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Dynamics of Antibody Nuclease Activity in Blood of Women during Pregnancy and Lactation

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Abstract—In human milk we previously found catalytic antibodies (abzymes) catalyzing hydrolysis of DNA, RNA, NMP, NDP, and NTP and also phosphorylation of proteins and lipids. In the present study we have analyzed nuclease activities of antibodies in blood of women during pregnancy and lactation. Blood of healthy male and female volunteers lacked catalytically active antibodies, whereas antibodies from blood of pregnant women hydrolyzed DNA and RNA and their relative activity varied over a wide range. Relative blood abzyme activities significantly increased after delivery and at the beginning of lactation. The highest abzyme activity was observed in blood of parturient women. Although the dynamics of changes in antibody DNase activity during pregnancy was rather individual for each woman, there was a common trend in the increase in antibody activity in the first and/or third trimester of the pregnancy. The DNase activity of IgG and IgM from blood of healthy pregnant women was 4-5 times less than that from pregnant women with pronounced autoimmune thyroiditis.

Key words: human milk and blood, pregnancy and lactation, immunoglobulins, natural abzymes, hydrolysis of DNA and RNA

During the last 15 years, the catalytic function of antibodies has been recognized. Such antibodies were called abzymes or catalytically active antibodies. There are more than 100 various reactions catalyzed by antibodies to stable analogs of transition state reaction complexes. These data have been considered in several reviews on design of effective catalytic antibodies and the development of a new scientific discipline, abzymology [1-7].

Natural abzymes exhibiting various catalytic activities are now known. These include proteolytic, phosphatase activities in patients with autoimmune diseases (e.g., bronchial asthma, systemic lupus erythematosus, autoimmune thyroiditis, polymyositis, polyarthritis, and multiple sclerosis) and some viral diseases (e.g., hepatitis, AIDS) (see for review [6, 8-11]).

Blood of patients with some autoimmune diseases contains increased concentrations of DNA and anti-DNA antibodies [10] which appear in blood as the result of apoptosis. Apoptotic cell antigens are recognized as the targets for autoantibodies [6-9]. Many anti-DNA antibodies are against nucleosome histone–DNA complexes, which appear as the result of apoptotic internucleosome cleavage. Apoptotic cells are the primary source of antigens and immunogens in systemic lupus erythematosus; these peculiarities of recognition, "understanding", processing, and/or presentation of apoptotic autoantigen by the antigen presenting cells may lead to autoimmune processes [6-9].

Autoimmune diseases are characterized by spontaneous production of primary antibodies to proteins, nucleic acids, and their complexes and then by production of secondary antibodies to primary antibodies, etc. [12, 13]. There is a notion that blood of patients with various autoimmune diseases may contain various antibodies, including antibodies to antigen with altered confor-

Abbreviations: AB) antibody; ON) oligonucleotide; DTT) dithiothreitol.

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mation (transition state analogs) or antiidiotypic antibodies. Their appearance may be explained on the basis of Jerne's model of an antiidiotypic network [14]. According to modern concepts, the presence of catalytic antibodies in the blood is a clear sign underlying the development of autoimmune processes in patients [9-11]. The existence of natural abzymes in donors without manifestations of any pathology of immune status was considered for a long time as improbable due to the absence of evident immunization. However, we initially described the presence of sIgA in milk of healthy parturient women; these antibodies catalyzed protein phosphorylation [15-17]. Later we found that small subfractions of polyclonal IgG and sIgA from human milk catalyzed hydrolysis of DNA, RNA [18-21], ribo- and deoxyribo-NMP, -NDP, and -NTP [22] and also cleavage of 5′-terminal phosphate of DNA and RNA (phosphatase) [20, 21] and polysaccharide hydrolysis [23]. Human milk antibodies are usually characterized by higher catalytic activity than most known abzymes from patients with autoimmune diseases [9-11]. It should be noted that abzymes were not found in healthy people and in patients with influenza, pneumonia, tuberculosis, tonsillitis, duodenal ulcer, and in some malignant tumors (e.g., uterus, breast, and intestine cancers) [9-11] which do not alter the immune status. This raised absolutely new questions on the origin of abzymes and on immune status of pregnant and lactating women. Taking into consideration these problems, we have analyzed the literature data on immune system of women during pregnancy and lactation. According to the literature data, blood of pregnant women (as well as blood of patients with autoimmune diseases) contains increased concentrations of DNA [24-26] and low concentrations of fetal cells [27]. There is correlation between increased incidences of fetal cells in mother's peripheral blood and such autoimmune disease as scleroderma [27]. However, manifestations of rheumatoid arthritis reduce or even disappear during pregnancy [26], but other autoimmune diseases (e.g., systemic lupus erythematosus, anti-phospholipid syndrome [28], etc.) exacerbate in pregnant women. Appearance of some autoimmune diseases was also found during pregnancy of healthy women. Delivery may cause sharp provocation of autoimmune pathology (autoimmune shock). In some women, postpartum autoimmune pathologies develop irrespectively to autoimmune shock. These include thyroiditis, renal insufficiency, hemolytic–uremic syndrome, idiopathic polymyositis, anti-phospholipid syndrome, and autoimmune myocarditis [27, 29]. Postpartum autoimmune diseases may appear right after delivery or later. Autoimmune thyroiditis is one of the most frequent autoimmune disorders. According to different studies, its frequency varies from 1.9 to 16.7% [30, 31]. Maximal manifestation of autoimmune thyroiditis is usually observed during the first 3-6 months, but not later than one year after delivery.

Thus, analysis of literature data provides convincing evidence for the existence of pregnancy- and/or delivery-provoked predisposition to autoimmune processes similar to that observed in patients with autoimmune diseases. The question is whether all these autoimmune processes in patients with autoimmune diseases and in parturient women have similar nature and whether they involve similar or different mechanisms. It should be mentioned that typical autoimmune diseases are chronic diseases characterized by periods of attack and remission, whereas pregnancy-associated signs of autoimmune disorders are often limited by pregnancy and postpartum period. For example, the concentration of autoantibodies to thyroglobulin is lower during pregnancy than that before pregnancy and after delivery; the lowest concentration is observed during the third trimester [32, 33]. Study of 545 pregnant women revealed antibodies to thyroid hormone and/or thyroid peroxidase (Tab+) in 102 (18.7%) of pregnant women and 33% of them suffered from postpartum thyroiditis. In pregnant women lacking antibodies to these autoantigens, only 3% of pregnant women had incidences of autoimmune thyroiditis [34]. In other words, disappearance of autoimmune disorders during the postpartum period is normal although in some cases "typical activation of autoimmune processes" in pregnant women may be smoothly or sharply (via autoimmune shock) transformed into typical chronic autoimmune process.

The behavior of the immune system during pregnancy is of great interest. Progress in immunology of pregnancy promotes development of transplantation methods; this needs detailed study of immunologic tolerance of mother and fetus. In spite of numerous data that have accumulated, this problem still requires clarification of many important points and some controversial results. Pregnancy is characterized by significant reorganization of the immune system, which does not disturb fetal development; moreover, it does protect the fetal organism against infection and embryo implantation triggers this process.

As stated above, in human milk of parturient women we found abzymes with various catalytic activities including unique protein and lipid kinase activities [35, 36]. However, abzymes from blood of pregnant and lactating women have not been investigated yet. Study of catalytic activities of antibodies during pregnancy and lactation of healthy women and those with autoimmune diseases may help to evaluate features of their immune status and specify or determine possible reasons and mechanisms underlying appearance of autoimmune disorders in pregnant and lactating women.

In the present study, we have analyzed DNA- and RNA-hydrolyzing activity of antibodies in blood of women before and after delivery and investigated the dynamics of catalytic activities of antibodies during pregnancy. The data indicate that relative activities of various abzymes may vary over a wide range (and even be absent

in some cases), but generally their level significantly increases after delivery and onset of lactation.

MATERIALS AND METHODS

Blood plasma of healthy pregnant and parturient women was obtained from Polyclinic No. 1, Central Clinical Hospital, Siberian Branch of the Russian Academy of Sciences (Novosibirsk). Human milk was obtained from various donors 0.3-5 months after onset of lactation. Blood plasma of women suffering pregnancy-induced autoimmune thyroiditis was obtained from the Novosibirsk Medical University. These women were observed at Novosibirsk city clinics.

Milk IgG and sIgA antibodies were isolated by affinity chromatography on protein A-Sepharose followed by chromatography on DEAE-cellulose and gel filtration under conditions of decomposition of the immune complexes (separation of IgG and sIgA antibodies) [18-21]. Before FPLC gel filtration, 250 μl of antibody solution (about 1 mg/ml) was mixed with 83 μl 3 M $MgCl_2$ and 166 μl 3 M NaCl, incubated for 30 min and centrifuged at 10,000 rpm for 10 min. High performance gel filtration was carried out using a Superdex 200 HR column 10/30 (100 × 300 mm) and Sprint Biocard chromatograph (Pharmacia, Sweden). The column was equilibrated with 20 mM Tris-HCl buffer, pH 7.5. A sample (antibody solution) was applied onto the column after addition of 2 ml of buffer containing 0.5 M $MgCl_2$ and 1 M NaCl. Proteins were eluted with 20 mM Tris-HCl buffer, pH 7.5, at a flow rate 0.2 ml/min.

Isolation of IgG from blood plasma. Plasma obtained after mixing of 2.0 ml of the whole blood with 0.5 ml of 4% citrate buffer followed by centrifugation at 5000 rpm for 10 min was mixed with saturated ammonium sulfate solution (1 : 1 v/v). Homogenous antibody preparations were obtained by the above described procedures for purification of milk abzymes, which included gel filtration (separation of IgG and IgM). Electrophoresis of proteins in the presence of sodium dodecyl sulfate was carried out by the method of Laemmli [18, 37]; silver staining of proteins was carried out as described [38].

Abzyme purification on protein A-Sepharose included so-called acidic shock; such treatment is often fatal for many enzymes, which are completely inactivated [9-11]. After such treatment, antibodies slowly recover during storage in neutral buffer at 4°C for 0.5-2 months. So, catalytic activity of antibodies was analyzed at least 1-3 weeks after the last purification stage. During this period some antibodies were partially destroyed with formation of free light and heavy chains.

DNA hydrolysis. Catalytic activity of antibodies was evaluated by conversion of supercoiled DNA of Bluescript plasmid into circular nicking and linear forms and short oligonucleotides (as in the case of phage λ DNA). The reaction mixture (final volume 20 μl) contained 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 150 ng of phage λ DNA (or Bluescript plasmid). The reaction initiated by adding 10^{-6}-$5 \cdot 10^{-7}$ M antibody was carried out at 37°C for 2 h. Reaction products were analyzed by electrophoresis in 1% agarose gel using the following buffer: 40 mM Tris-acetate, pH 7.5, 1 mM EDTA. For evaluation of DNA conversion, the gels were stained with ethidium bromide (0.5 μg/ml), washed with water, and photographed under UV light.

Hydrolysis of oligodeoxyribonuleotides (ON). Homogenous 5′-[^{32}P]ON were obtained as described in [39]; their concentrations were determined using molar absorbance coefficients [40]. The reaction medium (of total volume of 10-20 μl) contained 20 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 10^{-5}-$5 \cdot 10^{-6}$ M ON substrate. The reaction was started by adding 10^{-6}-$5 \cdot 10^{-7}$ M antibody. After incubation for 3-6 h, the reaction medium was mixed with an equal volume of 95% formamide containing 0.1 mM EDTA, 0.025% xylene cyanole, and 0.025% bromophenol blue. The reaction products were analyzed by electrophoresis in 20% polyacrylamide gel containing 7 M urea and autoradiographed for 15 min. The degree of hydrolysis was determined by the ratio of the sum of all products to initial labeled ON; gel sites corresponding to spots on the radioautograph were excised and the amount of labeled compounds was determined by the Cherenkov method.

In gel analysis of DNA-hydrolyzing activity of antibody (*in situ*) [18]. This testing was carried out by means of SDS-electrophoresis in polyacrylamide gel containing 5-20 μg/ml polymerized bovine spleen DNA. For intact antibodies, electrophoresis was carried out in gradient 5-15% polyacrylamide gel; 12% gel was used for analysis of reduced antibody preparations (light and heavy chains). The reduction procedure was carried out by incubating preparations in the presence of 1 mM dithiothreitol at 37°C for 20 min. After the electrophoretic separation of proteins, gels were washed with 4 M urea (for 1 h) and water (10 times for 7 min) to remove SDS. For reduction of antibody activity, gels were incubated in 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, 1 mM EDTA at 37°C for 16 h. After this procedure the gels were stained with ethidium bromide (0.5 μg/ml). Hydrolysis of DNA was developed as a dark spot on homogenous fluorescent background. Position of protein bands was identified after staining with Coomassie R-250.

Chemicals. Tris, 2-mercaptoethanol, 1,4-dithiothreitol (DTT), ammonium persulfate, EDTA, and agarose were from Serva (Germany); SDS was from Merck (Germany); N,N′-methylene-*bis*-acrylamide, acrylamide, bromophenol blue, and protein A-Sepharose CL-4B were from Pharmacia (Sweden); magnesium chloride was from Sigma (USA). Superdex 200 HR 30/10 was purchased from Amersham-Pharmacia-Biotech (Sweden); [γ-^{32}P]ATP (specific activity 0.7-0.8 Ci/mmol) was pro-

duced by Biosan (Novosibirsk, Russia). ON were kindly presented by G. A. Maksakova (NIBKh, Siberian Branch of the Russian Academy of Sciences). Phage T4 polynucleotide kinase (specific activity 10 U/μl) was from SibEnzyme (Novosibirsk, Russia); phage λ DNA was produced by NPO Vector (Novosibirsk, Russia). Bluescript plasmid was the generous gift by D. V. Bugreev (NIBKh, Siberian Branch of the Russian Academy of Sciences). All other chemicals of "specially pure grade" were produced by Reakhim (Russia).

RESULTS AND DISCUSSION

Our original method developed for abzyme purification from human milk and blood includes affinity chromatography on protein A-Sepharose, ion-exchange chromatography on DEAE-cellulose, and gel filtration on Superdex 200 HR [10]. Use of this method yields electrophoretically homogenous IgG, sIgA, and IgM antibody preparations. Figure 1 shows the result of typical electrophoretic analysis of IgG from blood of a pregnant woman. These abzyme preparations are homogenous before (Fig. 1, lane *2*) and after (Fig. 1, lane *5*) treatment with DTT. The IgG preparation contained only light (L) and heavy (H) chains of 25 and 50 kD, respectively.

Evidence for the existence of nuclease activity of antibodies. Experimental evidence underlying catalytic activity of an antibody should usually meet 13 rather strict criteria (see for review [10, 11]). Earlier we employed all these criteria for attribution of DNA- and/or RNA-hydrolyzing activities to IgG and sIgA antibodies from parturient women. Analysis of these criteria revealed lack of catalytic activity of antibodies isolated from blood of healthy donors (men and women). In this study, we investigated the possibility of the existence of catalytic activity of antibodies from milk and blood of healthy pregnant and lactating women. Employment of some of the 13 criteria revealed that antibodies from these groups of women can catalyze DNA hydrolysis (data not shown). It should be noted that the criteria employed gave definitive on the attribution of catalytic activity to antibodies rather than to any possible contamination. These include demonstration of catalytic activity of antibodies *in situ* after protein separation by SDS-electrophoresis in gel containing a substrate [18]. Previously we found that this is the strictest criterion; if it is applicable, other (less strict) criteria are applicable too [9-11].

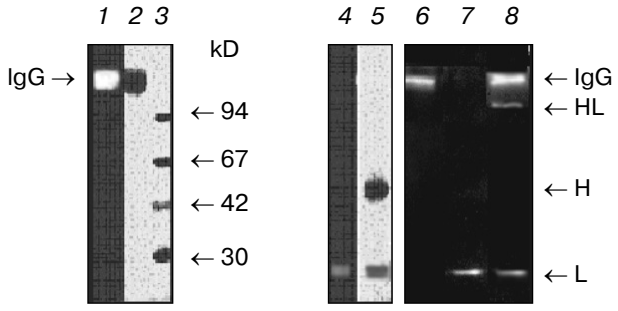

Fig. 1. Electrophoretic analysis of purified IgG preparations (Coomassie staining) isolated from human milk before (lane *2*) and after treatment with DTT (lane *5*). Lanes *1* and *4* correspond to analysis of milk IgG activity by the in gel method *in situ* (gel contains DNA, negatives are shown). Lanes *6*, *7*, and *8* correspond to analysis of activity of IgG from parturient women's blood by *in situ* method before (*6*) and after total (*7*) and also after partial (*8*) reduction of disulfide bonds with DTT. Lane *3* contains protein markers of known molecular mass.

Figure 1 shows that after SDS-electrophoresis of milk IgG in DNA-containing gel, hydrolysis of DNA is observed before reduction only in the sites corresponding to position of protein bands of initial IgG (Fig. 1, lane *1*). After disulfide bond reduction with DTT, sites of DNA hydrolysis corresponded to position of antibody light chain (Fig. 1, lane *4*). This unequivocally suggests that catalytic activity is an intrinsic property of milk IgG light chains.

DNase activity of human plasma antibodies of pregnant and lactating women was significantly lower than that of milk antibodies. Previously we demonstrated that treatment of abzymes with SDS cause their deep denaturation accompanied by loss of catalytic activity [9-11]. During subsequent renaturation, catalytic activity was restored in a small proportion of antibody molecules. So, only the most active antibody preparations could catalyze hydrolysis of Bluescript plasmid. Nevertheless, Fig. 1 shows, that DNase activity was detected in the case of IgG and IgG light chains from blood plasma of pregnant and lactating women.

Thus, in the present study we have demonstrated for the first time that not only milk but also blood plasma of pregnant and lactating women may contain DNA-hydrolyzing abzymes. As in the case of human milk IgG, DNase activity of blood plasma Ig is the intrinsic property of antibody light chain. This is consistent with literature data that in most cases catalytic sites of various abzymes are located in the variable parts of Ig light chains [10]. However, certain evidence exists that active site formation in natural abzymes and in antibodies with catalytic activity induced by transition state analog formation of active sites may involve not only light chains but also heavy chain [10]. For example, formation of DNA-hydrolyzing sIgA and ATP-hydrolyzing IgG of human milk involves both light and heavy chains [21, 22]. Perhaps, organization of active sites of natural abzymes may vary from one catalytic antibody to another, but DNA-hydrolyzing IgG from blood plasma of parturient women are related to the most common type of abzymes found in autoimmune diseases, when the active site is located in the light chain.

Dynamics of DNase activity of blood antibodies during pregnancy and post-parturient period. There were several reasons to compare relative catalytic activity of blood antibodies during pregnancy and also before and after onset of lactation. At the first glance, during pregnancy and after delivery women lack evident signs of autoimmune diseases. However, literature data suggest that during pregnancy women are subjected to "latent external" and "internal immunization", autoimmunization [11]. As stated above, blood of pregnant women contains increased concentrations of DNA and small amounts of fetal cells. It is also known that immunization of animals with various proteins 1-3 months before delivery (but not earlier!) was accompanied by accumulation of high levels of antibodies to these proteins in milk [41, 42]. According to results of our studies [43-45], levels of human milk abzymes increased by orders when women suffered from viral or allergic diseases during pregnancy (not far from delivery). In other words, pregnant women are characterized by special type of "immune response" to food antigens and various viruses or bacteria. This suggests that pregnant women have specific "immune memory" [11]. However, it remains unclear whether "latent external" and "internal" immunizations of women may be realized during pregnancy or only after onset of lactation.

Since DNA level increases in blood or pregnant women at the first and the third trimesters [24-26] we investigated DNA-hydrolyzing activity of blood antibodies in women during various trimesters of pregnancy. DNase activity of antibody preparations was determined in blood serum using phage λ DNA and Bluescript plasmid DNA. Figure 2 shows that blood serum DNase activity of IgG from donors of closing period of gestation (28-32 weeks) significantly differed depending on the donor. For example, antibodies from donors 1 and 2 exhibited high DNase activity, whereas in antibodies of donors 7 and 9 this catalytic activity was not found. Thus, it is clear that relative DNase activity is an individual feature of the certain donor.

Figure 3 shows the dependence of relative DNase activity of antibodies from four women on gestation period. In the first donor, increase in DNase activity was observed during the first trimester (8 weeks) and then this activity remained at the same level. In the case of the fourth donor, the activity remained unchanged over the whole gestation period. The tenth donor (as well as the first one) was characterized by pronounced increase in DNase activity during the first trimester (at 12-13 weeks), then the activity decreased (14-19 weeks) and increased again at the end of gestation. In the 11th donor initially low DNase activity sharply increased at gestation period of 18-26 weeks and then it sharply returned to the initial level.

These results clearly demonstrate that patterns of changes of DNase activity of antibodies do not coincide

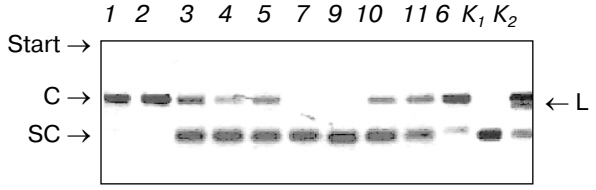

Fig. 2. DNase activity of IgG preparations from blood of pregnant women (28-32 weeks). Bluescript plasmid DNA was used as substrate. (Negatives are shown.) Lane numbers *1-11* correspond to donor number. K_1) DNA incubated in the absence of antibodies; K_2) milk antibody (positive control). Forms of plasmid: C) circular; L) linear; SC) supercoiled.

in different pregnant women during gestation. Nevertheless, in three of four cases higher catalytic activity of antibodies was found in the first than in the third trimesters (Fig. 3). This is consistent with observation that increased DNA content in blood of pregnant women is often observed during the first and the third trimesters of gestation [26], and the last gestation trimester is characterized by apoptosis typical for autoimmune diseases [46]. The second trimester is usually characterized by decreased DNA content in blood. Generally, the level of DNase activity in the third trimester is one order of magnitude higher than that observed during the first trimester.

In the same series of donors, we tested changes of catalytic activity of antibodies by hydrolysis of single

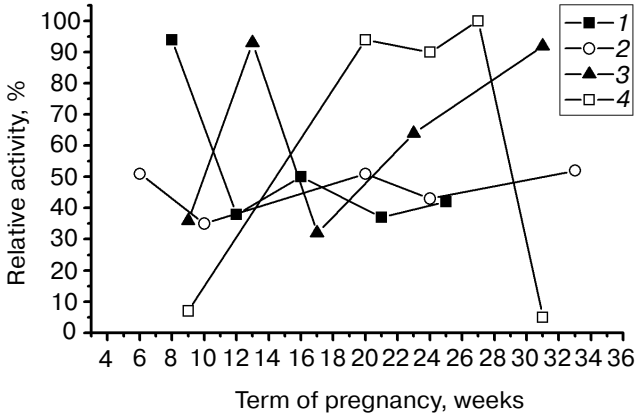

Fig. 3. Dynamics of relative DNA-hydrolyzing activity of antibodies from blood of four pregnant women (*1-4*). Concentrations of antibody preparations selected for determination of relative catalytic activity favored preferential formation of circular nicking form during DNA hydrolysis. The degree of hydrolysis was evaluated by transition of supercoiled DNA into the circular form (in %). The highest activity of antibody preparation among all antibody preparations tested was defined as 100%. At 10^{-7} M this antibody preparation caused 100% transition of supercoiled DNA into the circular form.

stranded oligonucleotides (data not shown). These experiments revealed that in one case oligonucleotide hydrolysis was not detected, but in the three other cases low catalytic activity of abzymes increased in the third trimester. This activity of blood abzymes was higher with $r(pU)_{10}$ rather than with $d(pT)_{10}$ employed as substrates. This is consistent with literature data that IgG from blood of patients with various autoimmune diseases can hydrolyze various types of RNA 10-1000-fold more effectively than oligodeoxyribonucleotides [47]. Moreover, even mouse monoclonal antibodies to various DNA sequences were 50-100-fold more active in hydrolysis of ribonucleotides than deoxyribonucleotides [48].

For studies of catalytic activity of antibodies we elected four donors; three of them characterized by detectable DNA-hydrolyzing activity at later gestation period. Blood of one woman lacked this activity before delivery. Figure 4 shows the dynamics of DNase activity at later stages of gestation and after delivery. Delivery caused marked increase in DNA hydrolyzing activity. Even in the case of complete absence of the catalytic activity of antibodies (donor 7) during gestation, DNase activity appeared after delivery. Comparison of DNase activities of antibodies in milk and plasma of the same parturient women revealed that milk abzymes are more active than blood abzymes (Fig. 5). Thus, it is clear that lactation is the major factor responsible for increase in abzyme activity; it has more pronounced effect on catalytic activity of milk rather than blood antibodies.

Abzyme production during gestation is very individual. This is consistent with literature data on individual characteristics of abzyme production in blood of patients with various autoimmune diseases. In these patients the repertoire of DNA- and RNA-hydrolyzing polyclonal IgG and IgM may be quite narrow or wide and very heterogeneous catalytically active antibodies may contain light chains of both type κ and λ. They can exhibit catalytic activity at various pH-optima, have different total charge and affinity to substrates, and demonstrate various dependence of catalytic activity on the presence of mono- and bivalent metal ions [10, 11, 47-61].

Since blood of healthy volunteers lacks detectable quantities of DNA-hydrolyzing antibodies and IgG of pregnant women are catalytically active, changes in DNase activity of antibodies during gestation may be considered as specific characteristics of altered immune status of (pregnant) women.

Effect of autoimmune reactions during pregnancy on the level of catalytically active antibodies in blood. Earlier we studied occurrence of DNA-hydrolyzing abzymes in 120 (male and female) patients with autoimmune thyroiditis [59, 60]. These studies revealed that relative level of catalytic activity of Ig-abzymes in DNA hydrolysis accompanied increase in blood concentration of thyroglobulin and antibodies against it. Antibodies possessing DNase activity were found in 65% of patients with autoimmune thyroiditis. All patients (100%) with reduced concentrations of blood thyroxine and triiodothyronine and high level of thyrotropin (hypothyroidism) were characterized by high level of catalytically

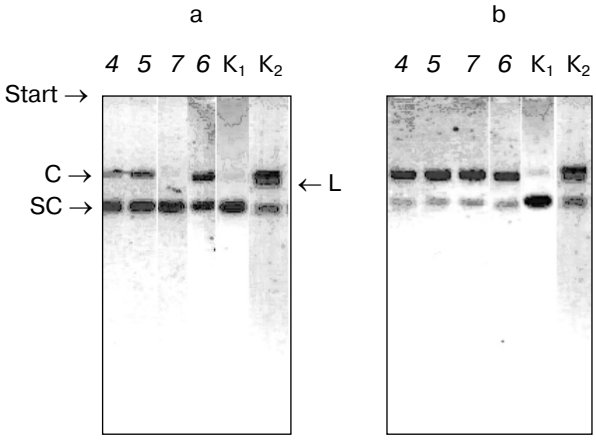

Fig. 4. Comparison of DNase activity of antibody preparations from blood of the same women before (a) and after (b) delivery. Bluescript plasmid DNA was used as substrate. (Negatives are shown.) Lane numbers correspond to number of preparations (*4*, *5*, *6*, and *7*). K_1) DNA incubated in the absence of antibodies; K_2) milk antibody (positive control). Forms of plasmid: C) circular; L) linear; SC) supercoiled.

Fig. 5. Comparison of DNase activity of antibody preparations from blood of the same woman before and after delivery and also in milk. Bluescript plasmid DNA was used as substrate. (Negatives are shown.) K) DNA incubated in the absence of antibodies; *1*) blood IgG during pregnancy; *2*) milk IgG; *3*) blood IgG after delivery.

active antibodies. Relative activity of antibodies may serve as a criterion reflecting intensity of autoimmune processes in these patients.

As demonstrated above, abzymes also appear in blood of pregnant women without evident manifestations of autoimmune pathologies such as autoimmune thyroiditis. So it was interesting to compare relative levels of abzyme activity in healthy woman and in pregnant women with autoimmune thyroiditis.

Using third trimester blood of 14 women with pregnancy induced autoimmune thyroiditis, we isolated antibodies on protein A-Sepharose and separated them by immunoglobulin classes by means of gel filtration. Figure 6 shows gel filtration profile and analysis of DNA-hydrolyzing activity of antibody fractions using plasmid DNA as substrate. In 4 of 14 women there was clearly distinguishable additional peak II corresponding to immunoglobulin class A (~29%). In antibodies from other women this peak was not detected and gel filtration profile basically corresponded to the profile typical for healthy pregnant women (Fig. 7). Figures 6 and 7 show results obtained for individual donors; the results for the whole group of donors are summarized in the table.

Increased synthesis of IgA correlated with pronounced DNase activity of IgA. Comparison of relative activity of IgG and IgM preparations from blood of healthy women and those with signs of autoimmune thyroiditis (14 donors) revealed that catalytic activity of antibodies in healthy women was 4.0-4.6 time less than that found in patients with autoimmune thyroiditis (table). Thus, development of pregnancy induced autoimmune thyroiditis accompanied by impaired functioning of the thyroid gland causes an increase in production of subfractions of polyclonal antibodies exhibiting catalytic activity.

The data suggest that pregnancy may be accompanied by activation of clones of immunocompetent cells producing autoantibodies including abzymes. However, this process is individual for each pregnant woman. In some cases abzymes (which were absent during pregnancy) appear after the beginning of lactation both in blood and milk and their activity in milk is significantly higher. During pregnancy the dynamics of accumulation of antibodies with DNase activity correlated with the dynamics of increase in blood DNA concentration [26] and cell apoptosis [46].

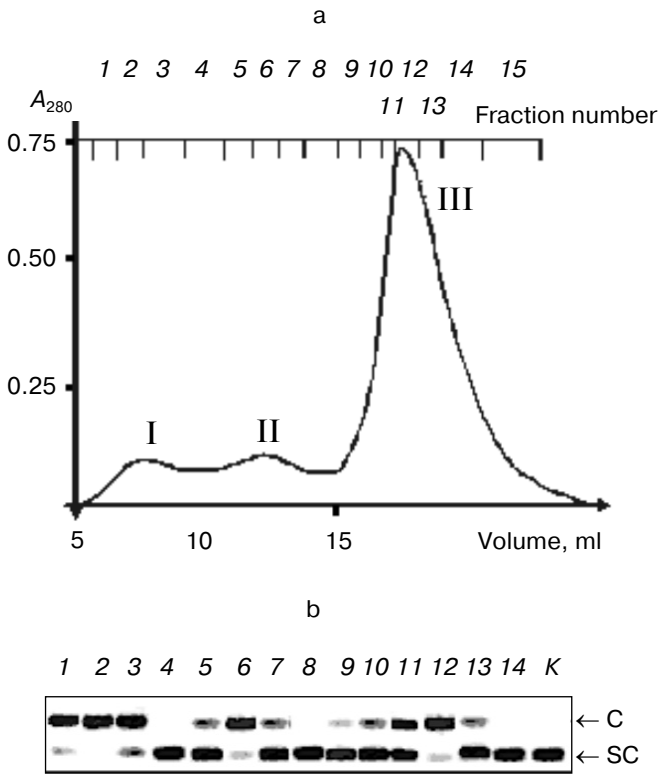

Fig. 6. Elution profile of high performance gel filtration on Superdex 200 HR 10/30 column of antibodies from blood of woman with parturient autoimmune thyroiditis (a) and DNase activity of fractions (b). Bluescript plasmid DNA was used as substrate. (Negatives are shown.) I) IgM; II) IgA; III) IgG; *1-15*) fraction numbers; K) DNA incubated in the absence of antibodies.

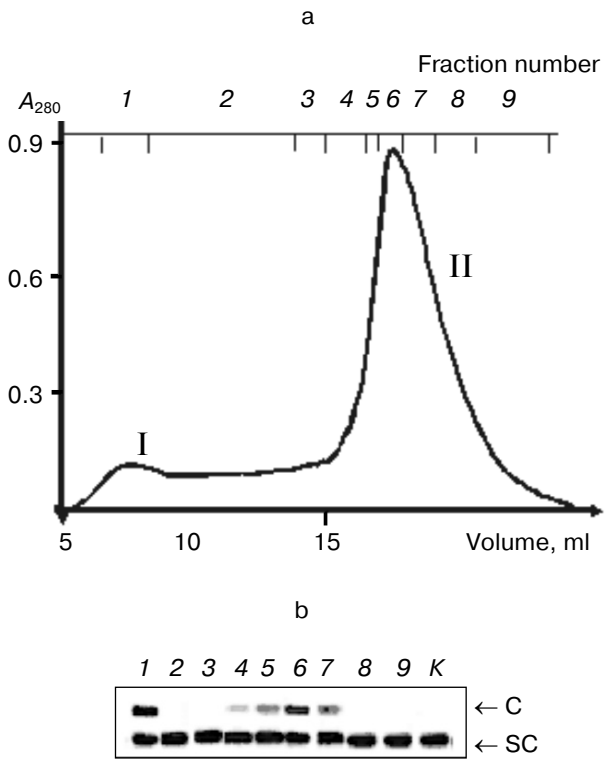

Fig. 7. Elution profile of high performance gel filtration on Superdex 200 HR 10/30 column of antibodies from blood of healthy woman (a) and DNase activity of fractions (b). Bluescript plasmid DNA was used as substrate. (Negatives are shown.) I) IgM; II) IgG; *1-9*) fraction numbers; K) DNA incubated in the absence of antibodies.

Relative DNA-hydrolyzing activity of IgG and IgM from blood of pregnant women in the third trimester

Preparation number	Donors	Relative IgG activity*, %	Mean value for IgG activity, %	Relative IgM activity*, %	Mean value for IgM activity, %
1	Healthy pregnant women	0.26		—	
4		0.46		—	
10		0.025	0.3 ± 0.2	21.0	15.0 ± 6.0
11		0.44		—	
12		0.5		9.0	
1	Women with pregnancy-induced autoimmune thyroiditis	2.5		100	
2		1.7		93	
3		0.84		76	
4		1.2		82	
5		2.0		92	
6		0.55		64	
7		1.05	1.2 ± 0.5	50	68.6 ± 21.6
8		0.9		91	
9		0.12		15	
10		0.5		35	
11		0.7		44	
12		0.6		53	
13		0.9		79	
14		1.0		87	

* Activity is expressed as % of control antibody preparation from human milk (100%).

It should be noted that autoantibodies (including some abzymes) may also be found in healthy donors [62, 63]. However, their concentration in healthy donors is significantly lower than in patients with autoimmune diseases and in healthy donors these autoantibodies exist as tight autoimmune complexes. So reliable testing of these autoantibodies represent a rather difficult task. The possibility of the existence of autoantibodies in healthy donors is very important for interpretation of results on abzymes obtained for patients with autoimmune diseases and in pregnant women as well. The development of theoretical considerations in abzymology resulted in a hypothesis on abzyme appearance on the basis of Jerne's model of an antiidiotypic network [14]. According to this model the first antibody AB1 (idiotype) elaborated in response to an antigen represents the antigen for production of secondary antibody AB2 (antiidiotype). The latter may also serve as the antigen for production of AB3, then AB4, AB5, etc. This network of idiotypes–antiidiotypes was shown to be actually realized in healthy humans and it maintains equilibrium of various antibodies [14]. Since abzymes may have antiidiotypic nature, it is possible that production of high quantities of abzymes in healthy persons is suppressed due to equilibrium in the idiotype–antiidiotype network, whereas in patients with autoimmune diseases impairments of the network equilibrium occur. This results in accumulation of free abzymes (which are not bound to idiotype–antiidiotype complexes) and increase in their concentrations. According to our data [10, 11], the initial period of autoimmune diseases is characterized by a set of blood abzymes with narrow substrate specificity. The spectrum of catalytic activity of these abzymes significantly enlarges during the development and attacks of these diseases. In other words, the development of such disease is characterized by constant induction of additional clones of cells producing abzymes with new spectra of substrate specificity. The main problem is which factors are responsible for impairment of the equilibrium in idiotype–antiidiotype network in patients with autoimmune diseases and in pregnant women and whether these factors differ in pregnancy and autoimmune diseases.

Many authors believe that the major reason for the development of autoimmune diseases consists in altered functional activity and differentiation of stem cells [64, 65]. According to our data, autoimmune MRL1pr/1pr mice are characterized by spontaneous increase in activity and directed differentiation of stem cells towards immunocompetent cells (to be published separately). Such increase in the activity and irreversible change in

stem cell differentiation in MRL1pr/1pr mice towards production of clones of cells producing abzymes with DNase activity may be easily induced by immunization of mice with DNA and its complexes with proteins. These data suggest that appearance of autoimmune diseases may also be attributed to altered activity and differentiation direction of the stem cell which is a common precursor for a part of hemopoietic and immunocompetent cells. Increase of blood concentrations of DNA, proteins, lipid, and other autoantigens occurs due to impaired functioning of some organs and/or damage of their cells and also due to cell apoptosis. Increase of thyroglobulin concentrations during thyroid gland diseases may result in specific changes of cell differentiation accompanied by increase of anti-thyroglobulin antibodies and also antibodies to some components of cells (DNA) damaged during autoimmune diseases. Since these chronic diseases are characterized by periods of attacks and remissions, it is possible that the impaired immune system functioning may be to some degree irreversible. In this connection, it is relevant to mentioned our studies on the effect of various drugs on autoimmune processes in patients with autoimmune thyroiditis [59, 60]. Treatment of patients with thyroxin widely used for therapy of thyroid gland diseases causes only temporary normalization of blood thyroxine concentration and general condition of patients. However, such treatment did not influence blood levels of DNA-hydrolyzing antibodies and autoimmune processes as well. Treatment of patients with immunosuppressant plaquenil caused significant reduction of blood level of catalytically active antibodies and decrease in blood concentrations of thyroglobulin and antibodies to this protein. Such treatment (without thyroid hormone therapy) also caused increase (up to normal level) of blood concentration of thyroid hormones, and decrease in thyrotropin concentration. Normalization of these biochemical parameters was accompanied by improvement of general conditions of patients. These results suggest that general suppression of the immune system in patients with autoimmune thyroiditis by using immunosuppressants reduces functioning of cell clones producing various abzymes.

So, it is interesting to consider which factors may promote induction of cells producing abzymes in pregnant women. Increase in DNA concentration and appearance of "foreign" embryonal in maternal blood may represent such factor(s). Pregnant women are also characterized by altered ratio between T- and B-lymphocytes and also between T-activators and T-suppressors. Decrease in T-lymphocytes and increase in B-lymphocytes in the first trimester may stimulate production of antibodies against the father's antigens (and possibly abzymes) which may function as facilitating antibodies [24]. The level of fetal cells in a woman's blood should strongly depend on the permeability of the placental barrier. Subsequent reduction of abzyme level in the second trimester correlates (to some extent) with reduced synthesis of antibodies against the father's antigens and change in ratio between T- and B-lymphocytes. In some cases increased antibody activity may be associated with selective interaction of T-suppressors with B-lymphocytes producing abzymes.

Evidently, pregnancy and the parturient period are accompanied by rearrangement of the immune system, which obviously occurs in at least two stages. Compatibility between the mother's body and (partially) "foreign" fetus organism requires rearrangement of the immune system towards its tolerance to the fetal organism and also preparation of the immune system for subsequent rearrangement which occurs simultaneously with onset of lactation. Triggering of specific immune memory of pregnant women, which accumulates "data" on all types of internal and external immunogens, is an important factor in the second stage of rearrangement of the immune system. (This immune memory stems from contacts with food antigens and or viruses and bacteria.) This triggers synthesis of antibodies against all antigens that appeared during the last months of pregnancy and were kept by the immune memory of the mother's body up to delivery. Increase in abzyme titer in blood and milk of women after delivery may represent only a part of a general process of antibody production triggered by the immune memory. Sometimes cardinal rearrangements of the second stage may cause parturient autoimmune shock which may even be fatal. However, appropriate treatment of such parturient women can neutralize such a "flash" of autoimmune reactions and after termination of lactation period it is even possible to annihilate signs of parturient autoimmune diseases. Nevertheless, in some cases it is impossible to suppress such a flash of autoimmune reactions and these parturient autoimmune disorders are gradually transformed into typical progressing autoimmune disease.

So, it is possible that autoimmune reactions in women during pregnancy and lactation and in patients with autoimmune diseases may share some common mechanisms. However, it is clear that mechanisms underlying triggering and arrest of these autoimmune processes differ. In parturient women it is possible to switch the immune system from "temporary autoimmune abnormality" to normal functioning without external treatments, whereas patients with autoimmune diseases lack such possibility. In women with weak immune systems, parturient rearrangement may cause irreversible switch of the immune system into the "mode" of autoimmune processes leading to chronic autoimmune diseases.

We have demonstrated that the development of autoimmune thyroiditis in pregnant women results in increase in relative DNA-hydrolyzing activity of abzymes. However, all patients lacked any signs of autoimmune shock and subsequent observation did not

reveal any evident symptoms of autoimmune processes. Thus, the reasonable question arises which level of catalytic activity may characterize abzymes from women with autoimmune parturient shock and how subsequent transition of immune system from normal to autoimmune "mode" of functioning occurs. We believe that more detailed comparison of autoimmune processes in patients with autoimmune diseases and in pregnant women will reveal natural factors responsible for the switch of the immune system of parturient women from normal to autoimmune mode and vice versa.

Thus, we conclude that latent and evident immunization "prepares" the immune system for production of various antibodies including abzymes. After delivery, these abzymes with mother's milk appear in a newborn baby. These milk catalytic antibodies [10, 11] may play a positive role in protection of the newborn baby against various environmental factors. It is also possible that abzymes represent a reserved form of catalytic resources which are realized under such specific conditions as pregnancy, autoimmune diseases, and possibly some other (yet unknown) cases. It is relevant to suggest that the potential of the immune system in generation of antibodies with various catalytic properties is not limited by the spectrum of catalytic activities found in abzymes.

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REFERENCES

1. Benkovic, S. J. (1992) *Annu. Rev. Biochem.*, **61**, 29-54.
2. Hilvert, D. (1992) *Pyre Appl. Chem.*, **64**, 1103-1113.
3. Stewart, J. D., and Benkovic, S. J (1993) *Chem. Soc. Rev.*, **22**, 213-219.
4. Suzuki, H. (1994) *J. Biochem.*, **115**, 623-628.
5. Schultz, P. G., and Lerner, R. A. (1995) *Science*, **269**, 1835-1842.
6. Schurov, D. V. (1997) *Mol. Biol. (Moscow)*, **32**, 5-15.
7. Nevinsky, G. A., Semenov, D. V., and Buneva, V. N. (2000) *Biochemistry (Moscow)*, **65**, 1233-1244.
8. Generalov, I. I., and Novikov, D. K. (1998) *Usp. Sovr. Biol.*, **118**, 178-193.
9. Nevinsky, G. A., Kanyshkova, T. G., Semenov, D. V., and Buneva, V. N. (2001) *Vest. RAMN*, No. 2, 38-45.
10. Nevinsky, G. A., Kanyshkova, T. G., and Buneva, V. N. (2000) *Biochemistry (Moscow)*, **65**, 1245-1255.
11. Nevinsky, G. A., Favorova, O. O., and Buneva, V. N. (2002) in *Protein–Protein Interactions. A Molecular Cloning Manual* (Golemis, E., ed.) Cold Spring Harbor, New York, pp. 523-534.
12. Raptis, L., and Menard, H. A. (1980) *J. Clin. Invest.*, **66**, 1391-1399.
13. Reimer, C., Raska, I., Tan, E. M., and Sheer, U. (1987) *Virchows Arch.*, **54**, 131-136.
14. Jerne, N. K. (1974) *Ann. Immunol.*, **125**, 373-398.
15. Kit, Y. Y., Semenov, D. V., and Nevinsky, G. A. (1995) *Mol. Biol. (Moscow)*, **29**, 893-906.
16. Kit, Y. Y., Semenov, D. V., and Nevinsky, G. A. (1996) *Biochem. Mol. Biol. Int.*, **39**, 521-527.
17. Nevinsky, G. A., Kit, Y. Y., Semenov, D. V., and Buneva, V. N. (1998) *Appl. Biochem. Biotechnol.*, **75**, 77-91.
18. Kanyshkova, T. G., Semenov, D. V., Vlassov, A. V., Khlimankov, D. Yu., Baranovskii, A. G., Shipitsin, M. V., Yamkovoi, V. I., Buneva, V. N., and Nevinsky, G. A. (1997) *Mol. Biol. (Moscow)*, **31**, 1082-1091.
19. Kanyshkova, T. G., Semenov, D. V., Khlimankov, D. Y., Buneva, V. N., and Nevinsky, G. A. (1997) *FEBS Lett.*, **416**, 23-27.
20. Buneva, V. N., Kanyshkova, T. G., Vlassov, A. V., Semenov, D. V., Khlimankov, D. Y., Breusova, L. R., and Nevinsky, G. A. (1998) *Appl. Biochem. Biotechnol.*, **75**, 63-76.
21. Nevinsky, G. A., Kanyshkova, T. G., Semenov, D. V., Vlassov, A. V., Gal'vita, A. V., and Buneva, V. N. (2000) *Appl. Biochem. Biotechnol.*, **83**, 115-129.
22. Semenov, D. V., Kanyshkova, T. G., Kit, Y. Y., Khlimankov, D. Yu., Akimzhanov, A. M., Gorbunov, D. A., Buneva, V. N., and Nevinsky, G. A. (1998) *Biochemistry (Moscow)*, **63**, 935-943.
23. Savel'ev, A. N., Eneyskaya, E. V., Shabalin, K. A., Filatov, M. V., and Neustroev, K. N. (1999) *Protein Peptide Lett.*, **6**, 179-184.
24. Mohan, C., Adams, S., Stanic, V., and Datta, S. K. (1993) *J. Exp. Med.*, **177**, 1367-1381.
25. Teodorescu-Exarcu, I. (ed.) (1981) in *Human Reproduction*, Medical Publishing House, Bucharest, pp. 703-725.
26. Kazakov, V. I., Bozhkov, V. M., Linde, V. A., Repina, M. A., and Mikhailov, V. M. (1995) *Tsitologiya*, **37**, 232-235.
27. Amino, N., Tada, H., and Hitaka, Y. (1999) *Thyroid.*, **9**, 705-713.
28. Hazes, J. M. (1997) *Ann. Rheum.*, **50**, 71-72.
29. Dayan, C. M., and Daniels, G. H. (1996) *New Engl. J. Med.*, **335**, 99-107.
30. Tanaka, A., Lindor, K., Ansari, A., and Gershwin, M. E. (2000) *Liver Transpl.*, **6**, 138-143.
31. Freeman, R., Rosen, H., and Thysen, B. (1986) *Arch. Int. Med.*, **146**, 1361-1364.
32. Fung, H. Y., Kologlu, M., Collison, K., John, R., Richards, C. J., Hall, R., and McGregor, A. M. (1988) *Br. Med. J.*, **296**, 241-244.
33. Parker, Rh., and Beierwaltes, W. H. (1961) *J. Clin. Endocrinol. Metab.*, **21**, 792-798.
34. Amino, N., Mori, H., and Iwatani, Y. (1986) *N. Engl. J. Med.*, **306**, 849-852.
35. Gorbunov, D. V., Semenov, D. V., Shipitsin, M. V., Kit, Y. Y., Kanyshkova, T. G., Buneva, V. N., and Nevinsky, G. A. (2000) *Rus. J. Immunol.*, **5**, 267-278.
36. Gorbunov, D. V., Semenov, D. V., Shipitsin, M. V., and Nevinsky, G. A. (2001) *Dokl. RAN*, **377**, 119-121.
37. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
38. Merril, C. R., Goldman, D., and van Keuren, M. L. (1982) *Electrophoresis*, **33**, 17-26.
39. Andrievskaya, O. A., Buneva, V. N., Zabara, V. G., Naumov, V. Ya., Yamkovoi, V. I., and Nevinsky, G. A. (1998) *Mol. Biol. (Moscow)*, **32**, 908-915.
40. Fasman, G. D. (ed.) (1975) *Handbook of Biochemistry and Molecular Biology. Nucleic Acids*, Vol. 1, CRC Press, New York, p. 589.

41. Fey, H. R., Burtler, R., and Marti, F. (1973) *Vox Sang.*, **25**, 245-253.
42. Mestecky, J., and McGhee, J. R. (1987) *Adv. Immunol.*, **40**, 153-245.
43. Kanyshkova, T. G., Semenov, D. V., Vlassov, A. V., Khlimankov, D. Yu., Baranovskii, A. G., Shipitsyn, M. V., Yamkovoi, V. I., Buneva, V. N., and Nevinsky, G. A. (1997) *Mol. Biol. (Moscow)*, **31**, 1082-1091.
44. Buneva, V. N., Kanyshkova, T. G., Vlassov, A. V., Semenov, D. V., Khlimankov, D. Y., Breusova, L. R., and Nevinsky, G. A. (1998) *Appl. Biochem. Biotechnol.*, **75**, 63-76.
45. Nevinsky, G. A., Kanyshkova, T. G., Semenov, D. V., Vlassov, A. V., Gal'vita, A. V., and Buneva, V. N. (2000) *Appl. Biochem. Biotechnol.*, **83**, 115-129.
46. Levin, J. S., and Koh, J. S. (1999) *Sem. Nephrol.*, **19**, 34-47.
47. Andrievskaya, O. A., Buneva, V. N., Baranovskii, A. G., Gal'vita, A. V., Benzo, E. S., Naumov, V. A., and Nevinsky, G. A. (2002) *Immunol. Lett.*, **81**, 191-198.
48. Andrievskaya, O. A., Kanyshkova, T. G., Yamkovoi, V. I., Buneva, V. N., and Nevinsky, G. A. (1997) *Dokl. RAN*, **355**, 401-403.
49. Buneva, V. N., Andrievskaya, O. A., Romannikova, I. V., Gololobov, G. V., Yadav, R. P., Yamkovoi, V. I., and Nevinsky, G. A. (1994) *Mol. Biol. (Moscow)*, **28**, 738-741.
50. Schurov, D. V., Makarevich, O. I., Lopaeva, O. A., Buneva, V. N., Nevinsky, G. A., and Gabibov, A. G. (1994) *Dokl. RAN*, **337**, 407-410.
51. Baranovskii, A. G., Matyushin, V. G., Vlassov, A. V., Zabara, V. G., Naumov, V. A., Buneva, V. N., and Nevinsky, G. A. (1997) *Biochemistry (Moscow)*, **62**, 1358-1366.
52. Vlassov, A. V., Andrievskaya, O. A., Kanyshkova, T. G., Baranovskii, A. G., Naumov, V. A., Breusov, A. A., Giege, R., Buneva, V. N., and Nevinsky, G. A. (1997) *Biochemistry (Moscow)*, **62**, 474-479.
53. Vlassov, A. V., Helm, M., Naumov, V. A., Breusov, A. A., Buneva, V. N., Florentz, C., Giege, R., and Nevinsky, G. A. (1999) *Mol. Biol. (Moscow)*, **33**, 866-872.
54. Vlassov, A., Florentz, C., Helm, M., Naumov, V., Buneva, V., Nevinsky, G., and Giege, R. (1998) *Nucleic Acids Res.*, **26**, 5243-5250.
55. Vlassov, A. V., Helm, M., Florentz, C., Naumov, V. A., Breusov, A. A., Buneva, V. N., Giege, R., and Nevinsky, G. A. (1999) *Russ. J. Immunol.*, **4**, 25-32.
56. Vlassov, A. V., Baranovskii, A. G., Kanyshkova, T. G., Prints, A. V., Zabara, V. G., Naumov, V. A., Breusov, A. A., Giege, R., Buneva, V. N., and Nevinsky, G. A. (1998) *Mol. Biol. (Moscow)*, **32**, 559-569.
57. Baranovskii, A. G., Kanyshkova, T. G., Mogelnitskii, A. S., Naumov, V. A., Buneva, V. N., Gusev, E. I., Boiko, A. N., Zargarova, T. A., Favorova, O. O., and Nevinsky, G. A. (1998) *Biochemistry (Moscow)*, **63**, 1239-1248.
58. Andrievskaya, O. A., Buneva, V. N., Naumov, V. A., and Nevinsky, G. A. (2000) *Med. Sci. Monit.*, **6**, 460-470.
59. Nevinsky, G. A., Breusov, A. A., Baranovskii, A. G., Prints, A. V., Kanyshkova, T. G., Gal'vita, A. V., Naumov, V. A., and Buneva, V. N. (2001) *Med. Sci. Monit.*, **7**, 201-211.
60. Breusov, A. A., Gal'vita, A. V., Benzo, E. S., Baranovskii, A. G., Prints, A. V., Naumov, V. A., Buneva, V. N., and Nevinsky, G. A. (2001) *Rus. J. Immunol.*, **6**, 17-28.
61. 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.
62. Lekakh, I. V., Rott, G. M., and Poverennyi, A. M. (1991) *Mol. Biol. (Moscow)*, **25**, 1391-1399.
63. Lekakh, I. V., and Poverennyi, A. M. (1996) *Mol. Biol. (Moscow)*, **30**, 707-713.
64. Ikehara, S. (1998) *Int. J. Mol. Med.*, **1**, 5-16.
65. Kushida, T., Inaba, M., Takeuchi, K., Ogawa, R., and Ikehara, S. (2000) *Blood*, **95**, 1862-1868.