Blood Serum DNA in Patients with Rheumatoid Arthritis is Considerably Enriched with Fragments of Ribosomal Repeats Containing Immunostimulatory CpG-Motifs

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We previously hypothesized that the sequence of transcribed region of human ribosomal repeats is selectively accumulated in circulating extracellular DNA due to its increased resistance to double-strand breaks caused by accumulation of single-chain breaks produced by nucleases. The contents of rDNA in blood serum DNA and in DNA from leukocytic nuclei both in healthy donors and in patients with rheumatoid arthritis were compared using dot hybridization method. By the content of non-methylated CpG-repeats, transcribed region of rDNA is identical to bacterial DNA, which is characterized by potent immunostimulatory effect. The transcribed region of rDNA (13.3 kb) contains more than 200 CpG-motifs capable of interacting with TLR9 receptors, which are the mediators of the cell immune response to the action of CpG-rich DNA fragments. The data suggest that DNA from dead cells circulating in the peripheral blood is enriched with sequences possessing potent immunostimulatory properties.

Key Words: *rheumatoid arthritis; TLR9; rDNA; DNA CpG-motifs*

The presence of extracellular nucleic acid in the peripheral blood of mammals induces various reactions in the immune system. DNA sequences containing unmethylated CpG-motifs interact with Toll-like receptors (TLR9) in the cells and up-regulate synthesis of cytokines via stimulation of B-lymphocytes and plasmacytoid dendritic cells [2,8,14, 15]. Therefore, unmethylated DNA-CpG motifs strengthen immunity during preventive and therapeutic vaccination [9]. At the same time, there are data on the development of severe autoimmune disturbances caused by circulation of unmethylated DNA-CpG motifs in high concentration [13]. According to most papers, bacterial DNA is the only source of unmethylated CpG-motifs activating the immune system. Mammalian genomic DNA has no pronounced immunostimulatory properties, which can be explained by low content of CpG-motifs in the genome [12]. The "neutrality" of DNA was demonstrated in studies of the effects of total genomic DNA on animals and on cultured human cells. In the organism, extracellular host DNA can differ significantly from nuclear DNA by the content of unmethylated CpG motifs. We previously hypothesized that various fragments of genomic DNA in humans can differently accumulate in the serum and plasma of the peripheral blood [2]. It was demonstrated that GC-enriched transcribed region of ribosomal repeat (TRrDNA) is resistant to the effect of double-strand breaks due to accumulation of

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single-strand breaks under conditions of nuclease hydrolysis, and can accumulate in the blood.

Our aim was 1) to compare the content of TRrDNA fragments in DNA isolated from blood serum and leukocytes of the same blood samples drawn from healthy donors and patients with rheumatoid arthritis and 2) to examine the sequence of the ribosomal repeat in humans for the presence of CpG-motifs effectively interacting with TLR9.

MATERIALS AND METHODS

We used blood samples drawn from healthy donors (n=14) and patients with rheumatoid arthritis (n=14). The diagnosis of rheumatoid arthritis was documented in accordance with guidelines of American Rheumatology Association (ARA) published in 1982. Blood titre of IgM rheumatoid factor was 1:80-1:320. The samples of peripheral blood (3 ml) were centrifuged and DNA was isolated from the serum (0.5 ml) and from precipitated leukocytes by phenol extraction [1]. DNA concentration was determined by fluorescence of DNA complexes with Hoechst 33528 fluorescent dye.

The contents of rDNA and the control sequence were determined using quantitative dot hybridization with biotinylated probes [1]. The probe pHRGRR-28S was used to measure the amount of TRrDNA in the samples. For comparative analysis, parallel samples were hybridized with sSat3 probe (human satellite 3 subfragment). The method was slightly modified. Five dots of DNA samples isolated from serum or leukocytes were placed onto a nitrocellulose filter (20 ng/dot). For construction of the calibration curve relating hybridization signal to the content of analyzed DNA repeats, the standard DNA samples containing fixed number of these repeats were also placed on the filter (20 ng/dot). After hybridization, biotin was detected with streptavidin-alkaline phosphatase conjugate (Calbiochem) followed by enzymatic reaction producing colored precipitate. The intensity of colored dots (hybridization signal) was determined using Images software, the number of repeats was determined using the linear calibration curve. The data were presented as the ratio R between the mean contents of the repeats in serum and cell DNA. DNA-SUN software was used to search for the TLR9 binding sites in human ribosomal repeats (HSU 13369, GeneBank).

RESULTS

At the first stage, DNA concentration was determined in the serum from healthy donors and patients with rheumatoid arthritis (Fig. 1): in patients this parameter

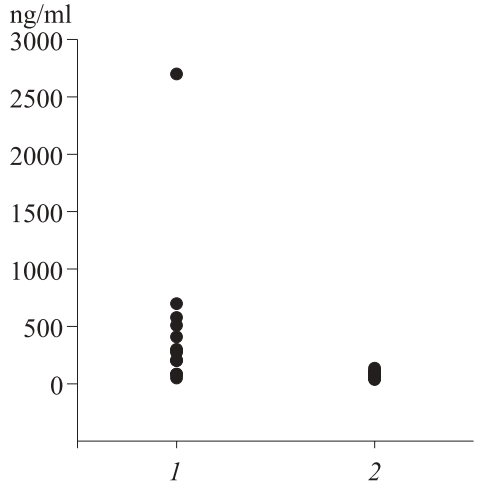

Fig. 1. DNA concentration in the serum of patients with rheumatoid arthritis (*1*, range 51-2007 ng/ml) and healthy donors (*2*, range 38-137 ng/ml). SE=7±5%.

more than 6-fold surpassed that in healthy individuals (471 and 79 ng/ml, respectively). Only 3 of 14 patients had normal level of serum DNA, in other patients serum content of DNA fragments varied from 203-2700 ng/ml. Our data agree with previous reports on increased content of extracellular DNA in the blood of patients with rheumatoid arthritis [10].

Then, we compared the content of 28S rRNA gene fragment (rDNA) and satellite 3 subfragment in serum and leukocytic DNA (Fig. 2, *a*). In humans, 300-700 copies of ribosomal repeat are present in the diploid genome of chromosomes 13, 14, 15, 21, and 22. Satellite 3 subfragment is present in 1q12 region of the first chromosome (hundreds of copies). To compare the number of repeats in serum and leukocytic DNA, the index R_{rDNA} was calculated as the ratio of numbers of repeats in 20 ng serum DNA and in 20 ng cellular DNA. In healthy donors R_{rDNA} index varied from 0.6 to 4.5 (mean 1.9). In 11 of 14 healthy donors, the number of ribosomal repeats in serum DNA 1.1-4.5-fold surpassed that in leukocytic DNA. In DNA samples from serum of patients with rheumatoid arthritis the content of rDNA 1.8-7.0-fold (3.8 on the average) surpassed the content of rDNA in leukocytic DNA. $R_{subSat3}$ was similar in both groups: 0.02-1.00 (mean 0.3) and 0.01-1.30 (mean 0.4) in control and experimental groups, respectively.

The product of index R by serum DNA concentration ($R \times C_{serumDNA}$) could assess changes in the content of repeats during simultaneous increase of serum DNA in patients compared to controls (Fig. 2, *b*). This parameter in patients far surpassed that in healthy donors (by ~14 times). In only 3 patients this parameter was low and approached the upper limit of this parameter for the control group. Similar

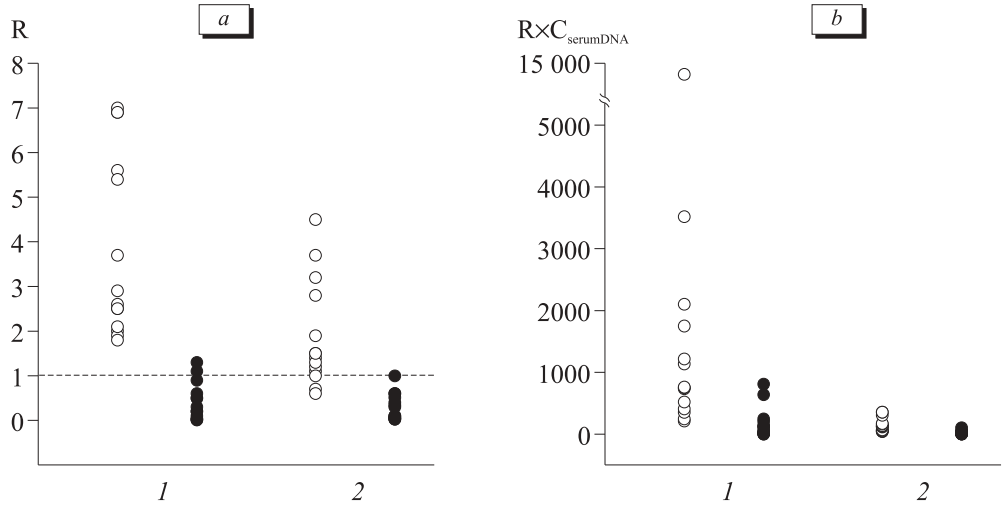

Fig. 2. Contents of rDNA (open circles) and satellite 3 (filled circles) in serum and nuclear lymphocytic DNA isolated from patients with rheumatoid arthritis (*1*) and healthy donors (*2*). *a*) R is the ratio of contents of the analyzed repeat in serum and cellular DNA. SE=16±5%. *b*) The value of *R* in relation to total serum DNA concentration (from Fig. 1).

parameter for satellite 3 subfragment was also higher in patients, but this increase was less pronounced.

In general, our study demonstrated more appreciable changes in the content of repeats in serum DNA compared to cellular DNA. Serum DNA is enriched with CpG-rich TRrDNA fragments; in patients with rheumatoid arthritis these changes are more pronounced compared to healthy donors, which agrees that published data. Previous studies showed that the content of CpG-dinucleotides in serum DNA from patients with lupus erythematosus far surpassed that in genomic DNA [11]. In healthy donors and patients with rheumatoid arthritis, the content of satellite 3 subfragment in serum DNA was several times lower than in nuclear DNA. This sequence contains a low number of CpG-dinucleotides (the same as in total genomic DNA).

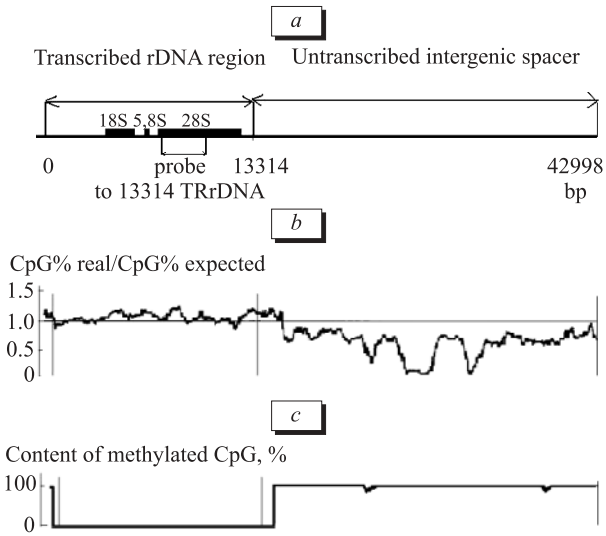

Fig. 3. The human ribosomal repeat (rDNA, *a*), the relative content of CpG-dinucleotide in rDNA (*b*), and the degree of CpG methylation in various regions of rDNA (*c*).

The qualitative change in the composition of serum DNA in comparison with cellular DNA can be explained by differences in solubility of chromatin fragments from dead cells and their capacity to circulate in the blood or remain bound to cell debris to be phagocytized by macrophages. Another explanation is different rate of DNA fragmentation by blood nucleases to short fragments excreted with the urine. The different rate of hydrolysis can be explained by the existence of protein complexes protecting some DNA sequences from nucleases or by intrinsic properties of these sequences. We previously showed that TRrDNA is more resistant to double-strand breaks due to accumulation of single-strand breaks (resulting from nuclease hydrolysis or chemical modification) than other sequences (satellite 3 subfragment, Alu-repeat, or histone genes) [2]. A possible mechanism of this phenomenon is elevated melting temperature of GC-rich rDNA with single-strand breaks.

Analysis of TRrDNA showed that the frequency of CpG-repeats approximates the expected value (Fig. 3, *b*), while the content of these repeats in total mammalian DNA is 20-fold below the expected value. Most CpG-repeats in human TRrDNA are unmethylated (Fig. 3, *c*) [6]. Within the distance of 13314 bp, TRrDNA contains 184 PuPuCGPyPy [11], 15 TCGTCG motifs [5], and 2 GTCGTT motifs [3], which determine DNA interaction with TLR9. For comparison, DNA untranscribed spacer region (similar by its composition to total genomic DNA) contains only 30 similar motifs per 30 kbp. Thus,

by the frequency of sequences interacting with TLR9, the transcribed region of rDNA is similar to bacterial DNA. Among other reasons, the increased content of TRrDNA fragments in the blood of patients with rheumatoid arthritis can favor the development of inflammation and the synthesis of autoantibodies thereby aggravating the disease. In healthy individuals, the same sequence is present in small amount sufficient to activate the protective functions of the immune system. Probably, the TRrDNA is not a unique CpG-reach sequence accumulating in the blood. It is known that CpG-islets located in the promoters of many active genes also contain a great number of unmethylated CpG-repeats [4].

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