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Changed Course of Cytotoxic Reaction as a Result of Modification of Target Cell Membrane

C. B. Cheknev and E. S. Gorozhanina

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We studied the sensitivity of K-562 erythromyeloblasts cultured for a long time in a nutrient media of common composition (control) or medium modified by proline-rich hexapeptide, to cytolysis mediated by natural killers. On day 2 of culturing the cytotoxicity of human natural killers towards targets maintained in the modified medium was almost 2-fold higher than in the control. By day 30 this difference was retained at the effector:target ratio of 50:1 and 12:1. By day 90 of the experiment activity of natural killers towards K-562 cells cultured in modified medium was lower than in the control. The results are discussed in the context of hydration of the target cell surface structures at the expense of bound water brought by proline-rich open type sequences into the distal regions of extracellular sites of adhesion receptors and target determinants recognized by natural killers.

Key Words: *cytotoxic reaction; target cell; exposure*

The outcome of natural cytotoxic reaction is determined by the functional activity of lymphocytes and by the target cell (TC) status (mobility of membrane glycoproteins and glycolipids, depending on the membrane viscosity, as well as expression of surface structures and receptors) [1,12].

The conformation characteristics of TC membrane structures can be modified by heparin and heparane sulfates, capable of generation and capture of oxygen metabolites utilized in natural killer-mediated (NK) cytolysis reactions [11,13] and of proline-rich compounds, forming, due to this, active centers binding water molecules and utilizing short-living oxygen radicals [2,4]. Among these compounds are oligopeptides located in the IgG molecule waist and determining (at the expense of variable hydrodynamic characteristics) the mobility of Fab fragments with respect to Fc site [2,3].

Laboratory of Cell-Cell Interactions, N. F. Gamaleya Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences. ***Address for correspondence:*** cheknev@riem.ru. S. B. Cheknev

The interactions between compounds, binding water molecules and capturing redox-active metabolites, and structures involved in the formation of the immune synapse [9] will necessarily modify the intercellular signaling process. This will take place at the expense of redistribution of the pro- and antioxidant effects determining the conditions of effector and accessory cell functioning in the microenvironment [10] and at the expense of induction of conformation changes in the mucin-like domains of extracellular sites of adhesion receptors [2,12], this modifying the strength of the structure formed in NK-TC contact.

The immunoregulatory characteristics of Cys-$(Pro)_3$-Glu-Leu (P-07) hexapeptide, reproducing the amino acid sequence between the 241st and 248th residues of the rabbit IgG heavy chain [5-7]. As enzymatic cleavage of proline-proline bonds is ineffective and not dynamic [2], proline-rich compounds can retain stability in buffered solutions for a rather long time and be used for studies of their effects in cell cultures maintained *in vitro* for a long time.

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MATERIALS AND METHODS

Human lymphocytes were isolated from peripheral venous blood of 12 donors (8 men and 4 women aged 19-36 years) in one-step Ficoll Paque density gradient (Pharmacia Fine Chemicals). NK cytotoxic activity against ^{3}H-uridine labeled K-562 human erythromyeloblasts (EMB) at effector:target ratios from 100:1 to 12:1 was measured by routine radiometry. Co-incubation of lymphocytes and TC was carried out for 14 h at 37°C in humid atmosphere with 5% CO_2. The reaction results were recorded with estimation of the cytotoxic index for each effector:target ratio and integral indicator of NK activity (area under the cytotoxicity curve).

K-562 EMB, used as targets, were cultured in complete nutrient medium based on RPMI-1640 (Flow Lab.), supplemented with 12% FCS (N. F. Gamaleya Institute of Epidemiology and Microbiology), 2 mM glutamine, and 40 μg/ml gentamicin (Pharmachim) in 10 mM Hepes buffer (Serva). In a parallel series, K-562 cells were cultured in nutrient medium modified by addition (for passage) of P-07 hexapeptide (0.05 μg/ml) exhibiting the most pronounced effect under conditions of lymphocyte-effector treatment [5]. The nutrient medium was enriched with hexapeptide during every reinoculation of the culture, this ensuring a more or less constant presence of P-07 in culture medium of experimental K-562 during the entire period of observation (up to 90 days). P-07 hexapeptide was a kind gift from A. Ya. Kul'berg, Corresponding Member of Russian Academy of Medical Sciences.

Cell viability in control and experimental cultures was evaluated by routine staining with 0.2% Trypan Blue. Proliferative activity of TC was evaluated using pulsed (1 h) label with ^{3}H-thymidine (40 h after cell passage) and ^{3}H-uridine (on days 2, 30, and 90 of the experiment). The cytotoxic activity of NK was tested in parallel.

The status of control and P-07-modified nutrient media conditioned by K-562 cells, was evaluated during the 1st, 2nd, 3rd, 24th, and 48th hours after primary passage of cultures using an UV spectrophotometer at λ=270-350 nm and common method for evaluation of antioxidant activity of samples (with $FeSO_4$ and thiobarbituric acid).

The significance of differences in the mean values was evaluated using Student's *t* test.

RESULTS

Proliferation of EMB during the first 40 h of K-562 cell culturing in the nutrient medium with P-07 increased to the level more than 3-fold ($p<0.1$) surpassing ^{3}H-thymidine incorporation into control cells. During the same period (on day 2 after culture passage) lymphocyte NK activity towards experimental K-562 cells was 69-86% higher in comparison with their activity towards control EMB. The most pronounced difference was observed at effector:target ratios of 100:1 and 50:1 ($p<0.1$; Fig. 1, *a*). Evaluation of the integral parameter also showed an almost 2-fold higher level of NK activity towards P-07-modified cells *vs.* control EMB ($p<0.05$; Fig. 1, *b*).

Experimental and control nutrient media conditioned by K-562 cells differed appreciably by their physico-chemical characteristics. The redox status, initially prooxidant in medium with P-07, and antioxidant in the control sample essentially changed over 2 days of culturing, becoming virtually neutral by the

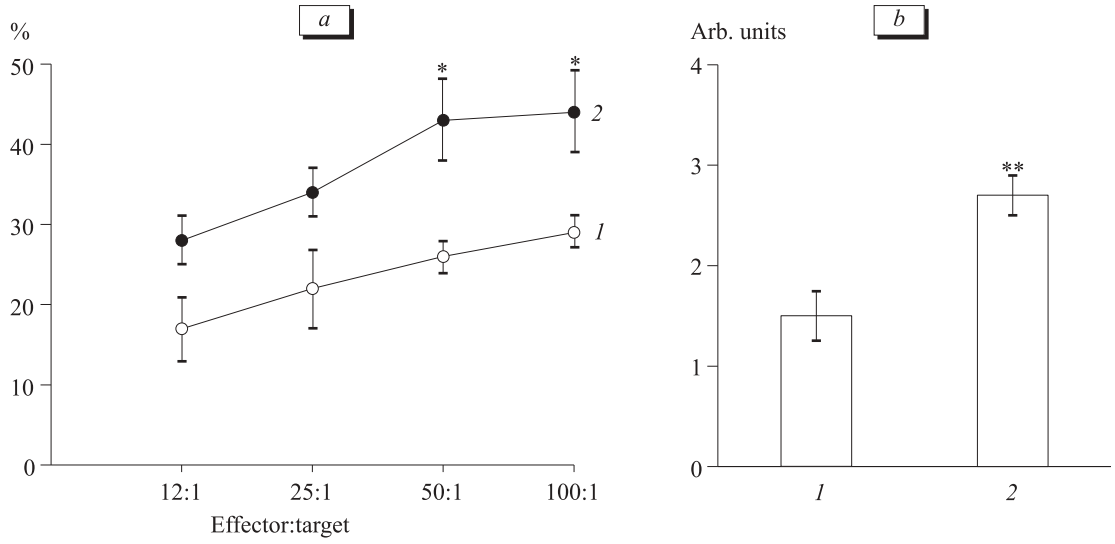

Fig. 1. Cytotoxic activity of natural killers towards K-562 cells, maintained during 48 h in nutrient medium with P-07 hexapeptide (*2*) and without it (*1*) (n=4, $M\pm m$). *a*) cytotoxic index; *b*) area under the curve. *$p<0.1$, **$p<0.05$ compared to *1*.

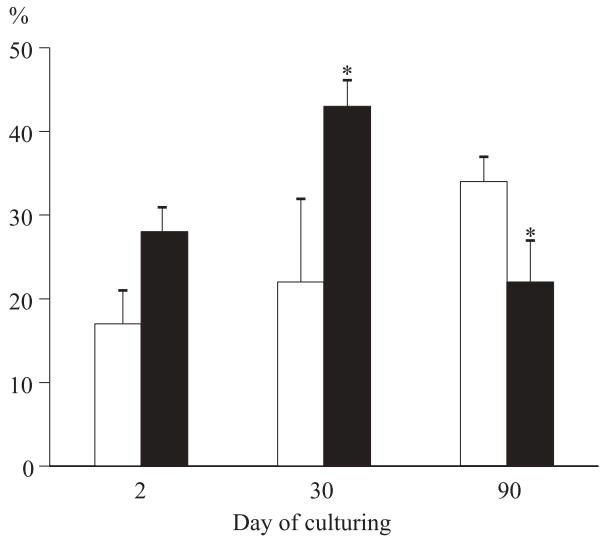

Fig. 2. Cytotoxic activity of natural killers towards K-562 cells maintained in nutrient medium containing P-07 hexapeptide (dark bars) and without it (light bars) (n=4, $M\pm m$). Effector:target ratio 12:1. Ordinate: cytotoxic index. *p<0.05 compared to previous term.

moment of cytotoxicity testing. The antioxidant activity of P-07-modified medium varied within a 2-fold lesser range of values than that of control samples. UV spectrophotometry showed that during 48 h of observation the absorption of the medium with P-07 changed by just 6%, while optical density of control samples was about 25%.

By day 30 of K-562 culturing *in vitro* a higher level of cytotoxicity towards EMB was retained in the medium with P-07, though this excess was less pronounced than on day 2. Lymphocyte NK activity towards TC in the medium with P-07 was 14-45% higher than the cytotoxicity towards control targets. A stable effect was observed at effector:target ratio 50:1 and 12:1.

By day 90 of the experiment NK activity towards the cells in medium with P-07 decreased and was 9-28% lower than that towards control EMB. The most pronounced decrease was observed at effector:target ratios 50:1 and 12:1.

The time course of ^{3}H-uridine incorporation into target RNA during the entire period of observation indicated a constantly increasing difference between the experimental and control EMB cultures (Table 1). The cells in medium with P-07 incorporated the isotope 1.3 times more actively than control cultures (p<0.05) on day 2, 1.5 times more actively on day 30, and 4.1 times more actively (p<0.05) on day 90 of the experiment (Table 1). Hence, after 30 days of culturing the synthetic processes surpass the medium level, optimal, as we know, for the realization of NK cytotoxicity towards K-562 cells.

TABLE 1. Incorporation of ^{3}H-Uridine (Pulse Label) in EMB Maintained in Control and P-07-Modified Nutrient Media (cpm, $M\pm m$, n=4)

Day of observation	Control medium	Medium with P-07
2	347.0±16.5	463.0±29.8*
30	946.0±189.6	1372.0±391.4
90	478.0±159.2	1965.0±408.7*

Note. *p<0.05 compared to control medium.

Evaluation of K-562 cells viability in the course of the experiment showed that P-07 stabilized EMB status. This parameter varied from 30 to 82% during 90 days of observation in the control medium and was characterized by sharp jumpwise changes, while in the medium with P-07 it varied from 52 to 86%, the fluctuations were less pronounced, and sharp shifts developed only by the end of the 3rd month of the experiment.

The results analyzed within the framework of common theory of protein hydrodynamics exhibit the capacity of proline-rich oligopeptides to bring bound water into the cell surface receptor structure, thus modifying the spatial packing. P-07 with its metal-dependent pseudoenzymatic activity [4] realizing it in the form of supramolecular aggregations [4] and reacting with serum proteins [7] seems to form hydrodynamically stable complexes saturated with water, with the TC receptors recognized by NK. After this restructuring the TC structure becomes more rigid and hence, more effectively recognized and bound by NK. NK activity increases.

With time, more and more surface structures of TC are involved in the interaction with P-07. The resultant excessive hydration of the membrane decreases its viscosity, destroys surface determinants, decreases the efficiency of target recognition and strength of its binding. NK cytotoxicity decreases.

The presence of P-07 in the NK:TC interaction system causes dissociation of the effector:target conjugates forming during the first 10 min of TC contact with NK [6]. As NK cytotoxicity markedly increased as a result of this dissociation, it was believed that "false" complexes (conjugates in which TC were not lyzed at all or were ineffectively lyzed because of inert NK participating in their formation) were destroyed in the presence of P-07 [6]. It seems that free sites are retained in such conjugates in the ligand—receptor complex, which serve as targets for attracting small open-type sequences intensely binding water. Presumably, conjugates formed by active NK have no sites of this type.

These findings suggest that modification of surface structures of TC membranes by prolonged exposure to P-07 decreases the efficiency of contact with TC for not only inert, but even for active NK, which results in decrease in their cytotoxicity.

Changes in cytolysis at low effector:target ratio (12:1), when it is possible, as we know, to trace the effects of an individual killer (Fig. 2), are demonstrative in this respect.

The cytotoxicity of NK during 1 month of EMB culturing in the medium with P-07 increased 1.5 times in comparison with activity determined on day 2 ($p<0.05$). During 2 subsequent months under conditions of lasting exposure to a hydrating compound NK activity decreased 1.9 times *vs.* the previous term ($p<0.05$) and became 1.3 times below the initial level (Fig. 2). The most pronounced cytolysis was recorded on day 30 of observation, when TC suspension was characterized by a medium level of isotope incorporation into RNA (Table 1).

Hence, addition of a membrane-active compound, represented by open-type oligopeptide sequence, binding water and bringing it into surface structures of EMB into culture of K-562 cells for a period of up to 1 month (9 passages) stabilized cell-conditioned nutrient medium, synchronized TC culture, and, presumably, preserved the distal sites of cell adhesion receptors and increased the sensitivity of TC to NK-mediated cytolysis.

This latter circumstance is very important, because, as was previously shown, P-07 can induce IFN production by blood lymphocytes [5]. IFN protects TC in the cytotoxic reaction [8]. P-07 stimulates NK and simultaneously completely cancels IFN production by lymphocytes reacting with TC [5]. These data indicate that the effect of proline-rich hexapeptide, stimulating cytolysis, is realized not only at the expense of discontinuation of cytokine production, protecting TC, but via direct modification of the EMB membrane. Thus *in vitro* reproducing the effect of IFN on cytotoxic lymphocytes, P-07 differs essentially from IFN by its effects towards TC.

Long-term (1-3 months) exposure to P-07 leads to excessive hydration of TC membrane, saturation of surface structures with bound water, deconformation of the distal sites of adhesion receptors, and destruction of determinants significant for lymphocytes, which rules out the possibility of their effective recognition, TC binding and subsequent cytolysis. Proline-rich hexapeptides under these conditions reproduce the effect of IFN towards TC, which can contribute to rational limitation or canceling of NK cytotoxicity towards the body's own intact cells in the active NK microenvironment involved in the cytotoxic interactions with transformed TC.

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