Nucleotide-hydrolyzing antibodies from the sera of autoimmune-prone MRL-lpr/lpr mice

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Abstract

Abzymes (Abzs) with different enzymic activities have been detected in the sera of patients with various autoimmune (AI) diseases and in AI mice. In this work, electrophoretically homogeneous IgGs were isolated from the sera of MRL-lpr/lpr mice spontaneously developing lupus-like AI pathology. It was shown for the first time that polyclonal IgGs (pIgGs) and their isolated heavy and light chains hydrolyze different nucleoside-5'-triphosphate (NTPs), nucleoside-5'-diphosphate (NDPs), adenosine monophosphate and deoxiadenosine-5'-monophosphate (dAMP), whereas antibodies from the sera of control healthy mice were catalytically inactive. Monoclonal mouse IgGs also effectively hydrolyze nucleotides. The data demonstrate that nucleotide-hydrolyzing activity is an intrinsic property of isolated mouse pIgG and monoclonal IgG. It was shown that various markers of AI pathologies (proteinuria and antibody titers to native and denatured DNA) demonstrating spontaneous development of AI reactions increased in animals with aging and correlated with an increase in Abz relative activity in hydrolysis of nucleotides. The highest increase in AI reaction markers and in Abz enzymic activity was found in mice immunized with a DNA–protein complex.

Introduction

Catalytic antibodies against reaction transition states [artificial abzymes (Abzs)] catalyzing >100 distinct chemical reactions are novel biological catalysts that attracted much interest in the last years and have been amply reviewed recently (1). In the last 15 years, the evidence has been reported for the existence of natural catalytic Abzs hydrolyzing DNA, RNA, polysaccharides, oligopeptides and proteins in the sera of patients with several autoimmune (AI) diseases [systemic lupus erythematosus (SLE), Hashimoto's thyroiditis, polyarthritis and multiple sclerosis], viral hepatitis and acquired human immunodeficiency syndrome (reviewed in references 2–5). According to the modern point of view, Abzs can be generated in two ways (1–5). On the one hand, they may be antibodies against analogs of transition states of catalytic reactions or even against substrates of enzymes acting as haptons and imitating transition states of the reactions. On the other hand, in AI diseases, anti-idiotypic antibodies can be induced by a primary antigen and may show some of its characteristics including catalytic activity. If an idiotypic Ig is directed against the active center of an enzyme, the respective site of an anti-idiotypic antibody may display features of an 'internal image' of the original active center.

In principle, Abzs can be produced not only in the organisms of AI patients but also in healthy humans. IgG Abzs cleaving vasoactive peptide (VIP) was observed not only in asthma patients but also in healthy donors (6). Later, the presence of polyreactive Abzs hydrolyzing thyroglobulin was revealed in patients with rheumatoid arthritis, but IgGs from some healthy humans also demonstrate detectable levels of thyroglobulin-hydrolyzing activity (7). Proteolytic Abzs have been found in patients with sepsis, which causes many deaths in intensive care units and results from a deleterious systemic host response to infection (8). However, according to our and literature data (1–5), healthy humans usually do not have Abzs or develop Abzs with low proteolytic, polysaccharide-, DNA- and RNA-hydrolyzing activities, these activities often on a borderline of the sensitivity of detection methods. In addition, there was no confirmed nuclease Abzs in the sera of patients with many different diseases (influenza, pneumonia, tuberculosis, tonsillitis, duodenal ulcer, several types of cancer and other diseases) with insignificant AI reactions (2–5, 9–12 and references therein).

We have shown that appearance of Abzs specifically hydrolyzing DNA or some other substrates is among the...
earliest and clear signs of AI reactions in a number of AI diseases (SLE, Hashimoto’s thyroiditis, polyarthritis and multiple sclerosis) and viral diseases with strong immune system disturbances (acquired immunodeficiency syndrome and hepatitis) (2–6). Catalytic activity of Abzs is usually high and very well detectable at the beginning of AI diseases when titers of antibodies to DNA or other auto-antigens are not increased significantly and correspond to their ranges for healthy donors (2–6). In addition, Abzs most probably play an important role in pathogenesis of AI diseases. Anti-VIP antibodies of patients with asthma hydrolyze VIP (13, 14). It has been suggested that respiratory tract dysfunction in bronchial asthma may stem from the protease activity of auto-Abzs, resulting in a deficit in VIP, which plays a major role in the pathophysiology (13, 15). DNase Abzs from SLE lymphoproliferative (16) and multiple sclerosis patients (4) are cytotoxic and induce cell death via apoptotic mechanism. DNA-hydrolyzing Bence-Jones proteins from multiple myeloma patients enter the cell nucleus and cause DNA fragmentation and cell apoptosis; catalytically active protein preparations were significantly cytotoxic and their activity was related to the progressive deterioration of the clinical status (17). Our data suggest a possibility that serine protease-like and metal-dependent proteolytic IgGs can play an important role in multiple sclerosis pathogenesis, hydrolyzing MBP of the myelin-proteolipid shell of axons (11 and references therein). Proteolytic IgGs from patients with sepsis may participate in the control of disseminated microvascular thrombosis and can play a role in recovery from the disease (8).

SLE is a frequently encountered AI disorder proceeding as a chronic pathology with temporary exacerbations and remissions. A very prominent feature of SLE is the presence of antibodies to denatured and especially native DNA in patients’ blood (18, 19). DNA and anti-DNA antibodies at increased concentrations are considered to arise from cell apoptosis (20). Apoptotic cell antigens are often recognized as auto-antibody targets. Many anti-DNA antibodies are directed against histone–DNA nucleosomal complexes appearing as a result of internucleosomal cleavage during apoptosis. Apoptotic cells are the primary source of antigens and immunogens in SLE, and specific features in recognition, perception, processing and/or presentation of apoptotic auto-antigen by antigen-presenting cells can cause AI processes (20). SLE is one of the most pronounced AI diseases in which DNase and RNase Abzs have been found to possess a very high activity (2–5, 21–23 and references therein). Thus, SLE represents a very promising model to study the mechanisms of natural Abzs’ generation and their role in etiology and pathogenesis of severe AI disturbances.

The MRL/lpr mouse strain is widely accepted as a convenient SLE model. MRL-lpr/lpr (MRL) mice spontaneously develop an AI disorder characterized by marked hypergammaglobulinemia, production of numerous auto-antibodies, circulating immune complex, glomerulonephritis and severe lymphadenopathy (23–25). A mutation in the lpr gene of these animals leads in homozygotes to a deficit in functional Fas ligand and dysregulation of apoptosis (24, 25). As a result, the mice develop SLE-like phenotype, including accumulation of double-negative T cells (CD4− CD8− B220+ TCR+) in peripheral lymphoid organs.

Recently, homogeneous IgGs were isolated from the sera of MRL mice (23). It was shown that, similar to SLE patients, the sera of these mice contain DNase IgGs. Convincing evidence that DNase activity is intrinsic to mice IgGs was provided using different approaches (23).

Using haptenic transition-state analogues, surprising results were obtained with a dramatically higher incidence of Abzs with amidase/esterase activity in AI mouse strains than in conventionally used normal mouse strains (26, 27). Different components of apoptotic cells, which are the primary source of antigens in SLE, can significantly widen the immune response in AI mice, leading to production of Abzs not only with DNase but also with other different activities. Recently, we have shown that spontaneous development of SLE in MRL mice leads to the production of IgGs hydrolyzing not only DNA but also polysaccharides (28).

In this study, we present the first evidence that polyclonal IgGs (pIgGs) from the sera of MRL mice contain subfractions hydrolyzing different nucleotides, nucleoside-5′-triphosphate (NTP), adenosine monophosphate (AMP) and deoxiadenosine-5′-monophosphate (dAMP). In order to detect a possible association of Abz generation with pathology development, we have analyzed a correlation of the relative activities (RAs) of nucleotide-hydrolyzing Abzs with markers of AI pathologies (proteinuria and antibody titers to native and denatured DNA) during various stages of the disease and after mouse immunization with DNA.

Materials and methods

Materials, chemicals and animals

Most chemicals, proteins and Protein A-Sepharose were from Sigma or Pharmacia; Triton X-100 was from Ferak and SDS from Merck.

AI-prone MRL mice (originated from Harlan, UK) and control non-AI BALB/c and (CBA × C57BL)F1 (CBA) at 2–7 months of age used in this study were housed in the colonies under standard pathogen-free conditions including a system for protection from bacterial and viral infections at the Institute of Cytology and Genetics (Siberian Division of Russian Academy of Sciences) mouse-breeding facility. MRL mice are characterized by spontaneous development of a lupus-like AI disorder (observed more often at the age ≧3–7 months with maximal DNase RAs and AI markers at 8–12 months of age) with many known indexes of AI diseases including proteinuria and anti-DNA antibodies and other characteristics (23, 28) mentioned above.

Immunization of mice, proteinuria assay and ELISA of anti-DNA antibodies

Determination of the total urine protein was carried out as in (23, 28). The titers of anti-DNA antibodies were determined using standard assay plates with immobilized double- or single-stranded DNA as described in (23, 28). After consecutive treatment of the wells with the blood sera and HRP-conjugated rabbit antibodies against mouse IgG, the reaction mixtures were incubated with tetraethyl benzidine and hydrogen peroxide. The reaction was stopped with sulfuric acid and
optical density (A_{450}) of the solutions was determined using a Labsystems Uniskan II plate reader. The relative concentrations of anti-DNA antibodies in the samples were expressed as a difference in the relative absorbance at 450 nm (average of three measurements) between experimental and control samples; controls using DNA without antibodies and with antibodies not interacting with DNA produced the same results.

Conditionally, healthy 3-month-old mice were immunized three times with 40 μg of DNA per mouse using a conjugate of calf thymus DNA with methylated BSA as in (23, 28). Later (1–1.3 months), these mice demonstrated pronounced visual and biochemical symptoms typical of SLE and increased level of proteinuria and antibodies to double- and single-stranded DNA; the blood of the mice was used for the antibody analysis 1.5–1.6 months after the first immunization.

**IgG purification**

Electrophoretically and immunologically homogeneous IgGs were obtained as described in (9–12, 23, 29, 30) with some modifications. The serum (3–4 ml) was loaded on a Protein A-Sepharose column equilibrated in Tris-buffered saline (TBS) buffer (0.15 M NaCl, 20 mM Tris–HCl, pH 7.5). Proteins adsorbed non-specifically were eluted with this buffer containing 1% Triton X-100 and 1.0 M NaCl. The total IgG + IgM + IgA fraction was eluted in 40 mM glycine-HCl (pH 2.6), the column fractions were collected into cooled tubes containing 50 μl of 5 M Tris–HCl (pH 9.0) and finally each fraction was additionally neutralized with this buffer, dialyzed against 10 mM Tris–HCl (pH 7.5) containing 0.1 KCl and concentrated for additional purification.

IgGs were separated from IgAs and IgMs by fast protein liquid chromatography (FPLC) gel filtration of total antibody fraction on a Superdex 200 HR 10/30 column (Pfizer, New York, NY, USA) equilibrated with TBS using a BioCAD workstation (Applied Biosystems, Foster City, CA, USA) analogously to human IgGs as described previously (29). Before gel filtration, the Ig samples were incubated in TBS containing 2.5 M MgCl₂ for 20 min at 20°C. TBS containing 2.5 M MgCl₂ and 1 M NaCl (2 ml) was applied on the column before the samples. Antibodies were eluted with TBS. In order to protect antibody preparations from bacterial and viral contaminations, they were filtered through a Millex syringe-driven filter units (0.2 μm) and kept in sterilized tubes. After 3–4 weeks of storage at 4°C for refolding, the antibodies were used in activity assays as described below. To exclude possible artifacts due to hypothetical traces of contaminating enzymes, the rigid criteria for assigning the DNase activity to IgGs were checked (23).

Chromatography of purified IgGs under the ‘acid shock’ conditions was carried out by FPLC gel filtration on Superdex 200 using 0.1 M glycine-HCl (pH 2.6) as described previously (11, 23, 29). Before gel filtration, the antibodies were pre-incubated in 0.1 M glycine-HCl, pH 2.6, for 30 min at 30°C. All fractions were collected to cooled tubes containing 50 μl of 0.5 M Tris–HCl (pH 9.0) and finally each fraction was additionally neutralized with this buffer and dialyzed against 20 mM Tris–HCl (pH 7.5), containing 0.1 M NaCl, filtered through a Millex syringe-driven filter units (0.2 μm) and after 1–3 weeks of storage at 4°C used in activity assays as described below.

Chromatography of purified IgGs on Sepharose-bearing rabbit IgGs against light chains of mouse IgGs was performed in the same way as the chromatography on Protein A-Sepharose (see above). All fractions were collected and processed as described above including the filtration through a Millex syringe-driven filter and kept in sterilized tubes for 1–20 days to allow protein refolding and the activity restoration.

The specific ATPase activity of IgGs just after FPLC gel filtration and chromatography on Protein A-Sepharose was ∼40 to 45% as compared with that before chromatographic steps (100%) and increased after 20 days of the storage to 75–85%, but the RA in the fractions was the same after 1, 7, and 20 days of the storage. The KM values for substrates determined under the conditions of pseudo first-order reaction in the case of IgGs before and after treatment with acidic buffer during chromatographic step were the same within the error of determination, while the kcat values for the hydrolysis of substrates increased similar to the RA of the IgG preparations.

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**Nucleotide-hydrolyzing activity assay**

Reaction mixtures (10–20 μl) contained the optimal concentrations of the standard compounds: 2 mM MgCl₂, 0.5 mM EDTA, 50 mM Tris–HCl, pH 7.5, 0.2 mM γ-[32P]NTP (or [32P]AMP, [32P]dAMP or 1 mM p-nitrophenyl phosphate) and 0.001–0.2 mg ml⁻¹ Igs. In some experiments, DNA, oligonucleotides and AMP were used as effectors in different concentrations. For determination of KM and Vmax (and apparent kcat) values, different concentrations of nucleotides were used. All measurements (initial rates) were taken within the linear regions of the time courses and antibody concentration curves. The reaction mixtures were incubated for 2 h at 30°C. For screening the column fractions during purification of IgG, 2–3 μl of each fraction was incubated in 20 μl of the standard reaction mixture containing 0.1 mM γ-[32P]ATP (10⁵ counts per minute). The products of nucleotide hydrolysis were analyzed by thin-layer chromatography in 0.25 M KH₂PO₄ (pH 7.0) on PEI-cellulose plates (Merck) or on Kieselgel F₂₅₄ plates (Merck) using the system dioxan–10% NH₄OH–H₂O (6:1:4).

After chromatography, the plates were dried and the positions of various [32P] products were identified using [32P] nucleotide standards and autoradiography. The autoradiographs of the plates were imaged by scanning using Gel-Pro software.

**SDS-PAGE analyses**

SDS-PAGE analysis of antibody fractions for homogeneity under non-reducing conditions was done in 4–15% gradient gels; for polypeptide separation, the electrophoresis was performed in a reducing 12.5% gel (in the presence of 0.1% SDS and 10 mM dithiothreitol). The polypeptides were visualized by silver staining (32–34).
For SDS-PAGE assay of ATPase activity, catalytic IgGs (7–10 μg) were pre-incubated at 30°C for 30 min under non-reducing (buffer A: 50 mM Tris–HCl, pH 7.5, 1% SDS and 10% glycerol) or mild reducing conditions (buffer A + 5% 2-mercaptopethanol). After the electrophoresis, SDS was removed by incubating the gel for 30 min at 30°C with H2O (five times). To restore the enzymatic activity after SDS-PAGE, SDS was removed by incubating the gel for 1 h at 22°C with K-phosphate (pH 6.8). The gel was washed five times with this buffer. Then, 3- to 4-mm cross-sections of longitudinal slices of the gel were cut out and incubated with 50 μl of 20 mM Tris–HCl, pH 7.5, containing 5 mM MgCl2 and 1 mM EDTA for two days at 4°C to allow protein refolding and eluting from the gel. The solutions were removed from the gels by centrifugation and used for assay of nucleotide hydrolysis as described above. Parallel longitudinal lanes were used for detecting the position of IgG in the gel by Coomassie R250 staining.

**DNA-hydrolyzing activity assay**

Oligonucleotide-hydrolyzing activity of IgGs was analyzed similar to (34) using reaction mixtures containing the above-mentioned standard components, 0.01–0.1 mg ml⁻¹ IgGs; 5⁻[³²P]d(pA)₁₀ or 5⁻[³²P]d(pT)₁₀ (1–100 μM) and their duplexes (5⁻[³²P]d(pA)₁₀ · d(pT)₁₀ and 5⁻[³²P]d(pT)₁₀ · d(pA)₁₀) were used. The reaction mixtures were incubated for 10–20 h at 30°C. The cleavage products were analyzed by 20% PAGE electrophoresis (7 M urea). The products of hydrolysis were visualized by autoradiography.

**Production, purification and analysis of mouse mAbs**

Hybridoma cells were produced by a standard method (35) modified according to the instruction of the manufacturer of polyethylene glycol 1500, Roche Diagnostics GmbH, using NSO line cells of mouse myeloma and lymphatic gland cells of a diseased 7-month-old MRL male with high proteinuria (7 mg ml⁻¹) and high IgG DNase and ATPase activities. The hybridoma supernatants were first screened for ATP-binding IgGs by ELISA (similar to the analysis of DNA-binding antibodies, see above) using BSA modified by ATP in the presence of water-soluble carbodiimide (~1 mole ATP per mole of BSA; 0.2 mg ml⁻¹ of modified BSA in 50 mM sodium carbonate, pH 9.6, 50 microliters per well) for 10 h at 20°C, washed with washing buffer (TBS, 0.05% Triton X-100, 0.01% sodium azide) and blocked with gelatin (0.2% w/v in washing buffer, 200 microliters per well) for 2 h at 20°C. The hybridoma supernatants (20 microliters per well) were added and the plates incubated for 10 h at 20°C. The binding of the antigen was detected (after a washing step) with a HRP-conjugated rabbit IgGs against mouse IgG; the reaction mixtures were incubated with tetraethyl benzidine and hydrogen peroxide. Binding of the ATP–BSA conjugate was selected in comparison with unmodified BSA. ATP–BSA conjugate-positive hybridomas were used for screening for ATPase IgGs using the method of the analysis of ATPase activity described above; 1–5 μl of supernatant was added to standard 10 μl reaction mixture. Four hybridoma supernatants demonstrated significantly higher ATPase activity than others or completely inactive samples. These hybridomas were used to obtain electrophoretically homogeneous monoclonal ATPase IgGs by affinity chromatography on Protein A-Sepharose as described above for plgGs. An assay of monoclonal IgGs was carried out similar to plgGs.

**Determination of the kinetic parameters**

The $K_M$ and $V_{max}$ (apparent $k_{cat}$) values were calculated from the kinetic data by least-squares non-linear fitting using Microcal Origin v5.0 software and presented as linear transformations using a Lineweaver–Burk plot (36), with apparent $k_{cat} = V_{max} \cdot \text{product formation, molar per second}/[\text{IgG}]$ (molar). Errors in the values were within 10–30%.

**Statistical analysis**

The results are reported as the mean and the standard deviation of at least three to four independent experiments for each mouse, averaged over at least five different animals. The number of preparations assayed for each age is shown in Table 1. Differences between the samples were analyzed by the Student’s $t$-test; $P \leq 0.05$ was considered statistically significant.

**Results and discussion**

Recently, we have shown that the sera of MRL mice with visual symptoms of AI pathology (pink spots, baldness of head and some areas of the back, worsening general conditions, etc.) contain DNase (23) and amylase Abs (28). It should be mentioned that ATPase Abs have been detected previously only in the milk and in the sera of lactating women (2–5, 29 and references therein).

**Purification of antibodies, application of the strict criteria**

To search for Abs in AI-prone MRL and two types of control non-AI mice, the electrophoretically homogenous plgGs were purified from the serum of individual animals using chromatography on Protein A-Sepharose and FPLC gel filtration similar to (23, 29). It should be mentioned that acidic treatment of antibodies during both stages of purification leads to a partial inactivation of their catalytic activity (2–5, 23). However, after all stages of IgG purification, all preparations from ill mice demonstrated detectable or high activity in the hydrolysis of different nucleotides (Fig. 1). Application of a set of strict criteria worked out previously (3–6) allowed us recently to conclude that the observed DNase and amylase activity is an intrinsic property of MRL mice IgGs and is not due to co-purifying enzymes (23, 28). Here we applied some of the previously used standard strict criteria (5, 23) for analysis of nucleotide-hydrolyzing activity of mouse IgGs. The most important of these are (i) electrophoretic homogeneity of IgGs (23, 28); (ii) complete absorption of the nucleotide-hydrolyzing activity by Sepharose-bearing antibodies against mouse IgG light chains, leading to a disappearance of the catalytic activity from the solution, and its recovery after acidic buffer (pH 2.6; Fig. 2A); (iii) FPLC gel filtration of IgGs under conditions of acidic shock (pH 2.6) did not lead to a disappearance of the activity, and the peak of the enzymic activity tracked exactly with IgGs (Fig. 2B) and (iv) It was shown that F(ab) fragments of plgGs are catalytically active and...
and Pi, while after 2 h of incubation Pi is the main product (39). Thus, catalytic antibodies may show very different contributions of variable domains of H and L chains to the structural organization of their active centers. In addition, the repertoires of monoclonal Abs in a polyclonal antibody (pAb) population are extremely variable for different AI diseases and from patient to patient (2–5). Therefore, the observed ATPase activity of separated by SDS-PAGE L and H chains of mice pIgGs (Fig. 3) may have different causes. First, it is possible that these pIgGs contain a mixture of Abs with only light or only heavy chains catalytically active. However, similar to mouse monoclonal DNase IgGs (39), the ATPase centers may be located at the interface between the light and heavy chains, and both separated chains can probably catalyze hydrolysis of nucleotides.

Enzymic properties of nucleotide-hydrolyzing Abzs

The properties of the ATPase IgGs from AI-prone MRL mice distincted them from other ATP-hydrolyzing enzymes. In contrast to known ATPases (30, 40 and references therein), IgG utilized not only ATP but also other NTPs, nucleoside-5’-diphosphate (NDPs) and NMPs as substrates with comparable efficiencies. Figure 1(D) demonstrates that the incubation of mixtures of [α-32P]NTPs, [α-32P]NDPs and [α-32P]NMPs with IgGs leads finally to a formation of only orthophosphate (P). Hydrolysis of [α-32P]NTP during first 10–30 min leads to formation of [α-32P]NDP as the main product of the reaction and also small amount of [32P]NMP and P, while after 2 h of incubation P is the main product of NTP hydrolysis. No formation of 32P-labeled diphosphate (PPi) or triphosphates (PPP) was observed. The efficiencies of IgG-dependent hydrolysis of [32P]AMP, [32P]dAMP and [γ-32P]NTP were comparable (for example Figs 1 and 4).

Taking into account that the specific activities of IgGs with ATP as substrate varied significantly between different mice (see below), we compared the kinetics of hydrolysis of ATP
and AMP using two preparations of catalytic pIgGs with a relatively moderate specific activity. The $K_M$ and $V_{\text{max}}$ (and apparent $k_{\text{cat}}$) values were measured using total concentration of pIgGs and conditions of the pseudo first-order reaction. In this case, catalytically inactive IgG subfractions interacting and non-interacting with substrates do not affect the $K_M$ values. The measured $k_{\text{cat}}$ values reflect a relative content of catalytically active and inactive subfractions in pAbs and an average value of $k_{\text{cat}}$ values characterizes the RA of different catalytic mAbs within polyclonal preparations and reflect the bottom of the apparent $k_{\text{cat}}$ values (5, 30, 33, 41). In the case of significant difference in $K_M$ or $k_{\text{cat}}$ values (≈4- to 5-fold) characterizing different catalytic antibody subfractions in pAbs, the dependencies of $V$ on $[S]$ usually are not consistent with simple Michaelis–Menten curves but correspond to a sum of two or more hyperbolic curves as well as $K_M$ or $k_{\text{cat}}$ values (2–5, 30, 33, 41). The initial rate data obtained at increasing AMP (Fig. 4A) and ATP (Fig. 4B) concentrations were consistent with Michaelis–Menten kinetics, suggesting that pIgGs contain only one major catalytic subfraction or more major ATPase IgG subfractions characterized by comparable $K_M$ and $k_{\text{cat}}$ values. The apparent $K_M$ and $V_{\text{max}}$ (1.5 μg ml$^{-1}$ or 10$^{-8}$ M IgGs were used) values for ATP in the case of two IgGs were found to be 1.7 ± 0.4 and 1.0 ± 0.2 mM and 3.1 ± 0.5 and 3.7 ± 0.6 μM min$^{-1}$ (apparent $k_{\text{cat}}$ = 5.2 ± 0.8 and 6.2 ± 1.0 s$^{-1}$), respectively. Affinity of these two IgGs for AMP in terms of $K_M$ values ($K_M = 0.9 ± 0.3$ and 0.77 ± 0.2 mM) was comparable with that for ATP, while the relative rates of AMP hydrolysis ($V_{\text{max}}$: 1.5 μg ml$^{-1}$ or 10$^{-8}$ M IgGs were used) were ~3.3 to 3.8 times lower than 0.83 ± 0.15 and 1.1 ± 0.19 μM min$^{-1}$ (apparent $k_{\text{cat}} = 1.4 ± 0.25$ and 1.9 ± 0.3 s$^{-1}$).
Interestingly, the relative affinity of nucleotide-hydrolyzing mouse IgGs for nucleotides is ~3- to 40-fold lower than that of human milk IgGs ($K_M = 44$–$300$ µM) (30). At the same time, mouse IgGs hydrolyze ATP and AMP 80- to 2600-fold faster than human milk plgGs; the $k_{cat}$ values for which (determined using total IgG concentration) vary, depending on the preparation, in a $(0.23–1.7) \times 10^{-2}$ s$^{-1}$ range (30). As we have shown previously, the fraction of Abzs with different catalytic activities usually does not exceed 1–5% of total IgGs (2–5). Since the specific activities were calculated using the total concentration of mouse pIgGs, the specific activities of the individual monoclonal subfractions with nucleotide-hydrolyzing activities in a pIgG pool may be significantly higher than those of the non-separated pIgGs (see below).

**Relationship between antibody DNase and ATPase activities**

We next addressed the question whether the same fractions of pIgGs can hydrolyze both DNA and mononucleotides. In order to estimate an average situation for IgGs from various mice, we have prepared an equimolar mixture of five different IgGs (designated IgGm) from five spontaneously diseased mice. First, we have analyzed the effect of AMP on the IgGm-dependent hydrolysis of $[^{32}P]ATP$ and supercoiled DNA (scDNA) (15 nM) using concentrations of these substrates comparable with their $K_M$ values. Figure 5(A) demonstrates that AMP effectively inhibited ATP hydrolysis, while even at saturating concentrations (30–50 $K_M$) AMP did not decrease the hydrolysis of scDNA. ATP effectively inhibited the

![Graph](https://example.com/graph.png)

**Fig. 4.** Determination of the $K_M$ and $V_{max}$ values for AMP (A) and ATP (B) in the reaction catalyzed by IgGs from one mouse using a Lineweaver–Burk plot; 1.5 µg ml$^{-1}$ IgGs were used. Reactions were performed as described in Materials and methods.
hydrolysis of \([^{32}P]\text{AMP}\) (1.5 mM; data not shown). scDNA and oligonucleotides without 3’-terminal phosphate group, \(d(Tp)_{10}\) or \(d(Ap)_{10}\) and their duplexes, did not inhibit the hydrolysis of \([\gamma^{32}P]\text{ATP}\) at their concentrations comparable with ~25 to 1000 \(K_M\) for these substrates (Fig. 5B). As mentioned above, both separated H and L chains possess ATPase activity, while hydrolysis of DNA was catalyzed only by L chains of pIgGs. Using two IgG preparations, we have shown that AMP is a competitive inhibitor toward ATP as a substrate with \(K_I\) values 0.9 ± 0.1 and 1.5 ± 0.2 mM depending on the antibody preparation (data not shown), which are comparable with \(K_d\) (and \(K_M\)) values of complexes of different enzymes with AMP that usually lie between 0.05 and 5 mM [36]. We conclude that mononucleotides and DNA are hydrolyzed by different subfractions of pIgGs, and only AMP effectively competes with ATP (and vice versa) for the nucleotide-binding site of the specific subfractions of pIgGs.

At concentrations from 1 µM to 0.5 mM (2–10^3 \(K_M\)), \(d(pT)_{10}\) and \(d(pA)_{10}\) containing a 5’-terminal phosphate did not inhibit hydrolysis of \([\gamma^{32}P]\text{ATP}\), while a significant increase in their concentration to 10–50 mM (comparable with ~7 to 50 \(K_M\) for AMP) decreased the ATP hydrolysis by 20–25% (Fig. 5B). This result can indicate that at very high concentrations, oligonucleotides containing 5’-phosphate can interact with IgG fractions hydrolyzing ATP and other mononucleotides. However, the plateau corresponding to 20–25% of the reaction inhibition shows that most probably not all subfractions of ATPase IgGs are capable of interacting with \(d(pN)_{n}\) and their duplexes even at very high concentrations.

A number of canonical phosphatases are known to remove terminal phosphates not only from \(d(pN)_{n}\) but also from NMPs, NDPs and NTPs [42]. Mouse pIgGs not only hydrolyze 5’-\([^{32}P]d(pT)_{10}\), 5’-\([^{32}P]d(pA)_{10}\) and their duplexes.

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Fig. 5. (A) An effect of AMP on the hydrolysis of scDNA (15 nM, curve 1) and \([\gamma^{32}P]\text{ATP}\) (1.5 mM; curve 2) catalyzed by pIgGm. (B) The influence of scDNA (curve 1) and \(d(pT)_{10}\) • \(d(pA)_{10}\) (curve 2) on the IgGm-dependent hydrolysis of \([\gamma^{32}P]\text{ATP}\) (1.5 mM). The initial rate of the substrate hydrolysis in the absence of effectors was taken for 100% (A and B). An average error in initial rate determination from three experiments for any concentration of effectors did not exceed 5–7%. (C) Analysis of ATPase activity of purified monoclonal IgGs by thin-layer chromatography on Kieselgel F254 plates (C) and IgG-dependent hydrolysis of 5’-\([^{32}P]d(pT)_{10}\) • \(d(pA)_{10}\) by PAGE (D) and autoradiography. (C) Before chromatography, standard reaction mixtures without antibodies (lane 1) or with four different preparations of monoclonal IgGs (1.2 × 10^{-3} mg ml^{-1}, lanes 2–5) were incubated at 30°C for 10 min in the presence of 0.1 mM \([\gamma^{32}P]\text{ATP}\). (D) Reaction mixtures containing 0.1 mM 5’-\([^{32}P]d(pT)_{10}\) • \(d(pA)_{10}\) were incubated at 30°C for 20 h without antibodies (lane 6) or in the presence of four different monoclonal IgGs (0.05 mg ml^{-1}, lanes 1–4) and pIgG from the sera of one diseased mouse (0.01 mg ml^{-1}, lane 5). Lane 7 corresponds to mixture of control \(^{32}P\)-labeled \(d(pT)_{2}\) and orthophosphate.
but also catalyze accumulation of $[^{32}P]$orthophosphate (Fig. 5D, lane 5). The relative rate of orthophosphate accumulation during incubation of S'-$[^{32}P]$d(pT)$_{10}$• d(pA)$_{10}$ (100 μM, saturated concentration of this ligand for DNase IgGs) in the presence of three different plgGs was calculated to be $\sim$(1 to 3) $\times$ 10$^5$-fold lower than that for $[^{32}P]$ATP (100 μM). Thus, a part of specific subfractions of plgGs hydrolyzing ATP may possess low phosphatase-like activity, but this IgG phosphatase activity is not typical. In contrast to known canonical phosphatases, catalytic plgGs were active only in the presence of Mg$^{2+}$ ions and did not show detectable hydrolysis of p-nitrophenyl phosphate (data not shown), a typical substrate of canonical phosphatases (42). In addition, mouse IgGs similar to ATPase IgGs from human milk (30) did not hydrolyze ATP analog, γ-anilide of ATP, with a blocked terminal phosphate.

Using hybridoma technology with following screening for mouse ATPase IgGs, we have found four clones demonstrating high and reproducible ATPase activities. Homogeneous preparations of these monoclonal IgGs were obtained by affinity chromatography on Protein A-Sepharose similar to pIgGs and preparations of these monoclonal IgGs were obtained by affinity chromatography on Protein A-Sepharose similar to pIgGs.

Dynamics of ATPase activities

Spontaneously arising AI pathology of MRL mice leads to the appearance of the visual markers of disease at 3–7 months of age or later (23). The pronounced AI disorder in mice is characterized by marked hypergammaglobulinemia, production of numerous auto-antibodies, glomerulonephritis, etc. (see above). Proteinuria (>3 mg of protein per milliliter of urine) may be considered as a marker of very profound AI reactions in mice, when kidney functions are significantly impaired. Abzs can usually be detected in human serum at the early stages of different AI diseases, when the change in auto-antibody concentration is not significant in comparison with the norm, and their RAs are increased with the progress of the pathology (2–5). Therefore, it was interesting to compare activities of ATPase Abzs of mice at various life periods, including pre-disease or developed pathology, and after animal immunization.

Some examples of $[^{32}P]$ATP hydrolysis by IgGs from different mice are given in Fig. 6. The complete hydrolysis of ATP during 2 h at a standard concentration of IgGs (0.1 mg ml$^{-1}$) was taken as 100% of the ATPase activity.

First, we have studied the relative Abz activities in healthy non-AI control BALB/c and CBA mice of various ages (3–7 months, Table 1) and formally healthy AI-prone 2- to 3-month old MRL mice. At 3–7 months, BALB/c and CBA mice showed no proteinuria (0.1–0.12 mg ml$^{-1}$ <3 mg ml$^{-1}$), very low concentrations of antibodies to native and denatured DNA (0.017–0.04 A$_{450}$) and non-detectable level of ATPase activity (Table 1).

Most of MRL males and females at 2–3 months of age demonstrated characteristics similar to those for control healthy BALB/c and CBA mice and the absence of detectable level of ATPase activity (Table 1); these MRL mice were considered formally healthy animals. Some formally healthy MRL males and females at 7 months of age demonstrating no visual symptoms of AI pathology were similarly characterized by low protein concentrations (~0.8 to 0.88 mg ml$^{-1}$) and slightly increased average values of anti-DNA antibody concentrations (0.08–0.2 A$_{450}$) compared with 3-month-old mice (Table 1). Interestingly, all 7-month-old formally healthy MRL males demonstrated detectable ATPase (range 0.1–2%, average 0.4%) activity (Table 1). Similar average relative ATPase (range 0.3–6; average 2.4%) activity was observed for 7-month-old healthy MRL females (Table 1).

MRL males and females of the same age with visual symptoms of spontaneous SLE demonstrated very high proteinuria (~5 to 8 mg ml$^{-1}$) but a moderate increase in the anti-DNA antibody concentrations (0.16–0.23 A$_{450}$; Table 1). At the same time, the average values of ATPase activity for Abzs from males (~4 to 314%, average 68.3%) were
increased significantly (Table 1). Similarly, a very significant increase in ATPase activity was observed for 7-month-old females with visual symptoms of spontaneous SLE, but this activity in average (~65.0%) was comparable with that for males (Table 1).

We have immunized healthy MRL males (2–3 months of age) with complex of DNA and methylated BSA. This led to a fast development of all markers of SLE including drastic proteinuria (~9.5 mg ml⁻¹), which was increased ~25-fold compared with healthy males but was comparable with that for males with spontaneous SLE (~8.0 mg ml⁻¹) (Table 1). Immunization of the males led to a maximal increase in the level of antibodies against native (average ~0.6 A₄₅₀) and denatured DNA (average ~1.0 A₄₅₀) in comparison with all young mice or ill males of 7 months of age (Table 1). The maximal increase in the relative ATPase activity was observed for immunized males (range 500–1887%), the average activity estimated as ~1333% (Table 1). Compared with healthy 7-month-old males, ATPase activity of the immunized mice was increased ~3330-fold (Table 1).

The analysis of time-dependent changes in proteinuria, anti-DNA antibodies and Abz activities before and after development of visible pathology markers permits us to propose that MRL mice at any age 1–1.5 months before development of profound spontaneous pathology are characterized in average by detectable but very moderate increases in concentrations of anti-DNA antibodies and urine proteins. Interestingly, only the increase in ATPase activity at a pre-disease stage in MRL mice was unambiguously significant (Table 1).

Although the mice were immunized with DNA, the ATPase activity of their antibodies also increased significantly (Table 1). These findings are in an agreement with previously published data that immunization of mice with AI diseases gives rise to an unexpectedly high increase in the number of clones secreting catalytic antibodies compared with normal mice (26,27).

It should be noted that we have previously observed production of Abzs with different enzymic activities at very early stages of several human AI pathologies and a significant increase in the activity 0.5–1.5 months before exacerbation during typical remission type of AI disease progress (2–5). Therefore, one cannot exclude that the increase in the ATPase Abz activity can be considered a reliable marker of the beginning of the spontaneous pathology (pre-disease conditions) in MRL mice and as an indicator of the debut stages or exacerbation of previously developed human AI diseases.

**References**

ATPase antibodies

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