# Fibronectin as a Compensating Factor in the Natural Cytotoxicity System in Interferon-Dependent Immunodeficiency

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The cytotoxicity of natural killer cells (NK) against $^3$H-uridine-labeled target cells (TC, human erythromyeloleukosis cells K-562) and the intensity of conjugate formation in the NK:TC system in the presence of γ-interferon, C-reactive protein, and human fibronectin are studied *in vitro* in 14 patients with multiple sclerosis. It is shown that γ-interferon and C-reactive protein decrease the cytotoxic activity of NK with a simultaneous stimulation of conjugate formation in the NK:TC system. The correlation between the studied parameters becomes weaker. Human fibronectin induces collateral changes in the activity of NK and in the number of effector:target conjugates formed in the natural cytotoxicity reaction.

**Key Words:** *fibronectin; natural cytotoxicity; compensation*

Once an interferon (IFN)-dependent immunodeficiency is revealed in any disease, the search for ways of pathogenetic correction utilizing IFN or its inducers gets underway. However, attempts to use IFN preparations in the therapy of multiple sclerosis (MS, which is characterized by reduced production of IFN by practically all cells involved in this process [1,2] and by IFN-dependent deficiency of the natural cytotoxicity (NCT) system associated with a delayed development of natural killer cells (NK) at the maturation and activation stages controlled by IFN [3]) at least in some patients lead to aggravation of the demyelinization process [12]. This perverted immune response may be determined both by altered sensitivity of NK (which play an important role in the regulatory balance in the immune system) to the regulatory influence of IFN [4] and by reduced production of the inhibitor of IFN influence on NK activity in this disease [5].

Despite the convincing experimental evidence on the functional relationship of the IFN system with fibronectin (FN), which is manifested, for example, in the increased synthesis of FN and elevation of its blood content under the influence of interleukin-6 [8], and with the synthesis and expression of FN by NCT effectors [15] that can adhere or provide for the chemotaxis toward FN [16,19] due to the presence of great numbers of the membrane integrin receptors VLA-4 and VLA-5 [16], the role of FN in the NCT reaction remains unclear. Since FN mediates nonspecific adhesion of lymphocytes and displays regulatory activity toward the NK function *in vitro* [6], it was reasonable to study the influence of FN on the cytotoxic activity of NK and the intensity of conjugate formation in the NCT reaction in the sera of patients suffering from MS with a simultaneous development of an IFN-dependent immunodefi-

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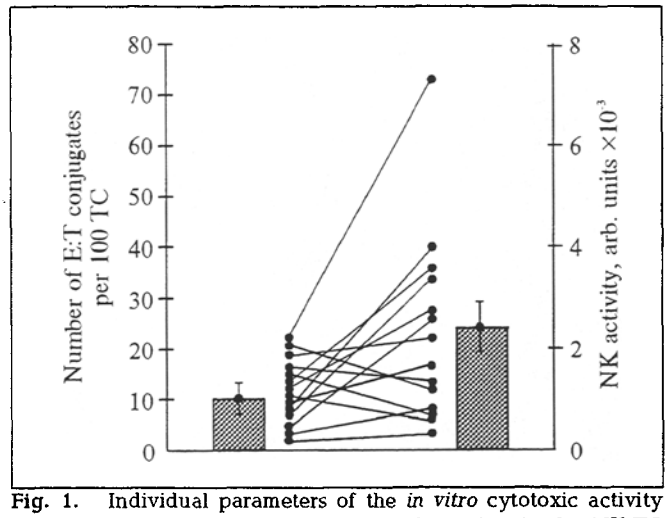


Fig. 1. Individual parameters of the *in vitro* cytotoxic activity of NK and the intensity of conjugate formation in the NK:TC system in MS patients.

ciency of NK [3] against the background of considerable FN deposition in the demyelinization foci [18]. The effectiveness of γ-IFN and C-reactive protein (CRP) that, unlike FN, potentiates the lytic potential of NK at the lethal blow stage [10,20], was studied in parallel.

Fourteen patients (13 women and 1 man aged 30-65 years) with remittent MS lasting from 3 months to 21 years (8.5 years on average) and various degrees of incapacitation according to the Kurtzke scale were included in the study. According to the classification [13], all the cases were regarded as definite MS.

## MATERIALS AND METHODS

Mononuclear cells (MNC) were isolated from peripheral venous blood in a one-step Ficoll-Verografin gradient. Mononuclears collected from the interphase ring were washed twice with medium 199 and resuspended ($10^7$ cells/ml) in complete growth medium (CGM) based on RPMI-1640 (Institute of Poliomyelitis and Viral Encephalitis, Russian Academy of Medical Sciences) supplemented with 12% fetal calf serum (F. M. Gamaleya Institute of Microbiology and Epidemiology, Russian Academy of Medical Sciences) and 40 μg/ml gentamicin (Pharmachim) in 1 M HEPES (Flow).

TABLE 1. Changes in the *in vitro* Cytotoxic Activity of NK in the Presence of Human γ-IFN, CRP, and FN in MS Patients (Arb. Units $\times10^3$, $M \pm m$)

| Preparation | *n* | Control | Experiment |
|---|---|---|---|
| γ-IFN | 6 | 2.0±1.1 | 1.9±1.1 |
| CRP | 7 | 2.7±0.5 | 1.8±0.3 |
| FN | 5 | 3.1±0.3 | 3.8±0.4 |

The cytotoxic activity of NK was determined as described [11] on standard human erythromyeloblastoma cells K-562 labeled with $^3$H-uridine (3 μCi/ml). The TC ($10^5$ cells/ml CGM) were incubated with MNC in round-bottom 96-well plates for 14 h at 37°C in a humidified atmosphere containing 5% $CO_2$. After incubation the cells were transferred onto Whatman fiberglass filters (2.5 μ pore diameter) in a 12-channel Titertech harvester. The residual radioactivity of each probe was measured in a Mark-II β-scintillation counter (1 min), using a toluol scintillator.

The cytotoxicity index (CI) for each 2 parallel wells with effector:target ratios (E:T) of 100:1, 50:1, 25:1, and 12:1 was calculated from a formula [11]. The area under the cytotoxicity curve was calculated as an integral parameter characterizing NK activity by a method described elsewhere [17], expressed in arbitrary units, and employed in the figures and tables.

For the calculation of E:T conjugates by the method of Halliotis *et al.* [9] MNC and TC were mixed at a E:T ratio of 6:1 in 0.4 ml CGM, incubated for 10 min at 37°C in a humidified atmosphere containing 5% $CO_2$, gently washed for 5 min with cooling, and carefully resuspended in 0.4 ml CGM. The E:T conjugates were counted in a Goryaev chamber and expressed per 100 TC.

In accordance with our previous data on the effects of immunoregulatory peptides on the *in vitro* activity of NK of healthy donors [6], gammaferon (recombinant human γ-IFN, Ferment Conglomerate, Ministry of the Biomedical Industry) was added in a dose of 50 IU/ml; CRP isolated by affinity chromatography from the ascitic fluid of oncological patients with the use of *Str. pneumoniae* C-polysaccharide as a ligand (N. F. Gamaleya Institute of Microbiology and Epidemiology) was added in a dose of 2.0 μg/ml, and human FN (Serva) in a dose of 0.1 μg/ml. The preparations were added in an MNC suspension immediately before its incubation with TC (14 h at 37°C in a humidified atmosphere containing 5% $CO_2$).

The results were analyzed using Student's *t* test; correlation coefficient (ρ) were calculated after Spearman.

## RESULTS

The *in vitro* cytotoxic activity of NK obtained from patients with MS was 2.4±0.4×$10^3$ arb. units (0.4×$10^3$-7.2з$10^3$ arb. units (Fig. 1). The mean number of E:T conjugates per 100 TC formed in this NK:TC system was 10.1±2.4 (5-20, Fig. 1). The correlation coefficient value (ρ =0.09) indicates

that few MNC which bound to TC during the first 10 min of contact are active effectors of NCT and are able to lyse the TC in the cytotoxicity test. As seen from Table 2, a greater proportion of the conjugates (20-25%) is represented by complexes consisting of one MNC and one TC, i.e., by LT-type conjugates [7]. Far fewer $LT_m$-type conjugates [7], consisting of one TC and 2 or more NCT effectors, and of $LT_n$-type conjugates, consisting of one lymphocyte and two or more TC [7], were formed upon incubation of nonfractionated MNC of MS patients with the TC.

From these findings it can be concluded that the deficiency of the IFN system in MS patients not only is determined by the 40% decrease in the *in vitro* cytotoxic activity of NK compared with healthy donors [1,2] but also is associated with the changes in the dynamics of conjugate formation in the NK:TC system, which reflects the effectiveness of TC recognition and the intensity of TC binding in the NCT reaction. The number of E:T conjugates formed by MNC of MS patients is 1.6-fold lower compared with the norm (16.2±3.3 conjugates per 100 TC), the decrease in the number of $LT_m$ conjugates formed by active NCT effectors being more pronounced [7]. The correlation coefficient (0.17-0.35 in the suspension of normal MNC) also decreases in the suspension of MNC from MS patients. This indicates that the number of MNC capable of lysing TC in the NCT in multiple sclerosis drops below 8.21% (the value characterizing the nonfractionated population of lymphocytes obtained from healthy donors [14]).

In the presence of γ-IFN the *in vitro* cytotoxic activity of the NK of MS patients decreases by 9% (Table 1, Fig. 2). Recognition and binding of TC in this case are characterized by a marked increase (68%) in the number of conjugates formed in the NK:TC system (Table 2, Fig. 2). It can be seen from Table 2 that in the presence of γ-IFN no $LT_m$ conjugates (i.e., those

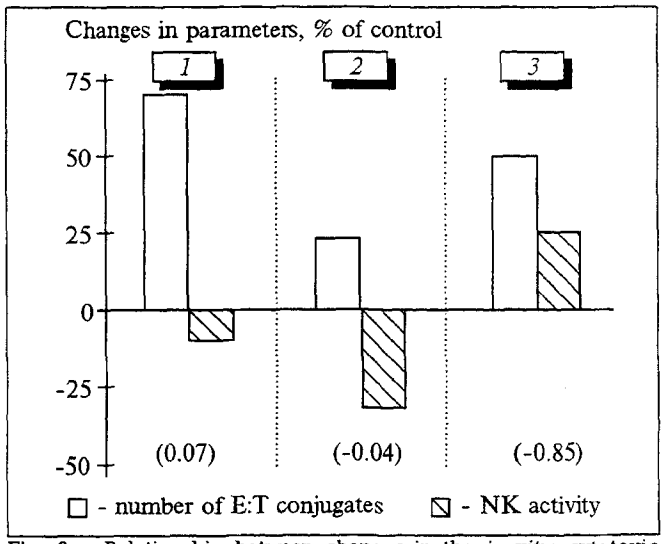


Fig. 2. Relationship between changes in the *in vitro* cytotoxic activity of NK and the intensity of conjugate formation in the NK:TC system in the presence of γ-IFN (*1*), CRP (*2*), and FN (*3*) in MS patients. The correlation coefficient values are given in parentheses.

formed by active effectors of NCT [7]) are formed in the NCT reaction, while the number of LT conjugates typical of a nonfractionated MNC population increases almost 2-fold. The correlation coefficient remains practically unchanged.

Consequently, even the absence of an inhibitor of the regulatory activity of IFN toward NK [5] in MS patients does not allow γ-IFN to realize its biological influence in the NCT system, an influence which is manifested not only in the stimulation of cytotoxic factor production at the "lethal blow" stage [20] but also in the stimulation of NK for TC cytolysis by inducing the dissociation of "false" E:T conjugates at the recognition and binding stage [6] occurring upon normal production of the proper inhibitor [5].

In the presence of CRP the *in vitro* cytotoxic activity of NK from MS patients decreases by 33% (Table 1, Fig. 2) with a simultaneous 22% increase in the intensity of conjugate formation in the

TABLE 2. Changes in the Intensity of Conjugate Formation in the NK:TC System in the Presence of human γ-IFN, CRP, and FN in MS Patients ($M \pm m$)

| Preparation | Total number of E:T conjugates | % of conjugates consisting of one TC and lymphocytes | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 and more |
| γ-IFN ($n=18$) | 10.28±2.64<br>17.23±4.57 | 25.8±7.01<br>49.2±12.9 | 0.84±0.84<br>0±0 | 1.67±1.06<br>0±0 |
| CRP ($n=21$) | 9.53±1.92<br>11.67±2.82 | 25.0±5.23<br>29.3±7.51 | 2.15±1.02<br>5.00±1.89 | 0.72±0.72<br>0±0 |
| FN ($n=15$) | 8.67±2.14<br>13.01±1.58 | 20.0±6.7<br>35.0±2.74 | 4.00±2.4<br>3.00±3.00 | 0±0<br>1.0±1.0 |

Note. The upper value is the control, the lower value is the preparation; the number of conjugates is calculated per 100 TC.

NK:TC system (Table 2, Fig. 2). The population of MNC from MS patients does not differ considerably from the control population in the composition of E:T conjugates: the changes in the numbers of LT and $LT_m$ complexes did not allow us to distinguish reliably the NCT effectors interacting with TC in the presence and in the absence of CRP. However, the loss of a positive correlation between cytotoxicity and intensity of conjugate formation ($\rho = -0.04$) indicates that in MS many more inactive NCT effectors are involved in the formation of E:T conjugates during the NCT reaction. It follows that in MS, which is characterized by the IFN dependence of immunodeficiency in NK [1-3], the sensitivity of NK not only to IFN, which realizes its influence at the lethal blow stage, but also to CRP, which similarly mediates its influence via the induction of NK cytotoxic factor [10], changes dramatically. Therefore, in MS CRP also cannot provide *in vitro* for the selection of functionally active NCT effectors via the dissociation of "false" E:T conjugates toward the corresponding IFN increase in the cytotoxic activity of the NK from healthy donors [6].

The addition of human FN to the NK:TC system dramatically changes the dynamics and direction of the NCT reaction. Without being involved in the production of NK cytotoxic factor but, in this case, only providing for nonspecific adhesion of MNC to TC and not altering appreciably the NCT reaction in the suspension of MNC from healthy donors, FN increases the *in vitro* cytotoxicity of NK from MS patients by 24% (Table 1, Fig. 2) with a simultaneous 50% increase in the number of E:T conjugates formed in the NCT reaction (Table 2, Fig. 2). The absence of functional similarity between the MNC population, which interacts with TC in the presence of FN, and the lymphocyte fraction enriched with lymphocytes forming $LT_m$ conjugates (i.e., with active effectors of NCT [7]), as well as the drop of the correlation coefficient to -0.85 show that the effectiveness of the cytotoxic reaction in this system increased due to stimulation of nonspecific adhesion caused by the expression of large numbers of the integrin receptors VLA-4 and VLA-5 [16] on the surface of NK, together with the ability of NK to provide for chemotaxis or to adhere to FN [16,19]. Thus, FN can be regarded as a factor compensating for the immunodeficiency in NK occurring in MS patients. Deposition of FN in demyelinization foci to a certain degree blocks its putative compensatory potential, and therefore the deficiency of the NCT system in MS preserves its functional significance in the pathogenesis of the immunoregulatory imbalance [1-3]. However, the available experimental data are insufficient to confirm this conclusion.

In MS, the presence of IFN-dependent immunodeficiency of NK [1-3] developing against the background of changes in the functioning of the system's responsible for cell-cell interactions at the level of nonspecific adhesion [18] may help to elucidate an important compensatory mechanism in the NCT system which normally secures effective TC lysis under the pathological conditions blocking a highly specific selection of active NCT effectors during contact with NK in the presence of γ-IFN and CRP [6]. The compensatory mechanism described in this study is associated with the involvement of additional adhesion molecules and factors in the cytotoxicity reaction and is realized via the initiation of more MNC for binding to TC (active effectors of NCT are likely to be present among these MNC). In an unaltered lytic cycle this mechanism providing for the effectiveness of the NCT reaction probably does not operate [6].

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