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Immunomodulating Characteristics of Cys-(Pro)$_3$-Glu-Leu Hexapeptide under Different Experimental Conditions

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Cys-(Pro)$_3$-Glu-Leu hexapeptide markedly stimulated activity of human natural killers during the cytotoxic test in the presence of autologous serum, but not fetal calf serum. This effect of hexapeptide on natural killer activity was physiological. Conditions are determined under which hexapeptide stimulates the cytotoxicity in a mode ensuring protection of the microenvironment from damage inflicted by own natural killer cytotoxic factors.

Key Words: *immunoregulation; hexapeptide*

Serum composition of the nutrient medium for the natural cytotoxicity test (NCT) is largely determined by conditions to which the target tumor cell culture is adapted. The serum used in the test (most often fetal calf serum, FCS) always contains culture growth factors in sufficient amounts.

In some experiments NCT on human lymphocytes should be performed with homologous or autologous serum (AS). This excludes the effect of fetal serum components, specifically, of fetal damper proteins, on the result of the test. This is important for the studies of the effects of immunoactive peptide compounds on activity of natural killers (NK). However, the result of the test under these conditions can considerably differ from the result recorded under standard experimental conditions. We previously showed that variations in serum composition of nutrient medium and duration of cell exposure in media of different composition led to more than 1.5-fold differences in NK activity [5].

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Synthetic Cys-(Pro)$_3$-Glu-Leu hexapeptide (P-07) is a structural analog of C-terminal fragment of pepsin Fab fragment heavy chain (Cγ2 domain) located near the waist of rabbit IgG molecule [2]. Its immunoregulatory effects manifest in stimulation of NK, mediated, among other things, by selection of active effectors at the stage of nonspecific adhesion in the lytic cycle, induction of interferon-γ production by lymphocytes, and arrest of interferon-γ production during NK-mediated cytolysis [6]. Pseudoenzyme activity of P-07 depending on the presence of Zn ions [4] determines close relationship between the manifestation of its immunoregulatory effects and the presence of compounds capable of binding metal cations in the microenvironment, *i. e* composition of the nutrient medium.

We evaluated the effects of P-07 on NCT of human lymphocytes in nutrient media of different serum composition under different conditions.

MATERIALS AND METHODS

Mononuclear cells were isolated from the peripheral venous blood collected from 12 donors (4 men and 8 women aged 18-45 years) in a Ficoll-Paque density gradient (Pharmacia Fine Chemicals). The cytotoxic ac-

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tivity of NK was evaluated using the conventional radiometric method against ^{3}H-uridine labeled K-562 human erythromyeloblasts in the effector:target ratio from 100:1 to 6:1. Lymphocytes were co-cultured with target cells for 14 h at 37°C in a humid atmosphere with 5% CO_2. The results were interpreted with estimation of the cytotoxic index for each effector:target ratio; the area under the cytotoxicity curve was considered as the integral NCT parameter and expressed in arbitrary units.

Hexapeptide P-07 was kindly provided by A. Ya. Kul'berg, Corresponding Member of Russian Academy of Medical Sciences. The agent was added to mononuclear cell suspension in doses of 0.05, 0.5, 5, and 50 μg/ml and exposed at 37°C in the presence of 5% CO_2 for 1 h before NCT, and removed by washout. The cells were treated with P-07 in complete nutrient medium based on RPMI-1640 (Amimed) and supplemented with 12% AS or FCS (Flow Lab.), other components and antibiotics were added according to standard formulation. During co-incubation of lymphocytes and target cells in the presence of AS, the medium contained 6% AS and 6% FCS, to which the target cell culture was adapted.

The significance of differences between the means was evaluated using Student's *t* test.

RESULTS

After treatment of human lymphocytes with P-07 in AS-based medium with subsequent incubation in nutrient medium containing AS and FCS NK activity increased from 5.3 to 6.1-6.5 arb. units (Fig. 1, *a*). The effect of the dose of 0.05 μg/ml was statistically significant ($p<0.05$).

Incubation of lymphocytes with target cells without AS reduced the effect of P-07 in doses of 0.05 and 50 μg/ml (Fig. 1, *b*). The retained activity manifested in increased NK cytotoxicity (from 4.0 to 4.9-5.0 arb. units). A reliable result can be obtained with P-07 dose higher by one order of magnitude.

The absence of AS in the nutrient medium, where lymphocytes were pre-treated with P-07 before incubation in FCS-based medium, abolished the immunostimulation effect (Fig. 1, *c*). NK activity increased only from 3.3 to 3.6-3.8 arb. units; no significant results were observed in this experimental series.

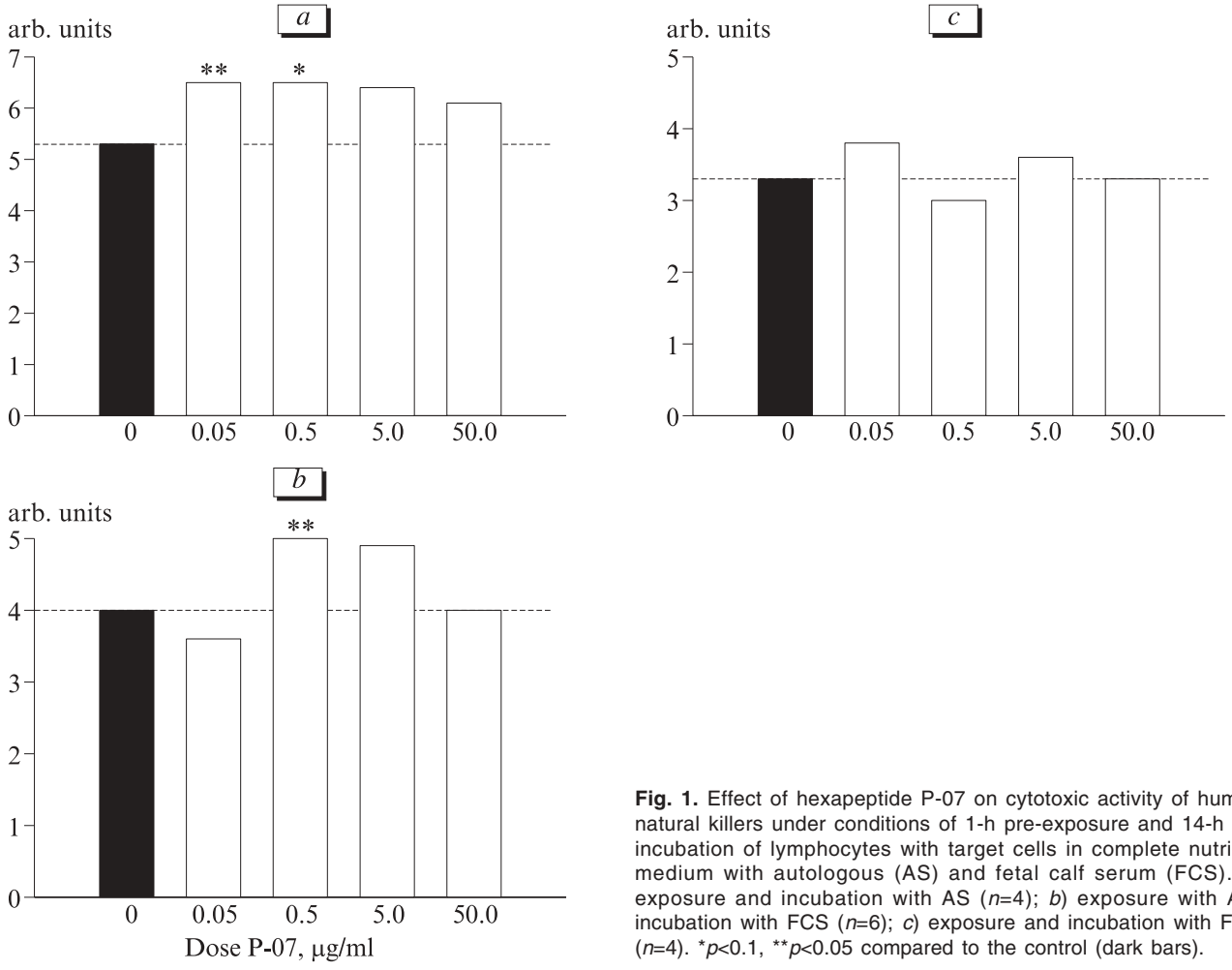

Fig. 1. Effect of hexapeptide P-07 on cytotoxic activity of human natural killers under conditions of 1-h pre-exposure and 14-h co-incubation of lymphocytes with target cells in complete nutrient medium with autologous (AS) and fetal calf serum (FCS). *a*) exposure and incubation with AS ($n=4$); *b*) exposure with AS, incubation with FCS ($n=6$); *c*) exposure and incubation with FCS ($n=4$). *$p<0.1$, **$p<0.05$ compared to the control (dark bars).

TABLE 1. Distribution of Hexapeptide P-07 Effects by the Effector:Target Ratio (Number of Cases)

Experimental conditions	Effector:target ratio			
	100:1 and 50:1		12:1 and 6:1	
	NK stimulation	NK inhibition	NK stimulation	NK inhibition
1 h AS+14 h AS	11	1	14	—
1 h AS+14 h FCS	18	8	9	8
1 h FCS+14 h FCS	—	2	10	—

Note. Only cases, when the absolute change in the cytotoxicity index was at least 5% were taken into consideration.

Hence, manifestations of the immunoregulatory effects of P-07 largely depend on the serum composition of the nutrient medium. We found that replacement of AS with FCS in the experimental system (and the resultant increase in the concentrations of fetal damper proteins, among other things) is paralleled by a decrease in P-07 effect on NK activity and even abolished this effect in nutrient medium based on FCS alone.

The effect of P-07 on lymphocyte cytotoxicity in a medium containing AS is physiological, due to similarity of P-07 to the natural products of partial cleavage of immunoglobulins [3]. The increase in activity of NK capable, as we know, to lyze (under certain conditions) own native cells, did not exceed 23-26% of the initial level.

Physiological regimen is formed in biological fluids and tissues of the body with participation of heparin and heparane sulfates involved in the generation and metabolism of active oxygen species [12,13]. Due to their amphipathic nature and metal binding capacity, these compounds can modify primembrane processes confined to metal cation transport and exchange in cell microenvironment [1,2,8,9]. Presumably, the same properties are intrinsic for P-07 and other products of natural catabolism of immunoglobulins, possessing proline nucleus binding H_2O molecules [2] and hence, appreciably modulating the conformation of cell receptors during interactions with them. If it be so, the regulatory effects of P-07 towards NK activity can be regarded in the context of its conformation interactions with the effector membrane structures responsible for recognition of target cells and mechanism of NCT triggering [10,11].

Damper proteins can bind to the same structures. Hence, P-07 effect is reduced or canceled in the presence of FCS because of shielding of membrane structures of reacting cells or because of involvement of P-07 in binding to FCS proteins, which, judging from our findings, is equiprobable.

Analysis of redistribution processes in NK population associated with variations in the intensity of the experimental system [7] indicates that the effect of P-07 is the most smooth when pretreatment of mononuclear cells by P-07 in AS-based medium is followed by incubation of lymphocytes with target cells in FCS-based medium. This protocol of the experiment allows general stimulation of NK in parallel with decrease of cytotoxicity at low effector:target ratios (Table 1), which is to be associated with increased production of interferon protecting the microenvironment from the cytotoxicity of host NK [7].

Hence, manifestation of immunoregulatory effects of Cys-$(Pro)_3$-Glu-Leu hexapeptide and, presumably, other proline-rich oligopeptides *in vitro* depends to a great extend on serum composition of the nutrient medium. The results admit a hypothesis about the involvement of certain components of the host biological fluids and tissues, functionally similar to fetal damper proteins, in the rational limitation of NCT.

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