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Systemic lupus erythematosus: Molecular cloning of fourteen recombinant DNase monoclonal kappa light chains with different catalytic properties

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ABSTRACT

Background: DNase antibodies can play an important role in the pathogenesis of different autoimmune pathologies.
Methods: An immunoglobulin light chain phagemid library derived from peripheral blood lymphocytes of patients with systemic lupus erythematosus (SLE) was used. The small pools of phage particles displaying DNA binding light chains with different for DNA were isolated by affinity chromatography on DNA-cellulose and the fraction eluted with 0.5 M NaCl was used for preparation of individual monoclonal light chains (MLChs, 28 kDa). Forty-five of 451 individual colonies were randomly chosen for a study of MLChs with DNase activity. The clones were expressed in *Escherichia coli* in a soluble form, and MLChs were purified by metal chelating chromatography followed by gel filtration, and studied in detail.
Results: Fifteen of 45 MLChs efficiently hydrolyzed DNA, and fourteen of them demonstrated various optimal concentrations of KCl or NaCl in a 1–100 mM range and showed one or two pH optima in a 4.8–9.1 range. All MLChs were dependent on divalent metal cations: the ratio of relative DNase activity in the presence of Mn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, and Co^{2+} was individual for each MLCh preparation. Fourteen MLChs demonstrated a comparable affinity for DNA (260–320 nM), but different k_{cat} values (0.02–0.7 min^{-1}).
Conclusions: These observations suggest an extreme diversity of DNase abzymes from SLE patients.
General significance: SLE light chain repertoire can serve as a source of new types of DNases.

1. Introduction

Catalytically active artificial antibodies (Abs) or abzymes (Abzs) against transition chemical states of different reactions were studied intensively (reviewed in [1–6]). During the past two decades it has become clear that auto-antibodies (auto-Abs) from the sera of patients with different autoimmune diseases can possess enzymatic activities and that their occurrence is a distinctive feature of autoimmune diseases (reviewed in [6–13]).

The first example of a natural polyclonal Abz was an IgG found in bronchial asthma patients which hydrolyzes intestinal vasoactive peptide [14], the second was an IgG with DNase activity from the sera of SLE patients [15], and the third was an IgG with RNase activity in SLE [16]. Similar to artificial abzymes against analogs of transition states of catalytic reactions [1–6], naturally occurring abzymes may be antibodies (Abs) raised directly against enzyme substrates acting as haptens and mimicking transition states of catalytic reactions [6–13]. On the other hand, antiidiotypic Abs can be induced in autoimmune diseases by a primary antigen and may show some of its features including the catalytic activity [17–21], for review also see [1–8].

Polyclonal natural IgG and/or IgA and IgM abzymes hydrolyzing DNA, RNA, polysaccharides, nucleotides, oligopeptides, and proteins from the sera of patients with several autoimmune diseases, such as systemic lupus erythematosus (SLE), Hashimoto's thyroiditis, polyarthritis, multiple sclerosis (MS), asthma, and rheumatoid arthritis, are described [22–38]. Abzymes can be found in viral diseases with a pronounced immune system disturbance (viral hepatitis, acquired immune deficiency syndrome, and thick bone encephalitis) [6–38]. Some healthy patients demonstrated abzymes with low proteolytic [14,29] and polysaccharide-hydrolyzing activities [27,28]. However, healthy humans and patients with many diseases with insignificant AI reactions usually lack abzymes or develop Abs with very low catalytic activities (especially with DNase activity), often on a borderline of the sensitivity of detection methods [6–13,22–26,31–38].

RNase and DNase IgG and/or IgM abzymes from the sera of patients with SLE, MS, other different autoimmune diseases, and autoimmune-prone MRL-lpr/lpr mice were found to be extremely diverse [6–13,22–25,35,37,38]. Different patients may have a relatively small or an

Abbreviations: Ab, antibody; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; MLCh, monoclonal light chain; MS, multiple sclerosis; SLE, systemic lupus erythematosus; RA, relative activity

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extremely large pool of polyclonal DNase abzymes. Abz pools can contain different proportions of light chains of κ- and λ-types, Abs demonstrating different pH optima, having different net charges, metal-independent or activated by different metal ions, and characterized by different substrate specificities [6–13]. It was shown that small fractions of IgGs of all four subclasses (IgG1–IgG4) from SLE and MS patients are catalytically active in the hydrolysis of DNA [13,36] and different proteins [37,38].

Importantly, all lengthy substrates interact with variable parts of both heavy and light chains of abzymes. The catalytic centers of abzymes are usually located on the light chain, while the heavy chain is mainly responsible for the specific antigen recognition and the increased antigen affinity for abzymes [6–13]. The isolated light chains hydrolyzed vasoactive intestinal peptide with the specific activity 32-fold greater than Fab fragments [39]. Interestingly, the abzyme-dependent hydrolysis of DNA and RNA by SDS-PAGE isolated light chains of IgGs (and/or IgA or IgM) from SLE, MS, asthma, and other autoimmune patients, as well as from MRL-lpr/lpr mice was observed; isolated light chains were more active than intact Abs [12,22–25,40–43]. A similar situation was observed for human milk IgGs and sIgAs with DNase and RNase activities [44,45]. In addition, it was shown that both heavy and light chains of sIgAs had affinity to DNA-cellulose but only light subunits hydrolyzed DNA and RNA [44].

The interesting goal of catalytic Abs research is not only to study such abzymes, but also to develop new patient and animal therapies that use the advantages offered by human and mammalian monoclonal catalytic Abs.

Prothrombin is the precursor of thrombin, a central enzyme in coagulation. Auto-Abs to prothrombin are associated with thromboembolism. Two light chains from patients with multiple myeloma with prothrombinase activity were identified [46,47].

Abs to the superantigenic determinant of HIV gp120 (gp120(SAg)) are potential protective agents against HIV infection. One MLCh from multiple myeloma patients specifically hydrolyzed gp120 protein [48]. The toxic effect of gp120 in neuronal cultures was reduced by about 100-fold by pre-treatment of the protein with the light chain. The light chain subunits of Abs cloned from lupus patients using phage library methods bind and hydrolyze gp120(SAg) independent of the heavy chains [49].

The catalytic light chain capable to degrade the active site of the urease of *Helicobacter pylori* and eradicate the bacterial infection in a mouse stomach was obtained [50]. A monoclonal abzyme, ECL2B-2, was obtained by immunizing with a peptide possessing a part of a sequence of a chemokine receptor, CCR-5, which is present as a membrane protein on the macrophage surface. It plays an important role in HIV infection [51]. The light chain had high catalytic activity.

Studies of abzymes in autoimmune patients and milk of human mothers show a very wide spectrum of natural abzymes which can be formed [6–13]. Their application in medicine, science and biotechnology requires production of human monoclonal abzymes, which is possible to obtain at this time using the phage display technology. First the light chain of the abzymes against vasoactive intestinal peptide (VIP) was expressed in bacteria, purified, and found to possess an intrinsic catalytic activity [52,53].

Deposition of beta-amyloid is considered an important early event in the pathogenesis of Alzheimer's disease. Clearance of beta-amyloid thus represents a potential therapeutic approach. Proteolytic recombinant Ab fragments for potential alpha-secretase activity were screened [54]. Two catalytic MLChs were identified.

A panel of human recombinant MLChs was screened for the ability to cleave a synthetic peptide corresponding to a neutralizing epitope of hepatitis C virus glycoprotein E1 [55]. One of the 39 light chains studied hydrolyzed the peptide.

SLE is usually considered to be related to patient's autoimmunization with DNA, since the sera of such patients usually contain DNA and anti-DNA Abs in high concentrations [56]. Compared with healthy donors, concentrations of DNA and anti-DNA Abs are higher not only in patients with SLE (36% of SLE patients), but also in MS (17–18%), primary Sjogren's syndrome (18%), Hashimoto's thyroiditis (23%), myasthenia gravis (6%), rheumatoid arthritis (7%) [57], autoimmune hepatitis [58], as well as lymphoproliferative [59] and some viral diseases (e.g., viral hepatitis [60] and AIDS [61]). On the example Abs from SLE [59], MS [9], and DNA-hydrolyzing Bence–Jones proteins from multiple myeloma patients [40] it was shown, that DNase abzymes are cytotoxic, enter the nucleus and cause DNA fragmentation, and induce cell death by apoptosis. These abzymes can play an important negative role in the pathogenesis of different autoimmune pathologies. A decrease in the relative activities of DNase abzymes from patients with Hashimoto thyroiditis correlates with normalization of the concentration of thyroid hormones and improvement of the patients' clinical status [26]. Interestingly, hydrolysis of DNA by DNases is considered a promising approach for the treatment of SLE (and probably other AI diseases) [62]. Thus, human DNase monoclonal Abs and their light chains may be potentially perspective agents against SLE and some other AI diseases.

There are several examples of monoclonal mouse IgGs with DNase activity. Monoclonal mouse anti-DNA autoantibody BV04-01 catalyzed hydrolysis of DNA in the presence of Mg^{2+} ions [63]. Pronounced cleavage specificity for both single- and double-stranded DNA was observed with efficient hydrolysis of CG-rich regions of double-stranded DNA. It was shown that the DNase center of a monoclonal IgGs from MRL-lpr/lpr mice is located at the interface between the light and heavy chains, and both L- and H-chains are able to hydrolyze DNA when separated [64]. Murine anti-DNA autoantibody MRL-4 with sequences of variable region genes highly homologous to those of autoantibody BV04-01. It was shown that despite significant homology to BV04-01, MRL-4 had no DNA-cleaving activity, but also reversion of its unusual P23 mutation to the germline alanine resulted in a dramatic loss of affinity to DNA [65]. Contrary to this effect, transfer of the P23 mutation to the BV04-01 has resulted in a significant drop in DNA binding and almost complete loss of catalytic activity.

Only Bence–Jones proteins from patients with multiple myeloma should be considered as human monoclonal abzymes [40].

We report in this article that 14 recombinant monoclonal light chains cloned from lupus patients using phage library method bind and hydrolyze DNA demonstrating very different enzymatic properties.

2. Materials and methods

2.1. Materials and chemicals

Most chemicals, proteins, IgG preparations, and the Superdex 200 HR 10/30 column were from Sigma, while chelating Sepharose from GE Healthcare. Glutarate crosslinked DNA-cellulose (stable to nucleases) was from NIKTI BAV (Russia).

2.2. Amplification of phage library

We have used human lupus kappa light chains library (from three patients; 10^{11} variants of different light chains) cloned in pCANTAB5His6 vector, which was obtained using standard methods [52,66–68]. This library was a gift from S. Paul and S. Plaque (University of Texas Houston Medical School, USA); all details of this library preparation were described earlier [66]. Amplification of the VCSM13 helper phage and determination of its titer were carried out according to [66]. For amplification of the phage library, 500 μl of the overnight culture of *Escherichia coli* TG1 was placed in a flask containing 50 ml of 2YTL medium; the mixture was shaken at 37 °C to $OD_{600} = 0.6–0.8$. Then 100 μl (10^9 phage particles) of the phage library was added to 5 ml of this culture, and the mixture was incubated for 30 min at 37 °C without shaking followed by 1 h with shaking. After the addition of 90 ml of 2YTL medium containing 50 μg/ml ampicillin, the cells were grown for 1 h with shaking and then 10 ml of phage VCSM13

(10^{11} phage particles) was added; the mixture was incubated for 30 min at 37 °C without shaking and then for 1 h with shaking. The cells were collected by centrifugation (10 min, 4000 rpm) at room temperature and the pellet was re-suspended in 100 ml of 2YTL medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. The cells were grown overnight and pelleted by centrifugation (1000 rpm, 10 min). A solution of PEG-8000 (20%) containing 2.5 M NaCl was added to a supernatant (1/5 of the final volume) and the mixture was incubated on ice for 3 h and then centrifuged (10 000 rpm, 30 min). The precipitate obtained was twice re-suspended in 1 ml of 10 mM Tris–HCl (pH 7.5) and the solution was centrifuged (12,000 rpm, 5 min). Finally the precipitate was re-suspended in 4 ml of 10 mM Tris–HCl (pH 7.5) and the titer (2×10^{12}) of phage library was estimated.

2.3. Chromatography of phage particles on DNA cellulose

Preparations of phage particles (1 ml containing 2×10^{12} phage particles) prepared using *E. coli* TG1 were loaded onto a DNA cellulose column (3 ml) equilibrated with 20 mM Tris–HCl (pH 7.5) containing 0.1 mM EDTA and the column was washed with the same buffer to zero optical density. For the control similar solution of phage particles corresponding to pCANTAB plasmid containing no library of light chains was used. The phage particles were eluted with the same buffer containing different concentrations of NaCl (0.05–3 M), and then with 50 mM glycine–HCl (pH 2.6) similar to purification of polyclonal Abs [22–26,69]. Phage particles were collected, concentrated, and each fraction was precipitated using PEG/NaCl as described above. The titers of phage particles were determined (see Fig. 1) and each fraction was assayed for DNase activity.

2.4. Preparation of monoclonal phage particles

For preparation of soluble MLChs we have used *E. coli* HB2151. An overnight culture of *E. coli* HB2151 (200 μl) was placed in a flask containing 80 ml of 2YTL medium and this mixture was incubated with shaking at 37 °C to $OD_{600} = 1.0$. The cells were centrifuged for 10 min (4000 rpm), and the pellet was re-suspended in 80 ml of 10 mM $MgSO_4$ for receiving a solution containing cells in initial concentration. The preparation of phage particles eluted from DNA cellulose with 0.5 M NaCl was diluted 100-fold in 2YTL, and 10 μl of this solution was added to 90 μl of the *E. coli* cells. The mixture was incubated for 30 min at 37 °C and uniformly distributed over a Petri dish with agarized 2YTL containing 40 μg/ml ampicillin; the dish was incubated overnight at 37 °C. For further analysis, 45 of 451 individual colonies from two dishes were randomly chosen.

To propagate individual colonies of phages, the material from each colony were grown overnight at 37 °C in a Petri dish as described above. The cells were scraped to a vial containing 1.5 ml 2YTL medium supplemented with ampicillin (50 μg/ml), and the mixture was shaken at 37 °C to $OD_{600} = 0.6$. Then 1.5 ml of 2YTL and isopropyl β-D-1-thiogalactopyranoside (IPTG) to the final concentration of 2 mM were added, and the mixture was shaken at 37 °C overnight. The suspension

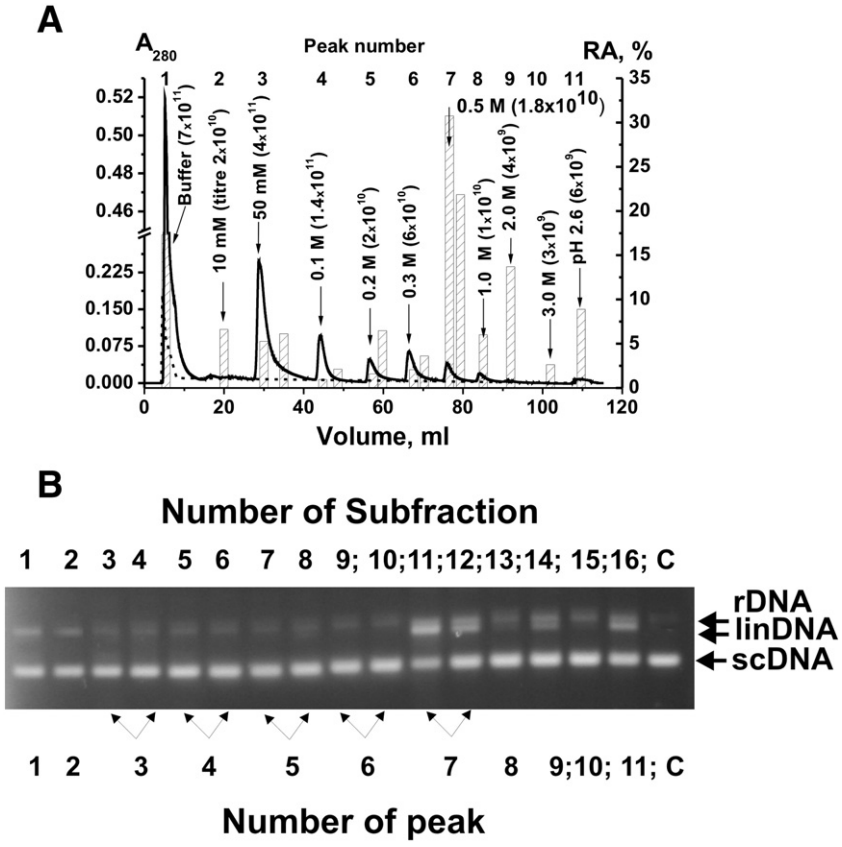

Fig. 1. Affinity chromatography of phage particle preparation on DNA-cellulose: (—) and (—), absorbance at 280 nm of the material corresponding to phage particles with and without kappa light chains cDNA, respectively. The bars indicate the relative activity (RA) of 16 phage particle fractions corresponding to peaks 1–11 eluted from the sorbent with different concentrations of NaCl and an acidic buffer (pH 2.6) (A); the reaction mixtures were incubated with the phage particles (2 μl) for 3 h at 37 °C. The titers of phage particles corresponding to the main different peaks are given in the parenthesis. The error in the initial rate determination from three experiments for any fraction did not exceed 6–10%. The DNA cleavage products in the case of small pools of phage particles with different affinity to DNA were analyzed by electrophoresis in 1% agarose (B); lanes 1–16 correspond to 16 fractions of peaks 1–11 (A). Lane C corresponds to scDNA incubated without phage particles. The arrows mark the positions of supercoiled (scDNA), relaxed (rDNA), and linear (linDNA) plasmid DNA. For details, see Materials and methods.

was centrifuged (1 min, 12,000 rpm); the supernatant containing phage particles were collected and used to obtain monoclonal phages.

2.5. Purification of monoclonal light chains

Supernatant (50 ml) containing MLCh was dialyzed twice for 3 h at 4 °C against 1 l of H_2O and then overnight against Buffer A consisting of 50 mM Tris–HCl (pH 7.2), 0.5 M NaCl, and 1 mM DTT. Affinity chromatography was performed on a HiTrap chelating Sepharose column (1 ml) charged with Ni^{2+} and equilibrated in buffer A. After loading MLCh, the column was washed with the same buffer to zero optical density in the eluate. The bound MLChs were eluted with a gradient of imidazole (0–1 M) in 50 mM Tris–HCl (pH 7.2). Optical density was measured in all fractions. The fractions containing MLChs were dialyzed against 20 mM Tris–HCl (pH 7.5) and then concentrated. According of SDS-PAGE analysis these preparations contain small admixtures of several proteins (probably from medium, *E. coli* cells or phage particles) interacting with HiTrap chelating Sepharose. For isolation of homogeneous preparations of MLCh we used gel-filtration. Gel filtration of this preparation was performed on a Superdex 200 HR 10/30 column equilibrated with 50 mM Tris–HCl (pH 7.5) containing 0.3 M NaCl as was described for human and rabbit IgGs [70–73]. Before gel filtration, the MLCh samples were incubated in TBS containing 2.0 M $MgCl_2$ for 30 min at 20 °C. The fractions containing 28 kDa MLCh were collected. 0.8–2.5 mg of MLChs was obtained from 1 l of the medium, depending on the preparation. Only fraction corresponding to the MLChs (not to small proteins admixtures) demonstrated DNase activity.

2.6. Western blotting and ELISA

The light chains of Abs corresponding to the combined fractions were analyzed by Western blotting onto a nitrocellulose membrane using horseradish peroxidase conjugated with mouse Abs against light chains of human Abs as in [25,36–38].

The interaction of the MLChs with DNA was analyzed in the presence of 1 mM EDTA using standard ELISA plates with immobilized double-stranded DNA as described in [25,36]. After adsorption of the MLChs and a consecutive treatment of samples with horseradish peroxidase conjugated with mouse antibodies against light chains of human Abs the reaction mixtures were incubated with tetraethyl benzidine and H_2O_2. The reaction was stopped with H_2SO_4 and optical density (A_{450}) was determined. The relative content of anti-DNA MLChs in the samples was expressed as a difference in the relative absorption at 450 nm between experimental and control samples; controls with DNA but without MLChs were used.

2.7. DNase activity assay

DNase activity was analyzed using scDNA as described earlier for DNase I, DNase II, and human serum catalytic Abs [22–26]. Initially, the reaction mixtures (20 μl) contained 14 μg/ml (4.7 nM) supercoiled pBluescript DNA, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM Tris–HCl (pH 7.5), 2–5 μl of the phage preparation after chromatography on DNA-cellulose (Fig. 1, see below) or 10–50 nM MLChs, and were incubated for 0.1–3 h at 37 °C. Later, the specific optimal conditions were used for every preparation of MLChs. Dependencies of the relative DNase activity (RA) on the concentration of metal ions were analyzed using $MnCl_2$, $MgCl_2$, $CaCl_2$, $CuCl_2$, $CoCl_2$, $NiCl_2$, or $ZnCl_2$ (each at 0.005–5 mM) as well as KCl and NaCl (0–150 mM).

The cleavage products were analyzed by electrophoresis in 1% agarose gel. The images of ethidium bromide-stained gels were captured on a Sony DSC-F717 camera and a relative amount of DNA in different bands was analyzed using ImageQuant v5.2 (Molecular Dynamics, GE Healthcare, UK).

Similar to DNase I and polyclonal abzymes from autoimmune patients [6–13], depending on the incubation time and type of metal ions, all of MLChs were capable to hydrolyze plasmid DNA forming single breaks in one strand of supercoiled DNA (relaxed DNA) and then multiple breaks yielding linear DNA. Finally, they hydrolyzed DNA into short and medium-length oligonucleotides. However, after such a deep hydrolysis of DNA, it was very difficult to estimate relative activity of MLChs. Therefore, to estimate the DNase activity quantitatively, we have found the concentration for each MLCh preparation and the time of incubation sufficient to convert scDNA into the relaxed form without further noticeable fragmentation after 0.2–5 h of incubation. Finally, the activities of the MLCh preparations were determined as a decrease in the percentage of DNA converted from the initial supercoiled form to the relaxed form (and sometimes additionally linear form), corrected for the distribution of DNA between these bands in the control (incubation of pBluescript in the absence of Abs).

All measurements (initial rates) were taken within the linear regions of the time courses (15–40% of DNA hydrolysis) and a complete transition of the supercoiled plasmid (14 μg/ml = 4.7 nM) to the nicked form was taken for 100% activity. If the activity was low (<5–10% of scDNA disappearance), the incubation was prolonged to 2–5 h, depending on the sample. If the degradation of supercoiled DNA after 0.5–2 h of incubation exceeded 50%, the concentration of Abs was lowered 2–100-fold, depending on the sample. This approach allowed a normalization of the relative activity: DNA (nM)/1 h/1 mg of MLChs.

In some experiments, the MLChs were extensively dialyzed against 50 mM Tris–HCl (pH 7.5) containing 0.1 M EDTA and 0.1 M EGTA and then three times against 1 mM Tris–HCl (pH 7.5); before the experiments, metal ions were removed from all solutions using a 5-ml Chelex column. The RAs of these preparations were assayed as described above using a standard reaction mixture. Dependencies of the RAs on the concentrations of metal ions were analyzed using $MnCl_2$, $MgCl_2$ (each at 0.005–5 mM).

Dependency of the activity on pH was analyzed using different buffer systems (50 mM): MES–NaOH (pH 5.3–6.6), Tris–HCl (pH 6.0–8.6) and glycine–NaOH (pH 9.0–10.0). The reaction mixture (20 μl) for DNase II contained 20 μg/ml supercoiled pBluescript DNA, 1 mM EDTA, 50 μg/ml gelatine, and one of above mentioned buffers of different pHs, while in the case of DNase I the same components and additionally 10 mM $MgCl_2$, 0.5 mM $CaCl_2$. The reaction was started by addition of DNase II and DNase I (10^{-2} Kunitz units/ml).

2.8. In situ analysis of DNase activity

DNase activity of MLChs was determined in situ after separation of proteins in 4–15% gradient SDS-PAGE gels containing 3 μg/ml calf thymus DNA as in [70–73]. For the analysis of DNase activity (and to remove SDS), the gels were washed at 25 °C for 4 h in 40 mM Tris–HCl, pH 7.5, containing 0.1% Triton X-100, 4 mM $MgCl_2$, and 0.2 mM $CaCl_2$ and then incubated in the same buffer containing 0.1 mM $CaCl_2$ but without Triton X-100 for 2 h followed with a fresh portion of the same buffer for 1–2 days at 30 °C. To visualize the regions of DNA hydrolysis, the gels were stained with ethidium bromide. The parallel longitudinal slices were used to detect the position of the MLCh in the gel by Coomassie R250 staining.

2.9. Determination of kinetic parameters

The K_m and V_{max} (k_{cat}) values were calculated from the dependencies of V versus [DNA] by least-squares non-linear fitting using Microcal Origin v5.0 software and presented as linear transformations using a Lineweaver–Burk plot [74]. All fifteen MLChs (5 nM) were used, the concentration of DNA was varied in the 30–200 nM range. Errors in the values determination were within 10–15%. The results are reported as mean ± S.E. of at least 2–3 independent experiments.

3. Results

3.1. Preparation and analysis of recombinant light chains

Polyclonal DNase and RNase IgGs from the sera of autoimmune patients, SLE mice, rabbits immunized with DNA, and human milk are usually very heterogeneous in their affinity for DNA and can be separated into many subfractions by chromatography on DNA-cellulose ([6–13,22–25,43–45,69–73] and references therein). It is known that MLChs (*E. coli* TG1) are presented on the surface of phage particles. Therefore, we first separated phage particles containing a pool of various MLChs with different affinity for DNA by chromatography on DNA-cellulose (Fig. 1A). The pool of MLChs of phage particles of the first peak having low affinity for this resin possessed detectable DNase activity. However, the same situation was observed earlier for intact polyclonal DNase abzymes from the sera of patients (and animals) with different diseases; some of them had a low affinity for DNA [6–13,22–25,43–45,69–73]. The complete pool of phage particles containing MLChs bound with DNA-cellulose were distributed between eleven peaks (16 fractions) eluted during chromatography (Fig. 1A) and all fractions corresponding to new small pools obtained were active in the hydrolysis of DNA (Fig. 1A and B). At the same time, we have not observed any detectable protein peaks having remarkable affinity for DNA-cellulose after similar affinity chromatography of phage particles corresponding to pCANTAB plasmid containing no cDNA of light chains (see Fig. 1A). It means that the pools of MLChs of all 16 fractions of phage particles with different affinities to DNA contain not only inactive but also catalytically active light chains with DNase activity. Interestingly, similar situation was observed for IgGs from autoimmune patients, diseased MRL-lpr/lpr mice and milk of healthy human mothers; when IgGs were eluted from DNA-cellulose by a NaCl gradient (0–3 M), 3 M $MgCl_2$, and acidic buffer the protein and DNase activity were distributed all over the chromatography profiles [6–13].

For preparation of individual colonies corresponding to recombinant MLChs, we have used *E. coli* HB2151 and phage particles eluted from DNA-cellulose with 0.5 M NaCl; this fraction demonstrated relatively high titer and DNase activity (peak 7, Fig. 1A). Two subfractions of peak 7 (Fig. 1A) were combined and phage particles of this mixture were growing on two Petri dishes with agar; 45 of 451 individual colonies obtained were chosen in a random way for study of DNase activity. It was shown that at least 15 of 45 single colonies of phage particles (~33%) are capable to hydrolyze DNA efficiently (see below).

On the end of recombinant MLChs there is a sequence of six histidine residues; this hexapeptide interacts efficiently with Ni^{2+} ions [66]. Recombinant MLChs corresponding to 15 single colonies demonstrating relatively high DNase activity and 30 single colonies without activity were used for purification of MLChs by chromatography on HiTrap™ chelating Sepharose charged with Ni^{2+} ions and by following gel filtration.

After purification, a mixture of equal amounts of 15 preparations of monoclonal MLChs (act-$MLCh_{mix}$) and 30 preparations without activity (inact-$MLCh_{mix}$) was prepared. The electrophoretical homogeneity of ~28-kDa act-$MLCh_{mix}$ was confirmed by SDS-PAGE with silver staining (Fig. 2A). It was shown, that all 15 purified monoclonal act-MLChs and 30 act-inact-MLChs preparations without activity demonstrated positive answer with mouse IgGs (conjugated with horseradish peroxidase) against human Abs light chains at Western blotting (for example, Fig. 2B and E) and positive ELISA answer using plates with immobilized double-stranded DNA. Fifteen act-MLCh preparations were active in the hydrolysis of DNA (for example, Fig. 2G), while 30 inact-MLCh preparations did not hydrolyze DNA noticeably (for example, Fig. 2H).

To exclude possible artifacts due to hypothetical traces of contaminating canonical enzymes, the $MLCh_{mix}$ was subjected to SDS-PAGE in a gel co-polymerized with calf thymus DNA, and their DNase activity was detected by incubating the gel in the standard reaction buffer (Fig. 2C, and F, lane 1). Ethidium bromide staining of the gels after the electrophoresis of the act-$MLCh_{mix}$ revealed sharp dark band against a fluorescent background of DNA in the gel zone corresponding only to $MLCh_{mix}$ and there were no other peaks of proteins or DNase activity (Fig. 2). The inact-$MLCh_{mix}$ preparation did not hydrolyze DNA (Fig. 2F, lane 2).

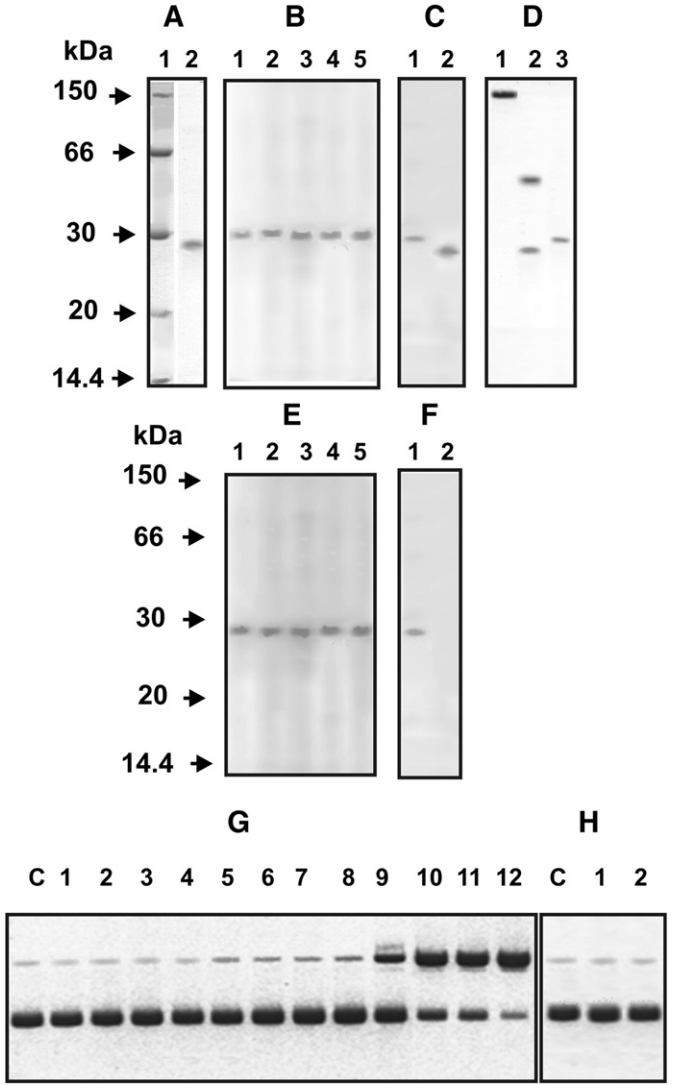

Fig. 2. SDS-PAGE analysis of act-$MLCh_{mix}$ (5 μg) homogeneity using a nonreducing 5–16% gradient gel followed by silver staining (lane 2); the arrows (lane 1) indicate the positions of molecular mass markers (A). Four individual preparations of act-MLChs (numbers 3, 7, 9 and 12; lanes 1–4) and act-$MLCh_{mix}$ with DNase activity (lane 5) (B), as well as five individual inact-MLCh (numbers 17, 21, 29, and 45; lanes 1–4) and inact-$MLCh_{mix}$ without DNase activity (lane 5) (E) were analyzed by Western blotting on a nitrocellulose membrane using murine IgGs against light chains of human Abs conjugated with horseradish peroxidase. In-gel assay of DNase activity of the act-$MLCh_{mix}$ (7 μg) (lane 1) and control SLE IgGs after incubation with DTT (lane 2) (C), as well as act-$MLCh_{mix}$ with (lane 1) and inact-$MLCh_{mix}$ without DNase activity (lane 2) (F). DNase activity was revealed by ethidium bromide staining as a dark band on the fluorescent background (C and F). A part of the gel containing DNA was stained with Coomassie R250 to show the position of intact SLE IgG (lane 1), its heavy and light chins after IgG reduction using DTT (lane 2), and the $MLCh_{mix}$ (lane 3) (D). DNase activity of different act-MLChs (20 nM) in the cleavage of scDNA was analyzed in the absence of externally added metal ions after (lanes 1–4) and before dialysis of the light chains against EDTA (lanes 5–8), as well as in the presence of externally added 5 mM $MgCl_2$ (lanes 9–12) (G). The reaction mixtures were incubated with act-$MLCh_{mix}$ (lanes 1, 5, and 9), MLCh-2 (lanes 2, 6, and 10), MLCh-3 (lanes 3, 7, and 11), and MLCh-9 (lanes 4, 8, and 12) (G). DNase activity of preparations of the inact-$MLCh_{mix}$ before (lane 1) and after addition of metal ions (lane 2) (H). Lane C corresponds to scDNA incubated in the presence 5 mM $MgCl_2$ but without MLChs (G and H). For details, see Materials and methods.

3.2. Recombinant DNase monoclonal light chains are Me^{2+} dependent

Many pro- and eukaryotic DNases including human DNase I [75,76] as well as polyclonal DNase abzymes from the sera of patients with different pathologies, and immunized rabbits [6–13,32–42,70–73] are Me^{2+}-dependent, while other DNases including DNase II [75,76] and some subfractions of DNase sIgAs from human milk [7–12,44,45] are Me^{2+}-independent. Therefore, we have first analyzed the RAs of the MLCh preparations before and after dialysis against EDTA + EGTA; after dialysis or in the presence of EDTA the MLChs completely lost DNase activity, but recovered this activity after addition of external $MgCl_2$ or $MnCl_2$ (for example, Fig. 2G).

In first set of experiments it was shown that the optimal concentrations of NaCl or KCl in the case of various act-MLChs may be very different. For some preparations two optima of these salts close to 10 or 80–100 mM were revealed Therefore, we have compared the dependences of DNase RAs for various act-MLCh preparations on the concentration of Mg^{2+} and Mn^{2+} in the presence of different fixed concentration of NaCl or KCl (10 and 80 or 100 mM). Fig. 3 demonstrates such dependences for several of act-MLChs analyzed. One can see that for all act-MLCh preparations in the case of all used concentrations of NaCl and KCl these dependencies reach plateau at 1.5–2.0 mM concentration of $MgCl_2$ and $MnCl_2$. Similar dependencies were observed for all 15 preparations of act-MLChs. Interestingly, for several act-MLCh preparations these dependencies had the shape-bell character; the inhibition of the reactions was usually observed at concentrations of Mg^{2+} and Mn^{2+} higher than 2–4 mM (Fig. 3A, B, and D). Therefore 2 mM concentration of $MgCl_2$ (and $MnCl_2$) was considered as optimal for all 15 act-MLCh preparations; it was independent on the concentrations of NaCl and KCl. At the same time, it is known that Na^+ and K^+ ions can influence on the spatial structures of nucleic acids, different enzymes, and antibodies. As a consequence they can in principle effect on the RAs of DNA-dependent enzymes and abzymes. In addition, dependences of RAs upon concentration of Na^+ and K^+ ions can give additional information about possible difference of 15 analyzed preparations of act-MLChs. Therefore, we have analyzed dependencies of the RAs of various act-MLChs on concentration of Na^+ (and K^+) ions at fixed 2 mM optimal concentration of $MgCl_2$ or $MnCl_2$. Several typical examples are given in Fig. 4. Interestingly, Na^+ and K^+ ions demonstrated different effects on the RAs of 14 of 15 act-MLCh preparations. For example, in the presence of Mg^{2+}, NaCl and KCl significantly increased the activity of MLCh-14 (Fig. 4A). Similarly effect of the increase in the RAs in the presence of NaCl was observed for MLCh-6 and MLCh-8 in the presence of $MnCl_2$ (Fig. 4B and F) and for MLCh-11 in the presence of $MgCl_2$ (Fig. 4C). At the same time, in the presence of $MgCl_2$, KCl at low concentrations (1–10 mM) did not effect on the activity of MLCh-11, while inhibited this Ab at its higher concentrations (15–150 mM) (Fig. 4C). In the presence of $MnCl_2$, KCl significantly inhibited the activity of MLCh-12 even at very low concentration of the salt (Fig. 4D). After small decrease in RA at low concentrations (1–10 mM) a remarkable activation of MLCh-8 was observed at high concentrations of KCl (80–100 mM) (Fig. 4F). At low concentration of KCl (Fig. 4B) or NaCl (Fig. 4D) a small activation of two MLChs was observed; at higher concentration of these salts MLCh-6 was significantly activated, while MLCh-12 significantly inhibited. Interestingly, several act-MLChs showed very complicated dependencies demonstrating two pronounced optima of KCl or NaCl concentrations. For example, in the presence of

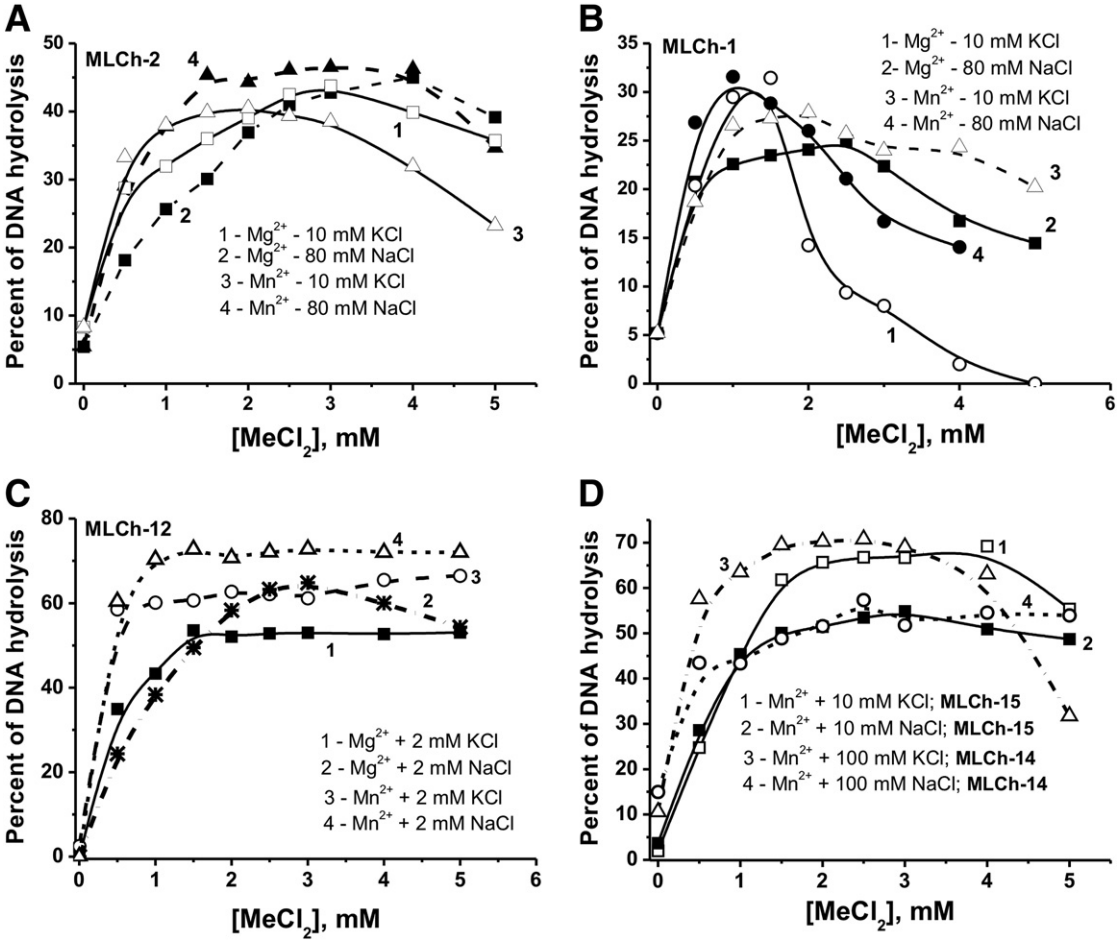

Fig. 3. Dependences of the relative DNase activity for various act-MLChs (20 nM) on the concentration of $MgCl_2$ and $MnCl_2$ at different fixed concentrations of NaCl and KCl (A–D). Numbers of act-MLChs and used concentrations of NaCl and KCl are given on panels A–D. A complete relaxation and linearization of plasmid f DNA was taken for 100%. The error in the rate determination at every Me^{2+} concentration from two experiments in each case did not exceed 8–10%. See Materials and methods for other details.

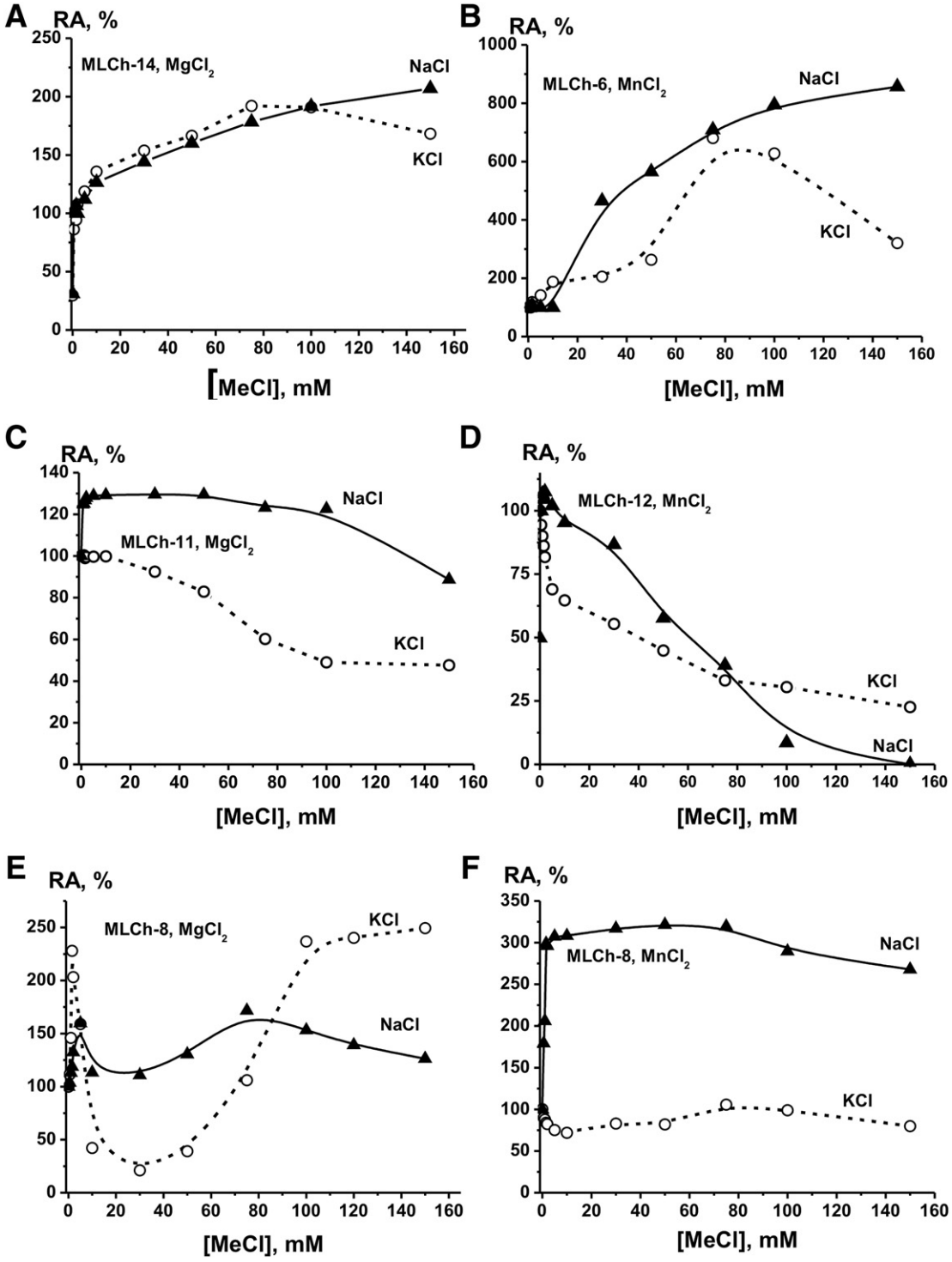

Fig. 4. Dependences of the relative DNase activity (RA) for several act-MLChs on the concentration of NaCl and KCl at fixed 2 mM concentration of $MgCl_2$ and $MnCl_2$ (A–F). Numbers of all act-MLChs (15 nM), the used $MgCl_2$ or $MnCl_2$ are given on panels A–D. The RAs in the absence of NaCl and KCl were taken for 100%. The error in the rate determination at every Me^+ concentration from two experiments in each case did not exceed 8–10%. See Materials and methods for other details.

$MgCl_2$ MLCh-8 had first optimum of NaCl and KCl at low (1–4 mM) and second at higher (80 or higher 100 mM, respectively) concentrations of one of these salts. Overall, 14 of 15 act-MLCh preparations demonstrated very specific dependencies on concentration of KCl and NaCl in the presence of $MgCl_2$ and $MnCl_2$. Only preparations of MLCh-3 and MLCh-5 showed completely coinciding dependencies. Optimal concentrations of KCl and NaCl for all 15 act-MLCh preparations are given in Table 1. These data allow distinguishing act-MLChs with comparable and different properties.

3.3. pH optima for different recombinant monoclonal light chains

The pH optima for various canonical DNases are very different, but all of them usually demonstrate only one pH optimum [75,76]. In

Table 1
The optimal concentrations of NaCl and KCl for individual recombinant MLChs in the presence of 2 mM $MnCl_2$ or 2 mM $MgCl_2$.

Number of MLCh preparation	$MnCl_2$		$MgCl_2$	
	[KCl], mM	[NaCl], mM	[KCl], mM	[NaCl], mM
1	0–0.5; >0.5 inhib.[a]	0–0.5; >0.5 inhib.	30.0; >plateau[b]	100.0; >120.0 inhib.[a]
2	100; >120 inhib.	100; >120 inhib.	75.0; >100 inhib.	15.0; >100.0 inhib.
3	5.0; >10 inhib.	5.0; >10.0 inhib.	0.0; >0.0 inhib.	0.0; >0.0 inhib.
4	2.5; >4.0 inhib.	7.5; >10.0 inhib.	5.0 > 10.0 inhib.	8.5; >15.0 inhib.
5	5.0; >10 inhib.	5.0; >10.0 inhib.	0.0 > 0.0 inhib.	0.0; >0.0 inhib.
6	1–2 and 75; >75 inhib.[c]	1–2 and 150	1.5–30 plateau; >40.0 inhib.	1–3 and 50.0; >100.0 inhib.
7	0.5; >1.0 inhib.	2.5; >3.0 inhib.	3.0; >4.0 inhib.	8.0; >10.0 inhib.
8	0–10 inhib.; 75; >80 inhib.	5–150 plateau	5.0 and 75.0; >80.0 inhib.	1.5; 2.0–75 inhib.; 100; >plateau[c]
9	20–25; >30 inhib.	2.0; >3.0 inhib.	10.0; >12.0 inhib.	5.0 and 75.0; >80.0 inhib.
10	2.5; plateau[b]	10.0; >15.0 inhib.	2.5; >5.0 inhib.	5.0; >7.0 inhib.
11	1–10 plateau; >10 inhib.	0.5–2.0 plateau; >2.0 inhib.	0–10.0 plateau; >10.0 inhib.	2–50 plateau; >60.0 inhib.
12	0.0; >0.0 inhib.	2.0 and 5–10; >10.0 inhib.	1–5; >10.0 inhib.	1–2 and 5–10; >10.0 inhib.
13	0.0; >0.0 inhib.	0.0 and 10.0; >10.0 inhib.	0.1–0.3; >0.7 inhib.	0–0.1; >0.1 inhib.
14	100–150 plateau	100–150 plateau	75.0; >100 inhib.	150.0
15	1.5; >2 inhib.	50.0; >50.0 inhib.	0.0 and 50.0; >50.0 inhib.	0–10 and 50.0; >50.0 inhib.

[a] For each value, a mean of two measurements is reported, optimal concentration(s) is given in bold; the mark (>any value inhib.) means that the dependence has bell-shaped character and that at higher concentrations of the salt the inhibition of the reaction is observed.
[b] The mark (30 (or any other value in bold); >plateau) means that optimal concentration corresponds to 30 mM and there is no remarkable inhibition up to maximal concentration (100–150 mM) of NaCl or KCl used.
[c] The mark (1.5; 2.0–75 inhib.; 100; >plateau (or other similar values)) means that there are two optimal concentrations at 1.5 and 100 mM salt and a significant decrease in the activity of analyzed MLCh at concentrations in the region 2.0–75 mM.

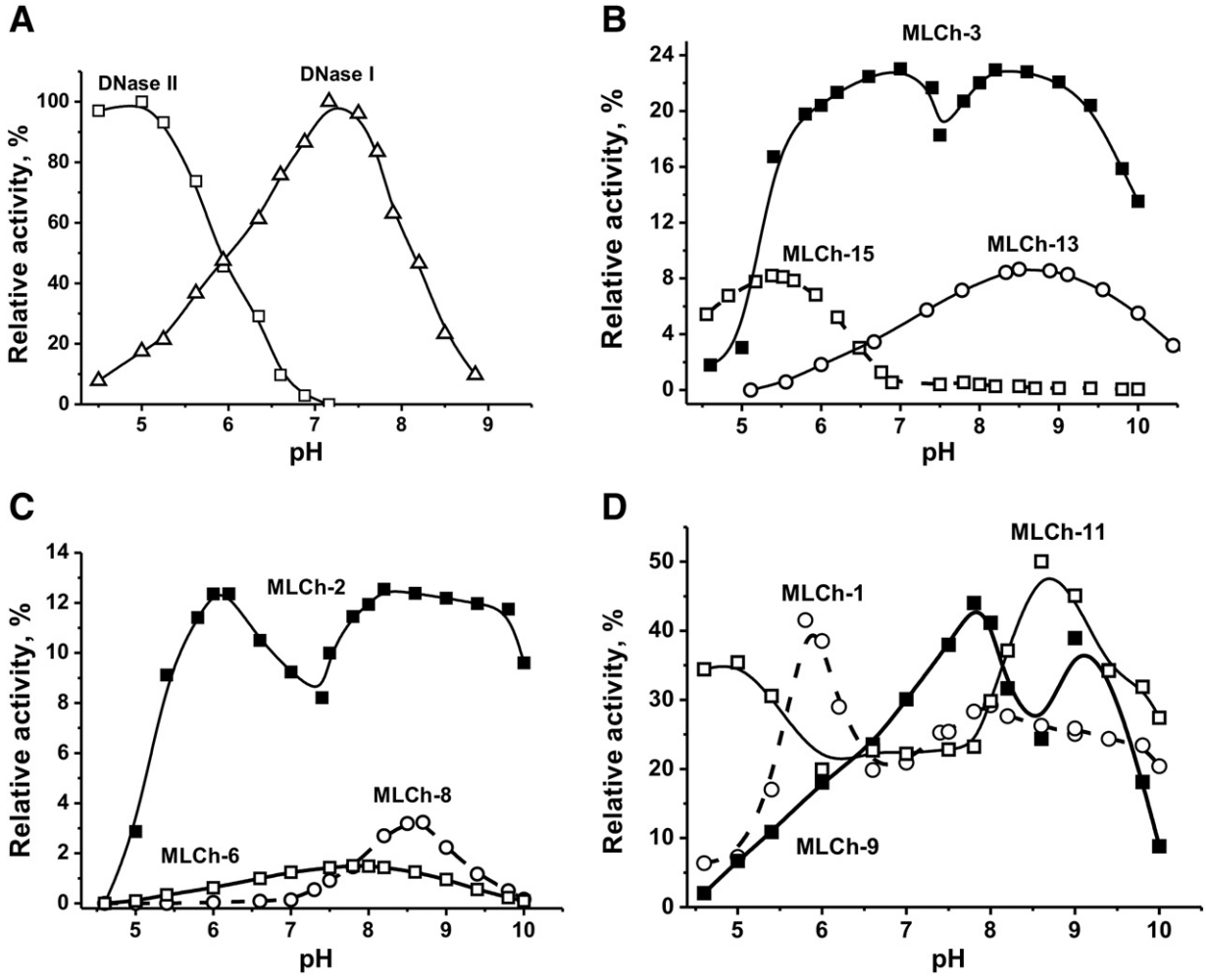

Fig. 5. Dependences of the relative DNase activity of human DNase I and DNase II (10^{-2} Kunitz units/ml) (A) and of nine act-MLChs (B–D) on pH of reaction mixture. The RAs for DNase I were estimated in the absence, while for DNase II, in the presence of optimal concentration of $MgCl_2$ (10 mM); the maximal DNase activity of DNase I and DNase II (A) was taken for 100%. The RAs of nine act-MLChs was measured using 2 mM $MeCl_2$ (the best Me^{2+} cofactor for every preparation of MLChs), optimal concentration of NaCl or KCl, and optimal pH of reaction mixture (see Table 3). Different reaction mixtures were incubated for 0.4–5.0 h and then all data were normalized to standard conditions; complete hydrolysis of scDNA (complete relaxation and linearization) for 30 min in the presence of various MLChs (10 nM) was taken for 100%. See Materials and methods for other details.

contrast to all canonical DNases, pool of polyclonal DNase abzymes from the sera patients with different diseases can contain from one to many monoclonal abzymes; some of them can demonstrate from 1 to 2–8 pronounced pH optima at range from 5 to 10 [6–13,22,23,25,36].

We have analyzed pH optima for 15 act-MLCh preparations in the presence of 2 mM $MgCl_2$ as well as for DNase I and DNase II. Several typical dependencies are given in Fig. 5. One can see that DNase I demonstrates only one optimum at pH 7.0–7.2, while DNase II has one optimum at pH 4.9–5.0 (Fig. 5A). Eight of fifteen act-MLChs also demonstrated only one pH optimum, but these optimal pHs were different for various MLChs. For example, MLCh-15 (Fig. 5B; pH optimum 5.4–5.6) and MLCh-7 (pH optimum 6.2–6.4) have acidic pH optimum, while six MLChs demonstrated one pH optimum in neutral or alkaline regions of pH from 7.5 to 8.7 (Fig. 5, Table 2). It was surprising, but six of fifteen MLChs demonstrated two different pH optima (Fig. 5, Table 2). Two of them, MLCh-3 and MLCh-5, have a couple of the same pH optima (Table 2).

It was possible to propose that two values of pH optima may be a consequence of accidental agglomeration of two different clones on Petri dishes. To obtain repeatedly chosen single colonies of phages, the phage materials corresponding to clones 1, 2, 3, 5, 9, 10, and 11 were re-grown in Petri dishes and five new single clones in every case were randomly chosen. Purified act-MLCh preparations corresponding to five new single mono-colonies of each initial clone used demonstrated the same double pH optima as in the case of initial clones. It means that act-MLChs with numbers 1, 2, 3, 5, 9, 10, and 11 were single after the first step of their selection and that these recombinant act-MLChs are characterized by two different pH optima.

3.4. Activation of DNase monoclonal light chains by different Me^{2+} ions

It was shown above that, similarly to DNase I, all fifteen act-MLChs are metal-dependent abzymes. It is known, that Mn^{2+}, Co^{2+}, Ni^{2+}, and Ca^{2+} ions activate DNase I in much smaller degree than Mg^{2+} ions [75,76]. We have compared the RAs of 15 act-MLChs in the presence of six different metal ions at their fixed 2 mM concentration, optimal concentrations of KCl or NaCl as well as optimal pHs (see Table 3). Fourteen of 15, except MLCh-3 and MLCh-5, demonstrated different ratios of the RAs in the presence of six different metal chlorides (Table 3). It should be mentioned that the maximal activity in the case of various MLChs was observed in the presence of different $MeCl_2$ salts, but in average the following order was observed: $MnCl_2 > CoCl_2 > MgCl_2 > NiCl_2 \approx CaCl2$ (Table 3). In the case of several metal ions the RAs were determined at two different pH optima. Interestingly, optimal Me^{2+}-cofactor in the case of MLCh-1 was the same at pHs 5.8 and 8.0. At the same time, for MLCh-2 the maximal activity was observed in the presence of Mn^{2+} at pH 6.1, but in the presence of Mg^{2+} at pH 8.2 (Table 3). Mn^{2+} and Ca^{2+} metal ions were the best activators of MLCh-3 and MLCh-5 at pHs 8.4 and 6.9, respectively. Cu^{2+} was the worst activator of all act-MLChs and several of preparations (numbers 1, 2, 3, 5, 6, 8, 9, and 11) were nearly completely inactive in the presence of $CuCl_2$. In addition, several other act-MLChs were practically inactive in the presence of some other metal ions: MLCh-9 (Ni^{2+}), MLCh-3 and MLCh-5 (Ca^{2+}, pH 8.4), MLCh-3, MLCh-5 (pH 6.9), and MLCh-8 (Co^{2+}). For all 15 act-MLChs apparent k_{cat} values were estimated (Table 3). Fourteen of fifteen act-MLCh preparations, except MLCh-3 and MLCh-5, demonstrated different apparent k_{cat} values. Overall, all MLChs demonstrated enzymatic properties quite different from the known DNases and each act-MLCh preparation showed a very specific ratio in the RAs in the presence of seven different metal ions (Table 3).

Table 2
The optimal pH values for DNase I, DNase II, and 15 recombinant individual MLChs.[a]

DNase or number of MLCh preparation	Optimal pH	
	pH_1	pH_2
DNase I	7.0–7.2	No second optimum
DNAse II	4.9–5.0	No second optimum
1	5.7–5.9	7.9–8.1[b]
2	6.0–6.2	8.2–8.3
3	6.9–7.0	8.2–8.5
4	7.5–7.6	No second optimum
5	6.9–7.0	8.2–8.5
6	7.8–8.0	No second optimum
7	6.2–6.4	No second optimum
8	8.5–8.6	No second optimum
9	7.8–8.0	8.9–9.1
10	6.1–6.3	8.5–8.7
11	4.8–5.0	8.6–8.7
12	7.7–7.9	No second optimum
13	8.5–8.7	No second optimum
14	7.9–8.1	No second optimum
15	5.4–5.6	No second optimum

[a] For each value, a mean of two measurements is reported.
[b] For different MLChs one or two pH optima were revealed.

3.5. Affinity of monoclonal light chains for DNA

Since for obtaining of the preparations of all recombinant MLChs we have used phage particles eluted from DNA-cellulose with 0.5 M NaCl (Fig. 1A), the affinity of all MLChs for DNA was expected to be comparable. The dependencies of the initial rates on the plasmid DNA concentration in the reaction catalyzed by 15 act-MLChs were analyzed. All these dependencies were consistent with Michaelis–Menten kinetics (for example, Fig. 6). The K_m values for all act-MLChs were comparable and varied in the 260–320 nM range. For example, for MLCh-1 the constants were $K_m = 270 \pm 20$ nM, $k_{cat} = 0.6 \pm 0.05$ min^{-1}; for MLCh-2, $K_m = 277 \pm 24$ nM, $k_{cat} = 0.1 \pm 0.01$ min^{-1}; for MLCh-3, $K_m = 280 \pm 28$ nM, $k_{cat} = 0.3 \pm 0.03$ min^{-1}, and for MLCh-11, $K_m = 310 \pm 30$ nM, $k_{cat} = 0.7 \pm 0.06$ min^{-1} (Fig. 6). Thus, all four K_m were nearly the same within the error of their determination. The k_{cat} values were different and varied in the 0.02–0.7 min^{-1} range (Table 3).

4. Discussion

An extreme diversity of polyclonal IgG, IgA, and IgM abzymes in their affinity for DNA was shown previously using different methods including determination of K_m values [6–13]. Interestingly, when polyclonal IgGs were eluted from DNA-cellulose by a NaCl gradient (0–3 M), the Ab optical density and DNase activity were distributed all over the chromatography profiles [22–25,43–45,69]. Several fractions were eluted only with 2–3 M $MgCl_2$ or with acidic buffer (pH 2.6) in the conditions destroying strong immunocomplexes. We have expected similar situation using chromatography on DNA-cellulose in the case of separation of phage particles containing kappa light chains on their surfaces.

Fig. 1A shows the distribution of the phage particles (and their DNase activity) all over the profile of the chromatography on DNA-cellulose. The data are indicative of the extreme diversity of SLE DNase recombinant kappa light chains in their affinity for DNA. In this article for a preparation of individual recombinant MLChs we have used phage particles eluted from DNA-cellulose with 0.5 M NaCl (Fig. 1A) and provided direct evidence that the SLE kappa light chains of Abs interact with DNA, hydrolyze DNA and are not contaminated by canonical DNases (Fig. 2).

Human and mammalian intact polyclonal Abs are known to interact with different metal ions. It was shown that human milk polyclonal DNase sIgA abzymes mainly Me^{2+}-independent and they were only slightly activated by Mg^{2+}, Mn^{2+}, or Zn^{2+}, and the cleavage of DNA substrates was inhibited by Ca^{2+} and Cu^{2+} [44]. In contrast to human milk DNase, sIgA, IgG, and IgA, abzymes from patients with autoimmune and viral diseases and animals are usually Me^{2+}-dependent [6–12]. For IgGs from MS patients, the effect of different metal ions on DNase activity decreased in the following order: $Mn^{2+} > Mg^{2+} > Zn^{2+} > Ca^{2+}$ [77]. The DNase activity of IgGs from tick-borne encephalitis decreased in the order: $Mn^{2+} \geq Co^{2+} \geq Mg^{2+} > Ca^{2+}$, while K^+, Na^+, Ni^{2+}, Zn^{2+},

Table 3
The relative activities of individual recombinant MLChs in the presence of various metal ions (2 mM) at optimal pHs and concentration of NaCl or KCl.

Number of MLChs	Mg^{2+}	Mn^{2+}	Zn^{2+}	Ni^{2+}	Co^{2+}	Cu^{2+}	Ca^{2+}	pH[c]	[NaCl] or [KCl], mM[d]	*App.* k_{cat}, min^{-1}[b]
1	92.7	**100**[a]	6.5	10.7	20.6	0.0	4.0	5.8[e]	100.0 Na^+	0.6 ± 0.04
	60.7	100.0	7.5	11.5	18.5	0.0	5.4	8.0[ξ]	100.0 Na^+	
2	69.6	100.0	55.4	83.8	73.0	0.0	10.1	6.1	80.0 Na^+	0.1 ± 0.01
	100.0	92.3	18.5	95.6	63.7	3.0	26.0	8.2	80.0 Na^+	
3	78.3	98.5	42.7	56.2	0.3	9.0	100.0	6.9	5.0 K^+	
	1.3	100.0	3.6	11.9	99.0	0.0	0.0	8.4	5.0 K^+	0.33 ± 0.03
4	51.0	100.0	80.0	56.0	24.0	5.0	26.0	7.5	7.5 Na^+	0.5 ± 0.04
5	78.3	98.5	42.7	56.2	0.0	9.0	100	6.9	5.0 K^+	0.3 ± 0.03
	1.3	100.0	3.6	11.9	99.0	0.0	0.0	8.4	5.0 K^+	0.34 ± 0.03
6	1.3	100.0	3.6	11.9	99.0	0.0	0.0	7.9	1.0 K^+	0.02 ± 0.003
7	75	70	80	85	100.0	5	21	6.3	2.5 Na^+	0.3 ± 0.04
8	58.5	100.0	18.3	19.7	0.0	0.0	16.2	8.5	5.0 Na^+	0.06 ± 0.07
9	100.0	85.2	15.9	0.0	85.4	0.0	75	7.8	1.5 Na^+	0.7 ± 0.08
10	25	20	15	100.0	17	2	39	8.6	10.0 Na^+	0.2 ± 0.02
11	80	85	43	24	100.0	0.0	8	8.6	5.0 K^+	0.7 ± 0.06
12	55.0	88.5	16.5	56.3	100.0	1.2	56.2	7.8	5.0 K^+	0.12 ± 0.01
13	34.5	25.0	16.9	83.3	100.0	4.8	41.9	8.6	0.1 K^+	0.13 ± 0.01
14	80.9	75.2	19.7	100.0	59.5	6.3	37.3	8.0	5.0 Na^+	0.11 ± 0.01
15	100.0	49.2	55.7	33.5	85.8	45.0	70.0	5.5	1.5 Na^+	0.12 ± 0.01

[a] The maximal relative activity (RA) in the presence of one of seven metal ions used was taken for 100% and given in bold; the error of the values determination from two independent experiments did not exceed 7–10%.
[b] The apparent k_{cat} values under optimal conditions were calculated as $k_{cat} = V_{max}$ (nM/min)/[MLCh] (nM).
[c] Optimal pH of reaction mixtures was used for every of MLCh preparation.
[d] Optimal concentrations of NaCl or KCl observed in the presence of $MgCl_2$ or $MnCl_2$ in the case of different MLCh preparations were used.
[e] The RAs for several preparations of MLChs were estimated in the case of two different pH optima.

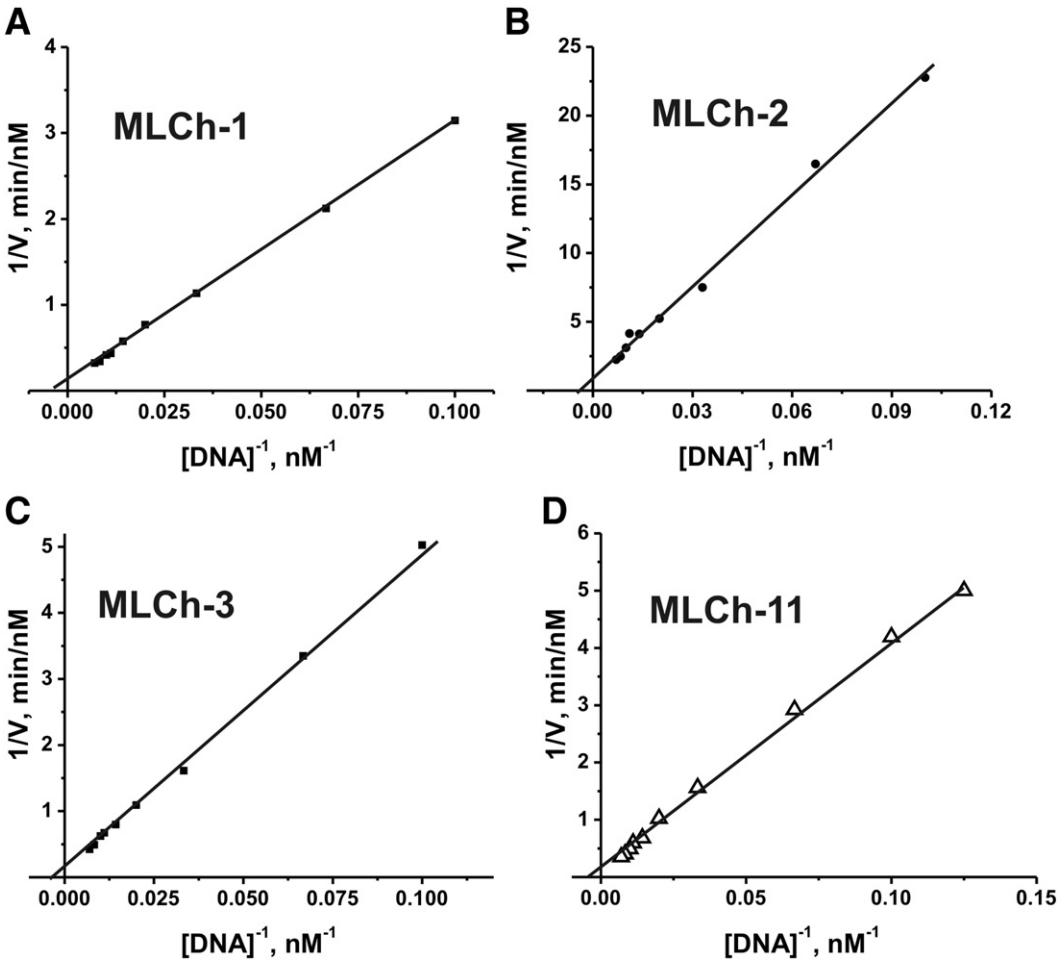

Fig. 6. Determination of the K_m and V_{max} values for plasmid DNA in the case of four different act-MLChs using the Lineweaver–Burk plot (A–D). Optimal conditions ($MeCl_2$; NaCl or KCl, pHs; see Table 3) for every act-MLChs (5 nM) were used. The error in the initial rate determination from two independent experiments at each substrate concentration did not exceed 7–10%.

and Cu^{2+} did not stimulate DNA hydrolysis [78]. Polyclonal IgGs from autoimmune MRL mice demonstrated the following specific order of DNase activity activation by different metal ions: $Mn^{2+} \geq Mg^{2+} > Ca^{2+} \geq Cu^{2+} > Co^{2+} \geq Ni^{2+} \geq Zn^{2+}$ [69].

As we have shown in this article, in the absence of externally added metal ions, purified act-MLChs possess very low activity and completely inactive in the presence in the reaction mixture EDTA or after their dialysis against EDTA + EGTA, but recovered their activity after the addition of external Me^{2+} ions. This result is in an agreement with a small content of Me^{2+} ions bound to various polyclonal Abs purified from the sera of human and animals by the standard procedure used in this study [79]. In addition, when EDTA was added to catalytic Abs hydrolyzing Gp41 peptide antigen of the HIV-1 envelope, it inhibited the degradation of the peptide [80].

DNase I is a Mg^{2+}-dependent nuclease [75,76]. Optimal concentration of Mg^{2+} is ~10 mM. Mg^{2+} ion is involved in the electrophilic catalysis of phosphodiester bond cleavage [75,76]. Interestingly, optimal concentration of Mg^{2+} and Mn^{2+} (2 mM; Fig. 3) in the case of all MLChs is approximately 5-fold lower than that for DNase I.

To find additional criteria to distinguish 15 act-MLChs we analyzed dependencies of their activity on the concentration of NaCl and KCl in the presence of a fixed 2 mM concentration of $MgCl_2$ and $MnCl_2$ (Fig. 4, Table 1). It was shown that these dependencies are very specific for 14 of 15 act-MLChs; they coincide only for MLCh-3 and MLCh-5.

The maximal DNase activity of various MLChs was observed in the presence of different metal ions: Mn^{2+} (MLChs — 1, 2, 3, 5, 4, 6, and 8), $CoCl_2$ (MLChs: 7, 11, 12, and 13), $MgCl_2$ (MLChs — 9 and 15), $NiCl_2$ (MLChs — 10 and 14), $CaCl_2$ (MLChs: 3 and 5 at pH 6.9) (Table 3). In addition, optimal metal ions in the case of MLChs having two different pH optima may be the same or different at different pHs (Table 3). The ratio of RAs corresponding different metal ions is individual for every preparation of MLChs except MLCh-3 and MLCh-5 (Table 3). Interestingly, Mg^{2+} ions demonstrate a weak activation of several MLChs (Table 3) and in contrast to DNase I, Mg^{2+} cannot be considered as the best metal cofactor of all act-MLChs. These data suggest that different Me^{2+} ions can play an important role in activation of SLE recombinant MLChs and corresponding to them intact Abs. In addition, Overall, monoclonal abzymes of autoimmune patients can be significantly different in their metal-dependent catalytic properties.

Many enzymes with hydrolytic functions more often use metal ions as cofactors [74]. Usually metal ions (like Mg^{2+}, Ca^{2+}, and Zn^{2+}) in the case of such enzymes coordinate water molecule and make it stronger nucleophile attacking hydrolyzed bond [74]. Different MLChs similar to DNase I are metal dependent and like DNase I hydrolyze scDNA produce first relaxed, then linear DNA, and finally short oligonucleotides. Therefore, a possible role of metal ions in the case of MLChs may be similar to their role for DNase I.

DNase I requires Ca^{2+} and Mg^{2+} for hydrolysis of double-stranded DNA. Co-crystal structures of DNase I with two different oligonucleotides have revealed the presence of several amino acid residues close to the scissile phosphate [81]. From data of X-ray analysis and site-directed mutagenesis it was possible only to propose that Glu39 chelates first metal ion, while Asp168 serves as a ligand for a second Mg^{2+} ion [81]. However, it is still not feasible to unequivocally assign a particular catalytic role to each amino acid/metal ion [81]. Using molecular dynamics simulations, it was recently shown that DNase I contains four ion-binding pockets [82]. Two of them strongly bind Ca^{2+} while the other two sites coordinate Mg^{2+} ions. One Ca^{2+} stabilizes the functional DNase I structure [82]. The presence of Mg^{2+} in close vicinity to the catalytic pocket of DNase I reinforces the idea of a cation-assisted hydrolytic mechanism. Poisson–Boltzmann-type electrostatic potential calculations demonstrate that the divalent cations collectively control the electrostatic fit between DNase I and DNA [82]. In addition, amino acid residues of DNase I chelating Mg^{2+} ions are not absolutely specific for Mg^{2+}; they can interact with other metal ions. The relative DNase activity is high in the presence of Mn^{2+} or Co^{2+}, but lower Ni^{2+} and Ca^{2+} ions [83]. Since various MLChs may be activated by different metal ions, one can suppose that they can interact with different metal ions by various amino acid residues, which are capable to chelate metal ions.

All known canonical human DNases have one pronounced pH optimum (for example, Fig. 5A). However, polyclonal abzymes from patients with different autoimmune diseases can demonstrate from 1 to 5–8 pH optima and from 1 to 6–7 or more K_m values [6–13]. At the early stages of autoimmune diseases the repertoire of abzymes is usually relatively small and characterized by only one or two K_m values for substrate (and one or two pH optima) but it greatly increases with the progress in the diseases leading to the generation of catalytically diverse abzymes with various levels of catalytic activity and many different K_m values and pH optima [6–13]. In contrast to all known DNases having one pronounced pH optimum, recombinant act-MLChs showed from one (MLChs — 4, 6, 7, 8, 12, 13, 14, and 15) to two optimal pH values (MLChs — 1, 2, 3, 5, 9, 10, and 11) (Table 2). The pH optima for several MLChs (7.5–8.6) were higher, while for other MLChs (5.4–6.4) were lower than that for DNase I (7.0–7.2). All acidic pH optima were higher than that for DNase II (4.8–5.0) (Table 2). In addition, in contrast to DNase II, all 15 act-MLChs were Me^{2+}-dependent.

All data about specific dependencies of RAs of various MLChs on ions of different metals (Table 3) including Na^+ and K^+ (Table 1) and optimal pHs (Table 2) indicate that 14 of 15 analyzed preparations correspond to individual recombinant MLChs.

The affinity of polyclonal DNase abzymes for DNA is usually high and corresponds to the typical affinity of Abs for nucleic acids [6–13]. The affinity of scDNA for DNase intact IgGs from rabbits immunized with DNA, RNA, DNase I, DNase II, and RNase A in terms of K_m values (0.5–60.8 nM) [70–73] is comparable with the affinity of plasmid scDNA for IgGs from SLE (K_m = 43–92 nM) [84] and MS patients (0.36 nM) [25]. The DNA affinity characterizing different subfractions of rabbit pIgGs [70–73] and abzymes from autoimmune patients [6–13] is about 3–4 orders of magnitude higher than that of bovine DNase I (K_m = 46–58 μM) [84].

The catalytic centers of intact Abs are usually located on the abzyme light chains, which contain only a part of DNA-binding site [6–13]. The heavy chains contain an additional part of the binding site of intact Abs and this part is responsible for the specific antigen recognition and the increased antigen affinity for abzymes [6–13]. Therefore, separated light chains usually characterized by 2–3 orders of magnitude lower affinity for DNA in comparison with that for intact abzymes [6–13]. Taking this into account, it was possible to expect that all MLChs corresponding to phage particles eluted from DNA-cellulose with 0.5 M NaCl (Fig. 1A) can possess comparable but not very high affinity for DNA.

The K_m values determined for 15 act-MLCh preparations were comparable: 260–320 nM (Fig. 6). Thus, the affinity of recombinant MLChs is approximately 10–100-fold lower than that for different intact DNase abzymes [6–13,24,70–73,84], but at the same time ~100-fold higher than that for DNase I [84].

The catalysis mediated by artificial abzymes is usually characterized by relatively lower reaction rates than for canonical enzymes [6–13]. The known k_{cat} values for natural abzymes from autoimmune patients catalyzing many different chemical reactions vary in the range of 0.00001–40 min^{-1} ([6–13,84] and refs therein). All fifteen MLChs demonstrated relatively high k_{cat} values (0.02–0.7 min^{-1}) (Fig. 6, Table 3). These k_{cat} values are significantly lower than those for canonical DNases (for example, k_{cat} for DNase I is (1–3) × 10^5 min^{-1} [84]), but comparable with those for restriction nucleases and repair enzymes [11].

Our previous findings showed that polyclonal Abs from the sera of patients with SLE, MS, and other autoimmune and viral diseases can contain many monoclonal DNase abzymes with very different enzymatic properties. Here we have shown for the first time catalytic diversity of SLE abzymes using preparations of fourteen recombinant MLChs, which

are characterized by individual pH dependencies, various k_{cat} values, and different dependencies on various Me^{2+}, and Me^{+} ions.

The question is why many antibodies with DNase activity exist in SLE patients. First, immunization of autoimmune mice results in a dramatically higher incidence of abzymes with a higher activity than in conventionally used normal mouse strains [85,86], so the formation of abzymes in autoimmune diseases may be much more profuse. The immune system can produce many different Ab variants in response to a single antigen. In autoimmune diseases the possible number of antigens that can elicit production of DNase abzymes is very high. An increased level of apoptosis in autoimmune patients may trigger production of abzymes directly to DNA and RNA and their complexes with various proteins. It is known that the immune response to DNA and RNA, especially in their complexes with proteins, partially depends on the nucleic acid sequence and length [87,88]. The number of different DNA and RNA molecules stimulating the formation of Abs and Abzs may be very high since DNA and RNA are significantly fragmented in the sera of humans, and even relatively small fragments of different sequences can elicit an immune response. In addition, antiidiotypic Abs against the active centers of different DNases and RNases can also possess catalytic activity; even Abs against human topoisomerase I are catalytically active in the hydrolysis of DNA [70–73]. It means that potentially many enzymes degrading DNA or RNA can also stimulate formation of DNase and/or RNase Abzs, which can be significantly different in their enzymatic properties. It was suggested that polyclonal DNase Abs of autoimmune patients may be a cocktail of Abs against complexes of DNA and RNA with proteins and antiidiotypic Abzs to different DNA-hydrolyzing enzymes [6–13].

It is known that the presence of anti-DNA Abs is the main important diagnostic index for SLE. High-affinity anti-DNA Abs have been recently identified as a major component of the intrathecal IgG in brain and cerebrospinal fluid of MS patients [89,90]. In addition, it was shown that DNase abzymes from SLE and multiple sclerosis patients are cytotoxic and induce apoptotic cell death [9]. The sera of patients with MS and SLE patients contain free light chains [89,90]. Taking these data in account, we propose that extreme diverse intact Abs and their free light chains with DNase activity may promote important neuropathologic mechanisms in SLE and MS pathogeneses.

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