# REVIEWS

# Signal Splitting as the Basis for Involvement of Natural Cytotoxicity System in Endogenous Biological Retranslation

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The ability of natural killers to split biological signal (dissociation of cytotoxic and interferon-producing activities) is the main precondition of the involvement of the natural cytotoxicity system in endogenous biological retranslation. Dissociation of the primary functions of natural cytotoxicity effectors is provided by enhanced interferon production against the background of decreased cytotoxicity of natural killers, or by enhanced natural cytotoxicity against the background of almost complete inhibition of interferon production. Dissociation of the primary functions of natural killers is a physiological phenomenon; it is induced and maintained by factors, which are not directly connected with induction or suppression of the immune response.

**Key Words:** *natural cytotoxicity; endogenous biological retranslation*

In 1997-1999 we presented a methodologic concept of endogenous biological retranslation (EBR) developed on the basis of comprehensive clinical, immunological, and experimental studies of the mechanisms of regulatory balance between natural cytotoxic reactions [14,15,17]. According to this concept, a number of intercellular interactions in man and higher animals are realized as retranslation: an effector cell receiving molecular signal (cytokine) responds by generation of the same signal (identical cytokine). The key point of this new concept is biological expediency of attenuation or limitation of other specific cell activities during induction of synthetic in a translational field. Thus, the increased energy consumption for retranslation of the molecular signal is accompanied by decreased metabolic expenditures for cell reactions not directly connected with EBR (proliferation, antigen presentation, phagocytosis, cytotoxicity, and others).

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Since the dynamics of translational intercellular interactions can mask the effect of biologically active compound changing specific target functions (exclude its recording), elaboration of new methods evaluating cytokine regulatory effects becomes actual. However, the effect can be realized via synthetic (translational) cell potential, cell associate or functional complex [15].

The hypothesis on the involvement of natural cytotoxicity (NCT) in EBR is based on:

- — high variability of functional activity of the natural killers (NK) manifested on the cell, organism (sex and age), and population levels [19,21,27];
- — wide-range sensitivity of these cells to immunoregulatory peptides of different origin, such as interferons (IFN), interleukins (IL), C-reactive protein, serum components [13,22,24-26];
- — autonomic activity of NK in immune reactions [1,4];
- — regulatory interactions between NCT and lymphoid and nonlymphoid cells not related to cytotoxicity [14,18];

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— responsiveness of NK to IFN, its production, and realization of cytotoxic potential not determined by IFN [8,9,19,22,23].

The latter property distinguishes NCT from other systems involved in the formation of adequate immune response at the physiological level of immunoregulation. Relatively autonomic realization of primary functions providing independent on antigen-specific reaction activity of effector cells allows to isolate translational field from the complex of immune processes. Generally, any biological system producing compounds identical to those affecting this system may be involved in EBR [15]. However, phylo- and ontogenetic pecularities of NCT development [14,15, 17], its general biology and physiology [14-18], highly efficient mechanisms of dissociation of cytotoxic and noncytotoxic NK activities [17,18] suggest that this system is the most ancient complex of intercellular interrelations. Activity of this complex is regulated via EBR involving NK-mediated reactions, which are more preferable than processes performed by other immunocompetent cell populations [14,15]. In any case, both well studied (previously not implicated in biological retranslation) EBR types in humans, such as priming of IFN production and NK-mediated cytolysis [15] are integrated into complex maintenance of the immune homeostasis and represent primary NCT functions.

Thus, the ability of NK to split biological signal, i.e. dissociation of their cytotoxic and IFN-producing activities, observed in experimental and clinical studies is the most important characteristic of these cells. This means that these very important for immunogenesis primary functions of NCT effectors are regulated by different mechanisms and can be realized independently, i.e. cytotoxic (supervision) and noncytotoxic (regulatory) components can be actively enhanced or attenuated with simultaneous attenuation or potentiation of energy supply of the "opposite" function.

Despite strong positive correlation between cytotoxicity and IFN production by NK from healthy donors during lymphocyte interactions with standard tumor target cells [7,19,20] NK activity can be independent and not associated with IFN production by effectors stimulated by K-562 target cells [33], while IFN production is sometimes not necessary for normal NCT functioning [42]. Activity of mouse peritoneal excudate cells against YAC-1 target cells is not associated with IFN release [56], while synthesis of IFN-α and IFN-γ by lymphoid cells does not correlate with their NCT [6]. Staphylococcal enterotoxin A enhances cytotoxicity of human NK after 3-h incubation, i.e. in the absence of IF-γ and IL-2 produced by staphylococcus-stimulated lymphocytes [51]. On the other hand, deficiency of Leu-CAM molecules sharply decreases lymphocyte NCT against the background of preserved IFN-γ production [50]. Cytotoxicity of human NK against T-cells of the HUT lineage infected with human immunodeficiency virus-1 does not depend on IFN-α release in culture medium [29].

These facts confirm the idea that NK-mediated cytolysis and IFN production are regulated by different NCT mechanisms and each of these primary cell functions can be stimulated or inhibited independently. Similarly, signal mechanism mediating T-cell cytotoxicity differs from that triggering IL-2 production [49]. These data prompted us to study biological foundations of the dissociation phenomenon, which might contribute to understanding of the key mechanism providing NCT involvement in EBR in humans and higher animals.

Dissociation of cytotoxicity and IFN production by NK during their interaction with target cells can be regarded as splitting of biological signal because both NK primary functions may serve as biological signals. Production of IFN in the cytolysis-associated cytokine in concentrations dramatically exceeding its physiological content in organs and tissues [31,32,41] provides its reception not only by NK and target cells involved in cytotoxic interaction, but also by many cells in the microenvironment. These processes enable generation and translation of a biological signal, which is mediated by IFN and possesses multiple pleiotropic and systemic effects.

Active spontaneous production of IFN-γ by cultured NK can be associated with noncytotoxic lymphocyte types [28]. This means that IFN production is not necessary associated with cytolysis and can be interpreted as independent biological signal similar to priming in the absence of target cells for cytotoxic action. Among purified NK fractions binders and killers can be isolated, which are characterized by predominant production of IFN-γ and tumor necrosis factor-α, respectively [40].

Cytolysis is accompanied by intense production and excretion of cytotoxic cytolysins/perforines by effector cells. These cytotoxic factors complexate with proteoglycans splited from the membranes of lymphocytes and other cells, which transport cytotoxic factors to the surface of the target cells [47,59]. Since binding of target cell to NCT effector is controlled at the level of nonspecific adhesion, NK can damage not only target cell but also other cells including lymphocyte. Therefore, these cells should be protected against membrane damage and subsequent destruction [48, 58,59]. Besides, the release of vital products from destructed target cell sharply changes its microenvironment. Proteolytic enzymes released from the target cells into intercellular matrix can activate protective mechanisms of the adjacent intact cells (antioxidant

mechanisms, production of prostaglandins, membrane reinforcement with sialic acids and chondroitin sulfate A and others). Finally, NK-mediated cytolysis is accompanied by production of a number of cytokines involved into NCT reactions (IFN, C-reactive protein, and tumor necrosis factor-α), which can affect adjacent cells not undergoing cytolysis [30, 37,40,45].

Thus, cytolysis can be considered as an event generating a polyvalent multicomponent biological signal. The perception and realization of this signal differ from the effects of IFN. Dissociation of NK cytotoxicity and IFN production presents splitting of biological processes induced by cytotoxic and noncytotoxic (regulatory) effects on the microenvironment of target cell, i.e. splitting of biological signal.

Experimental and clinical data suggest that dissociation of primary NK functions is the key mechanism of participation of NCT in EBR processes. This dissociation observed during interaction of NK with target cells opens possibilities for selective regulation of these primary cell functions.

Thus, prerequisites for dissociation of cytotoxicity and IFN production during NK interaction with target cells can be revealed in a standard test system based on serial dilutions of cell suspension [14,19]. Mild adaptation and compensation of the NCT system realized via dissociation of primary NK functions are provided by the factors and activity of the thymus, while its physiological involution is associated with a decrease in flexibility of NCT system [14,20].

In some diseases characterized by common pathogenetical mechanisms (systemic character, involvement of central and peripheral nervous system, regulatory imbalance, impaired interactions between lymphoid and nonlymphoid cells) immunodeficiency and immunoregulatory imbalance are compensated by different variants of dissociation of NK primary functions.

In patients with multiple sclerosis characterized by total decrease of NK cytotoxicity, dissociation was manifested individually as an increase or sharp attenuation of IFN production [19]. These shifts are connected, in particular, with changed contribution of monocytes to NCT reaction. These cells normally possess low NCT, but trigger IFN production during NK-target cell interaction. In the course of the disease their killing activity significantly increases, while IFN production proportionally decreases [8,14]. At the same time, proper cytotoxicity of NK decreases, while IFN production by NCT effector increases, which results in a pronounced immunoregulatory imbalance [11,12].

In patients with focal scleroderma, the decrease in NK cytotoxicity is not accompanied by changes in IFN production during interaction with target cells. IFN production remains normal and even tends to increase [19].

In patients with CNS disorders (angioneurotic dystonia, acute cerebral ischemia) and in some healthy donors, abnormal peaks of lymphocyte killing activity were observed at low NK/target cells ratio, which was not accompanied by enhanced IFN production [19]. High serum concentrations of IFN in these patients [19] accelerate maturation of precursors and induced (due to forced transition of IFN-dependent differentiation) the formation of functionally defective NCT effectors [14,19].

The described dissociation of NK primary functions can serve as compensatory mechanisms preventing hyperactivation of cytotoxic cells only to a certain level of immunodeficiency. Factors and mechanisms of dissociation can inhibit recovery of regulatory balance and promote autoagression and impairment of immune tolerance under conditions of sub- and decompensation associated with dysfunction of mechanisms maintaining the immune and total homeostasis.

Direct evidence on the connection between dissociation of NK functions and immunodefficiency-compensating mechanisms were obtained during examination of NK activity and IFN production in patients with recurrent genital herpes receiving IFN inducer ridostin. A strong negative correlation between NK cytotoxicity and IFN-α production was observed during remission or even reconvalescence in some patients treated with ridostin [23]. In patients receiving ridostin during remission, no recurrences were observed against the background of dissociation within subsequent 1-2 months [23].

These facts show that genetically determined dissociation of primary functions in NCT effectors reflects physiological state of the system and determines a wide spectrum of interactions between NK and lymphoid and nonlymphoid cells. Binding and recognition of target cells by activated lymphocytes imply not only identification of membrane class I antigens of the major histocompatibility complex triggering or excluding cytotoxic mechanisms [10,36,44], but also subsequent differentiation of NK activities in relation to the target cell. Thus, recognition of a target cell by NK implies receiving of the signal about the type of reaction (cytotoxic or regulatory) necessary for realization of this interaction. This signal determines the prevaling component of the cell-cell contact: cytolysis followed by destruction and elimination of the target cell or noncytotoxic regulatory effect involving the EBR mechanism.

Thus, any external stimulus causing similar shifts in NK activity and IFN production manifested in experiment as a strong correlation of primary NCT effector functions (IFN induction, total immunostimulation, activation of cytolysis) triggers the transition of the NCT system from the physiological state of disso-

ciation to active state characterized by reduced rational cell-cell interactions, adequate to varying microenvironment due to increased functional rigidity of the system. On the contrary, dissociation of cytotoxic activity and IFN production by NK is a physiological state of effectors providing variable reactions to exogenous stimuli and endogenous destabilizing factors.

In this case, dissociation of primary NK functions interpreted as the ability to split biological signal can be maintained only by physiological stimuli characterized by constant intensity and do not depend on activation or suppression of the immune response. The reveled mechanisms of NCT limitations [14,17] as well as the mechanisms preventing NK autocytotoxicity [18] are insufficient for induction and maintenance of dissociation in the NCT system. These mechanisms utilize active oxygen metabolites required for cytolysis [14,16] or dramatically attenuate the induction of cytotoxic factor production during programmed lysis of target cells [18,54,57]. Being involved in splitting of biological signal they also attenuate cytotoxic component of intercellular interactions, thus promoting synthetic and translational processes. However, these mechanisms (probably due to their NCT limiting function) cannot form dissociation, which would tend to cytolysis and inhibition of synthetic and translational effector potentials.

Study of physiological (noninducible) mechanisms providing such dissociation of primary NK functions could contribute to understanding of the role of signal splitting and dissociation of NK activity and IFN production in the processes maintaining immune balance on the basis of EBR.

This description of the mechanisms of physiological immunoregulation is based on general theory of protein hydrodynamics [3]. According to this theory, effector functions of immunoglobulins and other proteins mediating cell-cell interactions are largely determined by mucin-like glycans splited from external adhesion receptor fragments and other structures by surface proteases. These metal-binding redox-active proteoglycans modify conformation of immunoglobulins and other proteins of extracellular matrix containing at least minor proline- or hydroxiproline-enriched sequences. Proteoglycans interact with these sites and add water molecule forming highly labile hinge sites and facilitating exposure of free functional valencies into extramolecular space, thus providing realization of effector functions of the protein [3].

Redox-active metal-binding proteoglycans can mediate distant effects only by forming stable complexes, which dissociate only during contact with more conservative molecules (for example, immunoglobulins) or can be split by surface proteases during interaction with conformed membrane receptors. Products of partial IgG catabolism formed in the liver can interact with proteoglycans and transport them to the cell surface [34]. These products can be presented by antibody Fab-fragments interacting with serum proteins [2,4,34]. Fab-fragments are more resistant to proteolytic enzymes than Fc-fragments [35,43] and always present in the organism [2,4,5]. Trace amounts of antibody proteolysis in the circulation [5] can stabilize proteoglycans and realize wide spectrum of immunoregulatory functions traditionally refered to the effects of Fab-fragments (stimulation of proliferation of antibody-generating cells [5], production of factors of nonspecific response [43] and IFN-γ [53]).

These complexes including an amphipatic component (metal-binding proteoglycan) with varying conformation constantly present in the circulation dissociate on the cell surface. The conformation of proteoglycan can be stabilized by metal chelation and scavenging of active oxygen metabolites and destabilized by transfer of free electrons to water hydroxygroups and generation of oxygen radicals utilized in NK-mediated cytolysis [3,14,16], thus participating in NCT regulation. Another component of these complexes (product of partial antibody catabolism) can regulate IFN production [53]. The described complex formation can be the key mechanism of physiological dissociation of NK primary functions.

Indeed, cyclic functioning of NK manifested as successions of activated (inactive) and active states together with the presence of abundant cell surface receptors and adhesion molecules, which are most likely responsible for interaction with metal-chelating proteoglycan, forms constantly changing local (primembrane) microenvironment. Within this local space metal-containing proteoglycans can be stabilized or dissociate depending on NCT effector activity. These conformation changes induce biological signals of different (in relation to NCT effectors) functional directions.

It is known that C-terminal fragment of IgG heavy chain localized in the region of IgG molecule waist plays an important role in the immunoregulatory effect of antibody Fab-fragments [3,5]. This terminal proline-enriched domain involved in the organization of hinge region of IgG molecule, probably, binds proteoglycans, promotes stabilization and transfer of redox-active biopolimers and their localizing on the cell surface.

We examined synthetic hexapeptide P-07 (Cys-$(Pro)_3$-Glu-Leu) reproducing the structure of C-terminal region of pepsin-cleaved Fab-fragment (Cg2 domain) incorporated within the waist of rabbit IgG molecule [3]. The preparation was kindly provided by prof. A. Ya. Kul'berg, Russian Academy of Medical Sciences.

Hexapeptide P-07 significantly enhanced cytotoxicity of NK from healthy donors against K-562 erythromyeloblasts used as the standard target cells (Fig. 1). The effect recorded under conditions of 1-h treatment of mononuclear cells at functional effector:target ratio from 100:1 to 25:1 showed reverse dose dependency.

Table 1 shows that 72-h incubation of peripheral blood lymphocytes with P-07 induces IFN production. After incubation the peptide was removed from the supernatant [14], therefore antiviral activity of cultured cells did not depend on P-07. This activity corresponded to the effects of standard high-molecular-weight inducers of IFN production and dramatically decreased after passing the supernatant through a column with immobilized antibodies to human IFN-γ (Table 1). Residual activity was neutralized on a column with antibodies against human IFN-α and IFN-β. Blockade of protein synthesis with cycloheximide resulted in a proportional inhibiton of IFN production induced by P-07 and staphylococcal enterotoxin A.

Thus, synthetic analog of the short sequence of terminal domain of rabbit IgG Fab-fragment and similar synthetic structures (according to independent studies) activate cytotoxic mechanism and induce IFN-γ biosynthesis. Accumulation of IFN in the culture medium was observed not earlier than 24 h after induction, while NK cytotoxicity was realized during 14-h incubation of mononuclears with targets cells. This points to different mechanisms of regulation of these effector functions providing P-07-induced NK stimulation and IFN production.

**TABLE 1.** *In Vitro* IFN Production by Human Peripheral Blood Lymphocytes in the Presence of P-07 and Other Inducers

| Inducer | Dose, μg/ml | IF titer, IU/ml | |
|---|---|---|---|
| | | total | after adsorbtion by immobilized anti-IF-γ antibodies |
| P-07 | 0.5 | 5120 | 320 |
| SEA | 1.0 | 560 | 320 |
| Con A | 2.5 | 1280 | 160 |
| PHA | 10.0 | 1280 | 160 |
| Lentil | 10.0 | 1280 | 160 |
| Reaferon (adsorbtion control) | | 640 | 640 |
| Control | 0 | <4 | <4 |

**Note.** Con A: concanavalin A, SEA: staphyloccocal enterotoxin A, PHA: phytohemagglutinin.

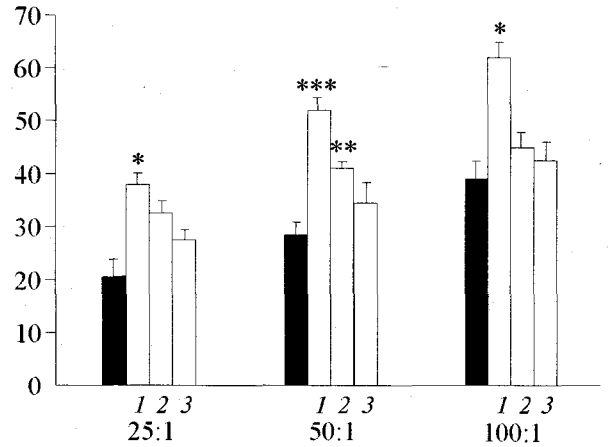


**Fig. 1.** Cytotoxic activity of natural killers in mononuclear cell suspension from healthy donors ($n$=6) in the presence of hexapeptide P-07 *in vitro*. Abscissa: effector:target cell ratio; ordinate: cytotoxic index, %. Filled bars: control, open bars: P-07 in doses of 0.05 (*1*), 0.5 (*2*), and 5.0 μg/ml (*3*); *$p$<0.1, **$p$<0.2, ***$p$<0.01 compared to the control.

Incubation of mononuclear cells with P-07 for 1 h completely inhibits IFN production in the cytotoxic test (Table 2), however, lymphocyte NCT significantly increases (Fig. 1).

Our findings proved the possibility of NCT reaction in a standard system without IFN production. Termination of IFN production is arrested by a factor comparable by its IFN-γ-stimulating capacities with effective high-molecular-weight inducers. Thus, signal splitting in the NCT system becomes biologically significant: in the presence of target cells and cell-activating factor (P-07) production of IFN is blocked and cytotoxic effect is realized via activation of circulating NK without additional maturation and activation of peripheral precursors.

On one hand, it prevents hyperactivation of NK, which can lyse normal cells together with transformed target cells [38,39,55]. On the other hand, it abolishes the well-established protective effect of IFN on target cells [46,52], which enhances the efficacy of cytolysis.

Signal splitting associated with complete inhibition of IFN production during cytotoxic interaction between NK and target cells reveals a principal immunoregulatory mechanism, which together with the mechanisms of NCT limitations suggests that:

— genetic program of NK determines equal possibilities of cytotoxic and noncytotoxic (regulatory) functioning;
— capacities for realization of cytotoxic and noncytotoxic (regulatory) functions developed in the course of evolution;
— biologically expedient splitting of primary NK functions prevents hyperactivation of circulating lymphocytes and determines high plasticity of the

NCT system in immunoregulatory and supervisory intercellular interactions;
— these cells can mutually enhance their primary functions under conditions of forced response or compensation of immunodeficiency;
— dissociation of cytotoxicity and IFN production by natural killers as well as its formation and maintenance by the products of intercellular metabolism are physiologically significant.

It can be hypothesized that products similar to P-07 formed during baseline immunoglobulin catabolism [34] are released and perform their transport functions in the circulation [2,4,5]. Then short terminal proline-enriched antibody Fab-fragments structurally similar to P-07 and/or complex-forming mucin-like metal-chelating proteoglycans saturated with water can serve as natural regulators of membrane redox systems maintaining redox exchange on the cell surface, thus determining the level of physiological activation of cytotoxic or regulatory (synthetic) potential of NCT effectors.

It is of no importance whether conformational changes on the membrane are regulated by polypeptide fixing and splitting proteoglycan, or by transported proteoglycan itself. The presence and the effect of glycans split from the cell surface cannot be excluded, because these molecules are normal cell metabolites. Examination of the ability of various structures to regulate redox exchange in the primembrane microenvironment can imply a special direction in studies of EBR realized by the NCT system in the organism of humans and animals.

It can concluded that the NCT system functions against the background of physiological intercellular metabolism modulating the dynamics and direction of primembrane processes. These processes determine the ability of effectors to dissociation of their primary functions. Each function can be realized independently. Forced or compensatory enhancement of one function or switching from one function to another can occur under conditions of reduction of the opposite potential. Therefore, during evolution the system aquired the ability to mediate numerous interactions between lymphoid and nonlymphoid cells and high potential for mild physiological adaptation and endogenous compensation, which determines its high biological efficacy and the possibility of involvement of NK into EBR.

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**TABLE 2.** IFN Production (Mean Values) in Natural Killers-Target Cells System Containing Hexapeptide P-07-Treated Mononuclear Cells as Effectors

| P-07, μg/ml | IF titer, IU/ml, at effector:target ratio of | | |
|---|---|---|---|
| | 100:1 | 50:1 | 25:1 |
| 0 | 128 | 64 | 32 |
| 0.05 | <4 | <4 | <4 |
| 0.5 | <4 | <4 | <4 |
| 5.0 | <4 | <4 | <4 |

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