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Antibodies against DNA Hydrolyze DNA and RNA

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Abstract—In this work, rabbits were immunized with a high polymer DNA complexed with methylated BSA (mBSA) and by mBSA. It is shown that electrophoretically homogeneous preparations of polyclonal antibodies (Ab) from non-immunized rabbits and animals immunized by mBSA do not exhibit catalytic activity. Ab from the blood of rabbits immunized with the DNA–mBSA complex hydrolyzed poly(C) and different RNAs with efficiency exceeding that towards DNA by approximately 3-4 orders of magnitude. Affinity chromatography of the IgG on DNA cellulose separated the Ab into fractions hydrolyzing both RNA and DNA, and for the first time fractions that hydrolyze only RNA were found. Kinetic parameters that characterize the RNA and DNA hydrolysis by initial Ab preparations and their fractions obtained by separation on an affinity sorbent are compared.

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Antibodies (Ab) to stable analogs of chemical reaction transient states as well as natural immunoglobulins (Ig) exhibiting catalytic activities have been named abzymes; they are quite well described in the literature (monograph [1] and references therein). The first examples of natural abzymes were the VIP (vasoactive intestinal polypeptide)-hydrolyzing IgGs isolated from the blood of patients suffering of bronchial asthma [2]. It is known, that the blood of volunteers may contain polyclonal auto-Ab to very different antigens [3, 4]. However, the amount of auto-Ab is significantly higher in the blood of patients with different autoimmune diseases (AID) [3, 4], while the presence in the blood of Ab with different catalytic activities is characteristic just of AID (see reviews [1, 5-9]). IgG and/or IgM as well as IgA hydrolyzing DNA, RNA, proteins, and polysaccharides, isolated from the blood of patients with different AID forms (systemic lupus erythematosus (SLE), Hashimoto's thyroiditis, polyarthritis, multiple sclerosis) as well as with lymph proliferative and some viral diseases (viral hepatitis, HIV infection [1, 5-9]) have been described. Ab from the blood of volunteers and patients with influenza, tonsillitis, duodenal ulcer, and some other diseases (uterine and intestinal cancer), not accompanied by pronounced disturbances in immune status, exhibited no noticeable DNase activity [10, 11].

According to current concepts, there are two ways for abzyme formation. On one side, similarly to the synthetic abzymes against stable analogs of chemical reaction transient states, natural abzymes might be produced just against the antigen that under certain conditions can mimic the transient state [1]. For example, abzymes specifically hydrolyzing VIP (asthma) [2], thyroglobulin (autoimmune thyroiditis) [12], and the main protein of myelin (multiple sclerosis) [13] are antibodies directly against these proteins. Ab can be produced directly against DNA and RNA in complexes with proteins, while nucleic acids *per se* are weak immunogens. In the case of SLE, antibodies against DNA and RNA are obtained after autoimmunization of mammals by DNA and RNA complexes with different proteins including histones emerging in the blood upon cell apoptosis [14]. These Ab may exhibit nuclease activity [9]. On the other side, anti-idiotypic antibodies can be abzymes [15-17]. If idiotypic antibodies are aimed against the enzyme active center, the corresponding secondary anti-idiotypic antibodies may have structural characteristics of the original enzyme active center. In accordance with Jerne theory, peculiari-

Abbreviations: Ab) antibodies; AID) autoimmune diseases; mBSA) methylated BSA; pAb) polyclonal Ab; pIgG) polyclonal immunoglobulin G; scDNA and rDNA) supercoiled and relaxed DNA, respectively; SLE) systemic lupus erythematosus; VIP) vasoactive intestinal polypeptide.

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ties of idiotypic and anti-idiotypic Ab formation were used to obtain monoclonal antibodies exhibiting acetylcholine esterase [17, 18], carboxypeptidase [19, 20], and β-lactamase [21] activities. It is supposed that the blood of SLE patients might contain DNA-hydrolyzing Ab against topoisomerase I [22]. It has been shown that immunization of rabbits using DNase I results in generation of idiotypic Ab1 [23]. Polyclonal anti-idiotypic Ab2 against isolated Ab1 exhibited DNase activity.

It is known that canonical DNases hydrolyze only DNA and do not show hydrolyzing activity towards RNA, whereas RNases are not able to hydrolyze DNA. At the same time, polyclonal IgG, IgA, sIgA, and IgM, isolated from the blood of patients with numerous AIDs and milk of healthy women, more efficiently hydrolyze RNA compared to DNA [2-6, 24-27]. It was shown [28] that sIgA of human milk hydrolyzes ribosomal RNA. Moreover, several different monoclonal IgGs against different B-DNA sequences, obtained using mouse SLE line, hydrolyzed RNA ~30-100 times more rapidly than DNA [29]. On the whole, it is obvious that polyclonal DNA-hydrolyzing Ab of AID patients might contain abzymes against DNA and different DNases [5-9]. At the same time, possible mechanisms of production of abzymes exhibiting RNase activity are still not completely clear. It is not clear whether immunization with DNA results in formation of only multifunctional abzymes able to hydrolyze both DNA and RNA, or parallel generation of Ig having only DNase or only RNase activity is possible. It is also not clear whether anti-idiotypic nature Ab with RNase activity can be produced. To answer this question, we immunized rabbits with DNase I, DNase II, RNase A, and by mBSA (methylated BSA) complexes with DNA and RNA and compared relative activities of the resulting Ab in hydrolysis of DNA and RNA. Formation of pIgG (polyclonal immunoglobulin) efficiently hydrolyzing both RNA and DNA was shown in all cases. The ratio of these activities and the Ab affinity to DNA and RNA strongly differ depending on the used antigen (data on each antigen will be published separately).

The goal of this work was to analyze enzymological features of Ab obtained in the case of immunization of rabbits with DNA.

MATERIALS AND METHODS

Reagents from Sigma (USA) and Pharmacia (Sweden) and also SDS from Merck (Germany) were used in this work. Isolation and purification of the pBluescript plasmid DNA were carried out according to the Qiagen (USA) protocol. Nuclease-resistant DNA-cellulose cross-linked by glutaraldehyde (NIKTI BAV, Russia) was used.

Immunization of rabbits. Healthy rabbits (three months old) were immunized with the DNA–mBSA complex; 200 μg of RNA-free highly polymeric DNA was injected per 200 g animal weight in physiological solution (0.85% NaCl, 0.02 M NaH_2PO_4, pH 7.2). Methylated BSA is a classic positively charged protein that forms stable complexes with nucleic acids and enhances the immunogenicity of the latter. A mixture of equal volumes of complete Freund's adjuvant and antigen solutions was used. The mixture was stirred until homogeneous gel formation and injected subcutaneously in the neck region. Two repeated immunizations were carried out with the mixture with incomplete Freund's adjuvant at 21 and 30 days. Immunization results were analyzed three months after the first immunization. The blood of unimmunized rabbits and animals immunized with mBSA was used as control.

Isolation of antibodies from rabbit blood. Purification of Ab from mouse blood was carried out using the modified technique developed previously for the abzyme isolation from the blood of AID patients [10, 11, 26, 27]. For protein precipitation, ammonium sulfate was added to the blood plasma to 50% saturation, the solution was kept at 4°C for 1-2 days, and the protein was separated by centrifugation (12,000 rpm, 10 min), the precipitate was dissolved in original volume of TBS (0.15 M NaCl, 20 mM Tris-HCl, pH 7.5). The resulting solution was applied onto the column of Protein A-Sepharose (5 ml) equilibrated with TBS and then washed with 15 ml TBS. Nonspecifically adsorbed proteins and lipids were eluted in TBS containing 0.5% Triton X-100 and 0.3 M NaCl. IgG fractions were eluted in 40 mM glycine-HCl, pH 2.6, and collected in cooled tubes containing 50 μl of 1 M Tris-HCl, pH 9.0, then solutions were titrated with this buffer to neutral pH. Protein from the center of the peak was concentrated and used for the next stage of purification.

IgG was separated from IgA and IgM by high-efficiency gel filtration on a Superdex-200 HR 10/30 column (100 × 300 mm) (Sprint Biocad chromatograph; Pharmacia, Sweden) equilibrated with 50 mM Tris-HCl, pH 7.5, similarly to that described in [30]. The sample (0.2-0.3 ml) was incubated for 30 min at 20°C in TBS containing 2.5 M $MgCl_2$. Prior to sample application onto the column, 2 ml buffer containing 3 M $MgCl_2$ was applied followed by Ab solution. Elution was carried out in TBS buffer (0.2 ml/min). For protection against contaminations, Ab fractions were filtered through a Millex filter (Millipore, USA) (pore diameter 0.2 μm) and used for analysis after storage in neutral buffer for one week at 4°C.

Affinity chromatography of purified IgG on Sepharose with immobilized monoclonal mouse IgG against light chains of rabbit IgG was carried out by analogy with Ab chromatography on Protein A-Sepharose (see above). The absence of IgA and IgM from IgG preparations was confirmed by immunoblotting as described in [26, 27].

Determination of Ab activity in DNA hydrolysis. Standard reaction mixture (20 μl) contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 13.3 μg/ml (4.43 nM) pBluescript plasmid DNA, and 0.005-0.2 mg/ml Ab. After incubation for 3-24 h at 30°C, 5 μl of 0.05% bromophenol blue solution containing 50% glycerol and 20 mM Tris-HCl, pH 7.5, were added to the reaction mixture. Reaction products were analyzed by electrophoresis in 0.8% agarose gel (electrophoresis buffer was 40 mM Tris-acetate, pH 7.5, 1 mM EDTA). After electrophoresis, DNA was stained by ethidium bromide solution (0.5 μg/ml). To determine relative activity, the gel was photographed and scanned using the GelPro software program (the linearity of measurements was checked using a gel containing known amounts of DNA). Relative activity was determined by lowering the DNA content in the scDNA (supercoiled DNA) band due to its transition to the relaxed form (rDNA). All measurements were carried out in pseudo-first-order reaction conditions using linear regions of the reaction rate dependence on the Ab concentration and time; the decrease in the original scDNA amount did not exceed 15-40%; ~95-100% rDNA molecules contained a single nick per molecule.

Determination of Ab activity in RNA hydrolysis. The RNase activity of Ab was analyzed spectrophotometrically using quartz cells (0.3- or 1-cm pathway); reaction mixtures (0.3-1.5 ml) contained 50 mM Tris-HCl, pH 7.5, 1 mM cCMP, or 50-100 μg/ml poly(N) (poly(U), poly(C), poly(A), poly(G)), or 50-100 μg/ml total yeast RNA, and 0.01-1.1 mg/ml (0.07-8 μM) pIgG or 1.6 nM pancreatic RNase A. In the case of poly(N) substrates, reaction mixtures were incubated for 0.1-10 h at 30°C or for 1-18 days with cCMP. Initial reaction rates were estimated based on the slopes of kinetic curves. Decrease in optical absorption at 282 nm was measured in the case of poly(U), poly(A), poly(G), and total RNA ($\Delta\varepsilon_{282}$ = 829 $M^{-1}\cdot cm^{-1}$ [31]), as well as absorption increase at 292 nm for cCMP [32] and at 250 nm ($\Delta\varepsilon_{250}$ = 2380 $M^{-1}\cdot cm^{-1}$ [33]) for poly(C), like in analysis of RNase activities. Concentration of the heterogeneous length poly(N) was expressed as concentration of corresponding mononucleotides [31, 33].

Electrophoretic analysis of Ab and their nuclease activities. Protein analysis was carried out according to Laemmli in 4-15% polyacrylamide gel gradient (0.1% SDS, with or without 10 mM dithiothreitol (DTT)). Proteins were incubated for 1 min at 100°C in 50 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, and 0.025% bromophenol blue, and applied onto the gel. After electrophoresis, proteins were stained with $AgNO_3$ [24-27].

DNase and RNase activities of Ab *in situ* were determined using SDS-electrophoresis in 4-15% polyacrylamide gel gradient containing 40 μg/ml yeast RNA or 3 μg/ml calf thymus DNA as described in [24-27]. For analysis of RNase activity, after electrophoresis the gel was incubated for 4 h in 20 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100 to remove SDS, and then it was incubated for four days at 30°C in 40 mM Tris-HCl, pH 7.5, for protein refolding and recovery of activity. To remove SDS from the DNA-containing gel, the latter was incubated for 4 h at 25°C in 40 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100, 4 mM $MgCl_2$, and 0.2 mM $CaCl_2$, then the gel was incubated for 2 h in the same buffer without Triton X-100, and after addition of fresh buffer the gel was incubated for three days at 30°C. Gels were stained with ethidium bromide to visualize regions with hydrolyzed DNA and RNA. Parallel longitudinal gel bands were used for detection of the protein band positions by Coomassie R250 staining.

Affinity chromatography of IgG on DNA-cellulose. The IgG preparation (1.5 ml, 0.7-1.0 mg/ml) was applied onto a column of DNA-cellulose (3 ml) equilibrated in advance with 50 mM Tris-HCl, pH 7.5. Then the column was washed with the same buffer to the disappearance of optical absorption, and Ab were eluted with 20 mM Tris-HCl, pH 7.5, containing NaCl at different concentrations (0.1-3 M), then with 50 mM glycine-HCl, pH 2.6. The fractions were neutralized as described above and dialyzed against 10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and then their relative activities in DNA or RNA hydrolysis were estimated.

Acid shock of antibodies. High-efficiency gel filtration of Ab in acid buffer, pH 2.6, was carried out after incubation of the Ab for 20 min in this buffer using the above-described technique. Immediately after gel filtration in acid buffer and solution neutralization, the Ab activity in DNA and RNA hydrolysis significantly decreased. Noticeable recovery was observed after keeping the neutral Ab solutions at 4°C for 1-2 weeks.

Determination of reaction kinetic parameters. All measurements were carried out within the linear regions of the reaction rate dependences on time and Ab concentration. The V_{max} and K_m (k_{cat}) values were estimated using nonlinear regression with the Microcal Origin v.5.0 software program and were presented in plots using Lineweaver–Burk double reciprocal coordinates [34]. The error in determination of these parameters did not exceed 10-30%.

RESULTS

DNase and RNase activities of pIgG antibodies from the blood of three rabbits immunized with DNA–mBSA complex were investigated. The pIgG of these three rabbits before immunization had no catalytic activity. The pIgG from the blood of another three non-immunized rabbits and from three animals immunized with mBSA were used as controls. The Ig were isolated by affinity chromatography of the rabbit blood proteins on Protein A-Sepharose under conditions favorable for the dissocia-

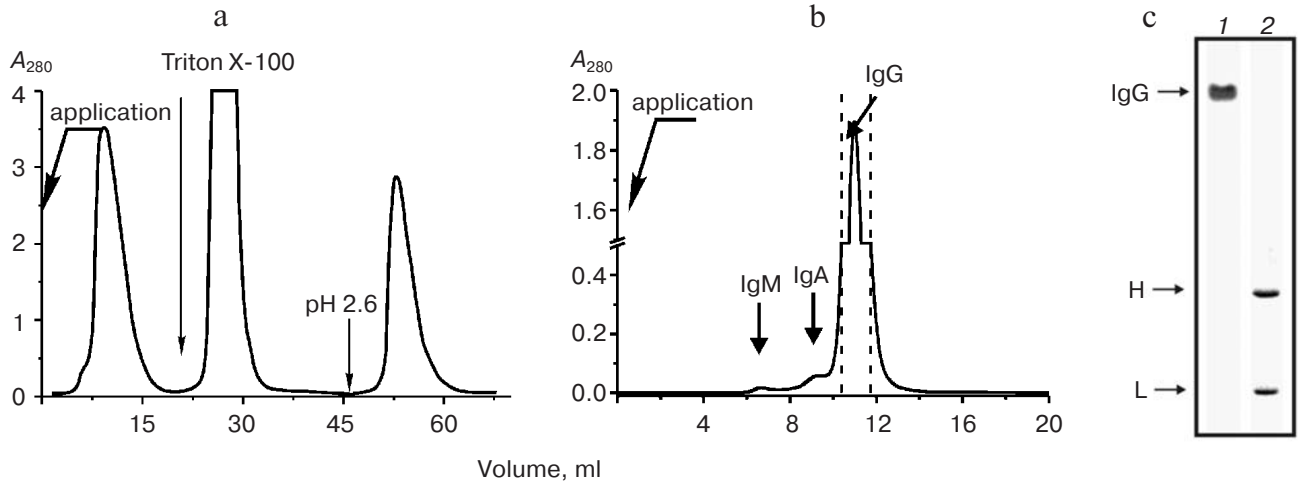

Fig. 1. Purification of pIgG preparation of the blood serum of a rabbit immunized with DNA–mBSA complex; purification was carried out by successive chromatography on a Protein A-Sepharose column (a) and FPLC gel filtration on a Superdex-200 column (b). Solid lines, optical density of eluate at 280 nm. c) Analysis of pIgG preparation (10 μg) homogeneity by SDS-electrophoresis in 4-15% gradient polyacrylamide gel: *1*, *2*) IgG before and after incubation with 10 mM DTT, respectively (silver-stained protein).

tion of nonspecific complex (Fig. 1a) with following IgG separation from IgA and IgM antibodies by FPLC gel filtration as described previously [30]. The IgG fraction corresponding to central part of the peak obtained after gel filtration was used in the study (Fig. 1b). The IgG preparations were electrophoretically homogeneous according to protein staining by silver (Fig. 1c).

It was shown that Ab from the blood of non-immunized rabbits and those immunized with mBSA do not hydrolyze either DNA or RNA (Figs. 2 and 3). The scDNA hydrolysis in the presence of Ab preparations from the blood of three rabbits immunized with DNA resulted in formation of only relaxed DNA form with its following hydrolysis, and no linear DNA was detected (Fig. 2a). The type of DNA hydrolysis was independent of Ab concentration, and the rate of hydrolysis increased linearly as the pIgG concentration and incubation time increased (Fig. 2). In the case of quantitative estimation of the relative activity of Ab, the concentration corresponding to the pseudo-first-order reaction was selected for each preparation, when scDNA was transformed only to relaxed form (15-40%) and no noticeable formation of fragmented DNA was observed (Fig. 2). The Ab relative activity was calculated based on the decrease in DNA content in the band corresponding to scDNA (Fig. 2). Relative activities of three Ab preparations in the scDNA hydrolysis (4.43 nM) were expressed in nanomoles of phosphodiester bonds hydrolyzed in 1 h per mg of pIgG. The following results were obtained: for IgG(1) – 5.3 ± 0.5, for IgG(2) – 7.2 ± 0.7, and for IgG(3) – 10.6 ± 1.5 (nM scDNA/h per mg protein).

Relative activity of three Ab preparations was estimated in poly(N) hydrolysis (Fig. 3) at fixed concentrations of poly(C) and poly(U). Relative activity of preparations in poly(N) hydrolysis was expressed in micromoles of poly(N) phosphodiester bonds hydrolyzed in 1 h per mg pIgG: IgG(1) – 52.0 ± 4.0, IgG(2) – 74.6 ± 3.0, and IgG(3) – 84.1 ± 6.5 (μM/h per mg for poly(C)); IgG(1) – 16.0 ± 2.1, IgG(2) – 21.8 ± 2.4, and IgG(3) – 29.4 ± 3.7 (μM/h per mg for poly(U)).

Evidence for antibody origin of catalytic activity. The proof of antibody origin of catalytic activity is usually based on verification of a great number of rather severe criteria described in the literature (for review see [1, 5-9]). One of them is electrophoretic homogeneity of Ab preparations in the case of protein staining by silver (Fig. 1c). It was also shown that IgG was quantitatively sorbed on Sepharose with immobilized mouse IgG against rabbit IgG and then were eluted by acidic buffer (pH 2.6); the protein absorption peak position coincided with those of DNase and RNase activities (Fig. 4a). In the case of gel filtration of Ab in acidic buffer (pH 2.6), profiles of protein absorption and DNase and RNase activities also coincided (Fig. 4b).

It should be noted that some criteria described in the literature are practically indicative of the unambiguous affiliation to Ab rather than to some contaminants. First, there are such criteria as *in situ* activity testing in a gel with the substrate polymerized into it [24-27, 30]. As shown by us previously, meeting this most strict criterion is accompanied by meeting the other less strict ones [5-9].

After gel electrophoresis in the presence of DNA (or RNA), the gel region in which the nucleic acid cleavage takes place was revealed as a dark spot against the uniformly fluorescent background. As seen in Fig. 5, only pIgG from the blood of rabbits immunized with DNA exhibit activity; electrophoretic mobility of these pIgG

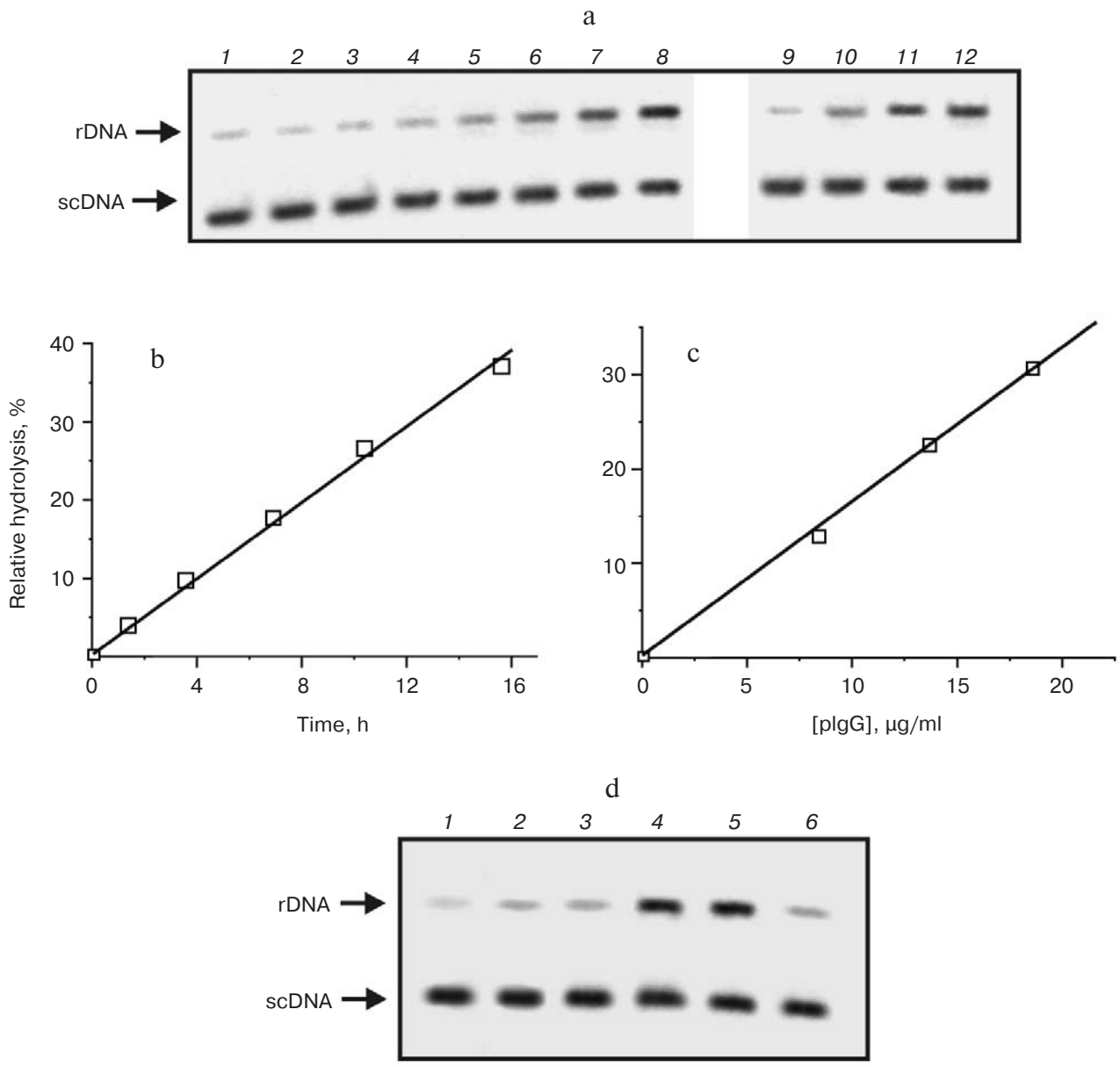

Fig. 2. a) Electrophoretic analysis of products of hydrolysis of 4.43 nM scDNA of pBluescript plasmid in agarose gel after incubation with pIgG from the blood of different rabbits (a). Lanes: *3*, *9*) DNA was incubated for 18 h without Ab; *1*, *2*) incubation for 18 h with 500 μg/ml Ab of a healthy rabbit and of that immunized with mBSA, respectively. DNA was incubated with 15 μg/ml pIgG(2) of a rabbit immunized with DNA–mBSA complex for 1.4, 3.57, 6.9, 10.4, and 15.6 h (lanes *4-8*, respectively) and for 10 h with 8.45, 13.7, and 18.6 μg/ml pIgG(2) (lanes *10-12*, respectively). b) Kinetic curve of scDNA relative hydrolysis; data from lanes *3-8* (Fig. 2a) were used. c) The relative hydrolysis rate dependence on pIgG(2) concentration; data from lanes *9-12* (Fig. 2a) were used. Complete hydrolysis of initial scDNA was taken as 100%. d) scDNA (9 nM) was incubated for 2 h 15 min without Ab (lane *1*) or with 0.4 μM pIgG(2) preparation not dialyzed against EDTA (lane *4*) (mixtures contained 5 mM $MgCl_2$) as well as for 48 h without Ab (lane *2*) and exogenous magnesium ions or with pIgG(2) preliminarily dialyzed against buffer containing 0.1 M EDTA (lane *3*), with initial not-dialyzed pIgG(2) (lane *5*), and with the same Ab preparation in the presence of 0.1 M EDTA (lane *6*).

(150 kD) is significantly lower than that of control human and bovine DNases I (~34-36 kD) as well as of RNase A (~14 kD). The absence of any protein contaminants as well as activity bands from gel regions not corresponding to IgG show that DNase and RNase activities are inherent properties of pIgG. Some other criteria including the Ab affinity to DNA, RNA, and DNA-cellulose also support this conclusion (see below).

Affinity chromatography of Ab on DNA-cellulose. It is known that DNase I and II are eluted from DNA-cellulose in a single peak [35, 36]. Chromatography of Ab from the blood of AID patients and milk of healthy donors

results in IgG separation into a great number of fractions in the presence of salts at different concentrations, or in IgG distribution along the whole profile in a salt concentration gradient [2-6, 37].

The affinity to DNA of pIgG preparations from rabbit blood was studied by affinity chromatography on DNA-cellulose. Autoantibodies to different antigens are present in tested amounts in the blood of patients with different AID forms as well as in the blood of healthy people and animals [3, 4]. Besides, not only auto-Ab against DNA, but those against phospholipids, polysaccharides, and DNA-dependent enzymes, histones, and

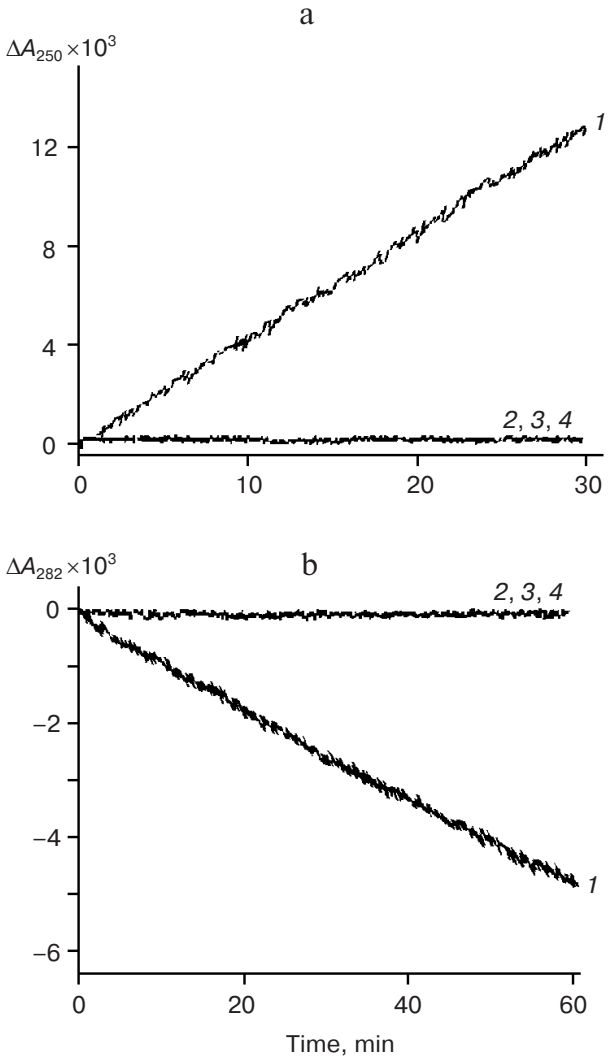

Fig. 3. Kinetic curves of optical density of poly(C) (50 μg/ml) at 250 nm (ΔA_{250}) (a) and of poly(U) (67 μg/ml) at 282 nm (ΔA_{282}) (b) in the presence of different pIgG preparations. Curves: *1*) poly(N) was incubated with Ab of a rabbit immunized with DNA–mBSA complex; *2*) without Ab; *3*) in the presence of pIgG from the blood of a non-immunized rabbit; *4*) in the presence of pIgG of an animal immunized with mBSA. In all cases, except curve *1* for poly(C) (0.138 mg/ml), Ab concentration of 1 mg/ml was used.

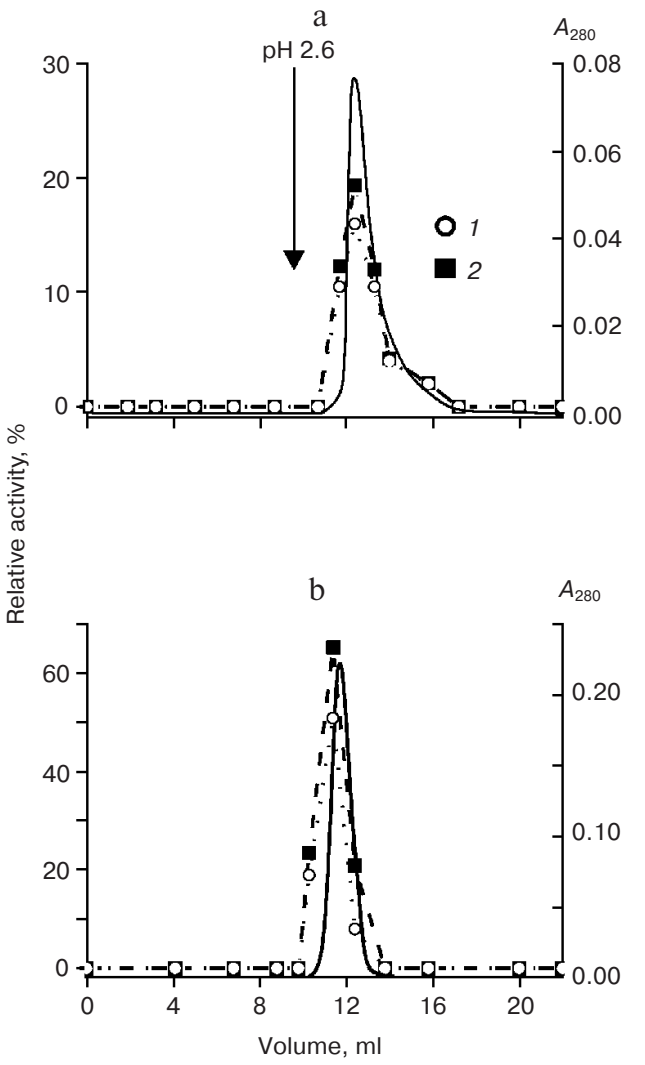

Fig. 4. Affinity chromatography of IgG(2) from the blood of a rabbit immunized with DNA–mBSA complex on a column with anti-IgG Sepharose (a) and FPLC high-efficiency gel filtration of IgG on a Superdex-200 column in acidic buffer, pH 2.6 (b). Solid line, optical density of eluate at 280 nm; *1*, *2*) relative efficiency of fractions in scDNA and poly(C) hydrolysis, respectively. The complete transition of 4.43 nM scDNA to rDNA in 18 h and complete hydrolysis of 140 μM poly(C) in 5 h incubation were taken as 100% relative activity. The error of two independent determinations of relative activity did not exceed 7-10%.

cell surface proteins, usually cross-reacting with DNA, can bind the DNA-sorbent [3, 4, 9]. Considering this, it was not surprising that depending on the Ab sample from healthy rabbits (three preparations), 25-31% Ab applied onto the column bound the sorbent. In this case, pIgG of all fractions were inactive in DNA and RNA hydrolysis (Fig. 6a). In the case of Ab from the blood of three rabbits immunized with DNA, the amount of pIgG specifically and nonspecifically interacting with DNA-cellulose increased to 77.5%. After chromatography, pIgG were eluted in four peaks (Fig. 6). Data on the relative protein

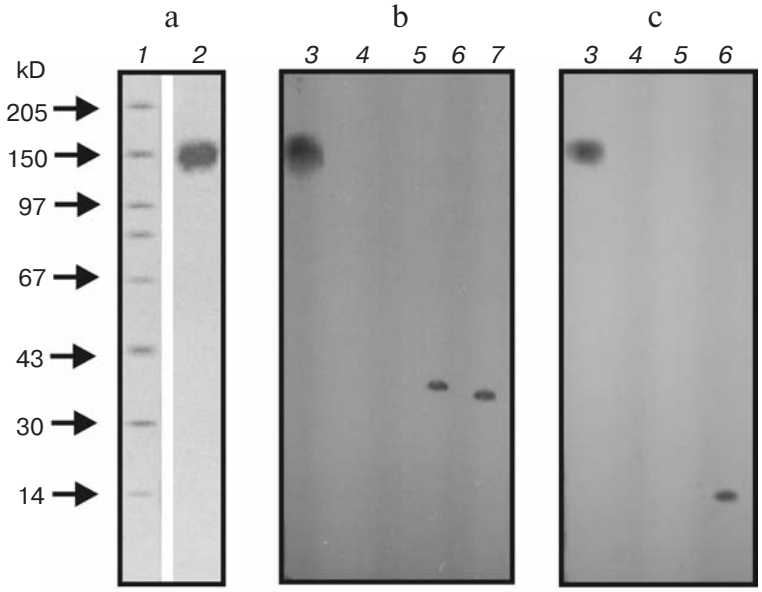

Fig. 5. *In situ* analysis of DNase (b) and RNase (c) activities of IgG and control nuclease activities after electrophoretic separation of proteins in 5-15% polyacrylamide gel containing, respectively, DNA and RNA polymerized into it. Lanes: *3*) pIgG of animals immunized with DNA–mBSA complex; *4*) pIgG of healthy animals; *5*) pIgG of rabbits immunized with mBSA; *6*, *7*) 1.0 standard Kunitz unit of DNase I from human urine and bovine liver, respectively (b); *6*) 15 ng pancreatic RNase A (c). Parallel gel bands were stained by Coomassie R250 (lane *2*); arrows point to positions of protein markers on lane *1* (a).

content in each peak for pIgG(2) are given in the table. It is interesting that for AID patients, Ab with high affinity to DNA-cellulose, which are eluted in 3 M NaCl and acidic buffer, are active in hydrolysis of both DNA and RNA [6, 37]. However, the main part of DNA-binding pIgG (~87.4%) containing DNA-hydrolyzing abzymes (in rabbits immunized with DNA) exhibited low affinity to DNA and was eluted upon the loading. Only low activity (~12.6%) corresponded to the second Ab peak eluted in 0.1 M NaCl (Fig. 6). A similar situation was observed for all three Ab preparations. It is interesting that for all three rabbit IgGs corresponding to three peaks (1-3) exhibited RNase activity (Fig. 6b). In this case, IgG of the third peak efficiently hydrolyzed only RNA and were completely inactive in DNA hydrolysis. pIgG of the fourth peak were inactive towards both DNA and RNA. It should be noted that the pIgG fraction corresponding to peak 3 was the largest by optical density (66.6%), but the RNase activity maximum corresponded to the tail of the peak (Fig. 6). One should assume that the pIgG at the

K_m and k_{cat} values characterizing interaction of initial pIgG preparation and its subfractions (separated by affinity chromatography on DNA cellulose) with scDNA and poly(C)

Peak number (Fig. 6)	Elution conditions	Relative content of Ab in immunized (healthy) animals, %	Relative DNase (RNase) activity, %	DNase activity, scDNA**		RNase activity, poly(C)**	
				K_m, nM	k_{cat}, min^{-1}	K_m, μM	k_{cat}, min^{-1}
0	before fractionation	100 (100)*	100 (100)*	58.9 ± 13.9	$(2.5 \pm 0.3) \cdot 10^{-4}$	33.9 ± 7.8	0.22 ± 0.02
1	0.0 M NaCl	22.5 (68.8)	87.4 (38.3)	68.1 ± 11.4	$(6.9 \pm 0.7) \cdot 10^{-4}$	73.2 ± 14.8	0.32 ± 0.03
2	0.1 M NaCl	5.7 (9.3)	12.6 (26.2)	21.5 ± 4.0	$(4.7 \pm 0.4) \cdot 10^{-4}$	28.5 ± 3.0	3.5 ± 0.4
3	0.5 M NaCl	66.6 (17.1)	0 (35.5)	0.0	0.0	25.2 ± 5.6	1.1 ± 0.1
4	3 M NaCl	5.2 (4.8)	0 (0)	0.0	0.0	0.0	0.0

* Error in determination in two independent experiments did not exceed 5-7%.
** Standard deviation of three independent determinations.

beginning and in the central part of the third peak are mainly canonical Ab against DNA, which are only able to bind DNA rather than to hydrolyze it. At the end of the third peak anti-DNA antibodies with RNase activity are eluted. The table shows distribution of protein density and DNase and RNase activities between the four peaks.

Explanation of simultaneous hydrolysis of DNA and RNA by pIgG(2) corresponding to peaks 1 and 2 seems difficult. Similar results were obtained for pIgG(1) and pIgG(3). On one hand, it might be that these Ab fractions, like mouse monoclonal Ab against DNA [29], can exhibit both activities. On the other hand, it is possible that these fractions are a mixture of pIgG able to hydrolyze both substrates as well as only DNA or only RNA, but due to similar affinity to DNA they are not separated by chromatography on DNA cellulose.

DNase activity of abzymes. It is known that mammalian DNase II is a metal-independent enzyme [38, 39], but DNase II is active only in the presence of Mg^{2+} or some other metal ions [40]. We showed previously that pIgG isolated by a standard technique contain a low amount of different metals, and due to the presence of these internal metal ions they are able to hydrolyze DNA at a low rate [37]. Dialysis of these Ab against EDTA-containing buffer or EDTA addition to the reaction mixture results in disappearance of this activity, which is restored after addition of external metal ions [37]. It is interesting that Ab from rabbit blood before dialysis in the absence of internal Mg^{2+} exhibited low DNase activity that completely disappeared after treatment with EDTA (Fig. 2d). Addition of 5 mM Mg^{2+} increased activity of pIgG ~17-20-fold.

All known type I DNases in the presence of Mg^{2+} (optimum 10 mM) hydrolyze DNA with emergence of relaxed and linear forms [40]. Unlike DNase I, initial rabbit pIgG and those after chromatography on DNA-cellulose hydrolyzed DNA in the presence of 5 mM Mg^{2+} with emergence of only relaxed form (Fig. 2) that then underwent deeper fragmentation without generation of linear form. The type of hydrolysis was independent of both the time of reaction and the Ab concentration. These data were indicative of essential difference in DNA cleavage by Ab compared to canonical DNases and Ab from the blood of AID patients and milk of healthy women after childbirth [24-27, 30].

We estimated kinetic parameters (K_m and k_{cat}) that characterize scDNA cleavage by pIgG(2) before and after their chromatography on DNA-cellulose. Fractions of each of four peaks after affinity chromatography, which exhibited maximal activity in hydrolysis of DNA and poly(C), were used. For pIgG fractions corresponding to peaks 1 and 2 (Fig. 7), initial reaction rates increased hyperbolically along with increase in the scDNA concentration, in accordance with Michaelis–Menten kinetics (Fig. 7). In terms of K_m values, the affinity of the first IgG fraction eluted upon application was approximately 3.2

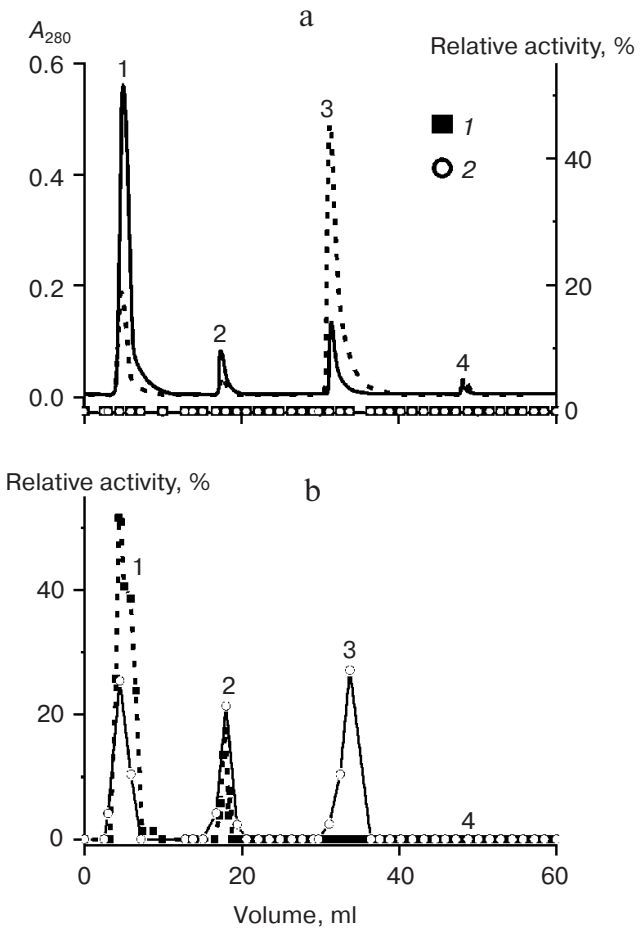

Fig. 6. Affinity chromatography of pIgG(2) on DNA cellulose. Optical density profiles (A_{280}) of Ab of a healthy rabbit (solid line) and of one immunized with DNA–mBSA complex (dashed line) and relative DNase (*1*) and RNase (*2*) activities of the healthy rabbit pIgG (a); relative activity of pIgG fractions in DNA (*1*) and RNA (*2*) hydrolysis (b). Complete transition of 4.43 nM scDNA to rDNA in 18 h and complete hydrolysis of 140 μM poly(C) in 5 h incubation were taken as 100% relative activity. The error of two independent determinations did not exceed 7-10%.

times lower than that for fraction 2, which was eluted in 0.1 M NaCl (table). Data in the table are indicative of pIgG pool heterogeneity in affinity to DNA. Considering this, the dependence of V on [S] for unfractionated IgG preparation should be a sum of hyperbolic curves characterizing individual fractions. However, the contribution of the second peak (Fig. 6) to total optical density and activity of total pIgG preparation is relatively low. In this connection, it is not surprising that the initial pIgG preparation is characterized by K_m (58.9 ± 13.9 nM) value close to that for the first Ab fraction with higher activity (68.1 ± 11.4 nM) eluted upon the loading. The k_{cat} value for initial Ab preparation is lower than that for catalytically active fractions with maximal activity (table), which correlates with the presence in this preparation of pIgG fractions with lower activity or those having no catalytic

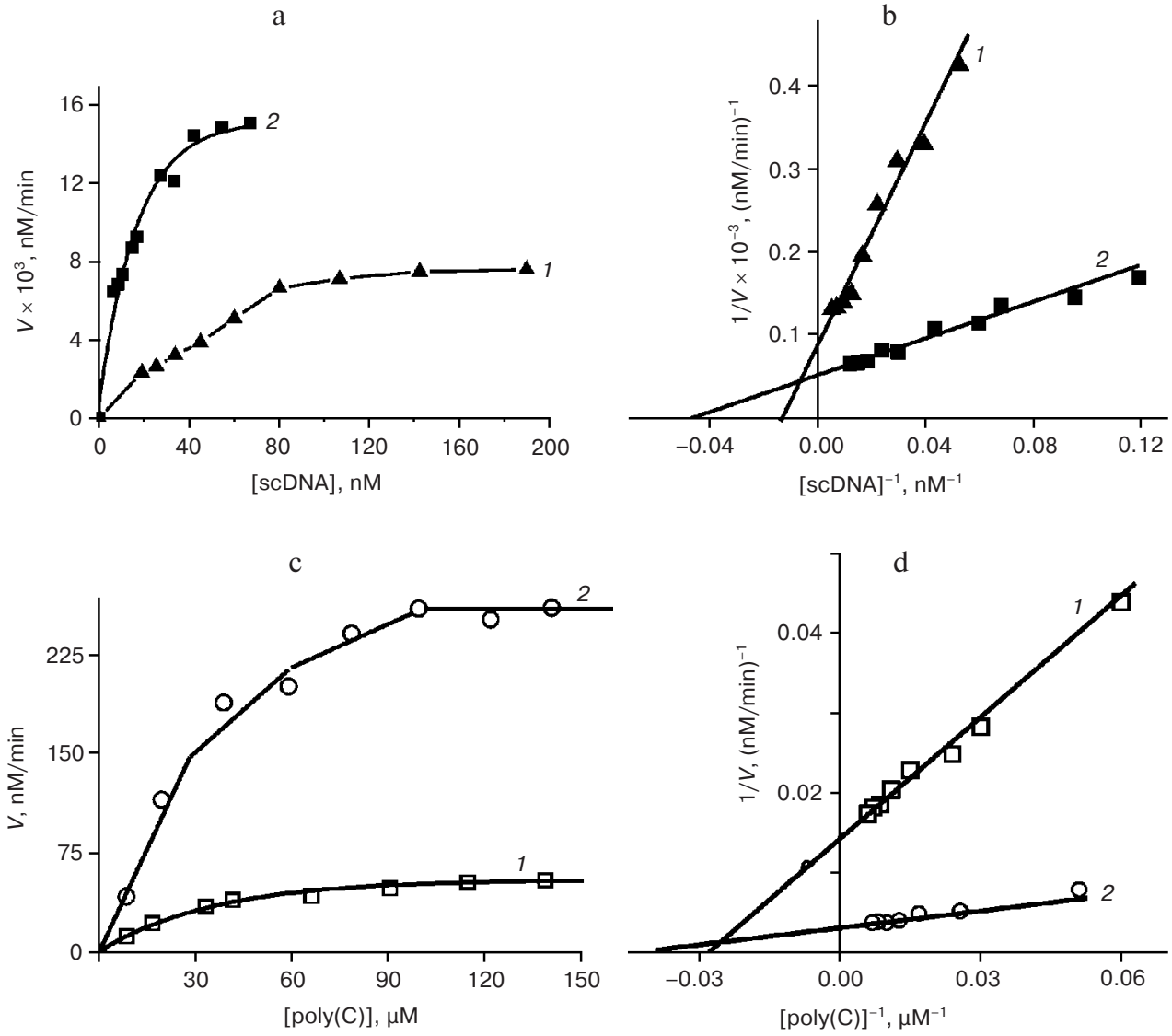

Fig. 7. Dependences of rates of scDNA hydrolysis by unfractionated 40 nM pIgG (*1*) and by the fraction corresponding to peak 2 (*2*) on scDNA concentration in coordinates V versus [S] (a) and $1/V$ versus 1/[S] (b). Dependences of rates of poly(C) hydrolysis by unfractionated 307-nM pIgG (*1*) and by the fraction corresponding to peak 3 (*2*) on poly(C) concentration in coordinates V versus [S] (c) and $1/V$ versus 1/[S] (d).

activity. Overall, catalytically active IgG fractions exhibit a high affinity to DNA (K_m = 21-68 nM), which is characteristic of Ab interaction with antigens. The DNase I affinity to scDNA (K_m = 46-58 μM [41]) is approximately three orders of magnitude lower than that for Ab. Taken together, the obtained data on the behavior of pIgG in affinity chromatography on DNA-cellulose, affinity to DNA, and the dependence of reaction products on metal ions are indicative of significant differences between Ab and canonical DNases.

RNase activity of abzymes. Two rabbit RNases, from liver and spleen, have been described; they are similar in enzymological characteristics and molecular mass (~14.3 kD) to bovine pancreatic RNase A [42]. Substrate specificity of five known ribonucleases from human blood is close to that for pancreatic RNase A [43]. Four of them, like RNase A, better hydrolyze poly(C) than poly(U), whereas the fifth prefers poly(U). Poly(A) and poly(G) are relatively poor substrates for these five RNases [43]. Natural Ab from the blood of AID patients hydrolyze poly(A) more efficiently than the above-mentioned canonical RNases [5-9]. cCMP is a very good substrate for all five RNases [43] and abzymes from the blood of AID patients [5-9]. Like known RNases, pIgG before and after fractionation on DNA-cellulose better hydrolyzed poly(C) than poly(U), but unlike canonical RNases and polyclonal Ab of AID patients they were completely inactive in hydrolysis of poly(A) and poly(G).

No noticeable hydrolysis of cCMP was detected even after incubation of the reaction mixture for 18 days. This shows that rabbit pIgGs catalyze only RNA cleavage but they are not able to catalyze hydrolysis of terminal 2′-3′-cyclophosphate intermediates. Thus, the mechanism of RNA hydrolysis by pIgG, exhibiting RNase activity, significantly differs from that for canonical RNases.

Dependences of the hydrolysis initial rate on poly(C) concentration for pIgG preparations corresponding to peaks 1-3 (Fig. 6) correlated with Michaelis–Menten dependences (Fig. 7). The affinity of pIgG, corresponding to the first peak obtained upon loading, to poly(C) in the range of K_m values was 2.6-2.9 times lower than that for Ab of the second and third peaks eluted in 0.1 and 0.5 M NaCl (table). Apparent k_{cat} values that characterize the pIgG factions with maximal activity from the second and third peaks were ~3-11 times higher than that for Ab from the first peak (table). According to these data, pIgG are heterogeneous in affinity to RNA, but due to the closeness of K_m values for different Ab fractions, unfractionated pIgG preparation was characterized by K_m close to the averaged values for individual fractions.

The initial pIgG preparation exhibited approximately three times lower affinity to poly(U) (K_m = 105.6 ± 16.4 μM) than to poly(C) (table) and hydrolyzed poly(C) approximately 2.8 times more rapidly than poly(U) (k_{cat} = 0.08 ± 0.009 min^{-1}). At saturating concentration of total yeast RNA (0.2 mg/ml), specific activity of unfractionated pIgG preparation was ~0.054% of that for bovine pancreatic RNase A (100%).

DISCUSSION

As shown previously, the specific abzyme fraction in total Ab pool from the blood of AID patients and milk of healthy women after childbirth usually does not exceed 1-7% [5-9]. In the case of directed immunization, the abzyme percentage might be antigen-dependent and somewhat higher. Affinity chromatography on DNA-cellulose is not able to efficiently separate DNA- and RNA-abzymes from DNA-binding Ab devoid of catalytic activity; specific activity of individual fractions grows no more than two-to-fivefold compared to the initial sample [5-9, 25, 26, 37]. Most likely, this is due to the ability of DNA to bind Ab against very different antigens (cross-reactivity) [3, 4, 38]. As shown previously, the efficiency of interaction of numerous different enzymes with specific and unspecific DNA and RNA most often differs by one and very rarely by two orders of magnitude [44]. At the same time, the difference in the rate of transformation of specific and unspecific RNA and DNA varies for studied enzymes from five to eight orders of magnitude. This shows that just the stage of catalysis (k_{cat}), that requires adjustment of reacting atomic orbitals from the substrate and enzyme side with accuracy of 10-15°, is the most important in the specific substrate selection by enzymes [44], rather than the efficiency of interaction; high nucleic acid affinity does not ensure the possibility of its catalytic transformation. DNA and RNA hydrolysis by separate pIgG fractions, obtained after Ab chromatography on DNA-cellulose, shows that all these fractions, except DNA-binding Ab, contain different amounts of specific abzyme subfractions exhibiting these nuclease activities. It was shown that unlike DNases I and II [35, 36], both the DNA- and RNA-abzymes from the blood of AID patients [5-9] are heterogeneous in affinity to DNA. A similar situation was found for pIgG in the case of rabbit immunization by DNA. However, unlike canonical DNases and abzymes from the blood of AID patients, pIgG of immunized rabbits are not able to hydrolyze scDNA with generation of its linear form.

The fractionation of pIgG on DNA-cellulose shows that some fractions are able to hydrolyze both DNA and RNA (Fig. 6). An unexpected result is that the rather large Ab fraction eluted in 0.5 M NaCl is active only towards RNA hydrolysis. However, unlike canonical RNases from mammalian blood and pIgG from the blood of patients with different AID forms [5-9], Ab from rabbit blood cannot rapidly hydrolyze poly(A), poly(G), and cCMP. This can be caused by activation, upon immunization and AID development, of various mechanisms involved in generation of different kinds of abzyme-synthesizing lymphocytes. The production of abzymes with DNase, RNase, and ATPase activities in healthy autoimmune MRL-lpr/lpr mice in the case of immunization with DNA is mainly associated with inhibition of cell apoptosis and increase in the lymphocyte proliferation rate in thymus, lymphoid tissues, and first of all, in spleen [45]. However, production of these abzymes in MRL-lpr/lpr mice with SLE is mainly associated with alteration of proliferation level and differentiation profile of the bone-marrow stem cells, which result in formation of different type bone-marrow progenitors of hemopoietic cells, and to a lesser extent it is associated with lymphocyte proliferation in other organs. In addition, in the case of AID production of anti-idiotypic Ab against active centers of DNase I [23] and other nucleases as antigenic determinants, which may also exhibit DNase and RNase activities, is possible. As shown previously, upon transition from non-autoimmune to autoimmune mouse lines, immunization results in sharp expansion of the repertoire of Ab with catalytic activities and increase in the relative specific activity of the antibody in the studied reactions [46, 47]. These factors may be mainly responsible for the observed distinctions in catalysis of DNA and RNA hydrolysis by antibodies from the blood of immunized healthy rabbits, on one side, and by Ab from the blood of patients with different AID forms and sick MRL-lpr/lpr mice, on the other.

It was shown previously that monoclonal mouse IgG against B-DNA of different primary structure hydrolyze

RNA 30-100 times more efficiently than DNA [29]. pIgG of different AID patients also hydrolyze RNA by 1-2 orders of magnitude better than DNA [5-9, 26, 27]. It is interesting that on the whole, rabbit pIgG against the DNA–mBSA complex hydrolyze RNA by 3-4 orders of magnitude better than DNA. However, in this case, unlike canonical RNases and RNA-abzymes from human blood, Ab from the blood of immunized rabbits do not hydrolyze cCMP. As is known, catalysis of RNA cleavage and following hydrolysis of the formed 2′,3′-cyclophosphate intermediate are carried out by two different histidine residues in the RNase active center. Since the high optical density peak 3 (Fig. 6) contains pIgG fractions able to hydrolyze only RNA but not cCMP or DNA, it is possible that active centers of these Abs contain only a single histidine residue able to stimulate hydrolysis of RNA, but not of DNA or cCMP. This hypothesis is supported by data [48] showing that insertion, using gene-directed mutagenesis, of a single histidine residue into variable regions of the IgG light chains, specifically binding poly(I), results in Ab specifically hydrolyzing this substrate.

Antibodies against transient state analogs are usually characterized by reaction rates of 10^2-10^6 orders of magnitude lower than those of canonical enzymes [1]. The known k_{cat} values for natural enzymes vary within the interval from 0.001 to 15.6 min^{-1} [2, 5-9, 12, 24-27, 37, 41, 49]. The K_m values for scDNA (21.5-68.1 nM) in the case of rabbit pIgG are comparable with K_m characterizing the scDNA interaction with Ab from the SLE patients (43-92 nM) [41] but are higher than K_d for one subfraction of pIgG from the blood of patients with multiple sclerosis (0.36 nM) [49]. The given K_m values are characteristic of highly efficient Ab interaction with antigens and are lower than those for DNase I by ~3-4 orders of magnitude (K_m = 46-58 μM) [41]. The k_{cat} values ((2.5-4.7)·10^{-4} min^{-1}) that characterize scDNA hydrolysis by rabbit pAb (table) are significantly lower than those for pIgG for SLE patients (k_{cat} = 14-40 min^{-1}) [41] or k_{cat} (all values are within the interval (2.7·10^{-2}-11.3 min^{-1})) in the case of $d(pN)_{10-13}$ oligonucleotide hydrolysis by polyclonal IgG, IgA, and IgM antibodies from the blood of SLE patients and milk of healthy women after childbirth [5-9, 24-27], but are comparable with these values for artificial abzymes [1].

The affinity of single- and double-stranded $(pN)_{10-13}$ (within the terms of K_m values) to abzymes from the blood of immunized rabbits (25.2-73.2 μM, see table) is comparable with that for Ab from the blood of SLE patients and milk of healthy women after childbirth and by ~1-2 orders of magnitude exceeds that for pancreatic RNase [5-9, 24-27]. The k_{cat} values for unfractionated and partially enriched pIgG preparations in poly(C) hydrolysis (0.22-3.5 min^{-1}, the table) are approximately 3-4 orders of magnitude higher than those in DNA hydrolysis and are comparable with those in $(pN)_{10-13}$ hydrolysis (3·10^{-3}-5.8 min^{-1}) by pIgG and pIgM from the blood of SLE patients and milk of healthy women after childbirth and are somewhat lower than those for characterized RNases from the blood of healthy donors (k_{cat} = 26-37 min^{-1}) [5-7, 24-27]. At the same time, these values are essentially lower than k_{cat} values characterizing $(pU)_2$-poly(U) hydrolysis (63.5-1100 min^{-1}) by bovine pancreatic RNase A [31].

Since chromatography on DNA cellulose does not enable efficient separation of Ab with and without catalytic activity and the amount of abzymes exhibiting some specific activity is relatively low [5-9], specific activities of monoclonal antibodies entering in rabbit pIgG might be significantly higher.

The data show that immunization with DNA stimulates the formation of antibodies hydrolyzing both DNA and RNA. This conclusion correlates with the fact that monoclonal mouse SLE IgG against different sequence DNA hydrolyze both DNA and RNA [29]. The question concerning the possibility of only DNA hydrolysis by anti-DNA antibodies is still open. The efficient hydrolysis of only RNA by antibodies against DNA was found for the first time in this work.

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