PHARMACOLOGY AND TOXICOLOGY

Experimental Study of Ultralow-Dose Antibodies to Cyclophosphamide on Cyclophosphamide Myelotoxicity

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The possibility of using ultralow-dose cyclophosphamide for reducing the myelotoxicity of cyclophosphamide, injected in the maximum permissible dose, was studied in mice. Combined treatment by the cytostatic and its ultralow-dose preparation led to a lesser suppression of the erythroid, lymphocytic, and particularly granulocytic hemopoiesis stems. This effect is explained by stimulation of the secretory activity of hemopoiesis-inducing microenvironment and hence, of the functional activity of granulocytopoiesis under the effect of ultralow-dose cyclophosphamide.

Key Words: *myelotoxicity; cyclophosphamide; ultralow doses; hemopoiesis; hemopoiesis-inducing microenvironment*

Low selective activity of the cytostatic effects of antitumor drugs, used in oncology, necessitates constant attention to the problem of these drugs toxicity towards normal organs with a high natural tempo of cell renewal [7,9]. The hemopoietic tissue is one of the most sensitive to antiblastoma agents, and therefore, hematological complications control during antitumor therapy is a pressing problem of modern oncology and hematology [2,3,8]. The problem of reducing the myelotoxicity of clinically used cytostatics is extremely important. The fact that drugs of this group in ultralow doses (ULD) modulate the specific activity of drugs used in therapeutic doses [10,11] attracts special interest. It was shown previously that the efficiency of cyclophosphamide (CP) cytostatic therapy can be amplified by using a combination of its therapeutic dose and ULD [1].

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We studied the effects of CP ULD on the myelotoxicity of CP injected in the maximum permissible dose.

MATERIALS AND METHODS

The study was carried out on 260 male CBA/CaLac mice aged 2 months (certified first-category conventional inbred mice from Breeding Center of Institute of Pharmacology). Mice were kept in accordance with the regulations adopted by the European Convention for Vertebrate Protection (Strasbourg, 1986). Myelosuppression was induced in animals by a single intraperitoneal injection of CP in a dose of 250 mg/kg. The animals were divided into 4 groups: 1) intragastric water, 0.2 ml/mouse, 30 min before CP injection; 2) oral ULD CP (mixture of homeopathic dilutions of antibodies to CP: C_{12}+ C_{30}+C_{200} (Materia Medica Holding), 0.2 ml/mouse, 30 min before CP; 3) water (0.2 ml/mouse) simultaneously with CP injection; and 4) ULD CP (0.2

ml/mouse) simultaneously with CP. Basal values were evaluated in intact mice.

Total counts of the peripheral blood reticulocytes, leukocytes, and the leukocytic formula were evaluated by the standard methods [6] in animals of all groups on days 2, 3, 5, 7, 10, and 13 of experiment. The peripheral component of the erythron (hemoglobin content, erythrocyte count, hematocrit, mean corpuscular concentration of hemoglobin) was evaluated by an automated hematological analyzer. During the same periods total content of myelocaryocytes per femoral bone was evaluated and their qualitative composition was studied in bone marrow smears. The content of erythroid (CFU-E), granulocytomacrophageal (CFU-GM), and stromal (CFU-F) precursors in the bone marrow was evaluated by cloning in methylcellulose-based semiviscous medium [5]. The intensity of hemopoietic precursors proliferation and differentiation was evaluated. The functional activity of hemopoiesis-inducing microenvironment (HIM) was evaluated by the intensity of secretion of factors, constituting the colony-stimulating and erythropoietic activities, by the HIM elements [5].

The data were statistically processed by methods of variation statistics using Student's *t* test. If the variant distribution in a sample differed from the normal, the significance of differences was evaluated by Mann—Whitney's nonparametrical test.

RESULTS

Analysis of the peripheral erythron parameters showed a significant reduction of mature erythrocyte content under the effect of CP in the blood of all experimental mice on days 7-10 after CP injection. On the other hand, erythrocyte count and hematocrit level in animals treated by ULD CP (before and

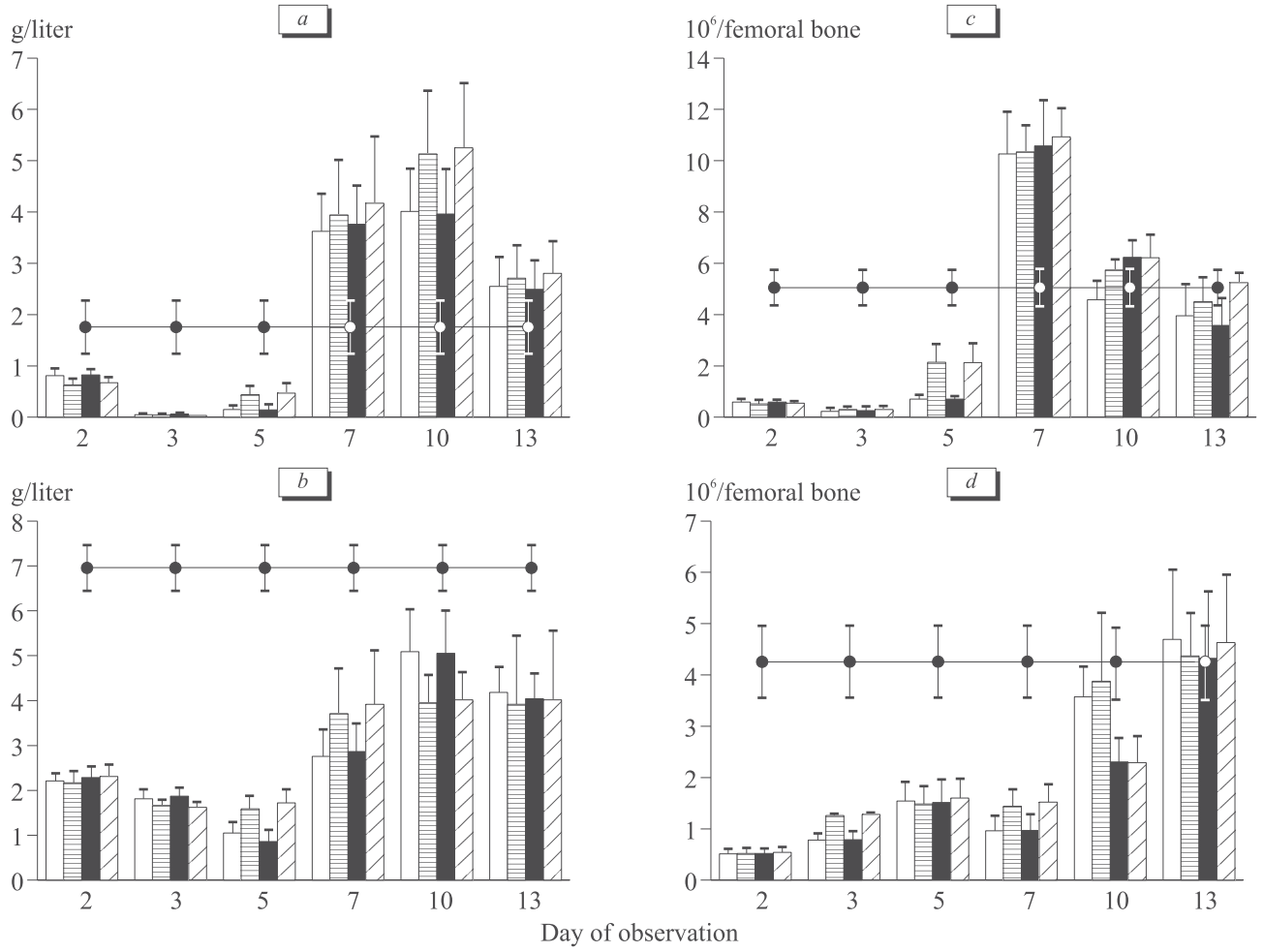

Fig. 1. The content of the peripheral blood segmented neutrophils (*a*) and lymphocytes (*b*), of bone marrow mature neutrophilic granulocytes (*c*) and lymphoid cells (*d*) in CBA/CaLac mice injected with water (light bars) and ULD CP (horizontal hatching) 30 min before CP or water (dark bars) and ULD CP simultaneously with CP (oblique hatching). Here and in Fig. 2: horizontal line shows the basal level. Confidence intervals at p=0.05.

simultaneously with CP) were significantly higher than in animals which received the solvent. Blood hemoglobin level reduced after the cytostatic injection starting from day 3 of experiment, while in animals treated additionally by ULD CP it started reducing only from day 7 and remained higher than in both reference groups. Reticulocytopenia, observed in all groups on days 2-7 of the study, was the most pronounced on day 2, but ULD CP treatment in combination with CP largely leveled the reduction of reticulocyte content in the blood during this period.

Measurements of the peripheral blood counts of leukocytes and their individual morphological forms showed leukopenia on days 2-7 after CP injection. On day 3 of experiment the count of white blood cells was somewhat lower in the group of animals treated by ULD CP in combination with CP. However as soon as on day 5 this value was 2-fold higher in mice which received the studied preparation than in the reference groups. This difference was due to higher blood level of neutrophils (mainly segmented), monocytes, and lymphocytes (Fig. 1). Later the blood levels of segmented neutrophils in the blood of all experimental animals were significantly higher than in intact mice, but on day 13 the counts of these cells normalized in mice treated by CP and the solvent and remained high in animals treated by the ULD preparation (Fig. 1).

These changes in the peripheral blood values reflected the changes in the bone marrow hemopoiesis. The ULD CP injected before and simultaneously with CP led to an increase in the count of lymphoid cells in hemopoietic tissue on day 3, of mature neutrophilic granulocytes and monocytes on day 5 of experiment in comparison with the values in animals which received water in addition to the cytostatic (Fig. 1). Later (on day 10) the total count of myelocaryocytes increased (at the expense of mature neutrophils) in animals injected with ULD CP before CP and on day 13 it increased in those injected with the drugs simultaneously (Fig. 1).

Study of the mechanisms of action of the ULD preparation showed a relationship between the above reactions of the blood system and the pools of hemopoietic precursors. For example, on days 2-5 of

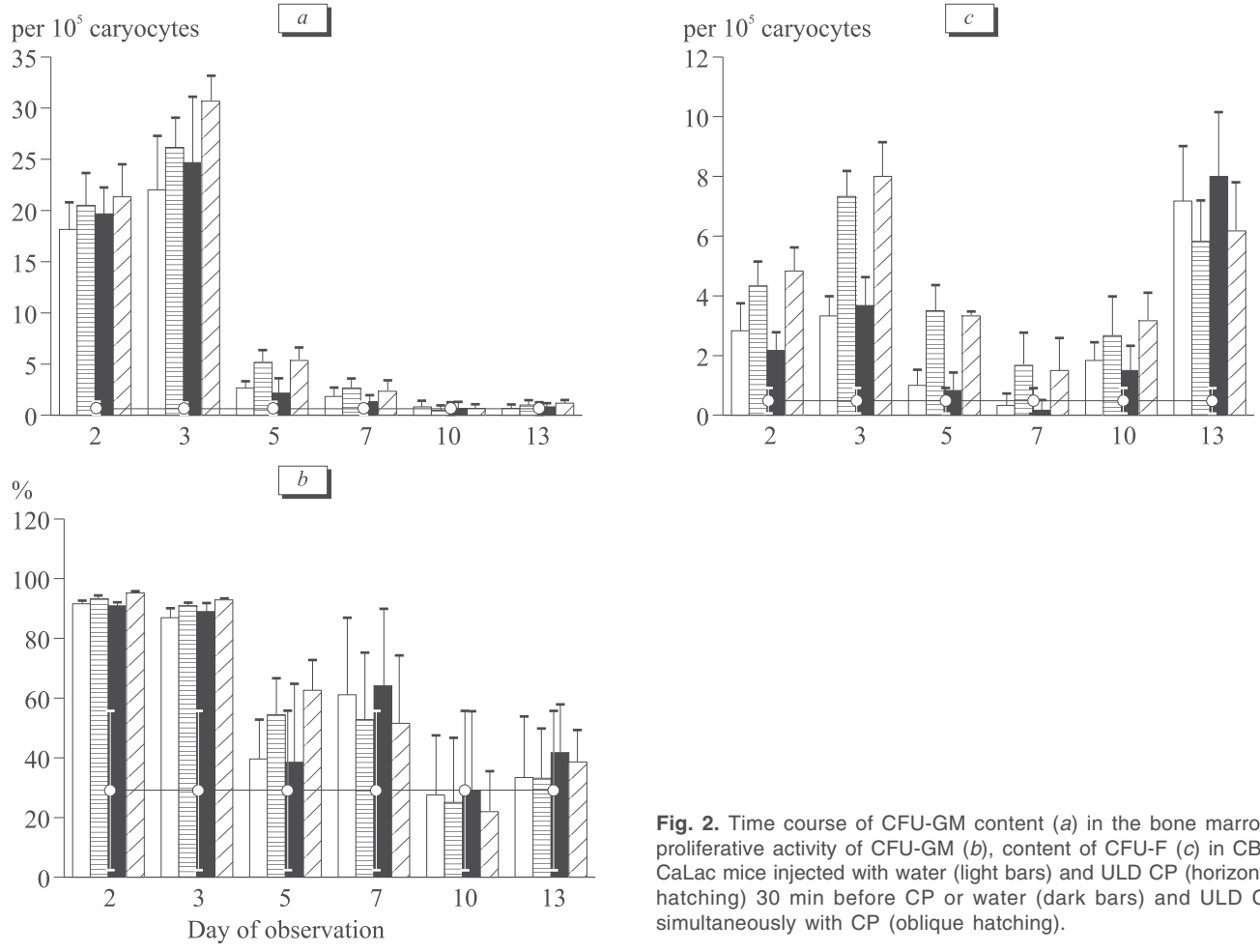

Fig. 2. Time course of CFU-GM content (*a*) in the bone marrow, proliferative activity of CFU-GM (*b*), content of CFU-F (*c*) in CBA/CaLac mice injected with water (light bars) and ULD CP (horizontal hatching) 30 min before CP or water (dark bars) and ULD CP simultaneously with CP (oblique hatching).

TABLE 1. Time Course of Colony-Stimulating Activity (CFU-GM/10^5 Test System Cells) of CBA/CaLac Mouse Bone Marrow Adhesive and Nonadhesive Cell Supernatants ($X \pm m$)

Day of experiment; group		Adhesive caryocytes	Nonadhesive caryocytes
Before treatment		0.17±0.17	0
2	1	1.00±0.37	0.33±0.21
	2	3.33±0.71*+	2.17±0.98
	3	1.17±0.48	0.33±0.21
	4	3.67±0.84*x	2.83±1.49
3	1	0.33±0.21	0.5±0.22
	2	0.50±0.34	0.33±0.33
	3	0.17±0.17	0.67±0.33
	4	0.33±0.21	0
5	1	0.17±0.17	0.33±0.21
	2	0.33±0.33	1.17±0.48
	3	0	0.33±0.33
	4	0.33±0.21	3.00±1.29
7	1	0	0.17±0.17
	2	0	0.50±0.34
	3	0	0.17±0.17
	4	0.17±0.17	0.33±0.21
10	1	0.17±0.17	0.17±0.17
	2	0	0.17±0.17
	3	0.33±0.21	0
	4	0.17±0.17	0
13	1	0.17±0.17	0
	2	0.17±0.17	0
	3	0.17±0.17	0
	4	0	0.17±0.17

Note. $p<0.05$ *vs.* *basal values, +group 1, xgroup 3.

experiment the general increase of the CFU-GM proliferative activity was paralleled by a more pronounced increase in this value in animals treated by ULD CP (higher in cases with simultaneous treatment by the preparation and CP; Fig. 2). Accordingly, these changes led to an increase in the content of granulomonocytic precursors in the bone marrow of animals in these groups, reaching the maximum differences in comparison with the values in the reference groups by day 5 of experiment. During the same period the content of morphologically differentiated neutrophils and monocytes in hemopoietic tissue increased statistically significantly (Fig. 1). The time course of erythropoietic stem cells in the bone marrow and their proliferative activity were characterized by opposite regularities. The intensity of CFU-E division and their counts were higher throughout the greater part of the period of observation in control mice which received water instead of ULD before and simultaneously with CP. No appreciable differences in the intensity of maturing of CFU-GM and CFU-E precursors were detected in the control and experimental animals.

The content of stromal precursors, similarly as of hemopoietic ones, increased many-fold in the bone marrow of experimental animals after injection of CP. Similarly to the CFU-GM precursors, the count of CFU-F increased more intensely in mice which received ULD CP in addition to CP (Fig. 2). A significant difference in comparison with the reference group was observed on days 2-7 of the study in animals treated by ULD preparation before CP and on days 2-10 in those which received it simultaneously with CP. Presumably, these facts reflect the morphogenetic relationship between fibroblasts and granulocytic stem cells in the hemopoietic tissue [4].

As the humoral hemopoietic growth factors, released by hemopoietic microenvironment cells, play the key role in hemopoiesis regulation, we studied the levels of erythropoietic and colony-stimulating activities in conditioned media from cells of different bone marrow fractions.

The production of colony-stimulating activity (its initial level was extremely low) by the microenvironment cells has changed negligibly under the effect of CP. On the other hand, the level of colony-stimulating activity increased significantly in the supernatants from bone marrow adhesive and nonadhesive cells from animals treated by ULD CP before and simultaneously with CP. These changes were statistically significant on day 2 for the bone marrow adhesive cells, presumably due to changes in the pool of bone marrow stromal cells (Table 1). Till day 7 of experiment this trend persisted without significant differences from the respective values in the reference groups. Study of the time course of HIM erythropoietic activity in the supernatants from adhesive and nonadhesive myelocaryocytes of animals treated by ULD CP before and simultaneously with CP showed no appreciable changes in their secretory function.

The study detected an obvious protective effect of ULD CP towards the blood system under conditions of myelosuppression caused by CP injection. It manifested by a less pronounced suppression of erythro-, lympho-, monocyto-, and particularly granulocytopoiesis under the effect of CP used in combination with its ULD and by a more intense recovery of these hemopoiesis stems (after ULD CP treatment by both protocols). These ef-

fects are explained by better preservation of hemopoiesis-inducing microenvironment due to ULD CP or by stimulation of its secretory activity (particularly of its adhesive component) and hence, of the functional activity of granulomonocytopoiesis precursor cells.

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