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Opposite Effects of 5-Hydroxymethyluracil on Mitogenic Response of T Cells Stimulated through T-Cell Receptor or through T-Cell Receptor and CD28 Co-Receptor Molecule

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The effect of a synthetic analog of "minor" pyrimidine base 5-hydroxymethyluracil (0.01-10 μg/ml) on the mitogenic response of donor peripheral blood T cells was studied *in vitro* under conditions of lymphocyte stimulation through T-cell receptor (antiCD3 monoclonal antibodies) or through T-cell receptor and CD28 co-receptor molecule (antiCD3 and antiCD28 monoclonal antibodies). 5-Hydroxymethyluracil suppressed the mitogenic response of T cells stimulated with antiCD3 antibodies, which was paralleled by an increase in the count of silent cells and decrease in the count of dividing lymphocytes, but not by stimulation of apoptosis of activated cells. Under conditions of integration of stimuli (lymphocyte stimulation with antiCD3 and antiCD28), 5-hydroxymethyluracil stimulated the mitogenic response, which was paralleled by suppression of activation apoptosis and increase in proliferative potential of cells.

Key Words: *hydroxymethyluracyl; T cells; mitogenic response*

5-Hydroxymethyluracil (2,4-dioxo-5-hydroxy-6-methyl-1,2,3,4-tetrahydropyrimidine; HMU) is a synthetic analog of a "minor" pyrimidine base. Persuasive experimental and clinical data demonstrated that in addition to low toxicity, HMU is characterized by a wide spectrum of pharmacological activities, including immunostimulatory activity, stimulation of regeneration, hemopoiesis, anti-inflammatory, antitoxic and antioxidant, radioprotective, anabolic effects, *etc.* [2,3]. The immunostimulatory effect of HMU manifests primarily against the background of immunosuppressions of different origin and depends on the initial level of immunoreactivity [2]. The immunostimulatory effect of synthetic pyrimidines is usually attributed to stimulation of nucleic acid metabolism and protein synthesis in cells (non-receptor mechanisms) [1]. However, receptor-mediated regulation of immunocompetent cell function by exogenous purine/pyrimidine bases and their synthetic analogs was also described [6].

No detailed studies aimed at detection of cellular mechanisms of immunotropic activity of HMU were carried out. We evaluated the effect of HMU on T cell mitogenesis *in vitro*.

MATERIALS AND METHODS

Peripheral lymphocytes from donor blood were studied. Mononuclears were isolated by the standard gradient centrifugation method (1000*g*, 30 min, 20°C) using HISTOPAQUE-1077 (Sigma). After isolation, the cells were washed twice in RPMI-1640 (Sigma) and resuspended in glutamine-supplemented RPMI-

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1640 with 10% inactivated FCS (Sigma) and 50 μg/ml gentamicin sulfate. Cell concentration was 10^6 cell/ml. Possible effect of monocytes on mitogenesis was minimized by reducing the count of adherent cells by 3-h adhesion to plastic (37°C, 5% CO_2). Nonadherent cells (95-99% lymphocytes according to analysis of light scatter parameters) were collected and incubated for 72 h in 96-well plates (Costar) at 37°C and 5% CO_2 without mitogens (spontaneous proliferation) or with 2 μg/ml antiCD3 monoclonal antibodies (MAb; clone ICO-90, Clonospectrum) or 2 μg/ml antiCD3 MAb+ 1 μg/ml antiCD28 MAb (eBioscience).

Hydroxymethyluracyl (Institute of Organic Chemistry, Ural Research Center of the Russian Academy of Sciences) was a kind gift from Prof. E. K. Alekhin, Head, Dept. Pharmacology of Bashkir Medical University. HMU (chemically pure substance) was first diluted in a minimum volume of DMSO and then in RPMI-1640 and added to the cultures together with the mitogen. DMSO concentration in the culture medium at maximum HMU concentration (10 μg/ml) was 0.01%; further increase in HMU concentration was impossible because of DMSO effect on functional activity of cells.

Lymphocyte mitogenesis was evaluated by spectrophotometric Alamar blue test with standard Alamar blue™ solution (Biosource) [9]. The intensity of apoptosis and cell cycle structure were analyzed by standard propidium iodide staining [4] followed by flow cytofluorometry on a Cytomics FC500 cytometer (Beckman Coulter). The relative content of cells in the hypodiploid (apoptosis), G1+G0, and S+G2+M peaks was evaluated.

The results were statistically processed using Student's test for conjugated sign pairs.

RESULTS

Addition of HMU in the maximum concentration (10 μg/ml) to the culture slightly increased proliferative activity of lymphocytes (no statistically significant difference from the control).

In T cell cultures stimulated with antiCD3 MAb (isolated stimulation of T cells through T-cell receptors, T-CR), mitogenesis tended to decrease in the presence of HMU (0.01 μg/ml). HMU in concentrations of 0.1-10 μg/ml significantly (though slightly) inhibited the mitogenic response of T cells. This inhibition was not related to enhanced apoptosis (Table 1), but mitogenic potential of cells decreased. For instance, a clear-cut trend to an increase in the count of silent (G0+G1 peak) cells was observed in cultures containing HMU. By contrast, the count of mitotically active cells (S+G2+M peak) tended to decrease in the presence of 0.01 and 0.1 μg/ml HMU and was significantly

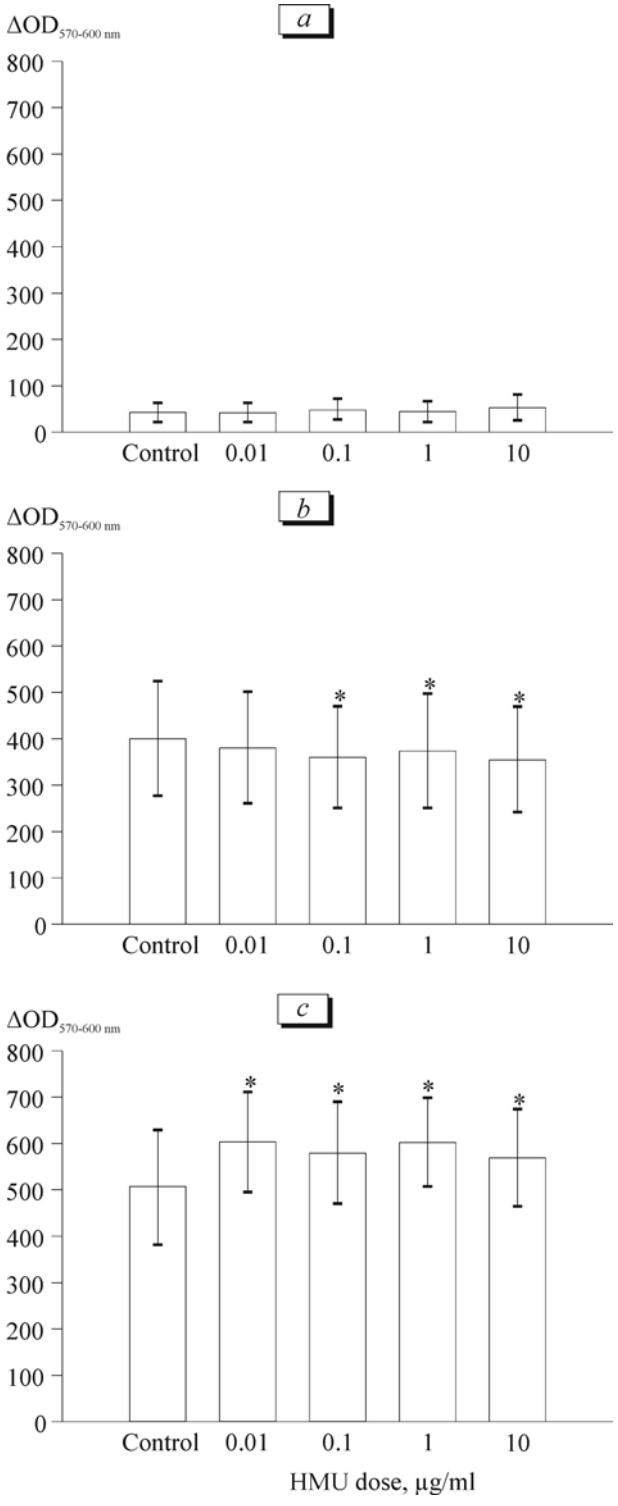

Fig. 1. Effect of HMU on spontaneous mitogenesis of donor peripheral blood lymphocytes and mitogenesis of T cells stimulated through T-CR (antiCD3 MAb) or through T-CR+co-stimulatory receptor CD28 (antiCD3+antiCD28 MAb). Colorimetric Alamar Blue test. *a*) spontaneous mitogenesis (data of 2 experiments, *n*=12); *b*) mitogenesis induced with antiCD3 MAb (*n*=8); *c*) mitogenesis induced with antiCD3 MAb and antiCD28 MAb (*n*=8). OD: optical density. **p*<0.05 compared to the control (no HMU). Ordinate: mitogenesis intensity.

TABLE 1. Effect of HMU on Apoptosis Intensity and Cell Cycle Structure of Donor Peripheral Blood Lymphocytes Stimulated with AntiCD3 MAb and after Integral Stimulation with AntiCD3 MAb and AntiCD28 MAb (*M*±*m*)

Culturing conditions		Stimulation with antiCD3 MAb (*n*=6)			Stimulation with antiCD3 MAb+ antiCD28 MAb (*n*=6)		
		apoptosis	G0+G1 peak	S+G2+M peak	apoptosis	G0+G1 peak	S+G2+M peak
Mitogen		4.9±1.4	67.2±7.5	26.1±6.9	9.1±0.5	71.2±1.9	18.3±1.7
HMU, μg/ml	0.01	4.7±1.4	71.4±14.7	24.0±7.6	8.3±0.4*	68.0±2.8	20.6±1.7*
	0.1	4.9±1.2	71.5±4.8	23.2±5.4	8.0±0.6*	70.2±2.0*	19.7±2.7*
	1	5.0±1.5	71.8±4.7	22.2±4.6	8.5±0.4*	70.8±0.4	18.8±1.0
	10	5.0±1.4	69.4±7.1	24.1±6.7*	8.4±0.6	70.5±1.6	19.7±1.4*

Note. *$p<0.05$ compared to mitogen alone.

lower than in control cultures in the presence of 1 and 10 μg/ml HMU.

An opposite effect of HMU was observed under conditions of T cell stimulation through T-CR and co-stimulatory CD28 molecule. The presence of HMU in culture medium markedly increased the mitogenic response of cells to HMU in all concentrations used (Fig. 1). In this case, HMU decreased the intensity of activation apoptosis of lymphocytes (significant differences from control cultures were observed in the presence of 0.01-1 μg/ml preparation). The relative content of silent cells and cells in the presynthetic phase of the cell cycle (G0+G1 peak) decreased (statistically significant difference in the presence of 0.1 μg/ml HMU). The count of mitotically active cells (S+G2+M peak) increased (statistically significant differences at 0.01, 0.1, and 10 μg/ml HMU).

It can be hypothesized that the inhibitory and stimulatory effects of HMU are caused by impairment of finely balanced pyrimidine base exchange during the cell cycle and accumulation of the "minor" pyrimidine base in the microenvironment. This hypothesis is confirmed by the data indicating that changes in the pools of intra- and extracellular metabolites and precursors of purine/pyrimidine metabolism in culture medium modulate activities of enzymes involved in the biosynthesis and catabolism of the major and minor metabolic chains, eventually changing the lymphocyte mitotic potential [7,10]. The direction of changes depends on cell type and mode of their stimulation. The effect of HMU can be also realized through subtypes P2Y and P2X7 purine/pyrimidine receptors, involved in the regulation of T cell differentiation, proliferation, and apoptosis. Exogenous purine and pyrimidine bases and their metabolites serve as ligands for these receptors [6]. Our data indicate that the effects of HMU are opposite in isolated stimulation of T cells through T-CR and in integration of the stimulatory signals from T-CR and co-stimulatory CD28 molecule. Similar results were reported previously [8]. These authors observed opposite responses of lymphocytes to Src kinase PP2 inhibitor stimulated with antiCD3 MAb and with antiCD3 MAb+antiCD28 MAb. It has been hypothesized that isolated stimulation of T-CR simulates lymphocyte contact with autoantigens and the "basal" stimulation essential for the maintenance of peripheral tolerance. Stimulation through T-CR and CD28 simulates an actual situation during T cell contact with a foreign antigen (dendritic cell) [5]. These cellular mechanisms can underlie a known differentiated effect of HMU on the immune response (humoral immune response, delayed type hypersensitivity, transplantation immunity) and phenotypical correction of immune response [2].

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