced. For instance, the content of CFU-GEMM attained 195% of the control level on day 3 of the experiment. Administration of non-conjugated G-CSF led to not only activation of "liable" compartment of progenitor cells (CFU-GM and CFU-G) [1,2], which was less pronounced than the effects of PG-G-CSF, but also significant stimulation of low-differentiated hemopoietic elements (CFU-GEMM; Fig. 2).

PG-G-CSF, in contrast to its non-modified analog, stimulates hemopoiesis via activation of immediate compensatory mechanisms due to the action on committed precursor cells, the buffer division of the regenerative potential of the hemopoietic tissue with easily renewable resources [1,2]. Little involvement of "deep reserve" elements of BM regeneration (polypotent hemopoietic cells) [2,3,5] maximally reduces the risk of possible exhaustion of this pool and adaptation failure (Fig. 2).

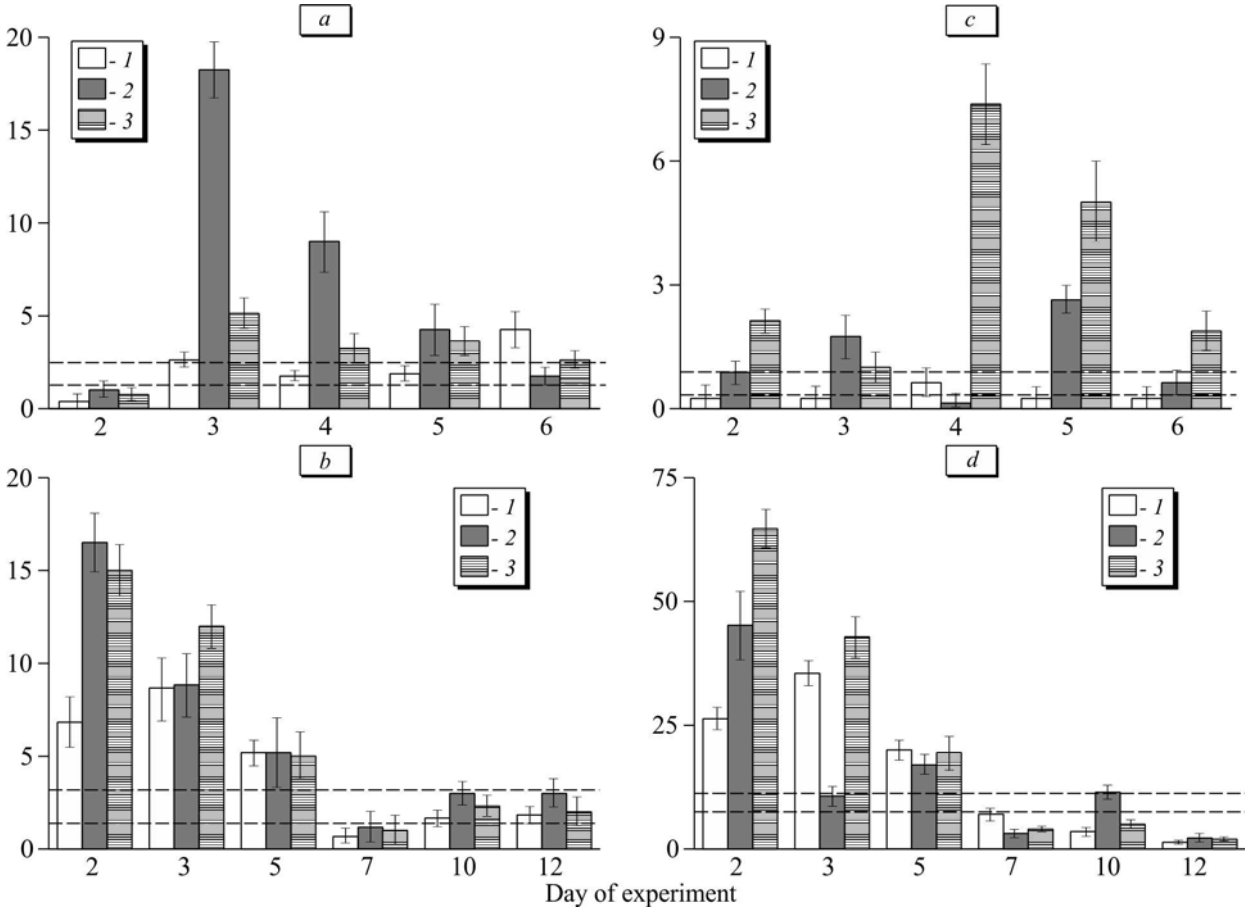

Fig. 2. Content of CFU-GEMM (*a*), CFU-GM (*b*), CFU-G (*c*), and CFU-F (*d*) in BM of CBA/CaLac mice after administration of cyclophosphamide (*1*) and non-modified G-CSF (*2*) and PG-G-CSF subcutaneously (*3*) against the background of modeled myelosuppression. Ordinate: per 10^6 myelokaryocytes (*a*); per 10^5 myelokaryocytes (*b*, *c*); per 2.5×10^5 myelokaryocytes (*d*).

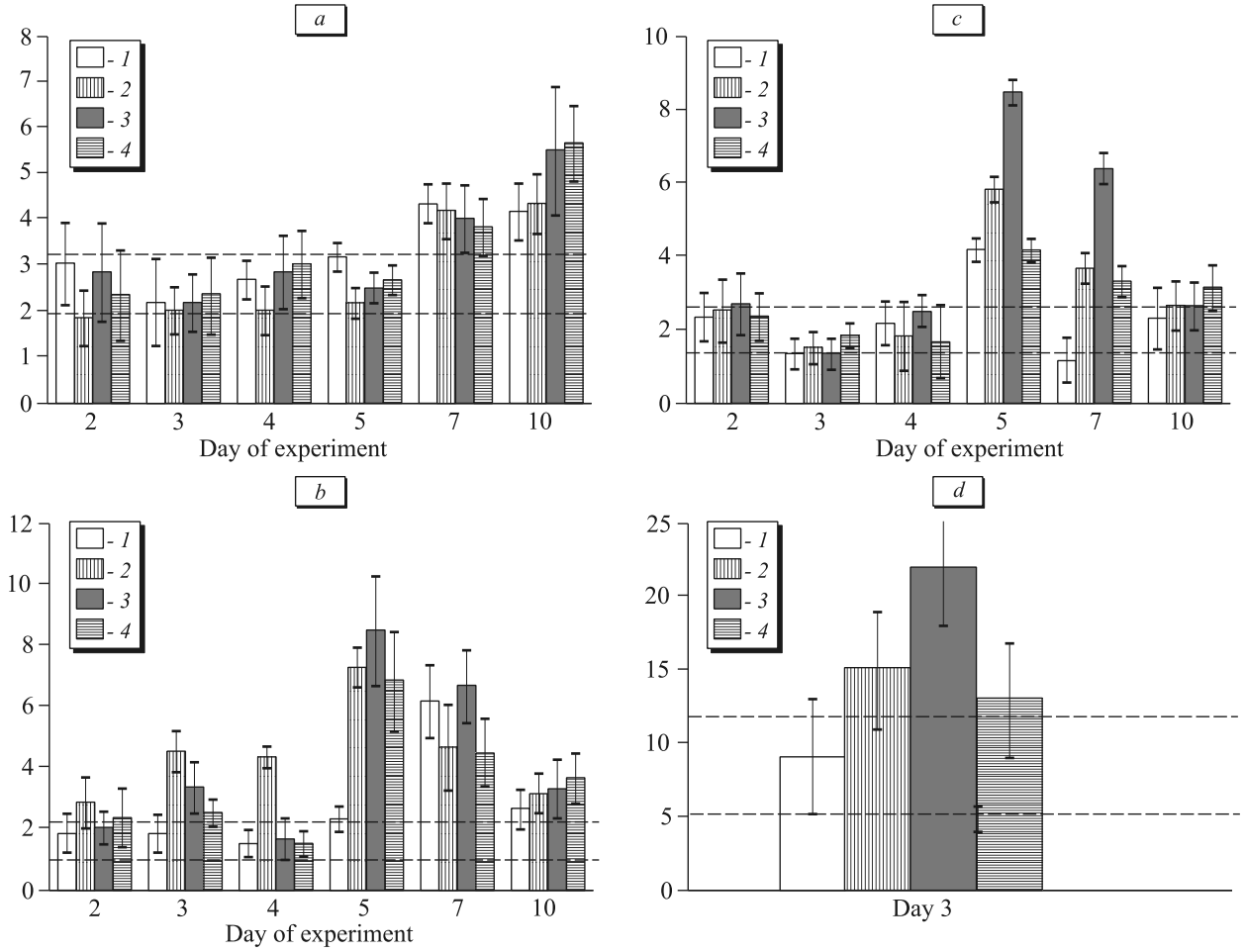

Fig. 3. Content of CFU-GM (*a*), CFU-E (*b*), CFU-F (*c*), and MSC (*d*) in the peripheral blood of CBA/CaLac mice after administration of cyclophosphamide (*1*) and non-modified G-CSF (*2*), PG-G-CSF subcutaneously (*3*), and PG-G-CSF *per os* (*4*) against the background of modeled myelosuppression. Ordinate: per 10^5 myelokaryocytes (*a, b*); per 2.5×10^5 mononuclears (*c*); per 10^6 mononuclears (*d*).

According to published data [7], G-CSF produces the granulocytopoiesis-stimulating effect *in situ* primarily via direct interaction with specific receptors on hemopoietic precursors. This was confirmed by *in vitro* experiments. Addition of standard G-CSF in various concentrations to BM tissue culture considerably increased the yield of granulocytic colonies and clusters. The effect linearly depended on the dose of the preparation: 2.75±0.49, 5.00±0.46, and 8.25±0.77 for G-CSF concentrations of 1, 5, and 20 ng/ml, respectively. At the same time, the intensity of colony formation indirectly reflecting mitotic activity of cells [5] in case of pegylated cytokine was less pronounced: the maximum difference (by 4 times) was observed for growth factor concentration of 5 ng/ml. However, maturation of hemopoietic precursors considerably increased to 222 and 236% from the corresponding values observed after addition of 5 and 20 ng/ml standard G-CSF, respectively.

These peculiarities of PG-G-CSF effect, including the rate of realization of proliferation and differentiation programs and the time required for attaining compensation and energy expenditures [1,2], undoubtedly make the use of pegylated factor pathogenetically more justified.

When studying the role of HIM in the formation of blood system reactions upon PG-G-CSF treatment, some peculiarities of its pharmacological effect were revealed. Considerable stimulation of CSA production by adherent BM cells (2159% of the control level on day 2 of the experiment) was observed, while after administration of non-conjugated G-CSF the increase in this parameter was noted only on day 12 of the experiment.

Analysis of the pool of mesenchymal progenitor cells (CFU-F), precursors of HIM stromal elements [2], also revealed more pronounced effect of PG-G-CFU on this compartment of the hemopoietic tissue in comparison with the standard cytokine preparation (Fig. 1). These findings attest to greater contribution of local mechanisms of homeostasis regulation (stimulation of HIM reparation at the expense of activation

of mesenchymal progenitor cells and improvement of feeder properties of stromal myelokaryocytes) to regeneration of the hemopoietic tissue [2,8] after administration of PG-G-CSF.

Evaluation of SC-mobilizing properties of the test preparations demonstrated considerable increase in the number of circulating CFU-GM, CFU-E, CFU-F, and MSC in all groups, including groups with peroral treatment (Fig. 3). In comparison with the non-modified analog, PG-G-CSF was more active in mobilizing earlier precursor cells: multipotent MSC and CFU-F containing both committed stromal elements and true SC [3,11]. The number of CFU-F in the peripheral blood after subcutaneous injection of PG-G-CSF surpassed the corresponding parameters in animals receiving non-modified cytokine by 46 and 74% on days 5 and 7, respectively, while the content of MSC was higher by 47% (day 3, Fig. 3).

These findings are of particular importance, because G-CSF is widely used for isolation of SC from the peripheral blood (for their further autologous or allogeneic transplantation to patients after chemo- or radiotherapy) [10]. In light of this, SC mobilization is the basis for realization of regeneratory properties of this cytokine [3,7,8]. Therefore, stimulation of the release of cells with maximum growth potential [4,8,12] undoubtedly attests to the prospects of PG-G-CFU use in the therapy of degenerative diseases. The possibility of using alternative (peroral) administration route is very important for minimization of the risk of complications related to protein nature of G-CSF [8,13], because pharmacological strategy of regenerative medicine implies long-term and repeated administration of the drug [3,8].

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