Dissociation of E:T Conjugates Upon Activation of Human Natural Killers by Factors Acting at the "Lethal Blow" Stage

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UDC 612.112.94.017.4:[616-006.885.04-092

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 117, № 3, pp. 280-285, March, 1994
Original article submitted August 12, 1993

It is demonstrated that in the presence of human recombinant γ-interferon and C-reactive protein the activity of natural killers increases 9-43%, whereas the number of effector:target cell conjugates formed at the stage of recognizing and binding of target cells decreases 14-80%. There is a weak positive correlation ($\rho = 0.35$) between the activity of natural killers and the number of effector:target cell conjugates.

Key Words: *natural killers; activation; conjugates; dissociation*

A positive correlation between the number of effector:target cell (E:T) conjugates which are formed at the stage of recognition and binding and the efficiency of subsequent lysis of target cells (TC) has been established in studies of the mechanisms responsible for the cytotoxic potential of human natural killers (NK). Unilateral changes in the intensity of E:T conjugate formation and activity of NK have been observed not only upon testing the background cytotoxicity of NK, but also when these cells are stimulated by leukotriene B4 [4] or inhibited by anti-gp90 (CD18) Fab-fragments [2] and anti-ICAM-1 antibodies [13].

Meanwhile, it has been shown that recognition and binding of TC in the NK-mediated cytotoxic reaction is accompanied by conformational changes in the plasma membranes of TC and natural cytotoxicity effector (NCTE) cells, providing for the exchange of receptors between NK and TC [9,17]. For example, trypsin-sensitive structures active at 4°C are transferred to TC during their conjugation with large granular lymphocytes (LGL), which appears to be a requisite step in TC lysis [9]. This transfer changes the direction of the receptor/antireceptor interactions in the NK:TC system [1] and may be significant in programming cell lysis and in triggering the mechanisms responsible for the initiation of the next step in this process, primarily, of the lethal blow, which is associated with the production of cytotoxicity factor by NK (CFNK) in the NK:TC system [18].

However, if the initial steps in the interaction between NCTE and TC are characterized by the transfer of surface structures [1,9] that is necessary for NK-mediated cytotoxicity, the positive correlation between the number of E:T conjugates and the cytotoxic activity of NK [2,4,13] may represent one of the ways of cytolysis activation, namely, by compelling the largest possible number of NCTE cells to interact with TC. The existence of other means of stimulating massive NK-mediated cytotoxicity presupposes the possibility of controlling TC recognition and binding of factors activating NK at the "lethal blow" stage. Interferon (IF)-γ and C-reactive protein were used as

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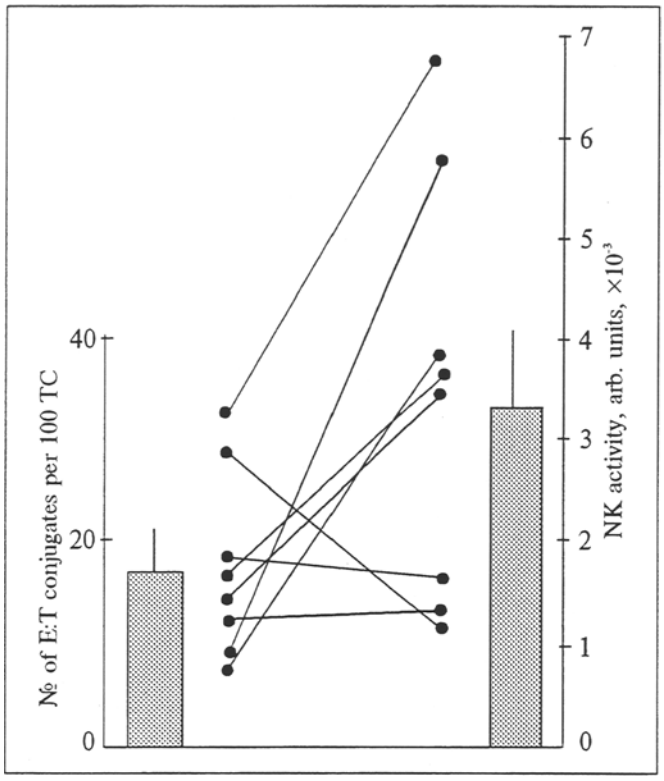

Fig. 1. Individual parameters of NK cytotoxicity and intensity of conjugate formation in the NK:TC system *in vitro*.

such factors in this study. These compounds do not activate NK during recognition or binding to TC [3,7,16,18].

MATERIALS AND METHODS

Peripheral blood mononuclear cells (MNC) were isolated from venous blood obtained from 8 healthy donors (2 men and 6 women aged 26-54 years) by one-step Ficoll-Verografin gradient (d=1.077 g/cm³) centrifugation (400 g, 45 min, 20°C). Cells were washed twice with medium 199 (400 g, 10 min, 20°C) and resuspended (10^7 cells/ml) in complete growth medium (CGM) containing 88 ml RPMI-1640 (Flow), 12 ml fetal calf serum (Flow), 2 mM glutamine, and 40 μg/ml gentamicin (Pharmachim) in 1 M HEPES (Flow).

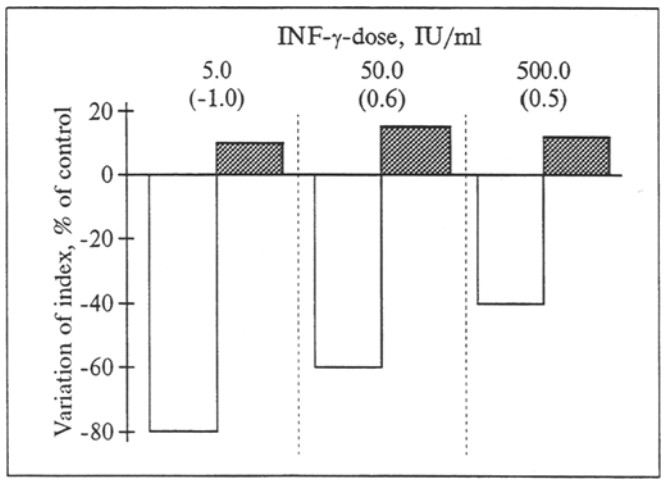

Fig. 2. Relationship between changes in NK cytotoxicity and intensity of conjugate formation in the NK:TC system in the presence of g-IF *in vitro*. Here and in Figs. 3 and 4 shaded bars: NK activity; white bars: number of E:T conjugates. Correlation coefficient values are given in parentheses.

The cytotoxic activity of NK was measured as described [8], using K-562 (human erythromyeloblasts) labeled with ^{3}H-uridine (3 μCi/ml cell suspension) as target cells. The TC (10^5 cells/ml CGM) were incubated with MNC (0.1 ml of each cell suspension) in 96-well plates for 14 h at 37°C in a humidified atmosphere containing 5% CO_2. After the incubation the resulting cell suspension was transferred to fiberglass filters (Whatman, pore diameter 2.5 μ). The filters were counted (1 min for each probe) in a toluol scintillator in a Packard β-counter. Target cells incubated without MNC under the same conditions served as a control.

The cytotoxicity index (CI) for 2 parallel probes at E:T ratios of 100:1, 50:1, 25:1, and 12:1 was calculated from the following formula:

CI= [1 - (cpm in experimental well/cpm in control well)]×100%.

The area under the curve was calculated by a described method [15], expressed in arbitrary units, and employed as an integral parameter in the figures and tables.

The number of E:T conjugates was determined by the method of Haliotis *et al.* [6]. Suspensions of TC and MNC were incubated at 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. Cells were centrifuged at 150 g for 5 min, carefully resuspended in 0.4 ml CGM, and the number of E:T conjugates was calculated under the light microscope in a Goryaev chamber. The number of conjugates was then expressed per 100 TC.

Gammaferon (human recombinant γ-IF, Ferment Conglomerate, Russia) was used at concentrations of 5, 50, and 500 IU/ml. C-reactive protein (Institute of Experimental Microbiology, Russian Academy of Medical Sciences) was used in concentrations of 0.02, 0.2, and 2 μg/ml. The protein was isolated by affinity chromatography from ascitic fluid of cancer patients, using glycerophosphocholine (CRP_1) or C-polysaccharide from *St. pneumoniae* (CRP_2). Both preparations were added to cell suspensions immediately before incubation for 14 h at 37°C in a humidified atmosphere containing 5% CO_2.

Statistical analysis was performed using Student's *t* test. Correlation coefficients (ρ) were calculated after Spearman.

RESULTS

The mean *in vitro* cytotoxic activity of NK isolated from donor blood was 3.4±0.72×10³ rel. units (1.1-6.65×10³ rel. units) (Fig. 1). The number of E:T conjugates varied from 8 to 32 per 100 TC (mean value 16.9±3.94). Judging from the correlation coefficient values (0.35), only the MNC that bind to TC within the first 10 min are active effectors of natural cytotoxicity, or probably LGL can lyse TC in the cytotoxicity test. As seen from Tables 2 and 4, conjugates of the LT type [5], i.e., those formed between 1 MNC and 1 TC, predominated. The proportion of the LT_m type [5] conjugates i.e., those consisting of 1 TC and 2 or more NCTE cells, was much smaller. There were no conjugates consisting of 1 lymphocyte and 2 or more TC (LT_n type [5]).

In the presence of γ-IF the *in vitro* cytotoxic activity of NK from healthy donors increased 9-15% (Table 1, Fig. 2), which was attributed to the ability of γ-IF to stimulate the production of CFNK at the "lethal blow" stage. The lowest dose of γ-IF (5 IU/ml) produced the maximum effect on the intensity of conjugate formation. The greatest increase occurred in the number of LT type conjugates, it was lower in the case of LT_m conjugates formed by 1 TC and more than 2 lymphocytes, while the number of LT_m conjugates consisting of 1 TC and 2 lymphocytes was virtually unchanged (Table 2). Taken together, these observations allowed us to consider the NK:TC system formed in the presence of γ-IF to correspond to a purified population of NK containing more than 98% $CD16^+$ cells, which are active NCTE cells [5]. In the presence of 50 and 500 IU/ml the γ-IF correlation coefficients increased to 0.6 and 0.5, respectively (0.35 in the control), indicating that greater numbers of active LGL, which can effectively lyse TC, function in the NK:TC system in the presence of γ-IF.

TABLE 1. Effect of γ-IF on Cytotoxic Activity of NK *in vitro*, $n=4$ ($M\pm m$)

Dose of g-IF, IU/ml	EC activity, arb. units ×10⁻³	
	control	γ-IF
5	4.7±1.0	5.1±0.46
50	4.9±0.75	5.6±0.48
500	4.9±0.75	5.5±0.5

The same regularities underlying the effect of γ-IF on the cytotoxicity of NK during recognition and binding to TC were observed in the experiments with C-reactive protein. It has been reported [7] that this protein plays a role in the regeneration of the CFNK, which is expressed on the surface of LGL [3]. In the presence of CRP_1 the activity of NK increased 22-36% (Table 3, Fig. 3), while the number of E:T conjugates decreased 28-58% (Table 4, Fig. 3). At a dose of 2 μg/ml the protein did not stimulate NK. CRP_2 increased NK activity 12-43% (Table 3, Fig. 4)

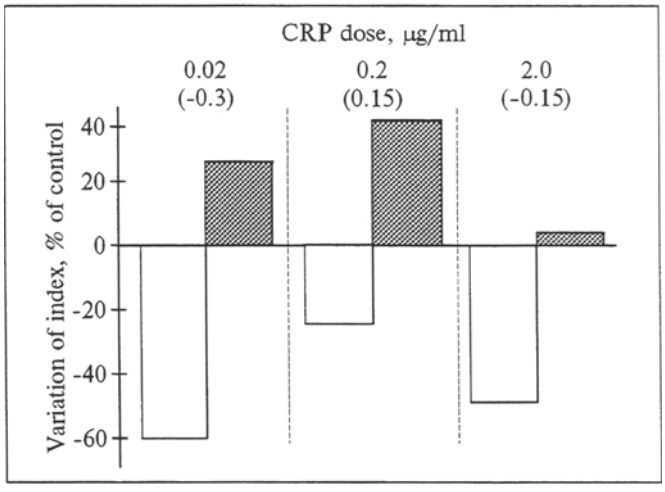

Fig. 3. Relationship between changes in NK cytotoxicity and intensity of conjugate formation in the NK:TC system in the presence of CRP_1 *in vitro*.

TABLE 2. Effect of g-IF on Conjugate Formation in the NK:TC System *in Vitro*

Dose of g-IF, IU/ml	Total number of E:T conjugates	% of conjugates including 1 TC and lymphocytes:		
		1	2	3 and more
5 ($n=6$)	8.33±3.33 1.67±1.67	17.5±2.56 0±0*	7.5±2.56 5±5	0±0 0±0
50 ($n=12$)	19.17±3.72 8.33±2.25	42.5±14.51 16.25±1.26	8.75±2.44 8.75±3.15	6.25±3.76 0±0
500 ($n=12$)	19.17±3.72 10.83±4.77	42.5±14.51 25.0±11.9	8.75±2.44 6.25±1.26	6.25±3.76 1.25±1.25

Note. Upper value – control; lower value – g-IF; asterisk indicates values statistically different from the control at $p<0.05$. Number of conjugates was calculated per 100 TC.

TABLE 3. Effect of CRP on NK Cytotoxicity *in vitro* (*M*±*m*)

Preparation	Dose, μg/ml	NK activity, arb. units×10^{-3}
CRP_1 (n=4)	0	1.8±0.52
	0.02	2.2±0.47
	0.2	2.5±0.35
	2.0	1.8±0.56
CRP_2 (n=3)	0	1.9±0.74
	0.02	2.7±0.9
	0.2	2.1±0.47
	2.0	2.2±0.6

and simultaneously lowered the rate of conjugate formation in the NK:TC system 14-52% (Table 4, Fig. 4). Inhibition of recognition and binding was dose-dependent (Fig. 4).

In contrast to γ-IF, CRP produced no appreciable effect on the number of LT_m complexes consisting of 1 TC and 2 MNC; however, a tendency toward a similarity between the results obtained in our NK:TC system with those reported for $CD16^+$-enriched lymphocyte populations [5] was observable.

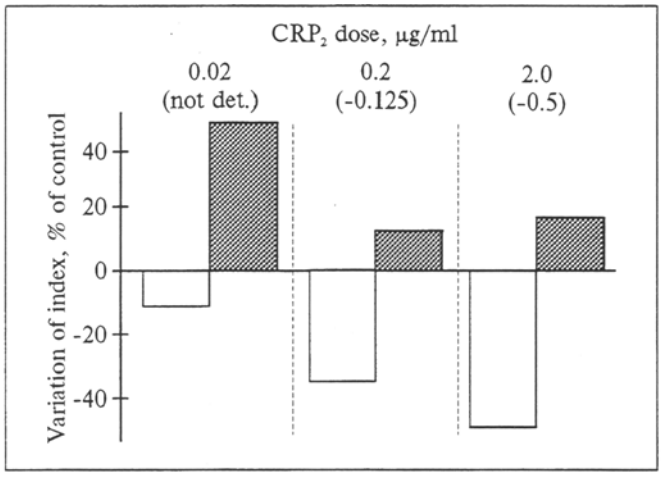

Fig. 4. Relationship between changes in NK cytotoxicity and intensity of conjugate formation in the NK:TC system in the presence of CRP_2 *in vitro*.

Our results provide the grounds for a fundamentally new concept of the mechanisms and dynamics of NCT-mediated cytolysis. It is likely that the unilateral changes in NK activity and the number of E:T conjugates formed during recognition and binding [2,4,13] represent the mechanism of cytotoxicity regulation which is realized by involving in TC binding or excluding from the NK:TC system the entire pool of nonspecific lymphocytes capable of forming E:T conjugates due to the considerable numbers of adhesion molecules, for example, ICAM-1 [13], expressed on their surface. This mechanism is ineffective and cannot provide for dynamic cytolysis of TC in a nonfractionated MNC population, since not only LGL or $CSD16^+$ cells [5] but also $CD3^+$ T cells [12], which do not mediate natural cytotoxicity, can form E:T conjugates. It has been reported that after the removal of $CD3^+$ lymphocytes from the reaction mixture, the number of MNC:K-562 cell conjugates increases, while it returns to the initial level after the addition of these cells [12].

The control over cytotoxicity exercised by the factors modulating the regeneration of CFNK at the "lethal blow" stage (CRP and γ-IF) is more important and more specific. Dissociation of E:T conjugates formed by inactive lymphocytes which cannot generate CFNK and provide for lysis of TC was observed in the presence of CRP and γ-IF.

As a result of dissociation of these "false" conjugates, some NCTE cells revert to the initial state, the population functionally "approaches" one

TABLE 4. Variation of Rate of Conjugate Formation in NK:TC System in the Presence of CRP *in vitro* (*M*±*m*)

Preparation	Dose, μg/ml	Total number of E:T conjugates	% of conjugates including 1 TC and lymphocytes		
			1	2	3 and more
CRP_1	0.02 (n=12)	13.89±4.47 / 5.83±1.93	26.67±7.28 / 12.5±3.24	15.0±2.89 / 5.0±2.04*	0±0 / 0±0
	0.2 (n=12)	13.89±4.47 / 10.0±3.02	26.67±7.28 / 20.0±4.09	15.0±2.89 / 10.0±4.09	0±0 / 0±0
	2.0 (n=12)	13.89±4.47 / 7.92±2.26	26.67±7.28 / 13.75±2.4	15.0±2.89 / 10.0±4.09	0±0 / 0±0
CRP_2	0.02 (n=3)	11.67±7.28 / 10.0±5.01	25 / 15	10 / 15	0 / 0
	0.2 (n=9)	13.89±4.47 / 9.44±3.48	26.67±7.28 / 18.33±6.02	15.0±2.89 / 10.0±5.02	0±0 / not det.
	2.0 (n=9)	13.89±4.47 / 6.67±2.77	26.67±7.28 / 15.0±5.01	15.0±2.89 / 5.0±2.89	0±0 / not det.

Note. Upper value – control; lower value – CRP; asterisk indicates values statistically different from the control at $p<0.1$; not det. – not determined; number of E:T conjugates calculated per 100 TC.

enriched in $CD16^+$ LGL [5], and the efficiency of cytolysis increases considerably. The result of the reaction is thus determined by the factors which activate the lytic potential of NK by generating CFNK but which become involved as early as at the stage of recognition and TC binding. Interleukin (IL)-1α, tumor necrosis factor-α [14], and interleukin-6 [11] (potent stimulators of NK) do not affect the formation of T:E conjugates, confirming that such activity is an intrinsic property of the regulators of CFNK production or of the lytic potential of NK. Fraction A of glycoprotein isolated from the K-562 cell membrane can dissociate E:T conjugates [17]; however, this may represent a variant of nonspecific protection of TC, since incubation of lymphocytes with TC in the presence of this factor reduces NK activity by 50-60% [17].

Thus, the initial stage of NK-mediated cytolysis is represented by complex membrane-associated processes, including reorganization of the effector membrane [1], activation of membrane-bound proteolytic enzymes in the lymphocyte [10], and transfer of the receptor/antireceptor structures onto the surface of TC [1,9]. The result of the reaction is determined not only by the potential of the effector but also by the activity of TC and their resistance to MNC [17]. Our results may indicate the existence of a natural nonspecific, but highly selective control of recognition and binding of TC, which is exerted by CRP constantly present on the plasma membrane of LGL [3] and by γ-IF produced by immune cells during any immune response.

Regulation of NK activity and efficacy of TC lysis by NCTE cells are characterized by a cascade of activating reactions involving IF and CRP that maintains the function of the active NK pool at a certain level: pre-NK + γ-IF - active NK (CRP expression) + TC - effective and ineffective E:T conjugates + γ-IF + CRP - dissociation of ineffective E:T conjugates - effective E:T conjugates + INF-γ + CRP - lysis of TC and production of INF-γ in the NK-TC system - recycling of NK - active NK - the next lytic cycle.

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