MICROBIOLOGY AND IMMUNOLOGY

Involvement of Fibronectin and Interacting Serum Components in the Regulation of Activity of Human Natural Killer Cells

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The cytotoxic activity of natural killer cells and the intensity of conjugate formation are studied *in vitro* in the natural cytotoxicity reaction against ^{3}H-uridine-labeled human erythromyeloleukotic cells K-562 in the presence of fibronectin, γ-globulin, and fibronectin/γ-globulin combination. It is demonstrated that fibronectin does not change natural cytotoxicity, γ-globulin increases the activity of human natural killer cells, and the fibronectin - γ-globulin combination increases both the intensity of conjugate formation and the cytotoxic activity of natural killer cells.

Key Words: *natural killer cells; fibronectin; regulation*

A broad spectrum of interactions of human natural killer cells (NK) with different target cells (TC) is strongly influenced by the high degree of expression of adhesion molecules (LFA-1 and LFA-3) [12] and integrins (VLA-4 and VLA-5) [7,16] by them. Expression of these molecules on the NK surface correlates with the intensity of effector:target (E:T) conjugate formation [12]. These molecular complexes mediate not only the interaction of NK with the pericellular matrix components and other cells but also the adhesion to fibronectin (FN) [16,18] which is synthesized and expressed by NK [7,15] as a factor significant in the realization of the cytotoxic potential of the effectors of natural cytotoxicity (NCT) [15].

The diversity of interactions between FN and different blood serum components implies that the effects of this protein in the organism are realized not by native but rather by modified molecules with considerably altered structural and functional characteristics due to partial catabolic cleavage involving R-proteins which exhibit affinity for FN [1], or resulting from the interaction of FN with aggregated IgG molecules which bind to both immobilized and dissolved FN [14]. Since serum IgG occur as complexes with proteins and protein components specifically bound to the C-terminal end of Fab-fragments [2], the above-mentioned interactions, including the formation of so-called natural antibodies during complexation of IgG with the products of partial catabolic cleavage of R-proteins [3], can change the delicate mechanisms of FN binding to the lymphocyte surface and the dynamics of cell-cell interactions realized via adhesion at the early stages, specifically, the reactions of antibody-dependent cellular cytotoxicity and lymphokine production [16].

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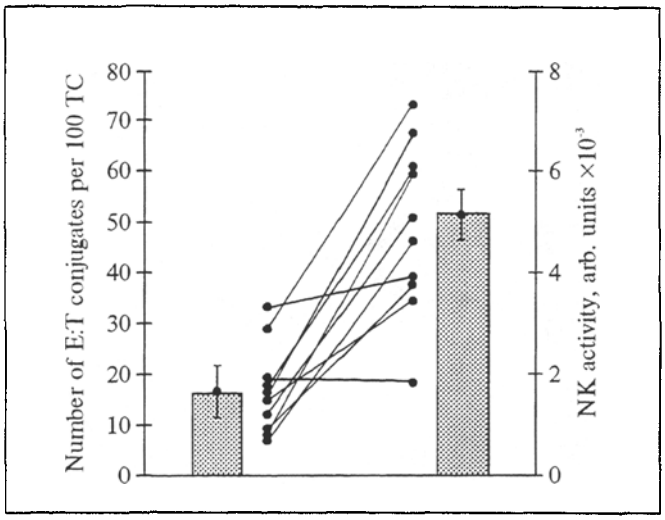

Fig. 1. Individual cytotoxic activities of NK and intensity of conjugate formation in the NK:TC system in healthy donors *in vitro*.

The objective of this study was to investigate the effects of human FN and serum components interacting with it on the activity of NK and the intensity of E:T conjugate formation in the NCT reaction.

MATERIALS AND METHODS

Mononuclear cells (MNC) were isolated on a Ficoll-Paque (Pharmacia Fine Chemicals) gradient from peripheral venous blood obtained from 38 healthy donors (7 men and 31 women aged 21-61 years). Cells 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 (Amimed) supplemented with 12% fetal calf serum (N. F. Gamaleya Institute of Microbiology and Epidemiology, Russian Academy of Medical Sciences) or tested human serum, 2 mM glutamine and 40 μg/ml gentamicin (Pharmachim) in 1 M HEPES buffer (Serva) .

The cytotoxic activity of NK was determined as described [9], using standard human erythromyeloblastoma K-562 cells labeled with ^{3}H-uridine (3 μCi/ml). The initial suspension (10^5 cells/ml CGM) of TC was incubated with MNC in round-bottom 96-well plates for 14 h at 37°C in a humidified atmosphere containing 5% CO_2. After the incubation the cells were transferred to fiberglass filters (2.5 μ pore diameter, Whatman) in a 12-channel Dynatech harvester. The residual radioactivity of each probe was measured in a Mark-II β-scintillation counter (1 min) in a toluol scintillator.

TABLE 1. Changes in the Cytotoxic Activity of NK (arb. units $\times 10^{-3}$) in the Presence of Human FN *in vitro* ($M \pm m$)

FN dose, μg/ml	*n*	Control	FN
0.01	4	4.2±0.85	3.9±0.55
0.1	5	4.0±0.69	4.2±0.75
1.0	4	4.2±0.85	4.6±0.96
10.0	6	4.4±0.67	4.1±0.73

The cytotoxicity index (CI) for each of 2 or 3 parallel wells with E:T ratios of 100:1, 50:1, 25:1, and 12:1 was calculated from a published formula [9]. The area under the cytotoxicity curve was calculated as an integral parameter characterizing the 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 a described method [8] the MNC and TC suspension were incubated in an E:T ratio of 6:1 in 0.4 ml CGM 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 calculated in a Goryaev chamber, and the number of conjugates was expressed per 100 TC.

Human FN (Serva) was used in doses 0.01, 0.1, 1.0, and 10.0 μg/ml; human serum γ-globulin (Serva) was used in doses 0.5, 5.0, and 50.0 μg/ml. The preparations were added immediately before coincubation of the cells in the cytotoxicity reaction.

In order to change the conditions of the NCT reaction, fetal calf serum was replaced by sera obtained from 13 healthy donors and 14 rheumatoid arthritis (RA) patients. The R-protein content in these sera was determined after Kul'berg *et al.* [3]: inhibition with test serum of the hemagglutination reaction between Rh^+ 1(0) erythrocytes and anti-R-serum obtained by immunization of rabbits with human R-proteins. The results were expressed as inverse titers of R-proteins, i.e., the maximum dilutions at which the serum inhibited the reaction.

The FN-binding antibodies adsorbing on the solid phase were determined by the immunoenzyme technique [19] with some modifications. Fibronectin was adsorbed on polystyrene (Nunc microplates) for 16-18 h at 4°C from a solution with an FN concentration of 1.0 μg/ml. Then (and after each conjugation) the plates were washed with phosphate-buffered saline containing 0.05% nonionic detergent. Blocking solution (0.05% bovine serum albumin, Serva) was used to prevent nonspecific binding. The tested sera at final dilutions 10^{-4} or 10^{-5} were conjugated in microplates for 1.5

h at 37°C. Anti-FN antibodies were revealed with commercial sheep anti-human IgG antiserum conjugated with horseradish peroxidase (N. F. Gamaleya Institute of Microbiology and Epidemiology) diluted 1:4000. Orthophenylenediamine was used as a substrate. The reaction was read in a Multiscan Plus P apparatus at 492 nm.

Before treatment of the sera with DOWEX (Serva), the latter was washed till zero light absorbance of the washing fluid. Serum (dilution 10^{-4} or 10^{-5}) was mixed with DOWEX at a volume ratio of 1:1, incubated for 10-15 min at room temperature, centrifuged for 10 min at 1000 rpm, and supernatant was carefully aspirated and analyzed.

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

RESULTS

The mean *in vitro* cytotoxic activity of NK of the studied donors was $4.6\pm0.5\times10^3$ arb. units, ranging from 1.8×10^3 to 6.65×10^3 arb. units (Fig. 1). The mean number of E:T conjugates formed in the NK:TC system varied from 7 to 32 (mean number 16.2±3.3 conjugates per 100 TC). The correlation coefficient (ρ=0.17) indicates that only some of the MNC which bound to TC during the first 10 minutes of contact are active effectors of NCT and are able to lyse TC in the cytotoxicity test. The largest proportion of E:T conjugates is presented by the complexes consisting of one MNC and one TC, i.e., the LT-type conjugates [6]. A nonfractionated MNC population has a much lesser ability to form LT_m-type conjugates [6] upon interaction with TC; these conjugates consist of 1 TC and 2 or more NCT effectors. Such complexes were virtually absent.

In the presence of FN, the *in vitro* cytotoxic activity of NK from healthy donors increased by not more than 10% (Table 1, Fig. 2, *a*), which confirms the hypothesis that R-proteins are present on the lymphocyte surface; these proteins are known to possess superoxide dismutase activity and can participate in FN cleavage [1]. The intensity of recognition and binding of TC by NCT effectors assessed by the number of conjugates formed in the NK:TC system did not change significantly (Table 2, Fig. 2, *a*). We did not reveal dose dependence in the FN effect. However, it should be noted that a tendency toward an increase in the formation of LT_m conjugates with a simultaneous decrease in the number of LT conjugates was observed for virtually all the tested doses of FN. It is likely that the ability of FN to mediate the nonspecific adhesion determines the involvement of numerous MNC in E:T conjugate formation, but these MNC are not active as NCT effectors. However, active NCT effectors do participate in conjugate formation in the presence of FN, as confirmed by the increased ρ values: 0.4-0.77 vs. 0.17 in the control (Fig. 2, *a*) for three of four tested FN doses.

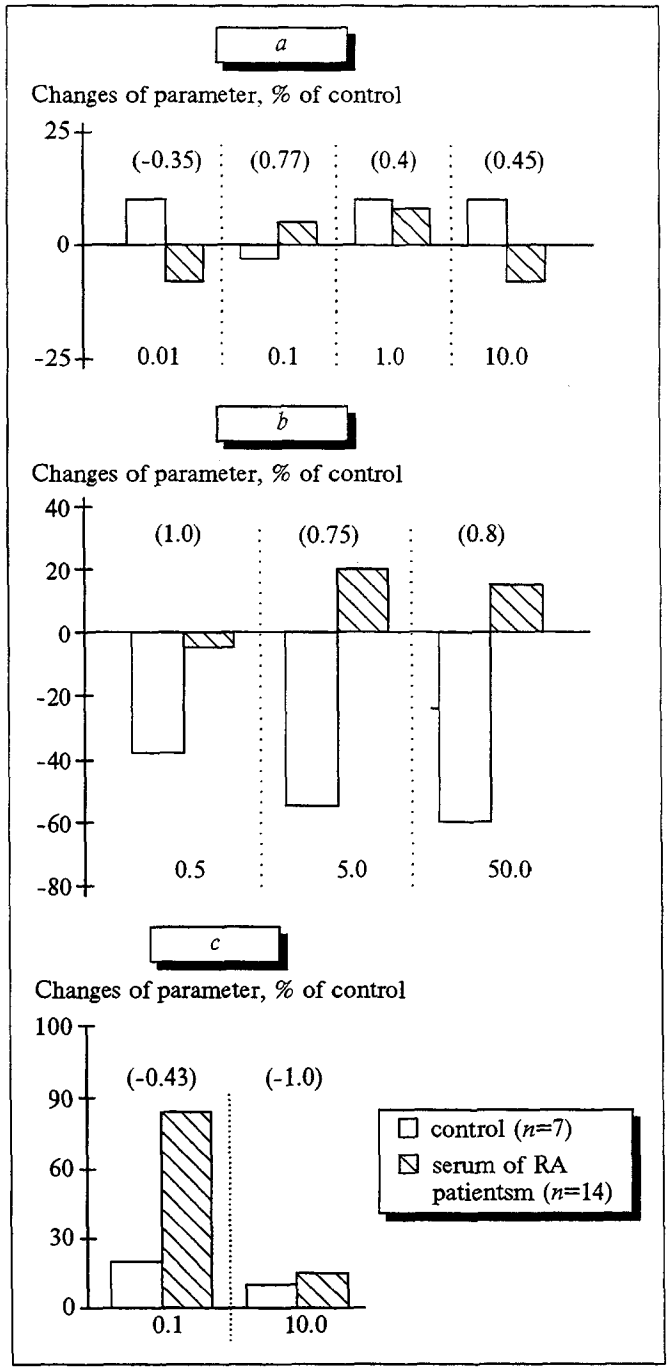

Fig. 2. Relationship between cytotoxic activity of NK and intensity of conjugate formation in the NK:TC system in the presence of human FN (*a*), γ-globulin (*b*), and their combination (*c*) *in vitro*. Abscissa – dose (μg/ml): *a*) FN; *b*) γ-globulin; *c*) FN (γ-globulin applied in a dose of 0.5 μg/ml); ρ values are givenin parentheneses

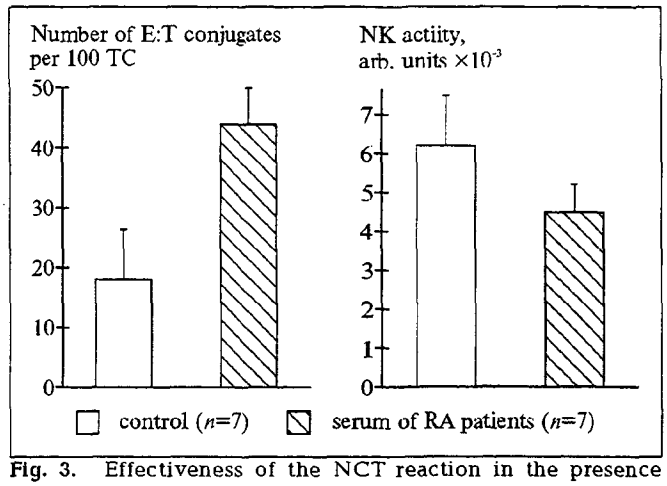

Fig. 3. Effectiveness of the NCT reaction in the presence of serum of RA patients *in vitro*. n=number of experiments; asterisk indicates values statistically significant at $p<0.05$ compared with the control.

In the presence of human γ-globulin (which interacts with human FN after aggregation [14] and, while circulating in the serum, forms complexes with catalytically active R-proteins [3] that participate in FN cleavage [1]) the activity of NK increased 15-21% (Table 3, Fig. 2, *b*), except in one case when FN was added in a dose of 0.5 μg/ml. At the same time, the intensity of conjugate formation in the NK:TC system decreased 37-61% (Table 4, Fig. 2, *b*). A direct dose dependence was observed during inhibition of conjugate formation by γ-globulin. Analysis of E:T conjugates formed in the presence of γ-globulin revealed the regularities observed previously in experiments with γ-interferon and C-reactive protein, when simultaneously with the decrease in the percentage of LT conjugates the number of LT_m conjugates (two lymphocytes per TC, Table 4) remained virtually the same, which in principle can be regarded as evidence of the functional similarity between the NCT effectors involved in TC binding in the presence of γ-globulin and the lymphocyte fraction

TABLE 3. Changes in the Cytotoxic Activity of NK (arb. units $\times10^{-3}$) in the Presence of Human γ-Globulin *in vitro* ($M\pm m$, $n=4$)

γ-Globulin dose, μg/ml	Control	γ-Globulin
0.5	4.7±0.7	4.4±0.7
5.0	4.9±0.8	5.9±0.3
50.0	4.9±0.8	5.6±0.5

enriched with $CD16^+$ cells that is characterized by a low content of LT conjugates [6]. In can be seen from Fig. 2, *b* that in this case the correlation coefficient ρ increases considerably to 0.75-1.0. This ponts up the high selectivity of effective E:T conjugates in the presence of γ-globulin, providing for the implication of effective NK in the NCT reaction and for a high probability of TC lysis after contact with these cells. The Fc-receptors for IgG on the NK surface [5] facilitating the interaction between MNC and allogenic TC in the antibody-dependent cytotoxicity reaction [16] may be responsible for this mechanism. When Fc-receptors on NK are blocked by monoclonal antibodies (IgG), the lysis of TC not of T-cell origin sensitive to NK, including the relatively resistant Daudi cells, increases markedly [10].

Combination of FN with human γ-globulin significantly increases the *in vitro* cytotoxic activity of NK of healthy donors (Table 5). The addition of FN in a dose of 0.1 μg/ml increased the CI by 86% (Fig. 2, *c*) with a simultaneous 10-20% increase in the number of E:T conjugates formed during the NCT reaction. The MNC population formed in the presence of FN and γ-globulin is functionally similar to the nonfractionated lymphocyte pool in the composition of the E:T conjugates [6]. There is no positive correlation between NK activity and the intensity of conjugate formation in the NK:TC system, as evidenced by

TABLE 2. Changes in the Intensity of Conjugate Formation in the NK:TC System in the Presence of Human FN *in vitro*

FN dose, μg/ml	n	Total number of E:T conjugates	% of conjugates consisting of one TC and lymphocytes		
			1	2	3 and more
0.01	12	12.9±4.2 14.2±4.4	30.0±5.8 26.2±9.0	6.25±2.4 12.5±6.0	2.5±2.5 3.7 ±3.7
0.1	15	13.7±3.5 13.3±3.4	28.0±4.9 25.0±6.7	11.0±4.9 11.0±3.3	2.0±2.0 4.0±2.4
1.0	12	12.9±4.2 14.2±4.7	30.0±5.8 32.5±7.5	6.2±2.4 6.2±2.4	2.5±2.5 3.7±3.7
10.0	18	14.2±3.3 15.6±2.5	28.3±4.0 22.5±3.6	9.2±4.5 19.2±3.3	5.0±3.4 5.0±2.2

Note. The upper line is the control; the lower line is FN; the number of conjugates is calculated per 100 TC.

TABLE 4. Changes in the Intensity of Conjugate Formation in the NK:TC System it the Presence of Human γ-Globulin *in vitro* ($M \pm m$, $n = 12$)

γ-Globulin dose, μg/ml	Total number of E:T conjugates	% of conjugates consisting of one TC and lymphocytes		
		1	2	3 and more
0.5	12.5±3.3 7.9±3.7	21.2±3.1 13.7±9.0	11.2±6.6 10.0±5.8	5±5 0±0
5.0	19.2±3.7 8.3±3.4	45.0±15.9 17.5±8.3	8.75±2.4 6.25±3.8	6.2±3.8 1.2±1.2
50.0	19.2±3.7 7.5±2.5	45.0±15.9 16.2±3.8	8.75±2.4 6.25±3.1	6.2±3.8 0±0

Note. The upper line is the control; the lower line is γ-globulin; the number of E:T conjugates is calculated per 100 TC.

correlation coefficient values ranging from -0.1 to -0.43. This may be attributed to the involvement (due to the presence of FN) of a great number of MNC not active in the NCT reaction, assuming that the molecular sites functionally important in the NCT reaction remain unchanged in the presence of FN and γ-globulin. In this case FN provides for nonspecific adhesion of MNC to TC, while γ-globulin provides for the selectivity of the effective E:T conjugates with a simultaneous dissociation of "false" complexes, as a result of which the cytotoxic activity of NK is increased.

In order to assess the influence on the NCT reaction of the factors interacting with FN in the serum and participating in the cleavage or in the modification of FN molecules, we studied *in vitro* the cytotoxic activity of NK from healthy donors and the intensity of conjugate formation in the NK:TC system in CGM supplemented with 12% serum of RA patients. This serum was characterized by the R-protein content, anti-FN antibodies, and by so-called natural anti-FN antibodies. Homologous serum of healthy donors served as a control. As seen from Table 6, the R-protein content in the sera of RA patients is significantly ($p<0.02$) higher (almost 4-fold), while there are no substantial differences in the anti-FN antibodies and their natural component (see treatment with DOWEX). In the presence of the patients' sera the cytotoxic activity of NK decreased 29%, while the intensity of E:T conjugate formation increased 2.5-fold (Fig. 3). Consequently, the accumulation of the catalytically active R-proteins and, probably, of the products of partial catabolic degradation of FN in the organism results in the formation of large numbers of "false" E:T conjugates containing NK that cannot lyse the TC. The

TABLE 5. Changes in the Cytotoxic Activity of NK and the Intensity of Conjugate Formation in the NK:TC System in the Presence of Human FN/Human γ-Globulin Combination *in vitro* ($M \pm m$)

Experimental conditions	NK activity, arb. units/10^{-3}	Total number of E:T conjugates	% of conjugates consisting of one TC and lymphocytes		
			1	2	3 and more
Experiment 1 (n=6)					
Control	4.7±1.5	16.7±5.6	25±5	15±5	10±10
FN, 10 μg/ml	4.0±2.1	18.3±4.8	30±0	20±0	5±5
γ-Globulin, 0.5 μg/ml	5.4±0.9	13.3±6.1	20±20	20±0	0±0
FN+ γ-globulin	5.3±0.4	18.3±6.0	20±10	10±10	25±15
Experiment 2 (n=3)					
Control	3.1±0.5	16.7±8.8	20±5	30±10	0±0
FN, 0.1 μg/ml	4.3±0.4	20.0±5.8	30±10	20±10	10±5
γ-globulin, 0.5 μg/ml	4.4±0.5	6.7±6.7	0±0**	20±5	0±0
FN+ γg-globulin	5.9±0.3*	20.0±10.0	30±10	30±5	0±0

Note. One asterisk indicates $p<0.05$, two asterisks indicate $p<0.02$ compared with the control; the number of E:T conjugates is calculated per 100 TC; the doses of human FN and γ-globulin in combination are the same as for their individual application.

TABLE 6. Contents of R–Protein and Anti–FN Antibodies in the Serum of RA Patients and Healthy Donors Added to an MNC Culture in the NCT Reaction ($M \pm m$)

Serum component	RA patients	Healthy donors
R–protein, $\log_2$ (titer^{-1})	16.7±0.3 (n=14) p<0.02	14.74±0.37 (n=13)
Anti–FN antibodies, light abs. units ×10^3	353.1±32.8* 165.0±8.8** (n=13)	414.2±22.25* 187.1±10.4** (n=12)
M_{ini}	1.16*	1.19*
M_{DOW}	1.32**	1.31**

Note. Serum dilutions: one asterisk, 10^{-4}; two asterisks 10^{-5}; M_{ini} and M_{DOW} are the content of anti–FN antibodies before and after treatment with DOWEX, respectively.

positive correlation is lost. The correlation coefficient ρ becomes equal to -0.12.

Since only 39.1% of 21% of the MNC forming conjugates with TC in the NCT reaction lyse TC [11], obviously the E:T complexes formed during accumulation of R-proteins in the serum and probably including the products of partial FN cleavage (which is characteristic of practically any pathological process and correlates with the disease severity [1]) act as some kind of "traps" which prevent active NK from functioning as an immunobiological control. In view of the fact that the expression of LFA-1 adhesion molecules correlates with the invasive potential of malignant cell lines [13] and that the lack of LFA-1, LFA-3 and ICAM-1 adhesion molecules in these cells allows them to "slip" from this control [4], the mechanism responsible for the blocking of NK function described in this study is a substantial risk factor of reduced antitumor reactivity in pathological states associated with changes in the dynamic equilibrium of cell-cell interactions at the level of nonspecific adhesion.

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