components increase the sensitivity of platelets to ADP by actively interacting with the cell membrane. This assumption does not contradict the findings of other researchers who demonstrated a stimulatory effect of peptoglycans of nontoxigenic DCB on cultured immune cells *in vitro*.

Thus, this study has shown that diphtheria toxin, diphtheria anatoxin, and codivac are agents that directly affect the functional activity of human platelets. In addition, it was found that diphtheria toxin and anatoxin reduce ADP-induced and total platelet aggregation, the decrease being dependent on preparation dose and incubation time. However, codivac, a glycopeptide from the cell wall of nontoxigenic DCB, stimulates platelet aggregation in all experimental series.

REFERENCES

1. V. V. Maleev, K. D. Lomazova, A. M. Polyakova, and O. S. Astrina, *Byull. Eksp. Biol Med.*, **102**, № 12, 678-681 (1986).
2. E. Neuere, *Z. Gesamte Inn. Med.*, **39**, № 6, 85-92 (1984).

Blast Transformation of Lymphocytes and the Activity of Natural Killer Cells in the Presence of γ-Globulin *In Vitro*

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The cytotoxic activity of natural killer cells against ^{3}H-uridine-labeled target cells (human erythromyeloleukosis cells K-562) and the intensity of spontaneous blast transformation are studied *in vitro* in the presence of human serum γ-globulin. It is shown that spontaneous blast transformation is 49-51% due to the presence of aggregated γ-globulin, while the aggregate-free γ-globulin fraction does not induce this reaction. The cytotoxic activity of natural killer cells *in vitro* declines in the presence of native γ-globulin, which is related to the influence of aggregated γ-globulin, the intensity of whose formation may increase upon a manyfold decrease in the γ-globulin content of the preparation.

Key Words: *blast transformation; natural killer cells; γ-globulin*

The receptors for the Fc portion of IgG (FcγR) or the CD16 antigens [1], the expression of which has been reported on the lymphocyte surface [6,9,16,17], are regarded, along with others, as potential receptors (structural elements) participating in the interaction between natural killer cells (NK) and target cells. It is believed that the FcγR-III expressed by NK [5], which is similar to the neutrophil FcγR but different from the B-cell FcγR [17], is the sole type of receptor providing for the antibody-dependent lysis of target cells [16]. The FcγR of NK (molecular weight 50-70 kD [9]) mediates the cell-to-cell contact upon lysis of antibody-covered target cells [17] and triggers the formation of NK cytotoxic factor [3].

Like most FcγR$^+$ cells, activated NK generate 40-50-kD soluble forms of FcγR-III [4,13] that can become involved in the natural cytotoxicity reaction which is presumed to occur at the early stages via the contact and transfer of receptor-

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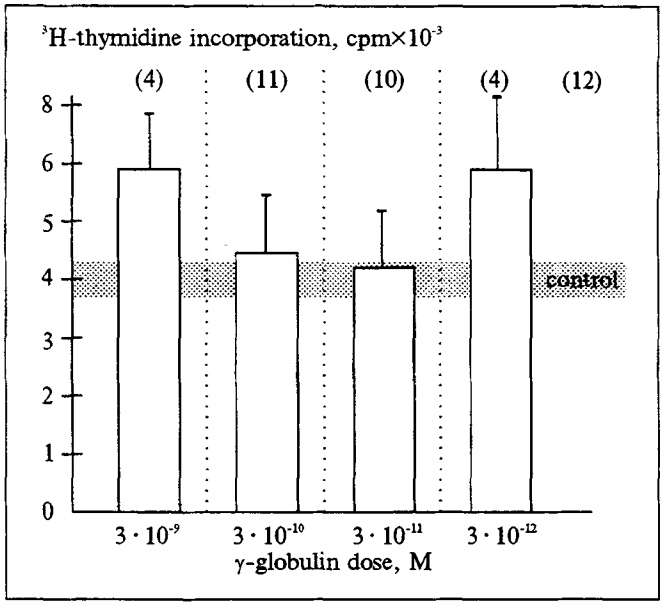

Fig. 1. Spontaneous LBT in the presence of native preparation of human γ-globulin in healthy donors *in vitro*. Here and in Fig. 3 the number of observations is indicated in parentheses.

antireceptor type structures on the surface of an opposite cell [1,8]. It is believed [8] that trypsin-sensitive structures transferred from the NK membrane to the target cell carry out the subsequent lysis of the target cells. From this viewpoint, γ-globulin, analogously to the complement receptors CR2 and CD21 (acceptors of soluble Fcε R-II, an FcγR ligand) [5], can be regarded along with fibronectin as a recognizing and/or recognized structure during the interaction between NK and target cell [1]. It is known that even after a short-term incubation with interleukin-2, up to 90% of human NK start expressing cytophilic IgG, whose presence on the effector cell membrane correlates with a decrease in natural cytotoxicity and whose dissociation leads to an increase in the effectiveness of cytolysis [21].

The mechanisms underlying the generation of soluble FcγR-III by proteolysis on the lymphocyte membrane [5] are similar to those providing for the formation of enzymatically active R-proteins that have an affinity for fibronectin and can participate in its cleavage [2]. Both soluble and immobilized fibronectin interacts only with aggregated human γ-globulins [18], suggesting the participation of aggregated γ-globulins in the regulation of the effectiveness of NK-mediated cytolysis of target cells at the stage of bilateral transfer of the receptor-antireceptor complexes between target cells and NK [1] and the stage of polyclonal activation of lymphocytes, which, like the generation of soluble FcγR-III, is accompanied by reorganization of the lymphocyte membrane and redistribution of cell receptors [5,13].

Our objective was to study the intensity of spontaneous lymphocyte blast transformation (LBT) and the cytotoxic activity of human NK in the presence of γ-globulin differing in aggregate content.

MATERIALS AND METHODS

Mononuclear cells (MNC) were isolated on a one-step Ficoll-Paque gradient (Pharmacia Fine Chemicals) from peripheral venous blood obtained from 25 donors (10 men and 15 women aged 20-55 years). Splenocytes were obtained from outbred male albino mice weighing 25-36 g (Central Breeding Farm of the Russian Academy of Medical Sciences).

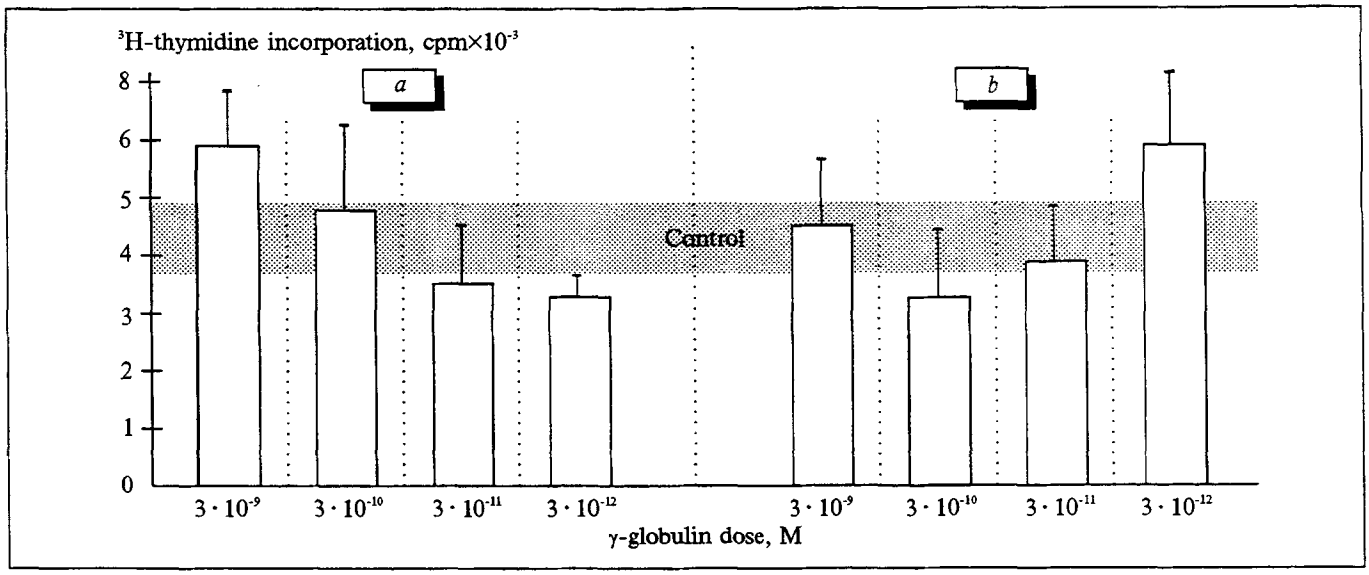

Fig. 2. Spontaneous LBT in the presence of aggregated human γ-globulin (*a*) and aggregate-free γ-globulin (*b*) in healthy donors *in vitro*. The values are the means of 6 determinations.

The cytotoxic activity of NK was determined as described [7] using standard human erythromyeloblastoma cells K-562 labeled with ^{3}H-uridine (3 μCi/ml). Splenocytes and MNC were washed twice with double Eagle's medium (400 g, 10 min, 20°C). The initial suspensions contained 10^7 splenocytes or MNC and 10^5 target cells in 1 ml complete growth medium based on RPMI-1640 (Flow) supplemented with 12% fetal calf serum (Flow), 2 mM glutamine, and 40 μg/ml gentamicin (Pharmachim) on 1 M HEPES buffer (Flow). Natural killer cells and target cells (0.1 ml of each suspension) were incubated in 96-well round-bottom microplates for 14 h at 37°C in a humidified atmosphere containing 5% CO_2. After the incubation the cells were transferred to Whatman fiberglass filters (2.5 μ pore diameter). The residual radioactivity was measured in a Mark-II or Packard scintillation β-counter. The cytotoxicity index for each of 2 or 3 parallel wells with effector:target ratios 100:1, 50:1, 25:1, and 12:1 was calculated from a formula [7]. The area under the cytotoxicity curve, calculated by a method described elsewhere [19] and expressed in arbitrary units (arb. units), was used in the figures and tables as an integral parameter characterizing NK activity.

In the reaction of spontaneous LBT, 0.2 ml of MNC suspension (10^6 cells/ml complete growth medium) and ^{3}H-thymidine (10 μCi/ml) were incubated for 18 h at 37°C in a humidified atmosphere containing 5% CO_2. After the incubation, transfer to fiberglass filters and measurement of residual radioactivity were performed as described above.

Human serum γ-globulin (Serva, dose range 3×10^{-9} - 3×10^{-13} M) was added to the MNC suspension immediately before transfer of the cells to the microplate wells with subsequent incubation during the course of the cytotoxicity or spontaneous blast transformation reaction for 14 and 18 h, respectively. For fractionation the γ-globulin preparation was centrifuged in a bucket rotor for 2 h at 20°C at 105,000 *g*. The upper two-thirds of the supernatant were used as the aggregate-free γ-globulin fraction. The lower third contained aggregated γ-globulin.

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

RESULTS

The addition of native human γ-globulin, which generally contains monomeric and aggregated forms of the protein, stimulates spontaneous LBT healthy donor *in vitro* (Fig. 1). The dose dependence of

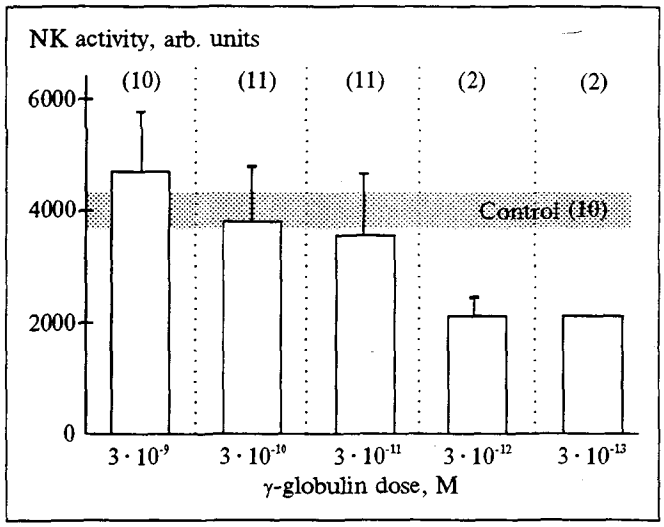

Fig. 3. Changes in the cytotoxic activity of NK in the presence of native preparation of human γ-globulin in healthy donors *in vitro*. One asterisk indicates values significantly different from the control at $p<0.02$, two asterisks at $p<0.001$. NK activity, arb. units

the effect is described by a bell-shaped curve. A commensurate, statistically significant stimulation of ^{3}H-thymidine incorporation in the lymphocytes was observed under the influence of the minimal and maximal doses of γ-globulin. Label incorporation increased from $4.0\pm0.52\times10^3$ to $5.9\pm0.50\times10^3$ cpm (49%) in the presence of 3×10^{-9} M γ-globulin, while in the presence of 3×10^{-12} M it increased to $5.9\pm0.87\times10^3$ cpm (51%) (Fig. 1). In intermediate doses the preparation had no effect on spontaneous LBT. However, whereas in the presence of 3×10^{-10} M γ-globulin the relationship between the effect of the preparation and the initial level of spontaneous LBT was weak and negative ($\rho = -0.23$), i.e., virtually absent, at 3×10^{-11} M this relationship was strong and positive ($\rho = 0.75$), which indicates the ability of γ-globulin to maintain under certain conditions the previously initiated LBT without any effect on the initiation of blast transformation per se.

Aggregation of γ-globulin and separation of the aggregate-free fraction reveal considerable differences

TABLE 1. Individual Parameters of Spontaneous LBT and NK Activity of MNC in Healthy Donors *in Vitro*

^{3}H-thymidine incorporation, cpm	NK activity, arb. units
1257	2425
1269	3693.8
1393	1525
1478	5081.3
1549	2187.5
1746	2962.5
2073	4506.3

TABLE 2. Individual Parameters of Spontaneous LBT and NK-Activity of Murine Splenocytes *in Vitro*

^{3}H-thymidine incorporation, cpm	NK activity, arb. units
1706	2456.3
1853	2431.3
2792	2800
2977	1837.5
2998	2306.3
3436	2906.3
3598	2393.3
4216	2943.8
4887	2318.8
5922	2487.5
5949	2275
6263	2956.3
6333	1850
6437	3025
6595	2606.3

in the effect of their presence in the MNC suspension on spontaneous LBT (Fig. 2). Aggregated γ-globulin (3×10^{-9} M) stimulated *in vitro* LBT in healthy donors, increasing ^{3}H-thymidine incorporation from $4.2\pm0.58\times10^3$ to $5.9\pm0.74\times10^3$ cpm (by 43%, Fig. 2, *a*), which corresponded to the action of the same dose of native preparation containing monomeric γ-globulin (Fig. 1). Upon subsequent dilution the effect was lost, while at 3×10^{-12} M label incorporation in MNC was reduced to $3.2\pm0.36\times10^3$ cpm, i.e., by 23% (Fig. 2, *a*). In contrast to native γ-globulin preparations (Fig. 1), aggregate-free γ-globulin fraction added to the MNC suspension in the maximum concentration (3×10^{-9} M) had no effect on spontaneous LBT (Fig. 2, *b*). A tendency toward the inhibition of LBT (label incorporation decreased to $3.3\pm0.73\times10^3$ cpm or by 20%) was observed in the presence of 3×10^{-10} M aggregate-free γ-globulin. When added in a dose of 3×10^{-12} M, aggregate-free γ-globulin stimulated *in vitro* spontaneous LBT in patients, increasing ^{3}H-thymidine incorporation into MNC to $5.5\pm1.41\times10^3$ cpm (33%, Fig. 2, *b*), which corresponded to the effect of the same dose of native γ-globulin (Fig. 1).

From the comparison of changes in the level of spontaneous LBT occurring in the presence of aggregated and aggregate-free human γ-globulin preparation in the MNC suspension (Fig. 2) with stimulation of LBT by native γ-globulin (bearing in mind that native preparation contains both monomers and aggregates of γ-globulin formed as a result of spontaneous aggregation) it can be concluded that aggregated γ-globulin stimulates *in vitro* spontaneous blast transformation of lymphocytes isolated from the blood of healthy donors. Most likely it stimulates spontaneous LBT in the presence of aggregate-free fractions, since a 1000-fold dilution of the preparation causes an increase in the intensity of spontaneous aggregation. Consequently, the γ-globulin dose effectively stimulating LBT cannot be regarded as aggregate-free, all the more so that its influence is comparable to that elicited by the effective dose of aggregated protein (Fig. 2).

However, if in the presence of aggregated γ-globulin there occurs a nonspecific polyclonal stimulation of MNC transformation into blast forms losing their specific functional activity, the highly differentiated activities of lymphocytes, including the cytotoxicity of NK, must be inhibited under similar conditions. The correlation between the baseline level of NK cytotoxicity in the suspension of donor MNC and the background activity was weak and positive: the correlation coefficient calculated from Table 1 was equal to 0.29. Similarly, the relationship between the corresponding parameters in the murine splenocyte suspension is characterized by $\rho = 0.21$ (Table 2), indicating that normal human and murine lymphocytes function at a level that determines an equally probable realization of highly differentiated function or nonspecific activation toward polyclonal transformation, depending on the inducing signal.

In the presence of native human γ-globulin in a dose of 3×10^{-9} M the *in vitro* cytotoxic activity of NK of healthy donors did not change, while in the presence of 3×10^{-10} and 3×10^{-11} M γ-globulin it tended to decrease (Fig. 3). At a γ-globulin concentration of 3×10^{-12} M, which significantly stimulated spontaneous LBT (Fig. 1), NK inhibition manifested itself in an activity decline from 4205 ± 458 to 2553 ± 129 arb. units (by 40%) and became statistically significant ($p<0.02$, Fig. 3). A further 10-fold dilution of the preparation (to a concentration of 3×10^{-13} M), together with the expected increase in the spontaneous aggregation, led to a further decrease in NK activity to 2000 ± 31 arb. units (53%) compared with the initial value ($p<0.001$, Fig. 3). It is noteworthy that the effects of γ-globulin observed at doses of 3×10^{-9}, 3×10^{-10}, and 3×10^{-11} M, unlike its effect on spontaneous LBT in the MNC suspension, were not associated with the initial NK activity of MNC: the ρ values were -0.09, 0.02, and 0.1, respectively. This indicates the effect of γ-globulin on MNC with initially high and low NK activity.

A statistically significant inhibition of NK cytotoxicity due to a direct effect of NK on FcγR effectors was observed in experiments with monomeric IgG_1 and IgG_3 [20]. A still more pronounced dose-dependent decrease in the NK activity of lymphocytes associated with the blockade of their FcμR was recorded under the influence of polyclonal IgM [15]. Monoclonal IgM proved to elicit a lower effect, while Fcμ fragments were more active than Fab fragments [15], which, on the contrary, can nonspecifically stimulate the immune response [12]. In a dose of 0.5 mg/ml, which is only one order of magnitude different from the plasma IgG content and is comparable to the IgM content, native IgG and IgM have no effect on NK activity [11]. This is consistent with the premise, based on our results, that FcR expressed on the MNC membrane at a low density [6,9] are saturated even at microgram concentrations of γ-globulin (Fig. 3). The induction of interleukin-6 production by aggregated (but not monomeric) IgG and Fc fragments may be an additional factor maintaining the baseline level of NK activity in the presence of aggregated IgG and Fc fragments [14].

On the other hand, increased lysis of most NK-sensitive target cells (including the relatively resistant Daudi cells) by anti-FcR monoclonal antibodies of the IgG class [10] implies a possible involvement of IgG in the cytotoxic interaction between NK and the target cells, which at certain stages proceeds along the lines of the intermembrane exchange pattern [1]. They can function as recognized and recognizing structures similarly, to a certain degree, to fibronectin [1] interacting with aggregated protein [18], which emerges as a factor restricting the cytotoxic reaction probably via stimulation of LBT.

REFERENCES

1. V. A. Kuznetsov, *Immunologiya,* № 3, 8-13 (1992).
2. A. Ya. Kul'berg, *Regulatory R-Proteins in Infectious and Other Diseases* [in Russian], Moscow (1990), pp. 3-9.
3. B. Bonavida, J. Katz, and M. Gottlieb, *J. Immunol,* **137**, № 4, 1157-1163 (1986).
4. W. H. Friedman, C. Bonnerot, M. Daeron, *et al., Immunol. Rev.,* **125**, 49-76 (1992).
5. W. H. Friedman, L-J. Teilland, C. Bouchard, *et al., J. Leukoc. Biol.,* **54**, № 5, 504-512 (1993).
6. C. J. Froelich and A. D. Bankhurst, *Clin. Exp. Immunol.,* **55**, № 3, 664-670 (1984).
7. Y. Hashimoto and H. Sudo, *Gann.,* **62**, № 2, 139-143 (1971).
8. J. Hiserodt, L. Britvan, and S. Targan, *Cell. Immunol.,* **83**, № 1, 43-51 (1984).
9. V. Horejsi and V. Bazil, *Biochem. J.,* **253**, 1-26 (1988).
10. M. Jondal, C. Kullman, M. Alter, and K. Ljunggren, *Cell. Immunol.,* **100**, № 1, 158-166 (1986).
11. K. Komiyama, S. S. Crego, K. Itoh, *et al., Ibid.,* **101**, № 1, 143-155 (1986).
12. A. J. Kulberg, L. M. Bartova, and D. N. Evnin, *Immunology,* **34**, 199-206 (1978).
13. L. L. Lanier, J. H. Philips, and R. Testi, *Europ. J. Immunol.,* **19**, 775-778 (1989).
14. Z.-D. Ling, H. J. Ziltener, B. T. Webb, and D. S. Matheson, *Cell. Immunol.,* **129**, № 1, 95-103 (1990).
15. M. Manciulea, L. Pricop, A. Sulica, and R. B. Herberman, *Molec. Immunol.,* **26**, № 12, 1087-1093 (1989).
16. B. Perussia and G. Trinchieri, *J. Immunol.,* **132**, № 3, 1410-1415 (1984).
17. B. Perussia, G. Trinchieri, A. Jackson, *et al., Ibid.,* **133**, № 1, 180-189 (1984).
18. M. Salvarrey and A. Rostagno, *Clin. Exp. Immunol.,* **76**, № 1, 92-98 (1989).
19. T. P. Sheeran, F. R. Jackson, P. T. Dawes, *et al., J. Immunol. Methods,* **115**, № 1, 95-98 (1988).