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Hemostimulating Effects of Immobilized Hyaluronidase and Their Mechanisms during Cytostatic-Induced Myelosuppression

A. M. Dygai, A. V. Artamonov*, A. A. Bekarev*, E. I. Vereschagin*, V. V. Zhdanov, G. N. Zyuz'kov, E. V. Udut, T. Yu. Khrichkova, E. V. Simanina, L. A. Stavrova, L. A. Miroshnichenko, A. V. Chaikovskiy, and P. G. Madonov*

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We studied the effect of immobilized hyaluronidase on hemopoiesis under conditions of cyclophosphamide-induced suppression. The preparation was shown to possess high hemostimulating activity. The stimulatory effect of hyaluronidase on the erithron was more pronounced than its effect on the granulocytic hemopoietic stem due to increase in functional activity of hemopoietic precursors and hemopoiesis-inducing microenvironment.

Key Words: *myelosuppression; immobilized hyaluronidase; erythropoiesis; granulocytopoiesis*

Hyaluronic acid (HA) is one of the major components of the intercellular matrix. An important role in metabolic transformations of this glycosaminoglycan is played by hyaluronidase, an enzyme gradually (step-by-step) reducing in the length of HA with the formation of polymers producing various effects on biological processes and cell functions [1,9-12]. Our previous experiments showed that the preparation of native hyaluronidase can produce a strong stimulatory effect on hemopoiesis [2,3,6]. However, the effective concentration of this enzyme far surpasses the therapeutic dose [6]. The technology of electron-beam synthesis allows us to obtain low-toxicity and safe protein compounds conjugated to a low-molecular-weight carrier [5].

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

Here we studied the hemostimulating effect and mechanism of the action of immobilized hyaluronidase under conditions of cyclophosphamide-induced myelosuppression.

MATERIALS AND METHODS

Experiments were performed on 60 male CBA/CaLac mice (class I conventional strain) aging 2 months and obtained from the nursery of the Institute of Pharmacology. The animals were divided into 2 groups. Myelosuppression was induced by single intraperitoneal injection of cyclophosphane in the maximum permissible dose (MPD 250 mg/kg, probit analysis). Group 1 mice received intragastrically 0.2 ml solution of immobilized hyaluronidase (iHD; Scientific Features Management Company) through a probe over 2 days after cytostatic treatment. Control animals received

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TABLE 1. Bone Marrow Hemopoiesis in CBA/CaLac Mice ($\times 10^6$/femur) Receiving Distilled Water or iHD after Single Treatment with Cyclophosphamide in MPD ($X \pm m$)

Period, days		Total number of myelokaryocytes	Immature neutrophilic granulocytes	Mature neutrophilic granulocytes	Eosinophilic granulocytes	Lymphoid cells	Monocytes	Erythroid cells
Baseline		10.92±0.83	1.10±0.10	3.72±0.31	0.47±0.09	3.38±0.30	0.48±0.06	1.77±0.32
3	control	2.74±0.16*	0.04±0.01*	0.11±0.04*	0	1.87±0.15*	0.55±0.04	0.18±0.05*
	iHD	2.37±0.11*	0.13±0.03*+	0.15±0.04*	0	1.36±0.10*+	0.42±0.09	0.31±0.08*
5	control	9.90±0.09	4.22±0.26*	1.45±0.26*	0.30±0.08	2.03±0.21*	0.86±0.08*	1.04±0.16
	iHD	13.27±0.98+	6.31±0.52*+	1.30±0.31*	0.12±0.08	2.28±0.36	1.29±0.15*	1.98±0.30+
7	control	13.20±0.57	2.78±0.30	7.71±0.37*	0.30±0.08	0.87±0.15*	0.96±0.08*	0.59±0.08*
	iHD	14.92±1.50*	2.46±0.32*	9.54±0.20*+	0.40±0.06	1.04±0.22*	0.68±0.10	0.81±0.21*
10	control	11.16±0.56	1.00±0.18	6.65±0.45*	0.76±0.45	1.82±0.25*	0.60±0.09	0.35±0.06*
	iHD	12.04±0.83	0.99±0.06	6.11±0.57*	0.33±0.10	2.39±0.50	0.94±0.21	1.29±0.19+

Note. $p<0.05$: *compared to the baseline; +compared to the control.

distilled water (0.2 ml orally) for 2 days. Baseline parameters were measured in intact mice.

The animals were killed by cervical dislocation under ether anesthesia on days 3, 5, 7, and 10 after cytostatic treatment. The state of the peripheral component of the erythron (hemoglobin concentration, erythrocyte count, hematocrit, and mean corpuscular hemoglobin concentration) and platelet count in animals of the treatment and control groups were evaluated on an ABACUS automatic blood analyzer (Diatron) operated in the veterinary mode. The number of leukocytes, content of various morphological types of leukocytes in the peripheral blood, and parameters of bone marrow hemopoiesis (total number of myelokaryocytes and myelogram) were measured by standard methods [7]. The content of committed precursor cells of erythropoiesis (CFU-E) and granulomonocytopoiesis (CFU-GM) in the bone marrow, erythropoietic activity, and colony-stimulating activity of conditioned media from adherent and nonadherent cells of the hemopoiesis-inducing microenvironment and blood plasma were evaluated by *in vitro* cloning of nonadherent myelokaryocytes in a semi-solid culture medium [4].

The results were analyzed by methods of variation statistics (Student's t test). The nonparametric Mann–Whitney test was used when the distribution of samples did not correspond to normal distribution [8].

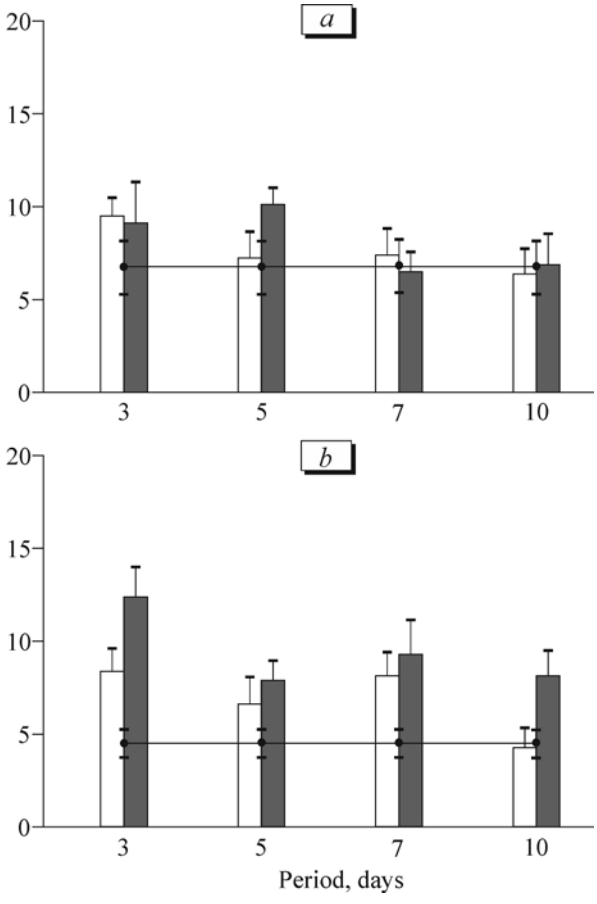

Fig. 1. Number of erythroid (*a*) and granulocytic precursor cells (per 10^5 nonadherent myelokaryocytes, *b*) in the bone marrow of CBA/CaLac mice receiving distilled water (light bars) or iHD (dark bars) after treatment with cyclophosphamide in a single dose of 250 mg/kg. Here and in Fig. 2: line, baseline parameters. Confidence intervals at $p=0.05$

RESULTS

Administration of cyclophosphamide in MPD led to suppression of all hemopoietic stems in animals. The number of morphologically distinct cells of the granu-

locytic, lymphocytic, and erythroid stem was shown to decrease in hemopoietic tissue (Table 1). These changes were accompanied by a decrease in the number of stab neutrophils, segmented neutrophils, lymphocytes, and erythrocytes and reduction of hemoglobin concentration in the peripheral blood. We revealed a compensatory increase in the content of CFU-GM and CFU-E in the bone marrow and elevation of secretory activity of adherent and nonadherent cells in the hemopoiesis-inducing microenvironment (Figs. 1 and 2).

The test preparation had a strong effect on hemopoiesis under conditions of cytostatic-induced suppression. The number of erythrocytes, concentration of hemoglobin, and hematocrit in animals of the treatment group on day 5 after administration of iHD were much higher than in the cytostatic control. Analysis of white blood cells showed that administration of iHD increased the number of stab neutrophils (by 2.6 times, day 5) and segmented neutrophils (by 1.5 times, day 7).

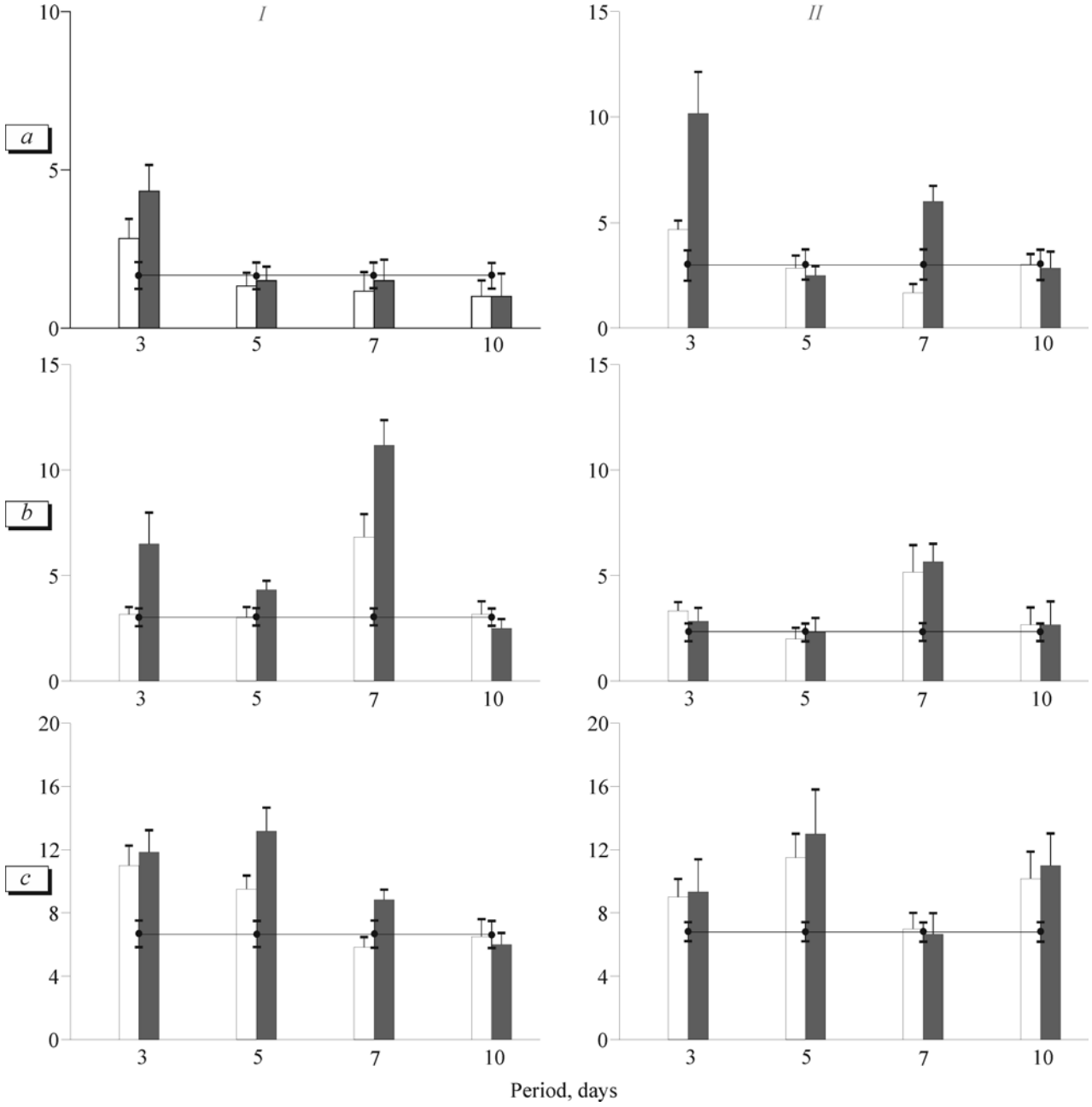

Fig. 2. Erythropoietic (*I*) and colony-stimulating activity (*II*) in supernatants of nonadherent (*a*) and adherent cells of the bone marrow (*b*) and peripheral blood (per 10^5 cells, *c*) from CBA/CaLac mice receiving distilled water (light bars) or iHD (dark bars) after treatment with cyclophosphamide in a single dose of 250 mg/kg.

These signs of the peripheral blood reflect changes in the bone marrow hemopoiesis. The number of erythrocytes in hemopoietic tissue was shown to increase on days 5 and 10 after treatment with iHD (by 1.9 and 3.7 times, respectively, compared to the control). Evaluation of the bone marrow granulocytic stem showed that the count of immature neutrophilic granulocytes increases on days 3-5 after administration of iHD. An increase in the number of mature neutrophils was observed on day 7. However, the total cellularity of hemopoietic tissue was shown to increase only on day 5 (Table 1).

The immobilized preparation produced a potent effect on colony-stimulating activity of the regenerating bone marrow. A 2-fold treatment with this form of the enzyme was followed by an increase in the number of erythroid (by 1.4 times, day 5) and granulomonocytic colonies in the methylcellulose medium (by 1.5 and 1.9 times on days 3 and 10, respectively; Fig. 1).

Humoral hemopoietic growth factors play the major role in the regulation of hemopoiesis. The erythropoietic and colony-stimulating activity was studied in conditioned media of cells from various fractions of the bone marrow and peripheral blood plasma.

The production of hemopoiesis-stimulating activity by microenvironmental cells was slightly increased under the influence of cyclophosphamide. However, colony-stimulating activity in supernatants of adherent bone marrow cells was shown to increase significantly on days 3 and 7 after administration of iHD (Fig. 2). iHD caused a statistically significant increase in erythropoietic activity of adherent (by 1.5 times, day 3) and nonadherent fractions of myelokaryocytes (by 2.05, 1.44, and 1.64 times on days 3, 5, and 7 respectively) and blood plasma (by 1.4 and 1.5 times on days 5 and 7, respectively, compared to the cytostatic control; Fig. 2).

Our results indicate that the preparation of iHD produced a strong hemostimulating effect under conditions of cyclophosphamide-induced myelosuppression. The action of iHD was most pronounced in the erythroid hemopoietic stem. The observed changes are associated not only with an increase in functional activity of hemopoietic precursors under the influence of low- and medium-molecular-weight forms of glucuronic acid (produced after treatment with the enzyme) [2,6], but also with stimulation of reparative processes in the hemopoiesis-inducing microenvironment (due to stimulation of proliferation and differentiation of mesenchymal progenitor cells) [3].

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