Stimulatory Effect of Fragments from Transcribed Region of Ribosomal Repeat on Human Peripheral Blood Lymphocytes

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Fragments from the transcribed region of the ribosomal repeat include considerable amounts of unmethylated CpG DNA motifs. These motifs activate immune cells via the interaction with Toll receptors. *In vitro* experiments confirmed the stimulatory effect of transcribed region of ribosomal repeat on human lymphocytes. Culturing of lymphocytes in a medium containing 2-20,000 ng/ml fragments from transcribed region of ribosomal repeat was accompanied by structural changes in the nucleus in a considerable number of cells. These changes manifested in translocation of pericentromeric heterochromatin from the membrane to the center of the nucleus and activation of the nucleolus and were accompanied by a significant increase in interleukin-6 production and slight stimulation of tumor necrosis factor-α synthesis. The transcribed region of the ribosomal repeat and *E. coli* DNA had various effects on quantitative parameters of lymphocytes. Our results suggest the existence of mechanisms of stimulation not mediated by the interaction of CpG DNA motifs with Toll receptors.

Key Words: *ribosomal DNA; CpG DNA motifs; TLR9; tumor necrosis factor-α; interleukin-6*

Cell death in the organism is accompanied by the appearance of DNA fragments in blood plasma. We described a previously unknown property of fragments from transcribed region of ribosomal repeat (TRrDNA): they are accumulated in extracellular peripheral blood DNA in healthy individuals and, especialy, in patients with rheumatoid arthritis (RA) [1]. The increase in the content of TRrDNA fragments in extracellular DNA is important for the immune system. The study of primary sequence in human TRrDNA revealed a considerable number of unmethylated CpG-containing motifs for binding to Toll receptors (TLR9) on antigen-presenting cells. This feature distinguishes TRrDNA from the total genomic DNA and makes it similar to bacterial DNA. The interaction of TRrDNA with TLR9 can result in the development of autoimmune disorders [11]. However, it is impossible to predict the effect of TRrDNA fragments on immune cells, because some regions of TRrDNA carry suppressor motifs (short repeats of a common structure (G)n [7]) that block the effect of TLR9-binding regions in the TRrDNA sequence.

Here we compared activation of human lymphocytes that were cultured with TRrDNA fragments, *E. coli* DNA, and human total genomic DNA. The degree of lymphocyte activation was estimated by structural changes in the nucleus, increase in

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nucleolar RNA transcription, and cytokine concentration (TNF-α, IL-6) in the culture medium.

MATERIALS AND METHODS

G_0 lymphocytes were isolated from the peripheral blood of 3 healthy donors and 2 patients with RA using a Ficoll—Verografin system. Lymphocytes (600,000-700,000 cells/ml) were cultured in RPMI-1640 medium (ICN) with 10% fetal bovine serum (HyClone) at 37°C.

The mixture of 2 linearized plasmids containing TRrDNA fragments (regions of 515-5321 and 9346-10,783 nucleotides; HSU 13369, GeneBank) incorporated into pBR322 vector (TRrDNA) or a mixture of *E. coli* DNA inserts from these plasmid (TRrDNA*) served as a source of TRrDNA fragments. *E. coli* DNA was isolated from strain MG 1655. Human genomic DNA was isolated from leukocytes of healthy donors. DNA samples were subjected to additional purification by treatment with Triton X-114 [6], electrophoresis in 1% low-melting-point agarose, and gel filtration on HW-85 carrier.

Structural changes in the nucleus were evaluated by the position of pericentromeric loci in chromosome 1 (1q12 region) of fixed lymphocyte nuclei [2]. *In situ* hybridization of biotinated probe with 1q12 was carried out. Biotin was visualized with avidin-fluorescein conjugate. The distribution of fluorescent signals was estimated by the normalized radius vector of the lymphocyte nucleus. We examined 500 nuclei in each series. The results were expressed as a frequency of perimembrane signals (normalized radius vector 0.8-1.0) [2]. Variations in activity of the nucleolus were determined in 150 nuclei. The number and total area of active sites in nucleoli were evaluated after selective staining of fixed nuclei with silver nitrate [3]. The study was performed using an Axioplan microscope and DC290 digital camera (Kodak). The images were analyzed using IBAS and InterEVM softwares.

IL-6 concentration in lymphocyte culture medium was measured by solid-phase enzyme immunoassay with Innogenetics reagents. TNF-α activity in the culture medium (IU/ml) was estimated by lysis of cytokine-sensitive mouse fibrosarcoma L-929 cells [14]. Human recombinant TNF-α served as the standard (DiaM).

RESULTS

DNA sequences containing unmethylated CpG motifs induced phosphorylation of JNK and SAPK via the interaction with TLR9. These protein kinases are activated during cells response to stress [8]. Lymphocyte stimulation under conditions of protein kinase activation is accompanied by structural changes in cell nuclei, which includes translocation of centromeric chromosomal loci from the membrane to the center of the nucleus and activation of the nucleolus [4,5]. These tests are highly sensitive for lymphocyte activation. Figure 1 shows the incidence of perimembrane 1q12 loci after lymphocyte culturing with different amounts of TRrDNA fragments, *E. coli* DNA, and human total genomic DNA.

In a special series, lymphocytes were incubated with pBR322 vector DNA. In a considerable number of cells, translocation of pericentromeric heterochromatin in chromosome 1 from the membrane to the center of the nucleus was noted at a TRrDNA concentration of 2 ng/ml. Similar changes were observed during culturing of lymphocytes with 20,000 ng/ml *E. coli* DNA. Total genomic DNA and vector DNA had little effect on this parameter. Kinetic studies showed that translocation of 1q12 loci to the center of the nucleus is completed after 2-h culturing of lymphocytes with TRrDNA in various concentrations.

Lymphocyte stimulation was accompanied by activation of the nucleolus, which reflects the increase in rRNA synthesis. The number and total area of silver-stained sites increase under these conditions [12]. Figure 2 illustrates changes in activity of the nucleolus during 3-h culturing of lymphocytes in a medium containing various DNA samples in a concentration of 200 ng/ml. TRrDNA significantly increased the number of nuclei with signs of

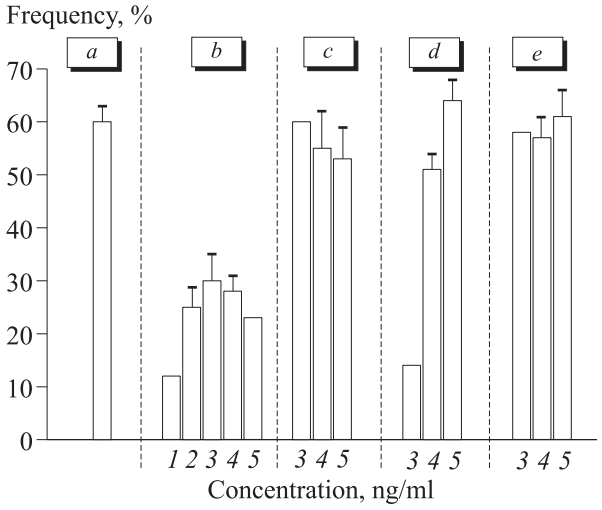

Fig. 1. Frequency of signals with normalized radius vectors (0.8-1.0) after hybridization of lymphocyte nuclei with a probe for the pericentromeric region of human chromosome 1 (1q12). Control (*a*), TRrDNA (*b*), pBR322 (*c*), *E. coli* (*d*), and total genomic DNA (*e*). 20,000 (*1*), 2000 (*2*), 200 (*3*), 20 (*4*), and 2 ng/ml (*5*).

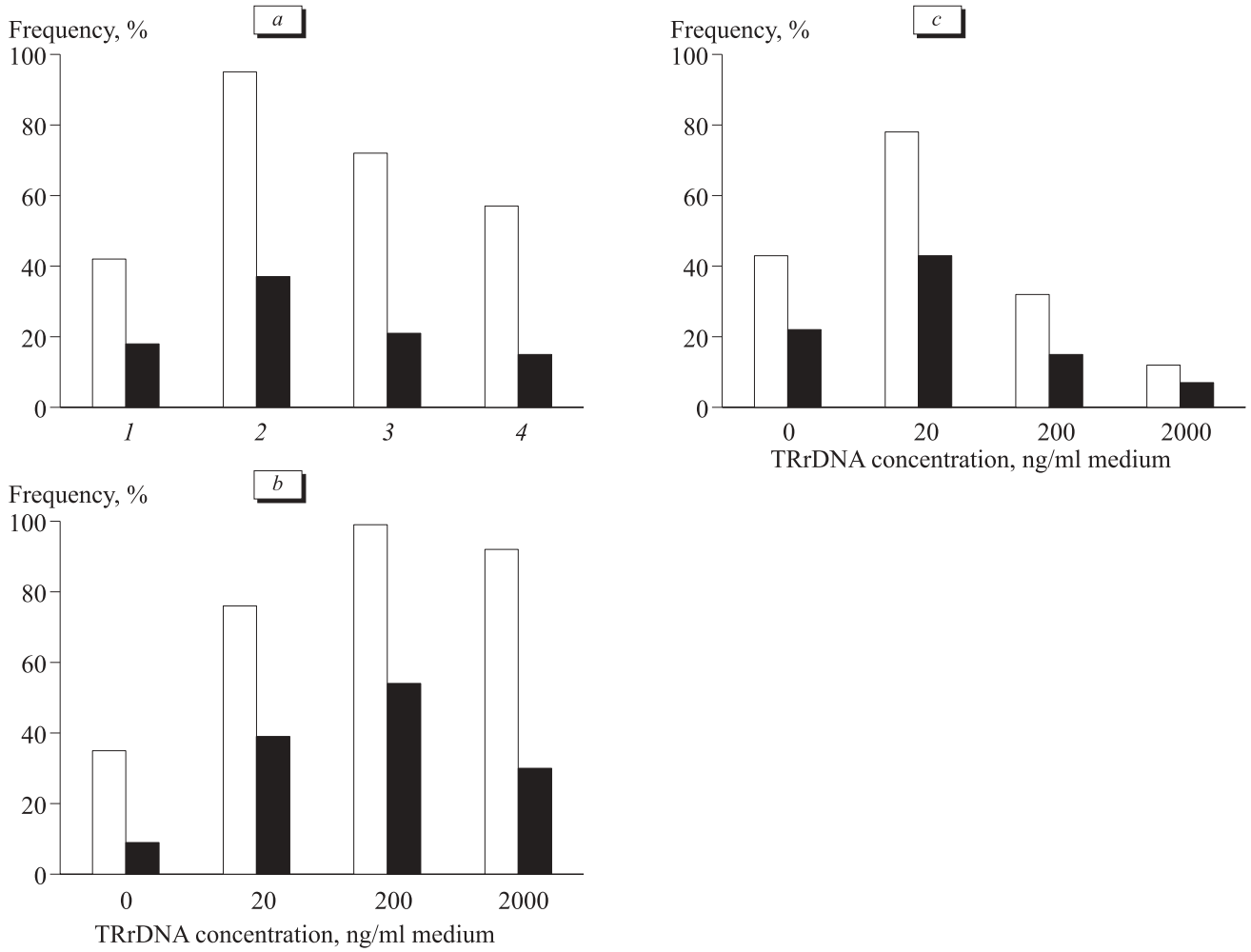

Fig. 2. Occurrence frequency for nuclei in which the area of nucleolar silver granules exceeds the mean control value (light bars); nuclei with not less than 3 silver-stained nucleoli (dark bars). (*a*) Three-hour culturing of lymphocytes in the presence of 200 ng/ml DNA: control (*1*), TRrDNA (*2*), pBR322 (*3*), *E. coli* (*4*). Culturing of lymphocytes with TRrDNA in various concentrations for 2 (*b*) or 4 h (*c*).

nucleolus activation. Activity of the nucleolus increased less significantly upon treatment with vector DNA and *E. coli* DNA. TRrDNA had a dose-dependent effect (Fig. 2, *b*, *c*). Two-hour incubation of cells with TRrDNA in concentration ≥200 ng/ml was accompanied by activation of the nucleolus in a considerable number of cells (Fig. 2, *b*). Opposite changes were revealed after 4-h treatment with TRrDNA in the same concentration. Activity of nucleoli significantly decreased compared to the control. These changes manifested in a decrease in the number and total area of silver granules (Fig. 2, *c*).

Cytokine synthesis is a sign of immune cell activation. We studied changes in the concentrations of IL-6 and TNF-α in the culture medium of lymphocytes. Published data show that these compounds determine various clinical symptoms of RA [10,13]. Much attention is paid to IL-6, because this cytokine produces various effects and is involved not only in the inflammatory process, but also in the regulation of endocrine function and metabolism [9]. We studied IL-6 synthesis in lymphocytes from healthy donors cultured with various samples of DNA in a concentration of 2 μg/ml for 24 h (Fig. 3, *a*). In contrast to *E. coli* DNA and plasmid vector DNA, TRrDNA stimulated the synthesis of abnormally large amounts of IL-6. Total genomic DNA did not induce IL-6 synthesis. A significant increase in IL-6 concentration was observed after 4-h culturing of lymphocytes in the presence of 200 ng/ml TRrDNA (Fig. 3, *b*). This effect was more pronounced in lymphocytes from RA patients (Fig. 3, *b*).

Culturing of lymphocytes from healthy donors in a medium containing 2 μg/ml DNA samples for 24 h was accompanied by an increase in the concentration of another cytokine (TNF-α). As differentiated from IL-6, *E. coli* DNA and vector DNA were more potent than TRrDNA in stimulating the synthesis of TNF-α. To exclude possible effect of the vector in TRrDNA plasmids on TNF-α synthe-

sis, we studied the ability of isolated inserts (TRrDNA*) to stimulate TNF-α synthesis in lymphocytes from another two healthy donors (compared to *E. coli* DNA). After 4-h culturing of lymphocytes from both donors with 50 ng/ml *E. coli* DNA, TNF-α activity was 18 times higher than in a medium containing 50 ng/ml TRrDNA*. Therefore, treatment of lymphocytes with TRrDNA fragments is accompanied by significant activation of IL-6 synthesis and slight stimulation of TNF-α production. It cannot be excluded that low activity of TNF-α results from high-intensity synthesis of IL-6. Published data show that IL-6 inhibits TNF-α secretion in lymphocytes [15].

Our results show that unmethylated CpG-rich sequence of TRrDNA *in vitro* stimulates human lymphocytes. Structural changes in chromatin in a considerable number of cells are observed over the 1st hours of lymphocyte culturing with TRrDNA. They manifested in translocation of pericentromeric loci in chromosome 1 from the membrane to the center of the nucleus and increase in the number and total area of silver-stained fibrillar centers in the nucleolus. Lymphocyte activation is accompanied by an increase in the production of IL-6 and TNF-α. Comparative study showed that *E. coli* DNA and TRrDNA in similar concentrations have various stimulatory effects on lymphocytes. After treatment with TRrDNA, structural reconstruction of the nucleus and activation of the nucleolus occurred in a greater number of cells under the influence of stimulating DNA fragments in lower concentrations. IL-6 production in the presence of TRrDNA was by one order of magnitude higher, while TNF-α activity was by one order of magnitude lower compared to those observed during culturing of lymphocytes with *E. coli* DNA. These differences were probably associated with a higher constant of bin-

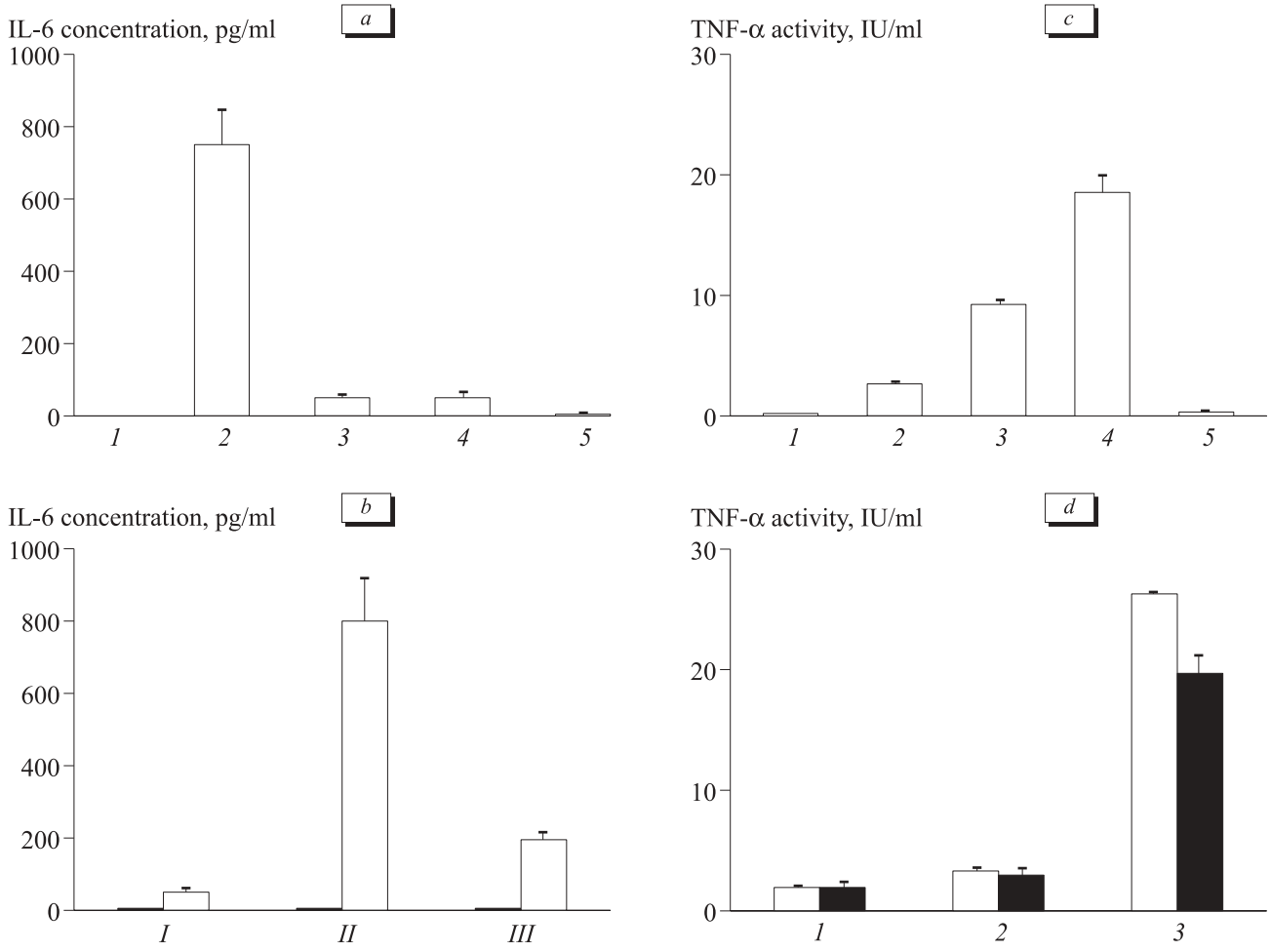

Fig. 3. IL-6 concentration (*a*, *b*) and TNF-α activity in the lymphocyte culture medium (*c*, *d*). Culturing of lymphocytes from a healthy donor with DNA samples in concentrations of 2 (*a*) or 0.2 μg/ml for 24 h (*c*): control (*1*), TRrDNA (*2*), pBR322 (*3*), *E. coli* (*4*), and total genomic DNA (*5*). (*b*) Incubation of lymphocytes from a healthy donor (*I*) and 2 patients (*II*, *III*) with (light bars) and without 0.2 μg/ml TRrDNA for 4 h (dark bars). (*d*) Culturing of lymphocytes from 2 healthy donors (light and dark bars) with 50 ng/ml TRrDNA* and *E. coli* DNA for 4 h: control (*1*), TRrDNA* (*2*), and *E. coli* (*3*).

ding of CpG motifs in TRrDNA to TLR9 receptors or more effective penetration of TRrDNA fragments into the cell. Rapid activation of TLR9 in competent cells is probably followed by the synthesis of considerable amounts of IL-6, which inhibits TNF-α synthesis [15]. It cannot be excluded that another mechanisms of cell activation in the presence of TRrDNA are not mediated by interaction with TLR9. We showed that TRrDNA fragments have an *in vitro* stimulatory effect on lymphocytes. It may be suggested that selective accumulation of these fragments in extracellular DNA [1] may modulate function of the immune system.

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REFERENCES

1. N. N. Veiko, N. O. Shubaeva, S. M. Ivanova, *et al*., *Byull. Eksp. Biol. Med*., **142**, No. 9, 282-285 (2006).
2. A. V. Karpukhin, A. G. Salimov, S. A. Karpukhin, *et al*., *Dokl. Ros. Akad. Nauk*, **341**, 553-557 (1995).
3. N. A. Lyapunova, I. A. Kravets-Mandron, and T. G. Tsvetkova, *Genetika*, **34**, 1298-1306 (1998).
4. D. M. Spitkovskii, N. N. Veiko, O. S. Moiseeva, *et al*., *Radiats. Biol. Radioekol*., **45**, 517-534 (2005).
5. D. M. Spitkovskii, N. N. Veiko, O. S. Moiseeva, *et al*., *Med. Genet*., No. 11, 494-504 (2005).
6. Y. Aida and M. J. Pabst, *Immunol. Methods*, **132**, 191 (1990).
7. R. F. Ashman, J. A. Goeken, J. Drahos, and P. Lenert, *Int. Immunol*., **17**, No. 4, 411-420 (2005).
8. G. Hartmann and A. M. Krieg, *J. Immunol*., **164**, 944-953 (2000).
9. T. Kishimoto, S. Akira, M. Narazaki, and T. Taga, *Blood*, **86**, 1243-1254 (1995).
10. S. Kotake, K. Sato, K. J. Kim, *et al*., *J. Bone Miner. Res*., **11**, 88-95 (1996).
11. A. M. Krieg, *J. Clin. Immunol*., **15**, No. 6, 284-292 (1995).
12. R. L. Ochs and K. Smetana, *Exp. Cell Res*., **184**, 552-557 (1989).
13. A. K. Palucka, J. P. Blanck, L. Bennett, *et al*., *Proc. Natl. Acad. Sci. USA*, **102**, No. 9, 3372-3377 (2005).
14. M. R. Ruff and G. E. Gifford, *Lymphokines*, New York (1981), pp. 235-241.
15. R. Schindler, J. Mancilla, S. Endres, *et al*., *Blood*, **75**, 40-47 (1990).