ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 11, pp. 1238-1246. © Pleiades Publishing, Inc., 2006.
Original Russian Text © T. A. Nevzorova, V. G. Vinter, O. A. Konovalova, M. Kh. Salakhov, 2006, published in Biokhimiya, 2006, Vol. 71, No. 11, pp. 1524-1533.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM06-064, September 30, 2006.

Mechanism of Action of DNA-Hydrolyzing Antibodies to DNA from Blood of Patients with Systemic Lupus Erythematosus

T. A. Nevzorova*, V. G. Vinter, O. A. Konovalova, and M. Kh. Salakhov

Kazan State University, ul. Kremlevskaya 18, 420008 Kazan, Russia; E-mail: Tatyana.Nevzorova@ksu.ru

Received March 23, 2006
Revision received July 10, 2006

Abstract—Four fractions of IgG antibodies to native DNA (nDNA) were obtained from blood of patients with systemic lupus erythematosus. These antibodies displayed a thermostable DNA-hydrolyzing activity and were different in affinity for DNA-cellulose and sorption on DEAE-cellulose. DNA-hydrolyzing antibodies to nDNA are metal-dependent endonucleases, cause mainly single-strand breaks in DNA, and are active over a wide range of pH. By atomic-force microscopy, three-dimensional images of DNA complexes with DNA-hydrolyzing antibodies to nDNA were obtained with nanometer resolution, and a nonprocessive action mechanism was shown for the DNase activity of antibodies to nDNA.

DOI: 10.1134/S0006297906110095

Key words: polyclonal catalytic antibodies, abzymes, systemic lupus erythematosus, IgG, hydrolysis of DNA, atomic-force microscopy

During the last twenty years, a new function of antibodies – the catalysis of various biochemical reactions – has been discovered [1-3]. By analogy with enzymes, such antibodies were called abzymes, or catalytic antibodies. Natural catalytic antibodies catalyze a wide spectrum of reactions. Abzymes of different Ig classes are generated in many diseases: autoimmune thyroiditis, polyarthritis [4, 5], multiple sclerosis [6], lymphoproliferative diseases [7, 8], different forms of viral hepatitis, AIDS and leukemia, experimental mouse autoimmune models, hemophilia, etc., as well as in milk and blood serum of virtually healthy women in labor [9, 10]. The presence in blood of highly active abzymes is usually considered to indicate autoimmune processes in the body [11, 12].

Systemic lupus erythematosus (SLE) is a severe autoimmune inflammatory disease of connective tissue with an unclear etiology. SLE is specified by high content in blood of IgG class antibodies to native DNA (nDNA), and determination of their titer is important for prognosis and diagnosis [13, 14]. Catalytically active DNA-hydrolyzing antibodies were first found among these antibodies to nDNA [15].

Abbreviations: AFM) atomic-force microscopy (microscope); ELISA) enzyme-linked immunosorbent assay; nDNA) native DNA; SLE) systemic lupus erythematosus.

* To whom correspondence should be addressed.

DNA-binding and DNA-hydrolyzing antibodies have been studied in many works [9-12, 16-19]. Many authors believe that IgG class antibodies to nDNA are responsible for development of the disease, whereas no consensus exists about the role of DNA-hydrolyzing antibodies. Researchers still have different opinions about the origin, features, structure, and action mechanisms of antibodies to nDNA, including those with nuclease activity. Data on immunochemical and enzymatic properties of the antibodies suggest the heterogeneity of the IgG antibodies to nDNA, but there is no agreement about the composition of these fractions.

Modern methods of investigation of the nuclease activity of antibodies (electrophoresis of DNA in agarose gel, linear dichroism method, electrophoresis in polyacrylamide gel containing a DNA substrate) indicate only indirectly the possible mechanisms of the antibody activity. The invention in 1986 of an atomic-force microscope (AFM) [20] allowed biologists not only to visualize macromolecules but also get information about their interaction, which is not always available when other approaches are used.

On studying two types of IgG isolated from blood serum of patients with SLE and different in the sorption on DEAE-cellulose, we have found that the DNase activity of antibodies to DNA is manifested nonprocessively, as discriminated from the activity of serum DNases [21].

The purpose of the present work was to study the DNA-hydrolyzing activity and the mechanism of interaction with DNA of four fractions of IgG antibodies to nDNA, which were obtained from blood serum of patients with SLE. These antibodies were heterogeneous in charge and displayed different affinities for DNA-cellulose.

MATERIALS AND METHODS

Blood sera of seven patients (five women from 17 to 52 and two men 48 and 62 years old) with the primarily established SLE in acute phase and not treated with prednisolone were received from hospitals of Kazan city. Blood sera of virtually healthy donors were used as the control.

Materials and reagents used were as follows: agarose NA, Sepharose CL-4B (Pharmacia, Sweden); DEAE-cellulose 23SH, microcrystalline cellulose, $MgCl_2$ (Serva, Germany); DNA from chicken erythrocytes, Bromophenol Blue (Reanal, Hungary); ethidium bromide (Koch-Light, England). Other reagents were pure for analysis and of special purity. Sterile buffer solutions were prepared using deionized water.

Fractions of IgG antibodies to nDNA were isolated from blood serum at 4°C. The preparation of IgG included salting out of IgG with $(NH_4)_2SO_4$, gel filtration on Acrilex P-6, and fractionation of the antibodies by ion-exchange chromatography on DEAE-cellulose [21]. The IgG fractions, such as that not bound with DEAE-cellulose (basic fraction I) and the bound fraction (acidic fraction II), were collected and dialyzed for 16 h against 500 volumes of 0.02 M Tris-HCl buffer (pH 7.5) supplemented with 0.05 M NaCl and 0.002 M EDTA (buffer B) at 4°C.

Antibodies to nDNA were finally isolated by affinity chromatography on microcrystalline nDNA-cellulose as a sorbent prepared as described by Litman [22]. Onto a column with nDNA-cellulose equilibrated with buffer B, the IgG fractions were placed separately after preincubation at 57°C for 45 min. The column was washed with buffer B free from proteins not interacting with the sorbent. The antibodies bound to nDNA-cellulose were eluted with 1 M NaCl in buffer B with the resulting fractions I*a* and II*a* and with 0.1 M glycine-HCl buffer (pH 2.3) with the resulting fractions I*b* and II*b*. The glycine-HCl fractions of the antibodies were immediately neutralized with 1 M Tris-HCl buffer (pH 8.0). The resulting fractions of antibodies to nDNA were concentrated, dialyzed for 48 h at 4°C against 10 mM Tris-HCl buffer (pH 7.5), and normalized by concentration.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the Laemmli method and subsequent protein staining with $AgNO_3$ [23, 24] was used to monitor the homogeneity of the antibody preparations at each stage of the antibody isolation. Values of p*I* of the IgG preparations were determined by **isoelectrofocusing** in polyacrylamide gel using a Multiphor device (LKB, Sweden) [25]. The antibody content and interaction with nDNA were assessed by **enzyme-linked immunosorbent assay** (ELISA) [21].

pBR-322 plasmid DNA was isolated and purified from *Escherichia coli* HB-101 cells by alkaline extraction with subsequent gel filtration on Sepharose CL-4B [26].

DNA-hydrolyzing activity of the antibodies to DNA was determined by conversion of the plasmid pBR-322 supercoiled DNA into the circular and linear forms. The dependence of the DNase activity of antibodies on the composition of the medium was studied by varying pH of the reaction mixture (from 5.0 to 9.8) and concentrations of bivalent metal ions.

The reaction mixture contained: 25 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$ (or 25 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA), 20 μg/ml of the pBR-322 plasmid DNA (containing 60-80% of supercoiled DNA) or chicken erythrocyte DNA. The reaction was initiated by addition of the antibodies to nDNA: once to the final concentration of 0.04-0.15 mg/ml or twice separately after incubation for 15 h to the final concentration in the mixture of 0.08-0.15 mg/ml.

In the course of incubation at 37°C, 10-μl aliquots were taken from the reaction mixtures in fixed time intervals for 1-24 h.

Kinetic parameters of hydrolysis of the pBR-322 plasmid DNA by the antibodies to nDNA (V_{max}, K_m, k_{cat}, k_{cat}/K_m) were determined by plotting [27, 28] using the Prism 4 program. The maximum concentration of abzymes to nDNA in the combined pool of the antibody fractions to nDNA was evaluated by kinetic approaches proposed by Brocklehurst [29, 30].

Results of the pBR-322 plasmid DNA hydrolysis were determined by electrophoresis in 0.7% agarose gel and subsequent DNA staining with ethidium bromide [26]. The gels were photographed, and densitograms were obtained using the Scion Image 4.0.2 (β) program. The content of supercoiled DNA was calculated with the coefficient 1.5 [31]. Hydrolysis of plasmid DNA and chicken erythrocyte DNA was assessed by atomic-force microscopy.

Atomic-force microscopy (AFM). Specimens were diluted in 25 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ to the chicken erythrocyte DNA concentration of 0.125-1.0 μg/ml, the plasmid DNA concentration of 2.5-5.0 μg/ml, or the antibody concentration of 25 μg/ml. The specimen studied (2 μl) was placed onto a freshly cleaved mica (1 × 1 cm), incubated for 3-5 min at room temperature, washed in 1 ml of deionized sterile water, and dried in air and then above silica gel.

DNA and antibodies were visualized in tapping mode in air at room temperature using a Solver P47H

AFM equipped with a Smena-B measuring head; silicon NSG11 cantilevers of 100 μm in length and with tip radius of 10 nm (NT-MDT, Russia) were used. In parallel with the measurement of the surface topography (height), changes in the cantilever oscillations (amplitude) were recorded. The scanning was performed with the resolution of 512 × 512 points and working amplitude of the cantilever oscillations of 9-23 nm [32].

The AFM images were processed and dimensions of the scanned objects were determined with Nova RC 1.0.26.578 software for NT-MDT probe microscopes. The length (nm) of DNA molecules was calculated using the DNA Processing Application 2.6 program. The length distribution of individual DNA molecules in the resulting samples was analyzed using a structural mean, the median. The difference significance was evaluated using nonparametric rank tests: Kruskell–Wallis, Mann–Whitney T-test, and the Dunn's test [33]. Each sample included more than 100 DNA molecules.

RESULTS

Considering the previously shown thermostability of the DNase activity, all antibody preparations obtained at different stages of isolation and fractionation were preheated for 45 min at 57°C [21]. Fractionation on DEAE-cellulose of antibodies from blood sera of patients with SLE resulted in two fractions of IgG containing DNA-binding and thermostable DNA-hydrolyzing antibodies, which were basic proteins with p*I* 7.16-8.3 (fraction I) and acidic proteins with p*I* lower than 7.0 (fraction II), different in sorption on the ion exchanger.

By affinity chromatography on nDNA-cellulose from each IgG fraction, two preparations of antibodies to nDNA were obtained which were different in affinity for the DNA-sorbent: subfractions *a* eluted from nDNA-cellulose with 1 M NaCl and subfractions *b* eluted with glycine-HCl buffer (pH 2.3). By PAGE and ELISA, all antibody fractions were shown to be IgG with molecular weight of 150 kD and actively interact with nDNA. The DNA-hydrolyzing activity of the antibodies to nDNA was thermostable.

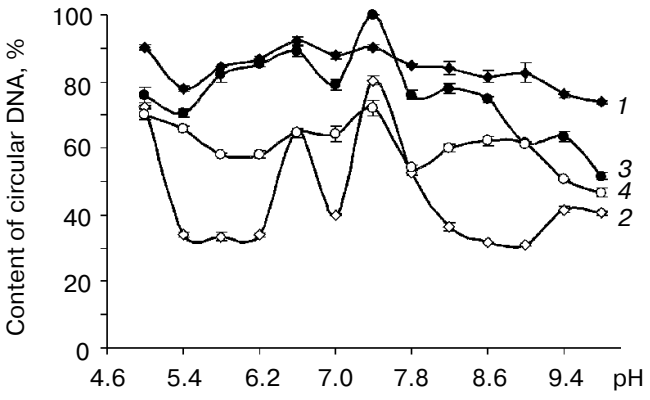

Fig. 1. The pH-dependence of the DNase activity of antibodies to nDNA of fractions I*a*, I*b*, II*a*, and II*b* (*1-4*, respectively).

Antibodies to nDNA in the resulting fractions were active in a wide range of pH values (Fig. 1); thus, their DNA-hydrolyzing activity did not depend on pH of the incubation medium. Nevertheless, the DNase activity of the I*b* fraction antibodies was the highest at pH values about 6.6 and 7.4 and the fraction II antibodies displayed an increased activity at pH of about 7.4 and weak activity maxima at low and high pH values.

All metal cations studied accelerated the DNA splitting by the antibodies, but to different extent. Mg^{2+} (and Mn^{2+} to a lesser extent) activated hydrolysis of the plasmid pBR-322 DNA by antibodies of fractions I and II at concentrations of 5 and 10 mM, respectively. However, increasing the concentration of these ions to 10 mM inhibited the activity of the I*b* fraction antibodies. Zn^{2+} and Ca^{2+} weakly activated the antibody-induced hydrolysis of DNA. Increasing the Ca^{2+} concentration inhibited the reaction catalyzed by fraction I antibodies but similarly to Mn^{2+} accelerated the DNA hydrolysis by fraction II*a* antibodies. But all antibodies to nDNA most actively hydrolyzed DNA in the presence of Co^{2+}. Afterwards, the DNase activities of the antibodies were compared with those of the described abzymes to DNA and enzymes functionally related with DNA under conditions close to optimal for the described enzymes.

To more completely characterize the DNA-hydrolyzing activity of the antibodies to nDNA, we studied kinetic parameters of hydrolysis of the plasmid pBR-322 DNA by the antibody preparations. The K_m values of the antibodies varied in the range from 0.01 to 0.1 μM. The fraction I antibodies had higher K_m values (0.05-0.1 μM) than the fraction II antibodies (0.01-0.05 μM). Values of the hydrolysis rate V_{max} (0.02-0.12 nM/min), rate constant k_{cat} ($(1.14\text{-}1.9)\cdot10^{-3}$ min^{-1}), and the hydrolysis efficiency k_{cat}/K_m (10^{-5} $nM^{-1}\cdot min^{-1}$) were low. Note that on determination of the k_{cat} and k_{cat}/K_m values the maximal calculated approximate contents of abzymes in the polyclonal antibody preparations to nDNA were used: 5-25% in fraction I*a*, 3-9.5% in I*b* fraction, 2-5.5% in II*a* fraction, and 1.5-3.5% in fraction II*b*.

The kinetics of supercoiled DNA hydrolysis by the antibodies to nDNA of different fractions were compared, and some similarities and differences were found. The antibodies hydrolyzed DNA within 12-15 h (Fig. 2, introduction of one portion of the antibodies). No considerable quantitative conformational changes occurred in DNA during further incubation. The anti-DNA antibodies of all fractions failed to hydrolyze supercoiled DNA totally even during incubation for 22 h and more.

The antibodies of subfractions *a* prepared by elution with 1 M NaCl from nDNA-cellulose hydrolyzed super-

coiled DNA more actively than the antibodies of subfractions *b* eluted from the affinity sorbent with glycine-HCl (pH 2.3), which resulted in 13-15% higher accumulation of circular DNA in the reaction products. Incubation of plasmid DNA with the antibodies of fractions I*a* and I*b* in twofold higher concentrations (Fig. 2, synchronous introduction of two portions of the antibodies) increased the hydrolysis of supercoiled DNA, but this effect was not detected in the case of antibodies to nDNA of fractions II*a* and II*b*. Open circular DNA molecules were resistant to the antibodies—there was no accumulation of linear forms of DNA.

Considering the incomplete hydrolysis of supercoiled DNA by the antibodies to DNA, the same amount of IgG antibodies to nDNA was added into the reaction medium after 15 h of incubation (Fig. 2, stepwise introduction of two portions of the antibodies). The plot shows that the repeated introduction of the antibodies resulted in additional decrease in the amount of supercoiled DNA and increase in the amount of circular DNA, and this effect was even more pronounced than the results of DNA hydrolysis by the antibodies introduced in the same total concentration at the beginning of the experiment (Fig. 2, synchronous introduction of two portions of the antibodies). Note that repeated introduction of the basic antibody fractions I*a* and I*b* caused the complete hydrolysis of supercoiled DNA after 6 and 9 h of incubation, respectively, whereas the acidic fractions II*a* and II*b* increased the hydrolysis of supercoiled DNA only by 4-10.5% 1 h after the second addition of the antibodies, and the further incubation did not change the contents of the conformations of plasmid DNA.

Based on these findings, it was suggested that the DNase-active antibodies should act nonprocessively [21]. To confirm the action mechanism of the DNA-hydrolyzing antibodies and evaluate their effect on chicken erythrocyte DNA and pBR-322 plasmid DNA, a new approach was used—atomic-force microscopy (AFM). Increasing the time of incubation of the DNA-hydrolyzing antibody with DNA from chicken erythrocytes resulted in an increase in the amount of low-molecular-weight DNA fragments.

The distribution of the lengths of DNA molecules was markedly asymmetric (the asymmetry coefficient for the unincubated DNA preparation was 0.77 and after incubation it was 0.92, which was above the critical value for the 1% significance level). Therefore, the data were compared using nonparametric methods. Changes in the mean lengths of DNA molecules in the control antibody-free preparations, one of which was incubated at 37°C, and the other was not incubated (724.5 and 706.6 nm, respectively), had no influence on results of the experiments, as manifested by the insignificant difference between them ($\alpha < 0.01$). The mean length of DNA molecules from chicken erythrocytes was significantly ($p > 0.95$) decreased after the incubation for 9 and 24 h with

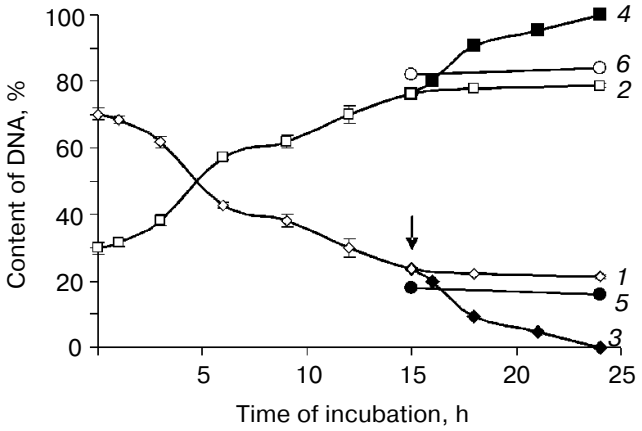

Fig. 2. Typical kinetic curves of plasmid pBR-322 DNA hydrolysis by antibodies to DNA. Introduction of one portion of the antibodies: *1*) supercoiled DNA; *2*) circular DNA. Stepwise introduction of two portions of the antibodies (the arrow indicates the time of the second introduction of the antibodies into the reaction medium): *3*) supercoiled DNA; *4*) circular DNA. Synchronous introduction of both portions of the antibodies: *5*) supercoiled DNA; *6*) circular DNA.

the fraction I antibodies to nDNA (509.6 to 273.3 nm and 566.3 to 238.7 nm for fractions I*a* and I*b*, respectively), and the variability range of the DNA lengths narrowed (Fig. 3a).

Incubation of DNA with the antibodies to nDNA of the fractions II*a* and II*b* also resulted in accumulation of low-molecular-weight DNA, but it was less pronounced. The incubation of DNA with the fraction II*a* antibodies caused a significant ($p > 0.95$) decrease in the mean length of DNA molecules to 649.8 and 469.7 nm after the incubation for 9 and 24 h, respectively (Fig. 3b). However, for the fraction II*b* antibodies the significant decrease in the mean length of DNA molecules during the incubation (from 685.7 to 548.8 nm) could be revealed only by pairwise comparison of the samples using the Mann–Whitney T-test. No differences were found when other tests were used.

The width of DNA molecules in the control preparations on AFM images varied from 8.3 to 19.8 nm (with the mean of 12.6 nm) and their height varied from 0.22 to 0.97 nm (with the mean of 0.5 nm). Molecules of the IgG antibodies on the AFM images were spherical or oval (which seemed to depend on the fit of the Y-shaped molecule onto the support), with diameters from 11.2 to 33 nm and heights from 0.4 to 1.4 nm with mean of 0.8 nm.

However, the diameter of the DNA individual molecule is known to be of about 2 nm. The lateral dimension (width) of the images of the macromolecules is overstated because of superposition of the tip shape onto the image, and the vertical dimensions (height) is understated because of the probe-caused deformation of the molecule. These artifacts are typical for AFM studies of mole-

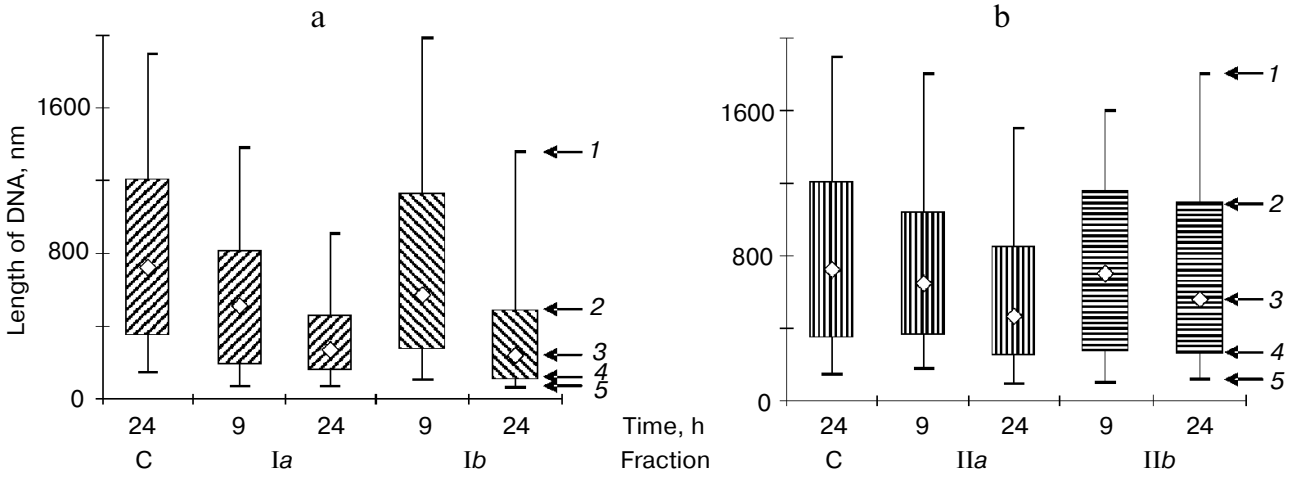

Fig. 3. Dependence of the length of DNA molecules incubated at 37°C with the antibodies to nDNA of fractions I*a* and I*b* (a) and II*a* and II*b* (b) on the time of incubation. C, DNA incubated without addition of antibodies; *1*) 95th percentile; *2*) 75th percentile; *3*) median; *4*) 25th percentile; *5*) 5th percentile.

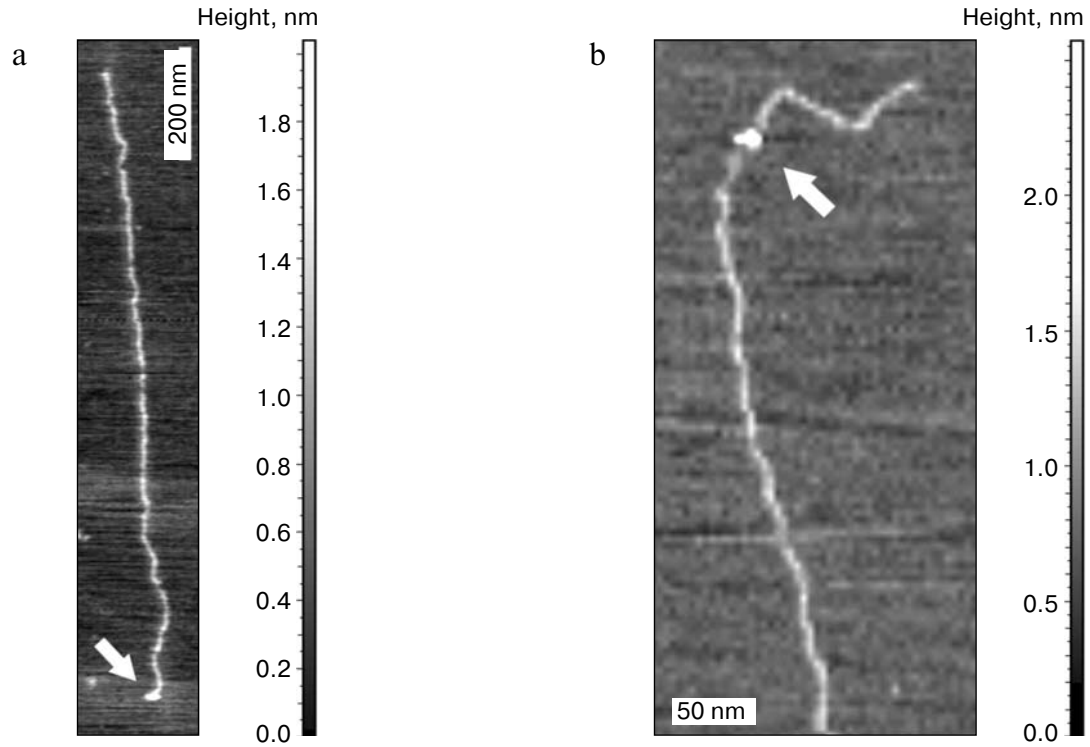

Fig. 4. Location of antibodies to nDNA (arrows) on molecules of chicken erythrocyte DNA after incubation with antibodies to nDNA at 37°C.

cules. Moreover, the height values of DNA scanned in air because of dehydration are, as a rule, lower than those of DNA scanned in fluid [34].

Incubation of the IgG class antibodies with chicken erythrocyte DNA resulted in location of the antibodies on DNA molecules. In most cases, after the incubation for 9 h the antibodies to nDNA were located in the middle of long molecules of DNA (Fig. 4). The antibodies were also visualized on the ends of the short and average length molecules, but such DNA molecules occurred consider-

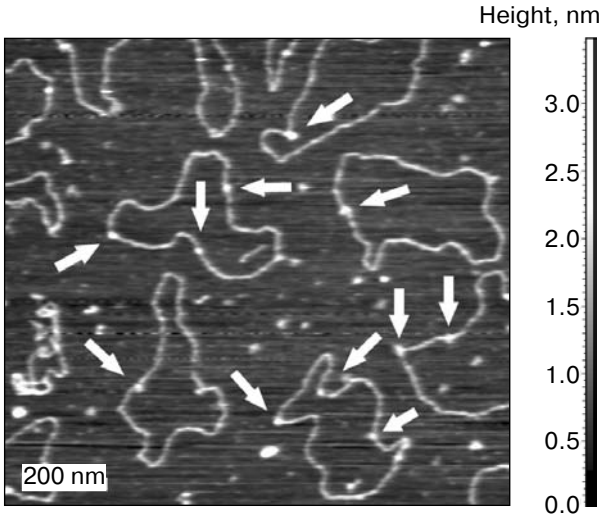

Fig. 5. Location of antibodies to nDNA (arrows) on pBR-322 plasmid DNA molecules after incubation with antibodies to nDNA at 37°C.

ably more seldom. After incubation for 24 h at 37°C, the number of short DNA molecules with the antibodies located on one or both ends was markedly increased. The DNA-bound antibodies were also visualized in the center and ends of the average length and long DNA molecules. The antibody locations on DNA were characterized by greater diameter (13.5-36.8 nm) and height (0.27-1.93 nm) than the free DNA molecules.

Incubation of DNA-hydrolyzing antibodies with pBR-322 plasmid DNA for 9 h increased the number of circular DNA molecules bound with one or several antibody molecules (Fig. 5). Scanning of the specimens revealed no more than five molecules of linear DNA with antibodies on one end. After incubation for 24 h the incidence of supercoiled DNA was significantly decreased, the amount of antibody-carrying circular DNA became markedly higher, and the incidence of linear DNA bound with antibodies to nDNA was slightly increased.

DISCUSSION

Pathologic antibodies to nDNA are known to be a fraction of IgG [16]. Therefore, in the first stage of the study attention was mainly paid to the preparation of IgG class DNA-hydrolyzing antibodies to nDNA from blood sera of primarily diagnosed patients with active SLE.

The study revealed heterogeneity of the IgG antibodies to nDNA. Similar data were also obtained by other authors [4, 7, 8, 35-38], but various chromatographic approaches allowed us to detect four fractions of the IgG antibodies to nDNA that displayed different affinities for the sorbent nDNA-cellulose, a thermostable DNA-hydrolyzing activity, heterogeneous charge, and difference in sorption on DEAE-cellulose. This finding could be explained by the presence of antibodies to different epitopes in DNA.

In the course of studies on the DNase activities of the antibody fractions to nDNA, they were found to differ in properties from one another and also from the serum DNases described in the literature.

The pH optima of the type I and type II blood DNases are in the range of 7.3-7.6 and about 5.2, respectively [39]. The weak pH dependence of the DNA-hydrolyzing activity of the antibodies seems to indicate heterogeneity of the antibody fractions to nDNA, and the differences are likely to be associated with the structure of the antigen-binding site of the antibodies. Moreover, the DNA-hydrolyzing antibodies to nDNA are not only thermostable, but also resistant to nonspecific pH-dependent denaturation. Nevertheless, the increased DNase activity of the antibodies recorded at pH of about 7.4 on comparison with the activity of blood serum DNases is close to the pH optimum of the type I DNase.

Type I DNases are known to hydrolyze DNA substrate only in the presence of 15 mM Mg^{2+} or Mn^{2+} [39]. It was shown later that according to the activation of DNase I, metal ions can be arranged as follows: $Mn^{2+} > Co^{2+} > Mg^{2+}$ [40, 41].

However, as differentiated from DNase I, DNA splitting by antibodies of all fractions was most strongly activated by the transition metal Co^{2+} at 10 mM concentration. No such observations are described in the literature, possibly because of the absence of similar studies. This effect indirectly indicates that the DNase activity of the antibodies also includes a nucleophilic attack by OH^-, which is activated by Co^{2+}.

Thus, the optimal conditions of DNA splitting by abzymes are significantly different from those for DNase I and DNase II.

Kinetic studies on hydrolysis of the pBR-322 plasmid supercoiled DNA by the antibody preparations to nDNA also confirmed the specific features found by us earlier [21]: fractions of the antibodies to nDNA were characterized by more prolonged time of hydrolysis (12-15 h) than serum DNases. The antibodies failed to hydrolyze supercoiled DNA totally even during incubation with DNA for 22 h and more. The antibodies to nDNA are likely to be endonucleases which cause single-strand breaks in molecules of supercoiled DNA and transform them into open circular molecules resistant to further action of the antibodies. This mechanism of the antibody action seems to explain the absence of linear DNA forms.

The K_m values of the antibody preparations were similar, but the antibody populations were differently eluted from the affinity matrix. It seems that the fractions of antibodies to DNA were different in their electrostatic and hydrophobic interactions with nDNA-cellulose. Nevertheless, the fraction I antibodies had higher K_m value than the fraction II antibodies.

The K_m values (0.01-0.1 μM) of all antibody preparations studied were several orders of magnitude lower than K_m values of the known human DNases [39], and such affinities are characteristic of antigen–antibody interactions. Thus, the affinity of the DNase-active antibodies for DNA was suggested to be very high. The K_m value obtained for the DNA-hydrolyzing antibodies was close to the value for some restrictases (e.g., *Eco*RI has 50 nM for pUC8 and 5 nM for pBR-322, *Rsr*I has 10 nM for pBR-322, etc.) [40] and to the K_m values for some abzymes to DNA obtained by other authors [7, 42], such as 16 nM for the oligonucleotide 5′-CCGAATTCGG-3′ [37] and 43 ± 5.8 nM for the pUC19 plasmid DNA [38].

The hydrolysis rates (V_{max}) and rate constants (k_{cat}) of the antibody fractions to nDNA were low but comparable with those of some earlier described abzymes [43, 44]. The hydrolysis efficiency k_{cat}/K_m was several orders lower than that of DNase, *Eco*RI, and even some of the described abzymes to DNA.

At present, there is no method for separating DNA-binding and DNA-hydrolyzing antibodies; therefore, the obtained preparations of antibodies to nDNA contained both DNA-hydrolyzing and catalytically inactive DNA-binding antibodies. Based on these findings, it was suggested that the k_{cat} values of abzymes could be higher, because they had been determined using the maximum calculated content of abzymes in the polyclonal preparations of antibodies to nDNA (approximately 1.5-25%), which is in agreement with the evaluation by other authors: 5-20% of abzymes of the total amount of antibodies after purification on DNA-cellulose [6]. Thus, the real efficiency of DNA hydrolysis by the antibody preparations could be considerably higher.

Polyclonal antibodies have now been shown to possess their own catalytic activities: phosphatase, protease, DNase, RNase, etc. [5, 38, 43]. Virtually all authors have recorded lower enzymatic activity of natural abzymes comparatively to activities of the corresponding enzymes, and this seems to be due to the high affinity of catalytic antibodies for antigens (0.1-10 μM) when high rates of reactions are impossible. Similarly to enzymes, antibodies are conformationally active and interact with DNA by the mechanism of induced correspondence [17, 45]. Conformational changes in molecules of both antibody and DNA [46] and also the high affinity of the antibody for DNA seem to be an explanation of the low rate of abzymes for DNA and the slow hydrolysis of the plasmid pBR-322 supercoiled DNA by antibodies to DNA.

Repeated introduction of antibodies into the reaction medium resulted in an additional decrease in the amount of supercoiled DNA and increase in the amount of open circular plasmid DNA. The incomplete hydrolysis of supercoiled DNA molecules in the incubation medium can be explained by generation of a stable immune antibody–DNA complex [21]. It seems that abzymes initially interact with DNA by mechanisms characteristic for production of immune antigen–antibody complexes and the enzymatic features of the antibodies are displayed later. But, as differentiated from enzymes, hydrolysis of the phosphodiester bond in DNA is not accompanied by liberation of the antibody of the DNA molecule.

The nonprocessive action with induction of a single-strand break is characteristic of topoisomerase I, but, as discriminated from the studied antibody, this mechanism is realized at the concentration of monovalent cations more than 150 mM [47]. To prove visually the nonprocessive action mechanism and evaluate the influence on a DNA molecule of the antibodies to nDNA, AFM was used.

Incubation of the antibody with chicken erythrocyte DNA increased the amount of short DNA fragments. Because the studies on plasmid supercoiled DNA have shown that the antibody-induced break is mainly single-strand, the production of short fragments of chicken erythrocyte DNA after the incubation with the antibodies is likely to be associated with the presence of single-strand breaks in the initial DNA preparation. On the other hand, supercoiled molecules of plasmid DNA are known to include a denatured region, which disappears on conversion to the circular form. Because after hydrolysis of the phosphodiester bond the antibody remains on DNA and circular DNA contains no denatured regions, the antibodies bind to nDNA but fail to hydrolyze it.

Thus, the DNA-hydrolyzing IgG antibodies to nDNA are shown to be endonucleases and prefer to hydrolyze denatured DNA, whereas the antibodies bind with double-stranded regions of DNA, and the antibody location on nDNA seems to depend on the nucleotide sequence. Moreover, the obtained DNA preparations are likely to contain abzymes responsible for splitting both DNA chains that can result in appearance among the reaction products of a small quantity of linear form of the plasmid DNA, which cannot be recorded by electrophoresis.

The highest activity was manifested by the abzymes to DNA prepared from fraction I. The antibodies of fractions I*a* and I*b* had higher values of K_m and isoelectric point (p*I* 7.16-8.3) and greater contents of abzymes, were likely to interact with DNA electrostatically, and could be the most pathogenic during the autoimmune process. The charge of pathogenic antibodies to nDNA is still controversial; nevertheless, pathologic IgG to nDNA is usually considered to be positively charged autoantibodies [16].

After the antibody incubation with DNA, the production of stable immune complexes of antibody with DNA was recorded by AFM, the size of which was greater than the size of individual DNA and antibody molecules. Thus, using AFM allowed us to visually establish the mechanism of action on nDNA of abzymes to DNA and show the nonprocessive character of the antibody action.

According to the literature, catalytically active sites of various abzymes are mainly located in the variable part

of light chains of Ig [6, 42, 43, 48]. In many antibodies to DNA, the ability to interact with DNA involves the heavy chain, whereas the light chain can increase, decrease, or completely inhibit this interaction and also promote the recognition of additional antigenic determinants [49-51].

The majority of authors believe that IgG class pathologic antibodies to nDNA have wide cross-reactivity. However, in some works a cross-reacting antigen not always inhibited the antibody binding to DNA [52-54]. Therefore, it was suggested that the cross interaction of antibodies with various antigens should occur in different regions of the antigen-binding site.

Based on our findings and the literature data, two regions are concluded to exist in the antigen-binding site of DNA-hydrolyzing antibodies: "anchor region" providing for the specificity of the antibody interaction with DNA, and the active site responsible for the enzymatic activity [21]. The presence of two sites of the antibody interaction with DNA in different regions of the IgG molecule seems to explain the observed nonprocessive action of the antibodies to DNA when after hydrolysis of the phosphodiester bond the antibody molecule remains bound to DNA.

It seems that different populations of antibodies to nDNA with DNA-hydrolyzing activity can have different origin and execute different functions, depending on the environmental conditions.

Because in SLE the activity of serum DNases is decreased [55, 56], some of them are likely to play the compensatory role instead of nucleases. Such antibodies possessing DNase activity can be responsible for the metabolic and protective functions in the body of patients with SLE. Abzymes to DNA can be involved in utilization of nucleosomal DNA of apoptotic cells after their being engulfed by macrophages. Because antibodies to DNA are capable of penetrating into the cell and nucleus [19], the hydrolyzing antibodies to nDNA with nonprocessive action mechanism can be involved in replication, repair, and recombination of DNA, as well as in the cell proliferation and apoptosis. The activity of abzymes to DNA can be modulated by the cell conditions. It is likely that natural abzymes can be antibodies with a unique hydrolytic site.

The authors are grateful to Doctor of Biology Z. I. Abramova (Kazan State University) for help in discussion of the results.

This work was supported by the Federal Center of Collective Use "Physicochemical Investigations of Substances and Materials" (city of Kazan).

REFERENCES

1. Tramontano, A., Janda, K. D., and Lerner, R. A. (1986) *Science*, **234**, 1566-1570.
2. Pollack, S. J., Jacobs, J. W., and Schultz, P. G. (1986) *Science*, **234**, 1570-1573.
3. Paul, S., Volle, D. J., Beach, C. M., Johnson, D. R., Powell, M. J., and Massey, R. J. (1989) *Science*, **244**, 1158-1162.
4. Vlasov, A. V., Baranovskii, A. G., Kanyshkova, T. G., Prints, A. V., Zabara, V. G., Naumov, V. A., Breusov, A. A., Giege, P., Buneva, V. N., and Nevinsky, G. A. (1998) *Mol. Biol. (Moscow)*, **32**, 559-569.
5. Paul, S., Li, L., Kalaga, R., O'Dell, J., Dannenbring, R. E., Jr., Swindells, S., Hinrichs, S., Caturegli, P., and Rose, N. R. (1997) *J. Immunol.*, **159**, 1530-1536.
6. Baranovskii, A. G., Kanyshkova, T. G., Mogil'nitskii, A. S., Naumov, V. A., Buneva, V. N., Gusev, E. I., Boiko, A. N., Zargarova, T. A., Favorova, T. A., and Nevinsky, G. A. (1998) *Biochemistry (Moscow)*, **63**, 1239-1248.
7. Aleksandrova, E. S. (1996) *Mol. Biol. (Moscow)*, **30**, 921-926.
8. Kozyr', A. V., Kolesnikov, A. V., Yakhnina, E. I., Aststurov, I. A., Varlamova, E. Yu., Kirillov, E. V., and Gabibov, A. G. (1996) *Byull. Eksp. Biol. Med.*, **2**, 204-206.
9. Nevinsky, G. A., and Buneva, V. N. (2002) *J. Immunol. Meth.*, **269**, 235-249.
10. Lacroix-Desmazes, S., Wootla, B., Delignat, S., Dasgupta, S., Nagaraja, V., Kazatchkine, M. D., and Kaveri, S. V. (2006) *Immunol. Lett.*, **103**, 3-7.
11. Nevinsky, G. A., and Buneva, V. N. (2003) *J. Cell. Mol. Med.*, **7**, 265-276.
12. Gabibov, A. G., Ponomarenko, N. A., Tretyak, E. B., Paltsev, M. A., and Suchkov, S. V. (2006) *Autoimmun. Rev.*, **5**, 324-330.
13. Arbuckle, M. R., James, J. A., Kohlhase, K. F., Rubertone, M. V., Dennis, G. J., and Harley, J. B. (2001) *Scand. J. Immunol.*, **54**, 211-219.
14. Bootsma, H., Spronk, P. E., Ter Borg, E. J., Hummel, E. J., de Boer, G., Limburg, P. C., and Kallenberg, C. G. M. (1997) *Ann. Rheum. Dis.*, **56**, 661-666.
15. Shuster, A. M., Gololobov, G. V., Kvashuk, O. A., Bogomolova, A. E., Smirnov, I. V., and Gabibov, A. G. (1992) *Science*, **256**, 665-667.
16. Foster, M. H., Cizman, B., and Madaio, M. P. (1993) *Lab. Invest.*, **69**, 494-507.
17. Jang, Y. J., and Stollar, B. D. (2003) *Cell. Mol. Life Sci.*, **60**, 309-320.
18. Kalsi, J., Ravirajan, C. T., Rahman, A., and Isenberg, D. A. (1999) *Expert Reviews in Molecular Medicine*, Access order: http//www-ermm.cbcu.cam.ac.uk/
19. Putterman, C. (2004) *Autoimmun. Rev.*, **3**, 7-11.
20. Binnig, G., Quate, C. F., and Gerber, Ch. (1986) *Phys. Rev. Lett.*, **56**, 930-933.
21. Nevzorova, T. A., Temnikov, D. A., and Vinter, V. G. (2003) *Biochemistry (Moscow)*, **68**, 1300-1306.
22. Litman, R. M. (1968) *J. Biol. Chem.*, **243**, 6222-6233.
23. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
24. Osterman, L. A. (1981) *Methods of Investigating Proteins and Nucleic Acids: Electrophoresis and Ultracentrifugation* [in Russian], Nauka, Moscow.
25. Rigetti, P. (1986) *Isoelectric Focusing* [Russian translation], Mir, Moscow.
26. Kuznetsova, N. N., and Vinter, V. G. (1997) *Methods of Gene Engineering* [in Russian], Bioinformservis, Moscow.
27. Varfolomeev, S. D., and Gurevich, K. G. (1999) *Biokinetics* [in Russian], FAIR-PRESS, Moscow.

28. Kelety, T. (1990) *Basic Enzyme Kinetics* [Russian translation], Mir, Moscow.
29. Brocklehurst, K., Resmini, M., and Topham, C. M. (2001) *Methods*, **24**, 153-167.
30. Topham, C. M., Gul, S., Resmini, M., Sonkaria, S., Gallacher, G., and Brocklehurst, K. (2000) *J. Theor. Biol.*, **204**, 239-256.
31. Ambroz, H. B., Bradshaw, T. K., Kemp, T. J., Kornacka, E. M., and Przybytniak, G. K. (2001) *J. Photochem. Photobiol. A: Chemistry*, **142**, 9-18.
32. Konovalova, O. A., Nevzorova, T. A., Vinter, V. G., and Salakhov, M. Kh. (2005) *Instruments and Experimental Techniques (Moscow)*, **48**, 802-806.
33. Lakin, G. F. (1990) *Biometry* [in Russian], Vysshaya Shkola, Moscow.
34. Moreno-Herrero, F., Colchero, J., and Baro, A. M. (2003) *Ultramicroscopy*, **96**, 167-174.
35. Kubota, T., Akatsuka, T., and Kanai, Y. (1985) *Clin. Exp. Immunol.*, **62**, 321-328.
36. Lekakh, I. V., Rott, G. M., and Poverennyi, A. M. (1991) *Mol. Biol.*, **25**, 1391-1399.
37. Gololobov, G. V., Bogomolova, A. E., Yadav, R. P., Ermolaeva, M. V., Belostotskaya, K. M., Prokaeva, T. B., Shuster, A. M., and Gabibov, A. G. (1993) *Biochemistry (Moscow)*, **58**, 183-185.
38. Gololobov, G. V., Chernova, E. A., Schourov, D. V., Smirnov, I. V., Kudelina, I. A., and Gabibov, A. G. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 254-257.
39. Shapot, V. S. (1968) *Nucleases* [in Russian], Meditsina, Moscow.
40. Burrell, M. M. (ed.) (1993) *Methods in Molecular Biology. Enzymes of Molecular Biology*, Humana Press, Totowa.
41. Baranovskii, A. G., Buneva, V. N., and Nevinsky, G. A. (2004) *Biochemistry (Moscow)*, **69**, 587-601.
42. Baranovskii, A. G., Ershova, N. A., Buneva, V. N., Kanyshkova, T. G., Mogelnitskii, A. S., Doronin, B. M., Boiko, A. N., Gusev, E. I., Favorova, O. O., and Nevinsky, G. A. (2001) *Immunol. Lett.*, **76**, 163-167.
43. Andrievskaya, O. A., Buneva, V. N., Baranovskii, A. G., Gal'vita, A. G., Benzo, E. S., Naumov, V. A., and Nevinsky, G. A. (2002) *Immunol. Lett.*, **81**, 191-198.
44. Kanyshkova, T. G., Semenov, D. V., Vlasov, A. V., Shipitsyn, M. V., Yamkovoi, V. I., Buneva, V. N., and Nevinsky, G. A. (1997) *Mol. Biol. (Moscow)*, **31**, 1082-1091.
45. Miyazaki, S., Shimura, J., Hirose, S., Sanokawa, R., Tsurui, H., Wakiya, M., Sugawara, H., and Shirai, T. (1997) *Int. Immunol.*, **9**, 771-777.
46. Pisetsky, D. S., and Gonzalez, T. C. (1999) *Clin. Exp. Immunol.*, **116**, 354-359.
47. Tereshchenko, O. D., and Khaidarova, N. V. (1983) *Usp. Sovr. Biol.*, **96**, 28-45.
48. Kanyshkova, T. G., Semenov, D. V., Khlimankov, D. Yu., Buneva, V. N., and Nevinsky, G. A. (1997) *FEBS Lett.*, **416**, 23-26.
49. Li, Z., Schettino, E. W., Padlan, E. A., Ikematsu, H., and Casali, P. (2000) *Eur. J. Immunol.*, **30**, 2015-2026.
50. Radic, M. Z., and Seal, S. N. (1997) *Methods*, **11**, 20-26.
51. Vargas, M. T., Gustilo, K. G., D'Andrea, D. M., Kalluri, R., Foster, M. H., and Madaio, M. P. (1997) *Methods*, **11**, 62-69.
52. Sharma, A., Isenberg, D. A., and Diamond, B. (2001) *J. Autoimmun.*, **16**, 479-484.
53. Caponi, L., Chimenti, D., Pratesi, F., and Migliorini, P. (2002) *Clin. Exp. Immunol.*, **130**, 541-547.
54. Pautova, L. V., Rykova, E. Yu., Laktionov, P. P., and Vlasov, V. V. (1996) *Mol. Biol. (Moscow)*, **30**, 941-950.
55. Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H. G., and Moroy, T. (2000) *Nature Genetics*, **25**, 177-181.
56. Tsukumo, S.-I., and Yasutomo, K. (2004) *Clin. Immunol.*, **113**, 14-18.