Peripheral Blood Serum from Healthy Donors Contains Antibodies against the Fragment of Transcribed Region of Ribosomal Repeat

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Enzyme immunoassay showed that blood serum from healthy donors contains specific high-affinity antibodies (apparent association constant $\geq 5\times10^9$ M^{-1}) against a fragment of transcribed region of ribosomal DNA repeat of human serum, which are present in a free form or are bound to extracellular DNA. Preheating of the serum at 55°C and high ionic strength (1.5 M NaCl) had no effect on the interaction of antibodies with this fragment. Competitive binding assay showed that these antibodies recognize DNA epitopes, which differ from the epitopes recognized by most anti-DNA antibodies in systemic lupus erythematosus.

Key Words: *rDNA; anti-DNA antibodies; enzyme immunoassay; systemic lupus erythematosus*

Fragments from the transcribed region of the ribosomal repeat (TRrDNA) are accumulated in extracellular DNA of the peripheral blood under normal conditions and, particularly, during autoimmune diseases [1]. Human TRrDNA includes considerable amounts of unmethylated CpG-containing motifs for binding to Toll receptors on antigen-presenting cells and *in vitro* stimulates human lymphocytes [1,2]. In this respect, TRrDNA is similar to bacterial DNA exhibiting high immunomodulatory activity [2,5]. Our previous studies showed that the exposure of lymphocytes to TRrDNA is accompanied by structural changes in the nuclei and increased synthesis of ribosomal RNA and cytokines [2]. Anti-DNA antibodies (ADA) to bacterial DNA were identified in blood serum from healthy donors. These antibodies differ from ADA during autoimmune disorders in the specificity to DNA epitopes and other properties [6,7]. It may be suggested that the stimulatory effect of extracellular fragments from TRrDNA is abolished after complex formation with the corresponding ADA.

Here we tested peripheral blood serum from healthy donors for the presence of antibodies against TRrDNA fragments. Several properties of antibodies against TRrDNA were evaluated.

MATERIALS AND METHODS

Blood serum was obtained from 10 healthy donors and 8 patients with systemic lupus erythematosus (SLE). The state of patients (women, 29.8 years) corresponded to SLE criteria [3]. The duration of disease was 5 years. The patients had SLE of grade 2 [3]. All patients received hormone therapy with prednisolone in a daily dose of 15-25 mg.

TRrDNA is a mixture of linearized plasmids (pBR322 vector) with the following inserts: rDNA regions (HSU 13369, GeneBank) from -515 to 5321

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(**ETS-18SrDNA**) and 9346-10,783 (**28SrDNA**). Plasmids pUC19, pBR322, **ETS-18SrDNA**, **28SrDNA**, and p601 (sequence of the cluster of histone genes H2A, H2B, H3, and H4 in pBR322) were isolated from *E. coli* cultures. These cultures were obtained from strain MG 1655. Genomic DNA was isolated from blood leukocytes. Phage λ DNA was manufactured by Fermentas. All samples of DNA were additionally purified [2].

In variant 1 of enzyme immunoassay (EIA), DNA in buffer A (1.5 M NaCl and 0.15 M sodium citrate, pH 7.0) was placed on the membrane (Hybound ExtraC, Amersham) moistened with buffer A (3-6 spots for sample, 2-172 ng per spot), dried, and heated at 85°C for 2 h. The filter was blocked with 1% bovine serum albumin in buffer B (0.01 M sodium phosphate, 0.15 M NaCl, and 0.05% Tween 20) at 37°C for 30 min, treated with the serum in buffer B (1:200, unless otherwise specified) at 37°C for 30 min, and washed with buffers A and B. The filter was then incubated in the presence of peroxidase-conjugated secondary antibodies against IgG (Vektor-best) at 37°C for 30 min. Incubation was performed in buffer B containing 1% non-fat dry milk. This filter was washed with buffer B and placed in the substrate solution (0.5 mg/ml diaminobenzidine, 0.5 μl/ml 30% H_2O_2, and 0.1 M imidazole). After formation of the sediment, the filter was washed with water. The intensity of colored spots (signal) was estimated by computer analysis of filter images (Images software, InterEVM).

Variant 2 of EIA was performed in a 96-well plate (Costar) as described elsewhere [7]. Preliminary studies of 5 serum samples from SLE patients with known content of antibodies against calf thymus DNA revealed a linear correlation between the mean signals from spots on the membrane (variant 1) and standard signals (variant 2).

Control and experimental samples of blood serum (20 μl) were mixed with 80 μl buffer (0.01 M Tris-HCl, pH 7.5, 0.05 M $MgCl_2$, and 0.01 M $CaCl_2$) and DNase I (50 μg/ml) or denatured DNase I (control) were added. The mixture was incubated at 25°C for 40 min and heated at 60°C for 20 min. The serum was diluted with buffer B (1:200), mixed with competitive DNA (100 μg/ml), and incubated at 25°C for 60 min to study competitive binding of antibodies to DNA. The control sample was incubated without serum.

RESULTS

EIA with DNA-binding membranes was performed for detection of serum antibodies against TRrDNA (Fig. 1). The advantage of this method is the ability of membranes to effectively bind DNA fragments >200 bp. The method provides rapid high-quality visual analysis of serum antibodies against various DNA samples (Fig. 1, *a*). Computer image analysis allows us to perform a quantitative study of the intensity of staining (signal) for each sample (Fig. 1, *b*). All DNA samples from the serum of SLE patients showed similar signals (Fig. 1), which is consistent with published data that ADA in SLE recognize a certain conformation of DNA independently on base sequence [6,7]. The intensity of signals decreased after washout with high ionic strength solution. The intensity of signals from genomic DNA decreased most significantly (Fig. 1, *a*). Analysis of blood serum from healthy donors revealed signals from TRrDNA that were resistant to 1.5 M NaCl and weak signals from denatured genomic DNA, which were not found after washout with saline solution (Fig. 1, *a*). ELISA on protamine sulfate-treated polystyrene plates confirmed the presence of anti-TRrDNA antibodies in the serum from healthy donors [7].

To confirm the specificity of ADA from the serum of healthy donors to DNA epitopes, we tested 8 samples of double-stranded DNA (Fig. 1, *c*) on one filter: plasmids **ETS-18SrDNA**, **28SrDNA**, pUC19, pBR322, and p601 (sequence of the cluster of histone genes H2A, H2B, H3, and H4 in pBR322) and DNA of humans, phage λ, and *E. coli*. Only 2 samples **ETS-18SrDNA** and *E. coli* DNA showed reproducible signal for donor serum and all eight samples interacted with serum ADA from a SLE patient (Fig. 1, *c*).

Special attention was paid to the interaction of serum ADA from a healthy donor with the **ETS-18SrDNA** fragment including a promoter region, external transcribed spacer, and 18SrDNA. The interaction of ADA with **ETS-18SrDNA** was studied qualitatively using a simplified model describing this interaction as a homogeneous steady-state one-step process with the homogeneous degree of binding allowing the use of Scatchard equation. These assumptions allowed us to estimate lower limits of the association constant for the complex of ADA and **ETS-18SrDNA** (5×10^9 M^{-1}). Heating of the serum at 55°C for 30 min had no effect on complex formation. Pretreatment of the serum sample with DNase I and inactivation of the enzyme by heating was followed by 1.4-fold intensification of the signal (Fig. 2, *b*). Hence, DNA antibodies against **ETS-18SrDNA** are present in a free form or bound to extracellular DNA in the serum of healthy donors.

Analysis of competitive binding of ADA to the DNA antigen on the filter and in the solution pro-

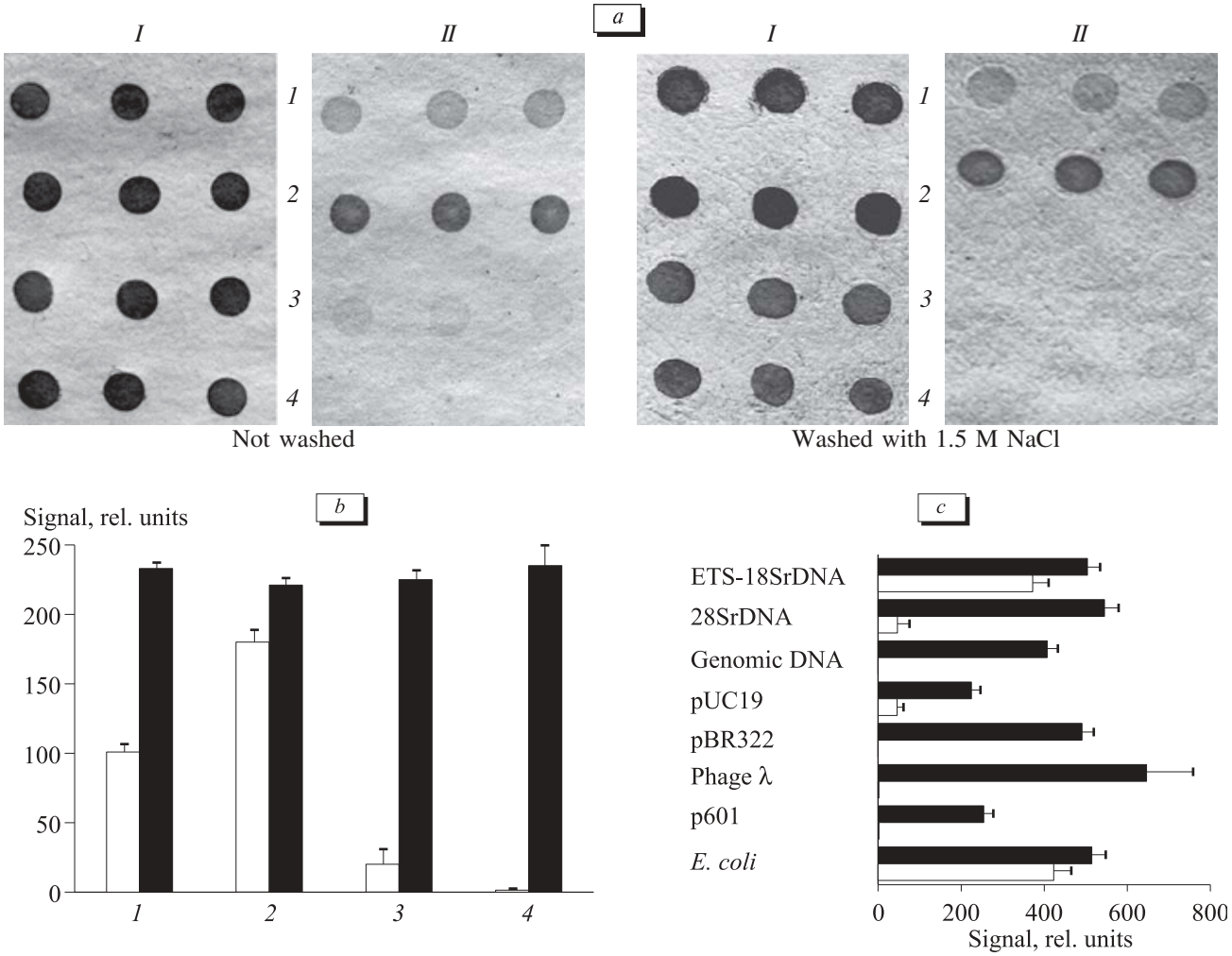

Fig 1. Evidence for the presence of anti-TRrDNA antibodies in the serum of healthy donors, which differ from ADA of SLE patients. *a*: DNA samples were put on filters (60 ng per spot, 3 spots for the sample). Filters I and II were treated with the serum from SLE patients (1:200) and healthy donors (1:200), respectively. *b*: Mean intensity of spots in filters treated with the serum from SLE patients (dark bars) and healthy donors (light bars). (*c*) The signals obtained after treatment of 8 DNA samples on one filter (100 ng, 3 spots for the sample) with the serum from SLE patients (dark bars) and healthy donors (light bars); dilution 1:200. Native sample of TRrDNA (*1*); denatured sample of TRrDNA (*2*); native genomic DNA (*3*); and denatured genomic DNA (*4*).

vided additional information about higher specificity of serum ADA from SLE patients compared to healthy donors (Fig. 3). pBR322, *E. coli* DNA, and **ETS-18SrDNA** were put on filters (40 ng per spot). pBR322 or human DNA served as competitive sequences for binding with ADA. The content of DNA-ADA complexes significantly decreased after treatment of DNA samples immobilized on the filter with serum from a SLE patient preincubated with pBR322. The decrease in the signal on samples of **ETS-18SrDNA** and *E. coli* DNA was several times less pronounced compared to that on pBR322 (Fig. 3, *a*). Study of the serum from a healthy donor showed that the intensity of signals for **ETS-18SrDNA** and *E. coli* DNA decreases insignificantly in the presence of competitive DNA (Fig. 3, *b*). Hence, pBR322 does not compete for biding to ADA under these conditions. Similar results were obtained in experiments with genomic DNA as a competitive agent. These data indicate that the major part of serum ADA in SLE patients binds to DNA epitopes with a certain conformation of DNA, which does not depend on the sequence of bases. Moreover, the serum of SLE patients contains antibodies specific to **ETS-18SrDNA** and *E. coli* DNA similar to those revealed in healthy donors. This explains less pronounced decrease in the signal, which was observed in the presence of competitive DNA on these samples (compared to pBR322).

It is now accepted that autoimmune disorders are associated with production of antibodies against host DNA fragments. The presence of ADA is an important sign of SLE. We showed for the first time that blood serum from healthy donors contains an-

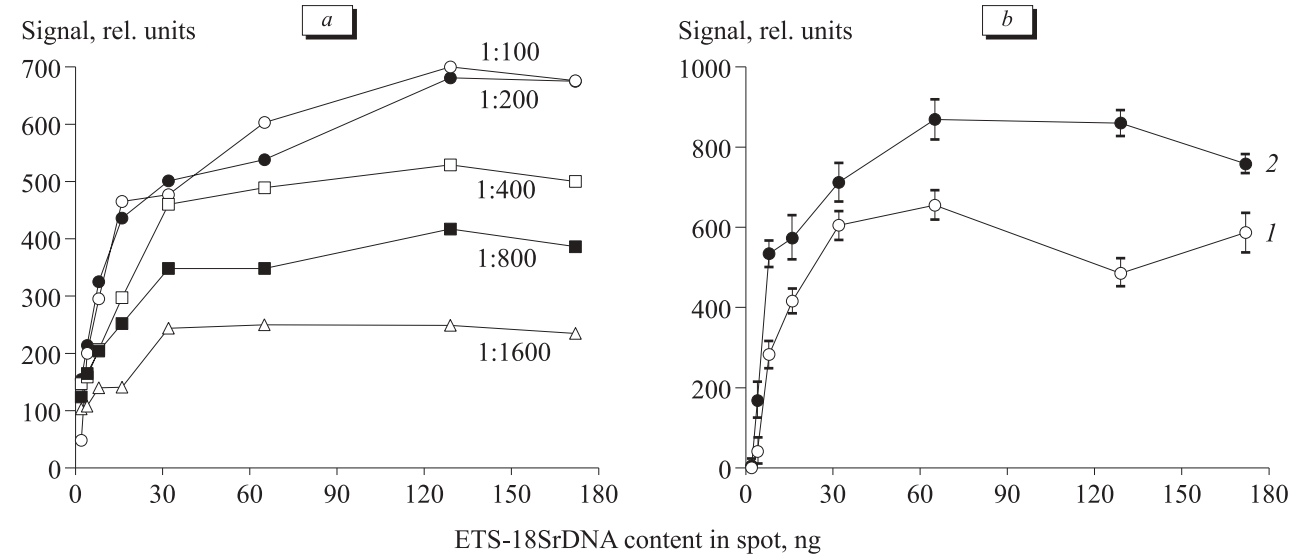

Fig. 2. Interaction of ADA from the serum of a healthy donor with **ETS-18SrDNA**. *a*: dependence of the signal on DNA content in the spot for various serum dilutions. Each point on curves is the mean for 5 DNA spots. Mean standard error 10±5% of estimated value. *b*: dependence of the signal on **ETS-18SrDNA** content in the spot for intact (*1*) and DNase I-treated serum samples (*2*); dilution 1:400.

tibodies against the fragment of human ribosomal repeat, which are present in a free form or bound to extracellular DNA. The apparent constant for the interaction of antibodies with TRrDNA is one order of magnitude higher than the constants for DNA-antibody complexes in SLE [4]. These complexes are resistant to high ionic strength. The amount of antibodies against TRrDNA varied in all healthy

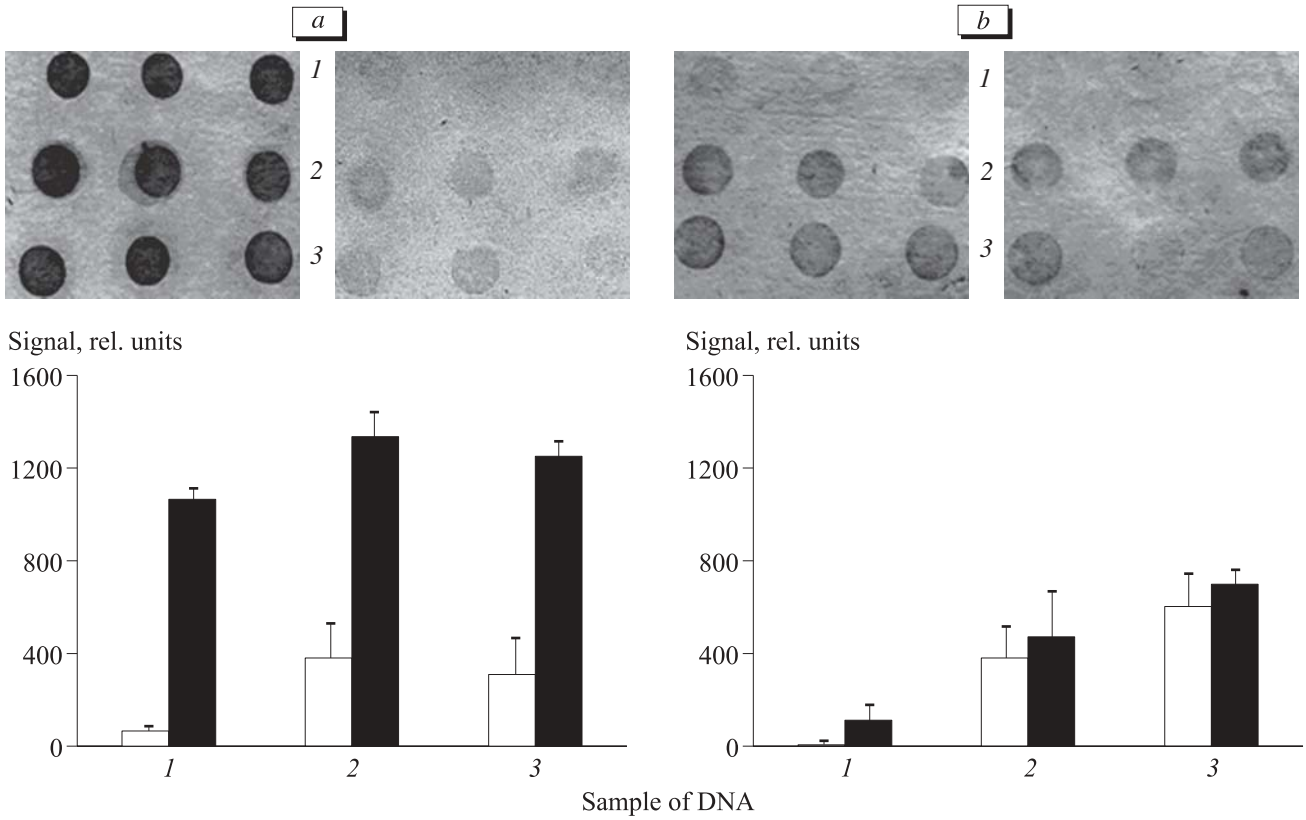

Fig. 3. Competitive interaction of serum ADA from SLE patient (*a*) and healthy donor (*b*) with DNA antigen on the filter and in the solution. Left filter and dark bars: treatment with serum samples (dilution 1:200) without addition of competitive DNA. Right filter and light bars: preincubation of serum samples with pBR322 (100 μg/ml).

donors. Our previous studies showed that TRrDNA fragments are potent immunostimulators [2]. Blockade of their activity with antibodies is probably an essential process. It can be hypothesized that changes in the ratio between the synthesis of specific antigens and amount of TRrDNA fragments released into the blood from cells due to enhanced apoptosis probably contribute to hyperstimulation of immune cells.

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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. N. N. Veiko, E. A. Kalashnikova, S. N. Kokarovtseva, *et al*., *Ibid*., **142**, No. 10, 409-413 (2006).
3. E. L. Nasonova, *Clinics and Immunopathology of Rheumatoid Diseases* [in Russian], Moscow (1997).
4. J. Ara and R. Ali, *Immunol. Lett*., **34**, No. 3, 195-200 (1992).
5. A. Dalpke, J. Frank, M. Peter, and K. Heeg, *Infect. Immun*., **74**, No. 2, 940-946 (2006).
6. D. Pisetsky, D. Frayton, and Z. Q. Wu, *J. Rheumatol*., **26**, 1934-1938 (1999).
7. D. S. Pisetsky and T. C. Gonzales, *Clin. Exp. Immunol*., **116**, No. 2, 354-359 (1999).