Effects and Mechanisms of Hemopoiesis-Stimulating Activity of Immobilized Oligonucleotides under Conditions of Cytostatic Myelosuppression

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Hemopoiesis-stimulating activity of immobilized oligonucleotide preparation was studied on the model of cytostatic myelosuppression induced by injection of cyclophosphamide and 5-fluorouracil. Immobilized oligonucleotides stimulated regeneration of erythro- and granulocytopoiesis in the bone marrow under conditions of cytostatic treatment. The counts of neutrophilic granulocytes and platelets in the peripheral blood increased. The stimulatory effect of the drug was more manifest in animals with active behavior. The mechanism of immobilized oligonucleotide effect was based on stimulation of functional activity of erythroid and granulocytic macrophage precursors.

Key Words: *immobilized oligonucleotides; hemopoiesis; hemopoietic precursors; cytostatics; individual sensitivity*

Nonspecific hemostimulants (zymosan, splenin, vitamins C, B_1, B_6, eleuterococcus extract, hormones, lithium salts), which proved to be ineffective in various hemopoiesis disorders, are now replaced by drugs based on recombinant human factors (granulocytic CSF, granulocytic macrophage CSF, erythropoietin, thrombopoietin) [3,6]. The target effects of growth factors on hemopoietic cells have determined their high activity and possibility of differentiated approach to therapy of cytostatic myelosuppressions. On the other hand, in some cases the use of some hemostimulants, *e.g.* granulocytic CSF and erythropoietin, is associated with the development of untoward side effects (osteomuscular pain, arthralgia, allergic reactions) [1,11,15].

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Oligonucleotide preparation is a promising drug stimulating various hemopoiesis stems without toxicity [4]. The nucleic acid preparation is involved in cell metabolism and accumulates in actively proliferating tissues (bone marrow, lymph nodes, spleen) [7]. These characteristics suggest its use not only in leuko- and thrombocytopenias, but also for preparation to and realization of transplantation, in skin ulcers and burns, infectious diseases, *etc.* [10]. However, poor delivery of oligonucleotides to the cell and rapid degradation by nucleases in lysosomes largely limit their clinical use [5]. A possible solution is the use of transport systems promoting penetration of the nucleotide material through histohematic barriers and cell membranes [8]. Nontoxic polyethylene glycol with low immunogenic activity is now more and more often used for addressed delivery of active molecules. Pegilated nucleic acids are more actively captured by cells than nonimmobilized molecules. In addition, the polymer

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improves the solubility and half-elimination of the nucleotide material [12-14].

We studied the hemopoiesis-stimulating activity of immobilized oligonucleotides (IMON) on models of cytostatic myelosuppressions.

MATERIALS AND METHODS

Experiments were carried out on CBA/CaLac mice (2-2.5 months; n=520), certified first-category conventional inbred mice obtained from Breeding Center of Institute of Pharmacology.

Cytostatic myelosuppression was induced by a single intraperitoneal injection of an alkylating agent (cyclophosphamide) in a dose of 200 mg/kg or a fluoropyrimidine antimetabolite (5-fluorouracil) in a dose of 83 mg/kg. One day after the cytostatic the animals received oral IMON in doses of 50, 100, 150, 200, and 250 mg/kg for 7 days (experimental group). The preparation consisted of sturgeon milt short highly purified DNA fragments with a molecular weight of 500-700 kDa immobilized on polyethylene glycol by electron radiosynthesis nanotechnology (molecular weight 400-6000 Da). Controls received an equivalent volume (0.2 ml) of saline under similar conditions (cytostatic control). Intact animals served for measurements of basal levels (intact control).

Peripheral blood parameters were measured by the standard hematological methods on days 2-13 after cytostatic treatment [2]. Platelet counts were measured on an ABACUS automated hematological analyzer (Diatron) in the veterinary mode. The animals were then sacrificed by CO_2 overdosage and bone marrow cells were counted. Activities of granulocytic macrophage (CFU-GM) and erythroid (CFU-E) colony growth in methylcellulose medium were studied by standard cultural methods [2]. The intensity of differentiation of hemopoietic precursors was evaluated by the index of maturation (number of clusters/number of colonies grown in the same well). Production of colony-stimulating and erythropoietic activities by the adherent and nonadherent fractions of the hemopoiesis-inducing microenvironment cells was evaluated by the formation of CFU-GM and CFU-E in a culture of intact mouse nonadherent myelokaryocytes [2].

The effects of IMON on the blood system of animals with initially high (group 1) and low (group 2) behavioral activity were studied by the method for hemopoiesis studies with consideration for individual higher nervous activity [9].

The results were processed by standard methods of variation statistics. The significance of differences was evaluated by parametric Student's t test or nonparametric Mann–Whitney U test. The data expressed in fractions were analyzed by Fisher's exact test.

RESULTS

Screening experiments showed that IMON in all studied doses (50, 100, 150, 200, and 250 mg/kg/day) significantly increased peripheral blood counts of total leukocytes under conditions of cytostatic myelosuppression induced by cyclophosphamide injection. The most pronounced and lasting effect was attained with IMON dose of 200 mg/kg (days 6, 8, 10).

Analysis of hemograms showed that oral IMON in a dose of 200 mg/kg after cyclophosphamide injection significantly increased peripheral blood counts of stab (on days 2, 4, 8, 10, 12) and segmented neutrophils (on days 2, 6, 8, 10). The parameters peaked on day 10 of observation (Fig. 1). On day 10 of drug therapy, lymphocyte counts in experimental group increased by 42.8% (p<0.05) in comparison with the cytostatic control.

High activity of IMON towards peripheral blood leukocytes prompted studies of the drug effects on bone marrow hemopoiesis. Addition of IMON elevated the bone marrow level of immature neutrophilic granulocytes by 47.2% (p<0.05) on day 8, of their mature forms by 15.3% (p<0.05) on day 4, and promoted accumulation of erythrokaryocytes on days 2 and 4 of the study (Fig. 1). In contrast to these events, the levels of lymphoid cells decreased on days 4 and 5.

Hence, IMON treatment stimulated bone marrow erythro- and granulocytopoiesis regeneration under conditions of cyclophosphamide treatment. The depression of the hemopoietic lymphoid stem was augmented.

Effective use of hemostimulants in clinical practice is impossible without understanding the mechanism of their action. A course of IMON during the development of myelosuppression in our study stimulated the formation of CFU-GM (day 2) and CFU-E (days 3, 4; Fig. 2). This was paralleled by an increase in the index of maturation of erythroid (day 3) and granulocyte macrophage (days 3, 4) precursors. The intensity of CFU-GM differentiation decreased to the initial level during active recovery of granulocytic stem cell content (day 6).

Humoral hemopoietic factors, produced by the hemopoiesis-inducing microenvironment cells, are involved in the regulation of proliferation and differentiation of hemopoietic precursors [3]. In our study, IMON reduced the level of erythropoietic activity in nonadherent myelokaryocyte supernatants on day 3 after cytostatic injection and did not modify the production of colony-stimulating activity by the hemopoietic microenvironment cells.

Hence, hemopoiesis-stimulating effect of IMON under conditions of cyclophosphamide treatment was caused by stimulation of hemopoietic precursors.

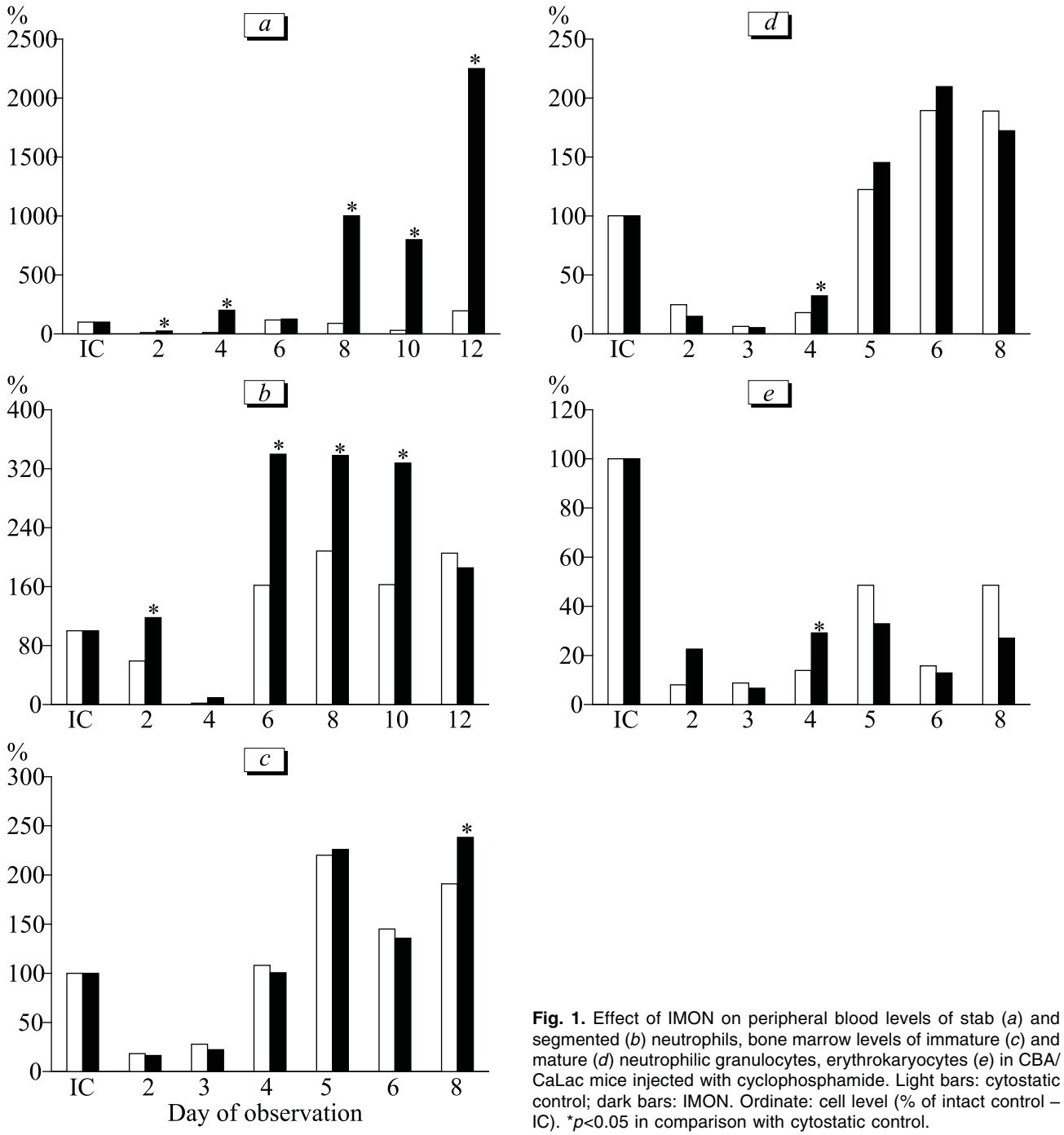

Fig. 1. Effect of IMON on peripheral blood levels of stab (*a*) and segmented (*b*) neutrophils, bone marrow levels of immature (*c*) and mature (*d*) neutrophilic granulocytes, erythrokaryocytes (*e*) in CBA/CaLac mice injected with cyclophosphamide. Light bars: cytostatic control; dark bars: IMON. Ordinate: cell level (% of intact control – IC). *$p<0.05$ in comparison with cytostatic control.

Stimulation of the erythroid and granulocytic hemopoiesis stems under the effect of IMON was observed on the model of cytostatic myelosuppression induced by 5-fluorouracil (Table 1). The level of peripheral blood platelets increased significantly on days 9 and 13 by 22.6-35.7% in comparison with the cytostatic control group. Hence, manifestation of hemostimulatory effect of IMON was not due to the cytostatic toxicity mechanism.

Ample clinical data accumulated by the present time indicate the absence of expected efficiency or overdosage of hemostimulants during therapy for leukopenias of different origin. In many cases it was caused by neglect of individual patient's characteristics. We previously developed a method for studies of hemopoiesis in experimental animals with consideration for individual features of their higher nervous activity [9]. Changes in the blood system under extreme conditions (immobilization stress, hypoxic trauma, experimental neurosis) depended on the initial behavioral activity and conditioned reflex habits [3].

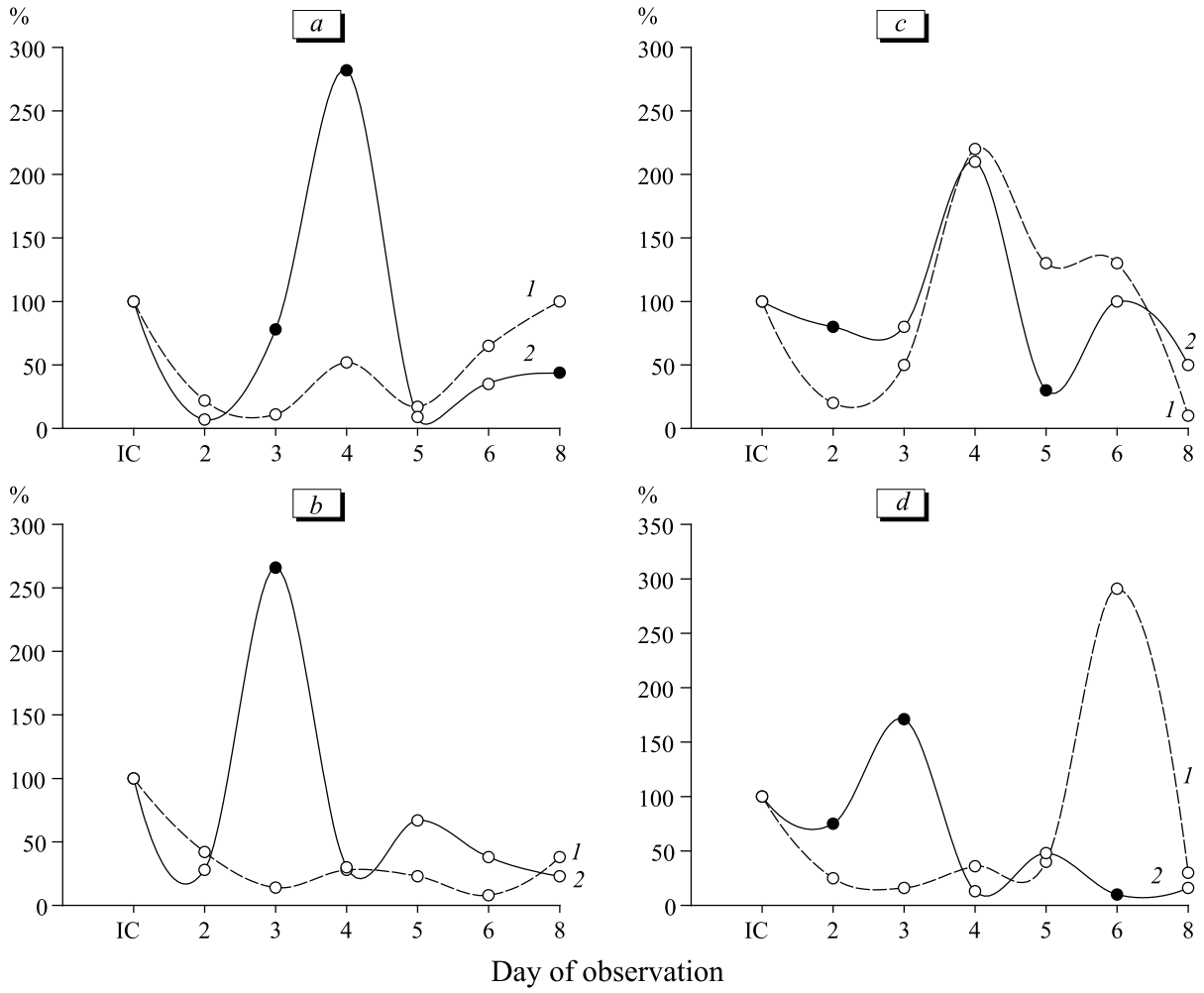

Fig. 2. Effect of IMON on the growth of CFU-E (*a*), CFU-GM (*b*), intensity of differentiation of erythroid (*c*) and granulocytic macrophage (*d*) precursors in nonadherent cell culture from the bone marrow of CBA/CaLac mice treated with cyclophosphamide. *1*) cytostatic control; *2*) IMON. Ordinate: level of precursors in the bone marrow, differentiation intensity (% of IC). Dark symbols: the parameter differed significantly from the cytostatic control ($p<0.05$).

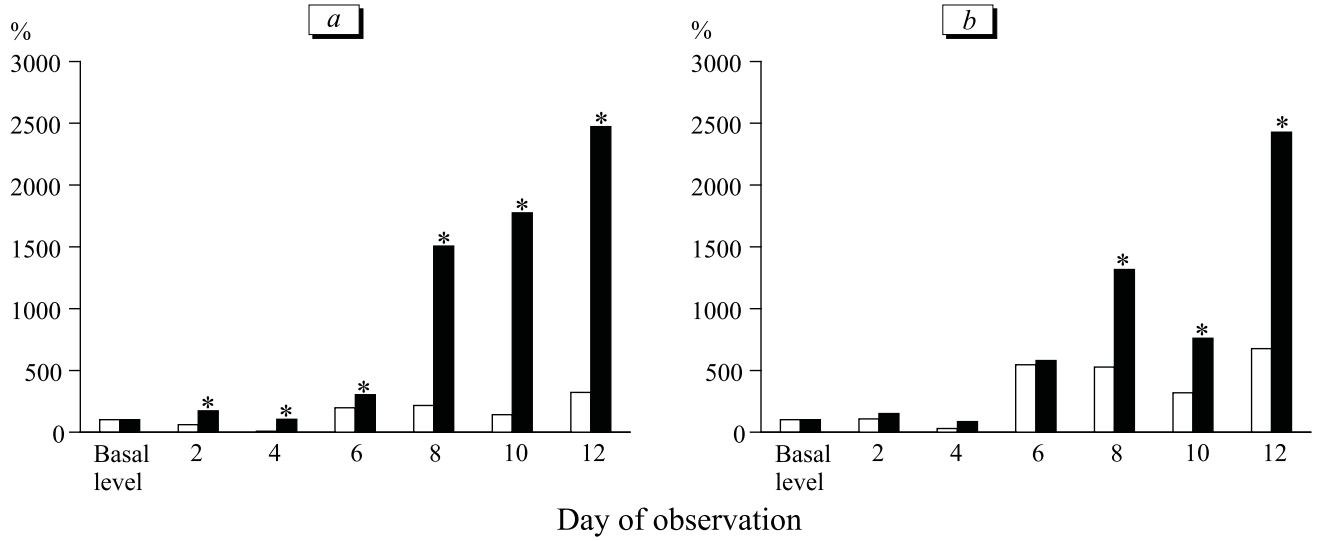

Fig. 3. Effects of IMON on peripheral blood levels of neutrophilic granulocytes in CBA/CaLac mice with active (*a*) and passive (*b*) behavior after cyclophosphamide treatment. Light bars: cytostatic control; dark bars: IMON. Ordinate: peripheral blood levels of neutrophilic granulocytes (% of basal levels). *$p<0.05$ in comparison with cytostatic control.

TABLE 1. Effects of IMON on Peripheral Blood and Bone Marrow Parameters of CBA/CaLac Mice Injected with 5-Fluorouracil ($X \pm m$)

Day of study		Peripheral blood ($\times 10^9$/liter)		Bone marrow (10^6 cell/femur)		
		stab neutrophilic granulocytes	segmented neutrophilic granulocytes	immature neutrophilic granulocytes	mature neutrophilic granulocytes	erythro-karyo-cytes
Intact control		0.17±0.05	3.86±0.61	1.82±0.13	4.54±0.12	2.75±0.32
3	I	0.02±0.01*	2.66±0.23*	0.08±0.02*	1.07±0.17*	0.02±0.01*
	II	0.08±0.02*+	3.87±0.33+	0.16±0.03*+	1.37±0.19*	0.17±0.03*+
5	I	0.22±0.08	3.51±0.64	1.07±0.36*	0.20±0.06*	1.49±0.35*
	II	0.35±0.07	5.52±0.77*+	1.99±0.36+	0.03±0.01*+	1.44±0.31*
7	I	0.24±0.05	1.54±0.37*	4.20±0.50*	1.56±0.24*	1.25±0.34*
	II	0.34±0.04*	3.27±0.58+	3.59±0.58*	2.91±0.35*+	1.48±0.28*
9	I	0.29±0.06	5.26±0.59	2.44±0.29*	3.75±0.14*	2.53±0.46
	II	0.56±0.07*	6.34±0.93*	2.10±0.30	4.02±0.38	1.71±0.20*
11	I	0.44±0.09*	3.88±0.31	1.62±0.13	4.92±0.30	2.16±0.29
	II	0.89±0.11*+	10.37±1.25*+	1.98±0.25	4.89±0.40	1.90±0.22
13	I	0.25±0.05	4.62±0.61	2.10±0.36	5.65±0.38	1.98±0.35
	II	0.45±0.06*	5.51±0.64*+	2.31±0.49	6.05±0.72*	1.29±0.37*

Note. *I*) animals under conditions of cytostatic treatment; *II*) animals treated with IMON after 5-fluorouracil. *p<0.05 in comparison with: *intact control, +cytostatic control.

Our experiments showed that myelosuppression was more manifest in animals with high behavioral activity and restoration of the granulocytic stem cellular composition was more intense in them than in animals with low activity (Fig. 3). Recovery of the granulocytic neutrophil count in the peripheral blood of animals under the effect of IMON in group 1 was observed throughout the entire period of observation, in group 2 on days 8, 10, 12. The process intensity in animals with active behavior was significantly higher than in passive animals.

High integration of the peripheral α-adrenergic structures in the regulation of hemopoietic precursors proliferation and differentiation determined pronounced shifts in the blood system in animals with active behavior under conditions of experimental neurotic exposure, not observed in animals with poor behavioral activity [3]. Published data and our findings attest to possible relationship between IMON effects under conditions of cytostatic myelosuppression and the adrenergic system. An obvious result of our findings is the need to take into consideration the characteristics of higher nervous activity in drug therapy in order to effectively correct the disorders in hemopoiesis processes in diseases of different kind.

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