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Characterization and selectivity of catalytic antibodies from human serum with RNase activity

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ABSTRACT

IgG purified from sera of several patients with systemic lupus erythematosus and hepatitis B are shown to present RNA hydrolyzing activities that are different from the weak RNase A-type activities found in the sera of healthy donors. Further investigation brings evidence for two intrinsic activities, one observed in low salt conditions and another specifically stimulated by Mg^{2+} ions and distinguishable from human sera RNases. Cleavage of RNA substrates by the latter activity is not sequence-specific but sensitive to both subtle conformational and/or drastic folding changes, as evidenced by comparative analysis of couples of structurally well-studied RNA substrates. These include yeast $tRNA^{Asp}$ and its *in vitro* transcript and human mitochondrial $tRNA^{Lys}$-derived *in vitro* transcripts. The discovery of catalytic antibodies with RNase activities is a first step towards creation of a new generation of tools for the investigation of RNA structure.

INTRODUCTION

In the last few years much interest has been devoted to the investigation of catalytically active antibodies. Antibodies able to catalyze a great variety of chemical transformations have been developed (1,2). This was done by immunization with haptens that resemble the transition state of reactions, a strategy that has its roots in the seminal contributions of Pauling (3) and that was later developed by Jenks (4). The existence of catalytic human autoantibodies has been reported for a peptidase activity against vasoactive intestinal peptides (5) and for a DNA nicking activity in the sera of patients with systemic lupus erythematosus (SLE) (6). The latter antibodies, thought to be anti-idiotypes with their active centers imitating those of nucleases, have been further carefully investigated (7,8). More recently, it was shown that antibodies isolated from the sera of SLE patients and purified according to Shuster *et al*. (6) also possess an RNA hydrolyzing activity (9).

The present paper investigates and compares the RNA hydrolyzing activity of IgG from sera of SLE and hepatitis B patients. The antibodies present two types of activity, one revealed in low salt conditions and another that can be specifically stimulated by magnesium ions. Antibodies purified from healthy donors and used as controls also present RNA hydrolyzing activities, but of an RNase A specificity and of weak amplitude. Using one IgG sample from an SLE patient, we demonstrate that RNase activity is an intrinsic property of the antibodies. Utilizing three couples of related tRNAs, already well characterized for their faint or large structural differences by classical structural investigations, we show that the activity of the antibodies is sensitive to RNA conformation, as evidenced by adequately different cleavage patterns. This characterization of an antibody-associated RNase activity sensitive towards conformational differences in tRNA substrates opens the possibility of development of a new generation of tools allowing structural investigation of RNAs, based on creation of monoclonal antibodies displaying these activities.

MATERIALS AND METHODS

Chemicals, enzymes and nucleic acids

All chemicals were of high grade quality. Imidazole was obtained from Boehringer-Mannheim (Mannheim, Germany). [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]ATP (3000 Ci/mmol) were from Amersham (Les Ullis, France). Bovine pancreatic ribonuclease (RNase A) was from Boehringer-Mannheim, RNase T1 from Worthington Biochemical Corp. (Freehold, NJ), bovine alkaline phosphatase (BAP) from Amersham and T4 polynucleotide kinase from US Biochemical (Cleveland, OH). (ATP,CTP):tRNA nucleotidyltransferase from *Escherichia coli*, a gift of Dr Anne Théobald-Dietrich (IBMC, Strasbourg, France), was prepared according to Cudny and Deutscher (10). Centricon-100 devices were from Amicon (Beverly, MA). Protein A–Sepharose was from Sigma (St Louis, MO) and Toyopearl HW55 fine from Toyo Soda (Tokyo, Japan). *In vitro* transcripts of human mitochondrial $tRNA^{Lys}$ (Kwt) and of two variants of this tRNA, designated Kmerrf and Krw (details in Discussion), were prepared as described previously (11). Yeast $tRNA^{Asp}$ and the corresponding

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in vitro transcript were gifts of Dr Anne Théobald-Dietrich (IBMC, Strasbourg, France) and were prepared as described (12).

Purification of antibodies

IgG were isolated basically as described (13). Antibodies in 2 ml of serum were precipitated three times with 40% saturated ammonium sulfate. This was followed by affinity chromatography on protein A–Sepharose. Samples were loaded on the column (6.5 × 30 mm) in 50 mM Tris–HCl, pH 7.5, 100 mM NaCl (buffer A). The column was washed with 10 vol of the same buffer, then with 2 vol of buffer A containing 1% Triton X-100, and again with 10 vol of buffer A. This procedure was repeated twice. Elution was performed with 100 mM glycine–HCl, pH 2.6. The fraction with high OD (~500 µl) was loaded on a Toyopearl HW55 gel filtration column (10 × 600 mm, flow rate 15 ml/h). Fractions corresponding by their molecular weight to IgG were collected, immediately neutralized with 1 M Tris–HCl, pH 10.0, and dialyzed against 500 vol of buffer A for 10 h. The protein concentrations of these samples, as estimated by OD measurements at 280 nm (1 OD = 0.75 mg/ml protein), were typically ~0.5–1 mg/ml. Samples were analyzed by SDS–PAGE, stained with silver or Coomassie blue according to standard procedures and kept at 4°C until use. Specific full-strength antibody-associated RNase activities appeared only 2–3 weeks after the antibody isolation procedure.

RNA cast gels

RNA cast gels were performed basically as described (14,15). rRNA was added to the separating gel solution (10%), to a final concentration of 0.3 mg/ml, along with the customary reagents prior to polymerization. Samples (5–15 µg) were incubated at 37°C for 20 min in SDS loading buffer and then fractionated by SDS–PAGE. After electrophoresis the gel was washed for 30 min in 50 mM Tris–HCl, pH 7.5, containing 30% isopropanol to remove SDS and renature proteins, and then three times with 50 mM Tris–HCl, pH 7.5. Subsequent incubation for *in situ* RNA digestion was carried out for 14–24 h at 37°C in the same buffer. The gel was then incubated with ethidium bromide for residual RNA staining.

Radiolabeling of tRNAs

tRNAs, either of natural origin or prepared by *in vitro* transcription, were labeled at their 3′- or 5′-ends according to established procedures (12). For 3′-end-labeling, the last adenosine was exchanged for [α-^{32}P]ATP with *E.coli* tRNA nucleotidyltransferase. For 5′-end-labeling, the non-labeled phosphates were first removed with BAP and then replaced with [γ-^{32}P]ATP using T4 polynucleotide kinase.

Cleavage of tRNA by antibodies and nucleases

Cleavage of tRNAs by purified antibodies or by RNase A was carried out at 37°C for 30 min in a final volume of 10 µl in 50 mM Tris–HCl, pH 7.5, in the presence of 0.1 mg/ml tRNA (50 000 Cerenkov counts/sample). Concentrations of samples with RNA hydrolyzing activity were 0.2–0.5 mg/ml for antibodies from healthy donors, 0.01–0.05 mg/ml for antibodies from patients with SLE or hepatitis B and 5×10^{-5} mg/ml for RNase A. These concentrations produce cuts of the same intensity for a given substrate. Various salt conditions (Na^+, Mg^{2+}) were as indicated in the figure legends. The reactions were stopped by precipitation with 100 µl of 2% $LiClO_4$ in acetone (16), centrifugation, washing with 250 µl of acetone and dissolution of the pellets in 4 M urea, 0.025% bromophenol blue and 0.025% xylene cyanol. Cleavage products were analyzed by electrophoresis on 15% polyacrylamide–8 M urea gels (1500 V for 2–3 h). Assignment of bands was performed by parallel runs of partial RNase T1 and imidazole digests of the tRNAs (16) and quantification by analysis on a BioImaging Analyser (Fujix BAS 2000 system; Fuji Photo Film Co. Ltd, Tokyo, Japan).

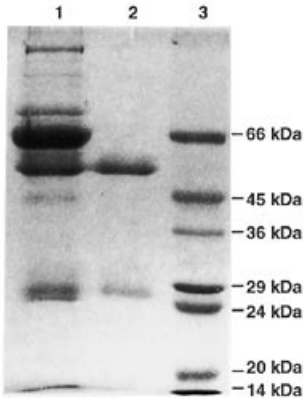

Figure 1. Purity of the SLE antibody preparation revealed on a SDS–polyacrylamide gel (10%) under dissociating conditions (2-mercaptoethanol) stained with Coomassie brilliant blue. Lane 1, serum of SLE patient after precipitation with ammonium sulfate (15 µg; Materials and Methods); lane 2, IgG (15 µg) isolated from this serum after three steps of purification (triple ammonium sulfate precipitation, protein A affinity chromatography, gel filtration); lane 3, molecular weight markers.

RESULTS

It has been shown in previous studies that human antibody preparations from sera of SLE and viral hepatitis patients present DNase activities (6–8,17) and it has been reported that IgG preparations exhibit RNase activity (9,13). In the present work we investigated the RNase activity of highly purified antibodies isolated from the sera of patients suffering from SLE (eight patients) and hepatitis B (six patients). RNase activities were compared with those of healthy donors (three donors). The results presented here consist of two parts.

Experiments described in the first part were designed to demonstrate that the RNase activity is an intrinsic property of the antibodies and to exclude the possibility that any observed RNase activity might stem from contamination of the antibodies by RNases from human serum or might even stem from tightly associated RNase–antibody complexes. For simplicity we will only display experiments that were carried out with an antibody sample from the serum of one SLE patient (except in Fig. 2) with controls from one healthy donor. We emphasize that all experiments undertaken to prove the catalytic activity of IgGs from SLE patients where also done for IgGs of healthy donors. Human serum RNases were already shown to have very much the same cleavage specificity as RNase A (15). Therefore, RNase A was used in some control experiments, to simulate the behavior of a possibly contaminating RNase from human serum.

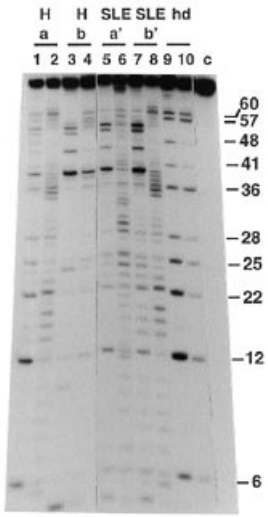

Figure 2. Presence of RNase activities in human antibodies. Hydrolysis patterns of a 5′-end-labeled *in vitro* transcript of human mitochondrial $tRNA^{Lys}$ designated KMERRF (nomenclature in text) by antibodies isolated from various human sera. Autorodiography of a 15% polyacrylamide denaturing gel. Lanes 1 and 2 and 3 and 4 correspond to cleavage patterns by two unrelated hepatitis B patients (H); lanes 5 and 6 and 7 and 8 to two unrelated SLE patients; lanes 9 and 10 to a healthy donor (hd). In lanes 1, 3, 5, 7 and 9 the hydrolysis has been performed in the absence of salts; in lanes 2, 4, 6, 8 and 10 it has been performed in the presence of 5 mM $MgCl_2$. Reactions were carried out as described in Materials and Methods. Concentrations of antibodies from H and SLE patients were 0.01–0.05 mg/ml and that of hd 0.2 mg/ml. Lane c is a control (incubation of RNA without antibodies). Cleavage positions within the tRNA are indicated along the right side of the panel. The sequence of the RNA is visualized in Figure 6.

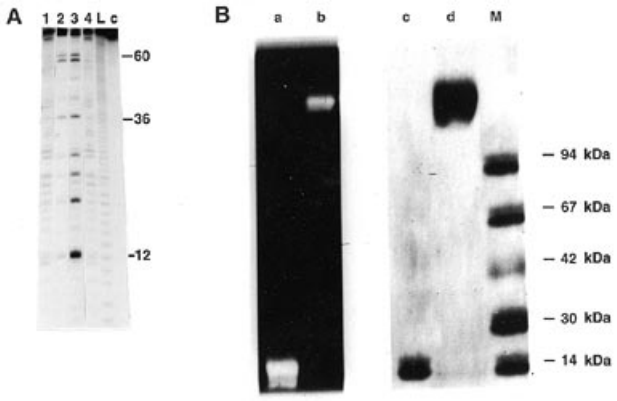

Figure 3. Characterization of the RNase activity of a SLE patient antibody preparation. (**A**) Assay for dissociation of putative contaminating RNases from antibodies on Centricon-100. Stock solutions of antibodies (0.5 mg/ml) were mixed with RNase A (5×10^{-4} mg/ml) in 50 µl, diluted up to 2 ml with 50 mM Tris–HCl, pH 7.5, and centrifuged at 1000 *g*. After addition of 2 ml of buffer and further centrifugation, the sample (~50 µl) was recovered with invert centrifugation at 300 *g*. The RNA hydrolyzing activity of this preparation (lane 4) using 5′-end-labeled KMERRF as substrate was compared with that of the same mixture of antibodies and RNase A not centrifuged on Centricon-100 (lane 3), with untreated RNase A (lane 2) and with untreated SLE antibodies (lane 1). All experiments were performed as described in Materials and Methods, in the presence of 5 mM $MgCl_2$. Concentration of SLE antibodies was 0.02 mg/ml. Lane L, partial hydrolysis by imidazole; lane c, control (incubation of RNA without antibodies). (**B**) RNA cast gel. (a) RNase A (0.05 µg) and (b) antibodies (5 µg) were run in a 5–15% gradient polyacrylamide gel cast with high molecular weight RNA. SDS was washed from the gel as described in Materials and Methods and RNase activity promoted by incubation at 37°C. After toluidine blue staining, RNase activity becomes visible as white bands indicating the hydrolysis of RNA. Size determination of RNase A (5 µg, lane c) and the antibody preparation (15 µg, lane d) was done by co-migration with molecular weight markers (M) and Coomassie staining. Note that overloading the gel with antibodies (d) does not reveal any contaminant.

Experiments in the second part were designed to decide whether the RNase activities were specific for the structure or for the sequence of the substrate RNA. Hence, we used three pairs of structurally well-characterized tRNA substrates with similar sequence but different structures. $tRNA^{Asp}$ from yeast has been compared with its *in vitro* transcript, both molecules differing only in stability but not in general structure (12). An *in vitro* transcript of human mitochondrial $tRNA^{Lys}$ (KWT) with an unusual hairpin structure (11) has been compared with a variant, KMERRF, with a point mutation (A55G) that differs only slightly in structure (18). KWT has also been compared with another variant carrying a point mutation (A9C), KRW, that differs greatly in structure (11).

Characterization of the antibody activity from the serum of a SLE patient

Purification. IgG isolation from human serum started from triple precipitation with ammonium sulfate (40%). To destroy any non-covalent complexes, affinity chromatography on protein A–Sepharose included specific treatment with Triton X-100 and gel filtration was performed at low pH conditions on a Toyopearl HW-55 column. Samples were analyzed by SDS–PAGE under dissociating conditions of electrophoresis. After Coomassie staining, only bands corresponding in molecular weight to light and heavy chains of IgG but no detectable contaminating proteins were observed (Fig. 1). No additional bands were detected on overloaded (see Fig. 3) or silver stained SDS gels (not shown).

Human serum antibody preparations display RNase activity. A systematic search for RNA hydrolyzing activities was performed by incubation of purified antibodies with end-labeled tRNAs as substrates. We found, as illustrated in Figure 2, that all antibody preparations tested present RNase activity. Antibodies from patients cleave the tRNA at a large number of positions and cleavage sites are specific to each patient (compare lanes 1, 3, 5 and 7). The preparation from a healthy donor cleaved the tRNA substrates at positions specific for RNase A, namely at CpA and UpA phosphodiester bonds. To observe any significant cleavage, however, this preparation had to be used in 10-fold higher concentration than antibodies from the various patients. Upon addition of 5 mM magnesium chloride, cleavage by antibodies from patients was much changed whereas cleavage by antibodies from a healthy donor was weakened at some positions. So, each pool of IgG from patients possesses at least two different types of activity, one revealed in the absence and one in the presence of Mg^{2+}. In what follows, the RNase activities of the antibodies from one SLE patient are characterized.

Size exclusion filtration. Since all known RNases from human serum have molecular weights <32 kDa (14), an attempt to dissociate any RNase activity from these antibodies was performed by filtration on Centricon-100 devices. These filters pass molecules of molecular weight <100 kDa. Thus, IgG (150 kDa)

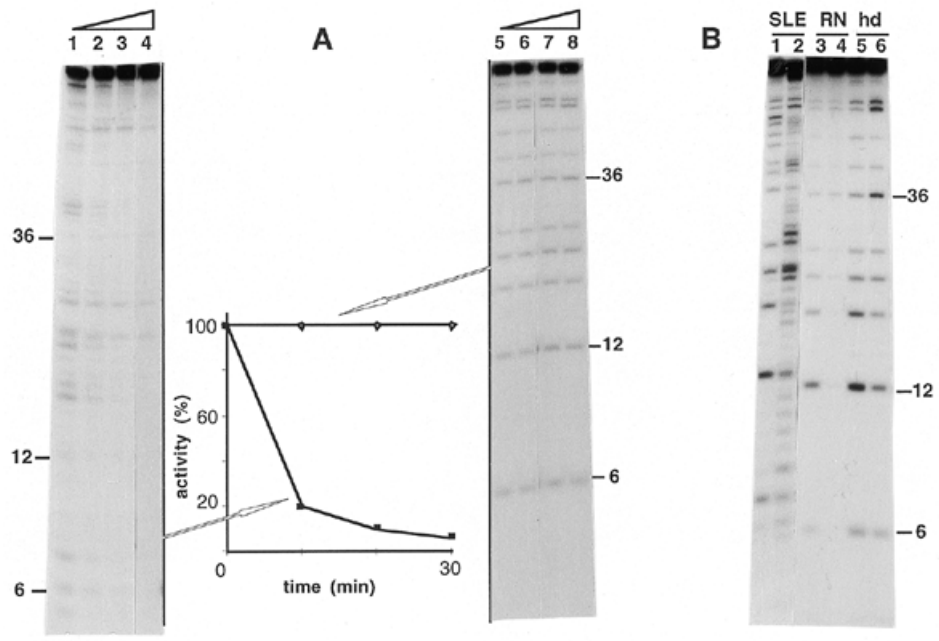

Figure 4. Temperature and Mg^{2+} effects on the RNase activity of a SLE antibody preparation on $tRNA^{Lys}$ cleavage. (**A**) Comparative effect of temperature inhibition of RNA hydrolysis by antibodies and RNase A. Stock solutions of SLE antibodies (0.5 mg/ml) and RNase A (0.5 mg/ml) were preincubated at 45°C for 10, 20 or 30 min and their activity tested on 5′-end-labeled KRW. Lanes 1–4, hydrolysis by antibodies; lanes 5–8, hydrolysis by RNase A. Lanes 1 and 5, without preincubation; lanes 2 and 6, 10 min; lanes 3 and 7, 20 min; lanes 4 and 8, 30 min preincubation. All reactions were carried out as described in Materials and Methods. Concentration of SLE antibodies in reaction mixtures was 0.02 mg/ml. The inset gives a quantitative view of the dependence of the RNA hydrolyzing activities upon preincubation at 45°C. The total amount of radioactivity of the cleavage products and of the uncleaved RNA substrate has been estimated on a BioImager. (**B**) Comparative Mg^{2+} effects on RNA hydrolysis by antibodies and RNase A. The substrate molecule was 5′-end-labeled KWT. Lanes 1 and 2, hydrolysis by SLE antibodies; lanes 3 and 4, hydrolysis by RNase A; lanes 5 and 6, hydrolysis by healthy donor antibodies. Lanes 1, 3 and 5, hydrolysis in the absence of $MgCl_2$; lanes 2, 4 and 6, hydrolysis in the presence of 5 mM $MgCl_2$. All reactions were carried out as described in Materials and Methods. Concentrations of antibodies were 0.05 mg/ml (SLE patient) and 0.4 mg/ml (healthy donor).

are expected to remain on the filter whereas the low molecular weight nucleases (14 kDa for RNase A) are expected to be recovered in the flow-through. To dissociate potentially present non-covalent protein complexes, additional experiments were carried out in the presence of 0.1% Triton X-100. As seen in Figure 3A, from an equivalent mixture of SLE antibodies and RNase A, only the SLE-specific activity was retained after filtration. These data show that it is indeed possible to separate antibodies from low molecular weight contaminants and accordingly strongly suggest that the RNA hydrolyzing activity studied is correlated with the antibodies themselves rather than with contaminants.

RNA cast gel experiment. To prove that there are no complexed RNases in the catalytically active IgG fraction, an RNA cast gel experiment has been performed. The denaturing properties of SDS–PAGE are expected to preclude any complex formation, like a nuclease complexed to an antibody or any other protein. In this experiment, an SDS gel was cast including high molecular weight RNA in the gel solution. After electrophoresis, the denaturing SDS was washed out and the gel was incubated in a Tris buffer to promote RNase activity. RNase activity was revealed by the absence of RNA in the bands after staining with ethidium bromide (Fig. 3B). RNase activity was observed in only one band, corresponding in molecular weight to IgG. The control experiments with RNase A are not shown.

Thermal denaturation. Further evidence allowing distinction of antibody activity from RNase activity comes from comparative thermal denaturation experiments of RNase A and antibodies (Fig. 4A). RNases are usually temperature stable (19), whereas antibodies are temperature labile (20). Human serum RNases, in particular, have been shown to be temperature stable even at 55°C and they demonstrated maximal hydrolysis activities at this temperature (15). Thermal denaturation of antibodies by pre-incubation at 45°C for increasing periods (up to 30 min) prior to the cleavage reaction assay resulted in progressive abolition of the Mg^{2+}-stimulated activity (Fig. 4A), which is thus demonstrated to be temperature sensitive. No temperature inhibition of RNase A was found.

Influence of Mg^{2+}, Na^+ and pH. To better characterize the RNA hydrolyzing activity of the antibody preparations, the effects of salts have been investigated. Indeed, it is known that salts affect several RNase activities (19). Thus, the effects of NaCl (100–200 mM) and of $MgCl_2$ (1–10 mM) have been examined. While Na^+ had approximately the same slight inhibiting effect on RNase A and antibodies (not shown), Mg^{2+} had differential effects (Fig. 4B). RNase was strongly inhibited, while antibodies isolated from the serum of the healthy donor were only slightly inhibited and antibodies from the SLE patient showed large changes in specificity. In this latter case, some positions of the RNA were less efficiently cleaved than in the absence of magnesium ions and new cuts appeared. An apparent discrepancy between Figures 2 and 4B originates from an overlap of both the Mg-dependent and Mg-independent RNase activities. Optimal Mg^{2+} concentration was found to be 5 mM. Thus, magnesium allows the demonstration

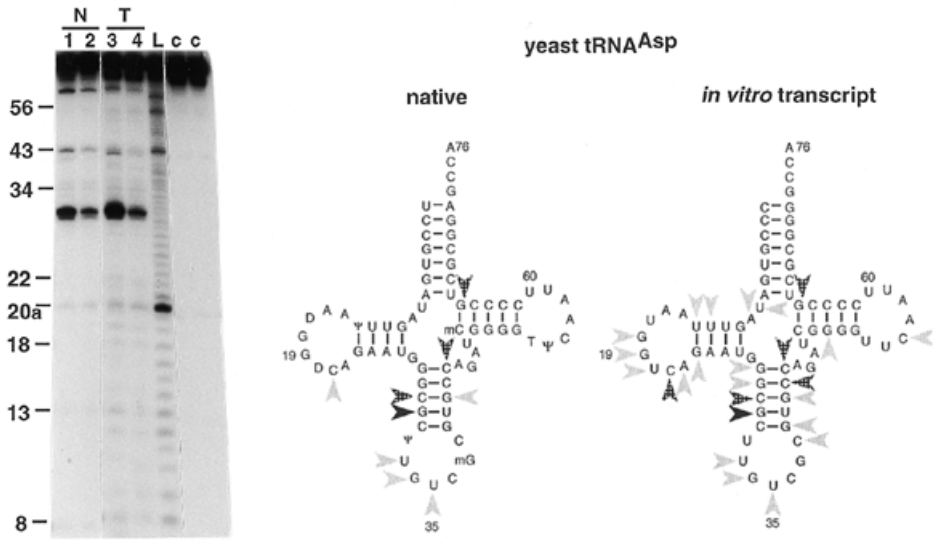

Figure 5. Cleavage patterns of 5′-end-labeled tRNA^{Asp} (N) and the corresponding *in vitro* transcript (T) by antibodies isolated from the serum of an SLE patient. (Left) Autoradiography of a 15% denaturing polyacrylamide gel. Lanes 1 and 2, native tRNA; lanes 3 and 4, *in vitro* transcribed tRNA. Lanes 1 and 3, hydrolysis in the presence of 5 mM $MgCl_2$; lanes 2 and 4, hydrolysis in the presence of 5 mM $MgCl_2$ and 100 mM NaCl. Reactions were carried out as described in Materials and Methods. Concentration of SLE antibodies was 0.05 mg/ml. Lane L, partial hydrolysis by imidazole; lanes c, control incubations of native tRNA and its transcript without antibodies, in the presence of $MgCl_2$. (Right) Localization of the cleavage sites observed in the presence of $MgCl_2$, within the cloverleaf structure of the investigated tRNAs. Darkness of arrows is proportional to the intensities of cuts. Numbering is according to Perret *et al*. (12).

of an RNA hydrolytic activity in antibodies distinct from classical RNase A-type serum RNase activities. As to pH, the optimal activity observed in low salt conditions was at pH 7.5 and for the Mg^{2+}-stimulated activity at pH 6.5 (not shown).

Structural selectivity of the Mg^{2+}-stimulated RNase activity of antibodies from the serum of an SLE patient

Comparative cleavage patterns of native yeast tRNA^{Asp} and the corresponding in vitro transcript by antibodies from an SLE patient. A comparison of the cleavage patterns of yeast tRNA^{Asp} and its *in vitro* transcript by antibodies isolated from a SLE patient is summarized in Figure 5. The Mg^{2+}-stimulated catalytic activity cleaves native tRNA^{Asp} at nine positions (20a, 29, 30, 33–35, 41, 43 and 65), with the strongest cut at G30. In the case of *in vitro* transcripts all cuts are intensified and several additional faint cuts appear as compared with native tRNA (at positions 8, 9, 12, 13, 18, 19–22, 27, 28, 38, 39, 42, 51 and 56).

Comparative cleavage patterns of human mitochondrial tRNA^{Lys} in vitro transcripts by antibodies from an SLE patient. Comparative statistical cleavage patterns of KWT and KMERRF by antibodies isolated from an SLE patient or a healthy donor are displayed in Figure 6. In the absence of magnesium, healthy donor antibodies cleave both tRNAs at the same Py-A sequences (at positions 6, 12, 22, 25, 28, 36, 41, 48, 57, 60, 66 and 68). The SLE preparations weakly cleaved additionally at positions 39 and 53. No differences between transcripts were observed (compare lanes 1 and 6 and 4 and 9).

Cleavage patterns obtained from the Mg^{2+}-induced activity of SLE antibodies show only slight differences between KWT and KMERRF (compare lanes 2 and 7). In KMERRF, cuts at positions 32 and 33 are slightly intensified and new faint cuts appear at positions 53 and 54 as compared with KWT. In KWT, cuts at positions 12, 25, 57 and 60 are slightly intensified as compared with KMERRF. No activation by Mg^{2+} is seen in the healthy donor control, where points of cleavage are of RNase A type.

The cleavage patterns of the second couple of *in vitro* transcripts derived from human mitochondrial tRNA^{Lys}, KWT and KRW, are presented in Figure 7. In the absence of magnesium, again no differences were observed (compare lanes 1 and 4), but upon addition of Mg^{2+} striking differences appeared between these RNAs (compare lanes 2 and 5). In the case of KRW, four additional cuts occurred (at positions 14, 51, 53 and 64) and a large number of cuts were strongly intensified as opposed to KWT (at positions 5–7, 15, 21, 23, 24, 39 and 40). In KWT, more intensive cuts occurred at positions 29 and 41 as opposed to KRW.

DISCUSSION

Four major conclusions can be drawn from this study. First, antibodies from human sera possess intrinsic RNase activity. Second, antibodies of healthy donors exhibit a weak RNase A-type activity and antibodies from SLE and hepatitis B patients possess two different types of activities, one observed under low salt conditions and another in the presence of magnesium ions. Third, the cleavage specificity is individual-dependent. Fourth, Mg^{2+} stimulated activities are sensitive to the conformation of RNA.

Catalytic antibodies with RNase activity in human sera

In this study it is shown that antibody-associated RNase activities can be found in the sera of SLE and hepatitis B patients as well as in that of healthy donors. However, whereas purified antibodies from the sera of healthy donors show RNase A-type activity of low efficiency (cleavage of Py-A sequences) which is inhibited by Mg^{2+}, those from patients show at least two different activities, one occurring in low salt conditions and one dependent on

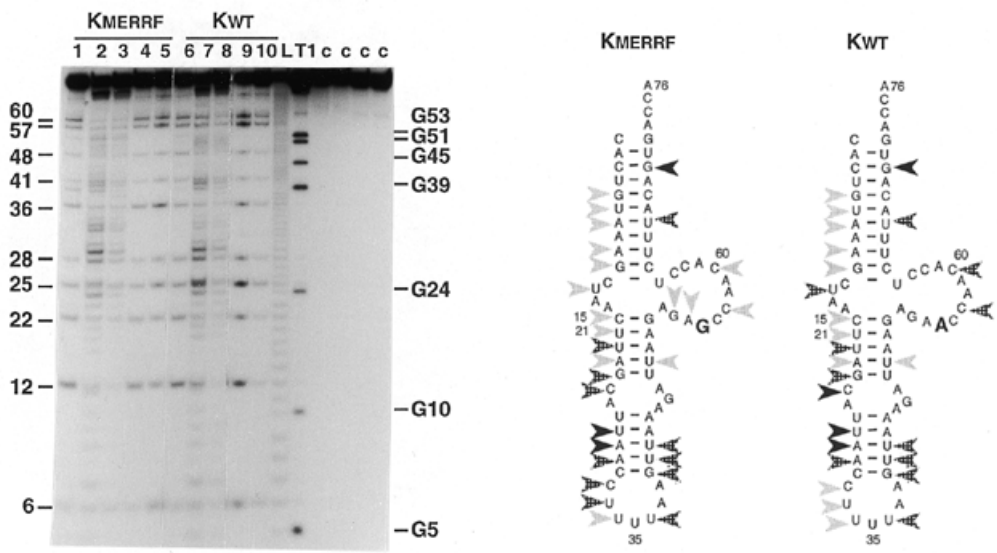

Figure 6. Cleavage patterns of 5′-end-labeled KMERRF and KWT by antibodies isolated from the serum of a SLE patient and from a healthy donor. (Left) Autoradiography of a 15% denaturing polyacrylamide gel. Lanes 1–5, KMERRF; lanes 6–10, KWT. Lanes 1–3 and 6–8, SLE patient; lanes 4–5 and 9–10, healthy donor. Lanes 1, 4, 6 and 9, hydrolysis in the absence of salts; lanes 2, 5, 7 and 10, in the presence of 5 mM $MgCl_2$; lanes 3 and 8, in the presence of 5 mM $MgCl_2$ and 100 mM NaCl. Reactions were carried out as described in Materials and Methods. Concentration of SLE antibodies was 0.05 mg/ml and that of healthy donor 0.4 mg/ml. Lane L, partial hydrolysis by imidazole; lane T1, partial hydrolysis by RNase T1; lanes c, control incubations of RNA without antibodies, from left to right KMERRF in the absence and presence of $MgCl_2$, KWT in the absence and presence of $MgCl_2$. (Right) Localization of cleavage sites observed in the presence of $MgCl_2$, within the secondary structures of the investigated RNAs. Arrows as in Figure 5. Numbering is according to Helm *et al.* (11).

the presence of Mg^{2+} ions. The fact that Mg ions greatly stabilize tRNA structures and render them less susceptible to cleaving agents speaks against the possibility that the increased cleavage reflects major conformational changes in the tRNA. Investigation of SLE antibodies shows the Mg^{2+}-dependent RNA hydrolyzing activity to be an intrinsic property of the immunoglobulins and rules out contaminating RNases. Arguments, obtained by systematic comparison with RNase A, are as follows: (i) the antibody purification procedure yields only two detectable bands on SDS gels under dissociating conditions of electrophoresis, corresponding to light and heavy chains of IgG; (ii) the activity is linked to proteins of molecular weight >100 kDa, as shown by size exclusion filtration, and thus higher than that of known serum RNases (14,15); (iii) activity in RNA cast gels is superimposed on proteins of the molecular weight of IgG; (iv) activity is sensitive to temperature and declines above 45°C, in contrast to serum RNases, which are more temperature-resistant (15); (v) cleavage specificity (no preference for Py-A or any other sequence) is completely different from that of RNase A and thus from human serum RNases, which are of very close specificities (15). Altogether these arguments lead to the conclusion that the RNA hydrolyzing activity present in the purified antibodies from an SLE patient is not due to contaminating RNase(s), but is intrinsically linked to the antibodies themselves. This conclusion also holds true for the low RNase activity present in healthy donor IgG. An additional argument comes from the observation that each antibody preparation tested has an individual specificity (Fig. 2). This can be correlated with the great variety of antibodies in each individual. It is much more difficult to expect that different individuals have different sets of RNases. Similar conclusions were reached when studying antibodies purified from hepatitis B patients.

Interestingly, the RNase activities evolve in time after purification of the antibodies. After the isolation procedure all antibodies demonstrate only low activity of RNase A type. In the case of healthy donors this activity is slightly enhanced after 2–3 weeks. For SLE and hepatitis B patients it also takes 2–3 weeks until their antibodies express specific full-strength cleaving activities. Initial limited activities are probably linked to the acidic treatment of the antibodies in which epitopes may become disturbed and only regain their active structures slowly.

Potential use of antibodies as tools for investigation of RNA structure

Nucleases are known to be either sequence-specific (e.g. RNase T1 is specific for guanosines, RNase A is specific for Py-A sequences) or specific for structural features (e.g. nuclease S1 cleaves exclusively single-stranded regions whereas RNase V1 is sensitive to structured or higher ordered domains of an RNA; reviewed in 21). To determine to what extent the Mg^{2+}-stimulated RNase activity of SLE antibodies was either sequence- or structure-specific, five structurally well-defined RNAs were submitted to cleavage. Natural and/or *in vitro* transcribed yeast $tRNA^{Asp}$ and human mitochondrial $tRNA^{Lys}$ were chosen for the following reasons. Yeast $tRNA^{Asp}$ is one of the best-studied tRNAs. Not only is its crystal structure known (22), but also its solution structure has been investigated with a large number of probes (23–25). Moreover, comparative chemical probing of this tRNA (fully modified) and its *in vitro* transcribed (unmodified) version has been performed (12). Similar global structures for both molecules were found but with an increased stability for the modified molecule. The secondary structures of *in vitro*

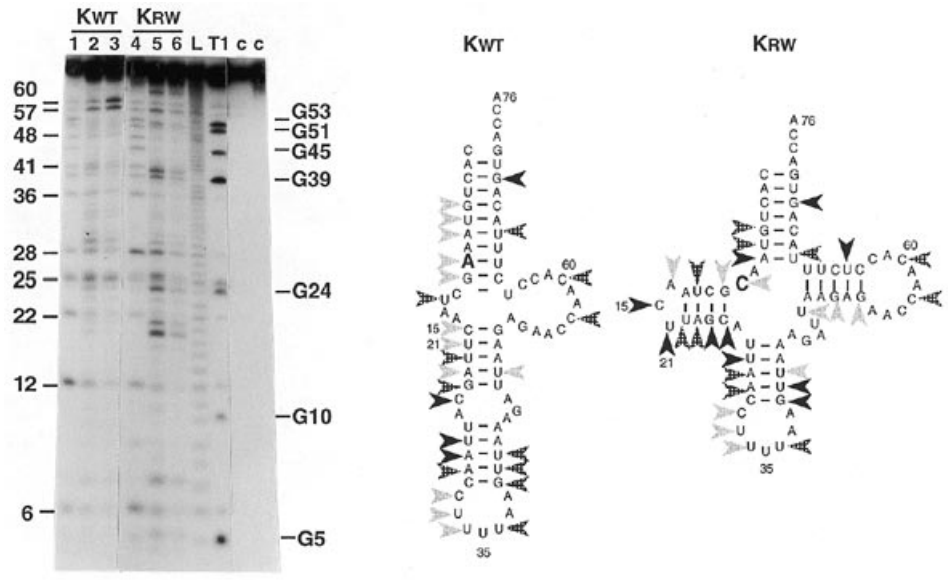

Figure 7. Cleavage patterns of 5′-end-labeled KWT and KRW by antibodies isolated from the serum of an SLE patient. (Left) Autoradiography of a 15% denaturing polyacrylamide gel. Lanes 1–3, KWT; lanes 4–6, KRW. Lanes 1 and 4, hydrolysis in the absence of salts; lanes 2 and 5, hydrolysis in the presence of 5 mM $MgCl_2$; lanes 3 and 6, hydrolysis in the presence of 5 mM $MgCl_2$ and 100 mM NaCl. Reactions were carried out as described in Materials and Methods. Concentration of SLE antibodies was 0.05 mg/ml. Lane L, partial hydrolysis by imidazole; lane T1, partial hydrolysis by RNase T1; lanes c, control incubations of KWT and KRW, without antibodies, in the presence of $MgCl_2$. (Right) Localization of cleavage sites observed in the presence of $MgCl_2$, within the secondary structures of the investigated RNAs. Arrows as in Figure 5. Numbering is according to Helm *et al.* (11).

transcribed $tRNA^{Lys}$, either of wild-type sequence (KWT) or with single point mutations (A55G for KMERRF and A9C for KRW) are also well established (11,18). KWT and KMERRF both fold into an extended bulged hairpin and differ only to a very small extent in the near neighborhood of the mutated positions (18). KRW has undergone a very large structural change. This molecule folds into a cloverleaf, as expected for tRNAs (11). Thus, comparison of the RNase activity of SLE antibodies on these RNAs should allow the establishment of whether the catalytic activities are sensitive to structural features or not.

The herein characterized SLE antibody Mg^{2+}-stimulated catalytic activity does not display sequence specificity, but is rather sensitive to structural features of the investigated RNA substrates. Yeast natural $tRNA^{Asp}$ has fewer cleavage points than the corresponding *in vitro* transcript, which was shown previously to be structurally less stable (12). KMERRF and KWT, which are known to have only slight structural differences, show very similar cleavage patterns with the antibody-specific activity with only minor differences. On the other hand, the cleavage patterns of KRW and KWT, which have similar sequences but very different structures, differ to a larger degree. The differences which are observed between cleavage patterns of given pairs of substrates correlate well with the structural knowledge of these molecules (11,12,18). Thus, the catalytic antibodies are able to discriminate between both subtle and large structural changes in RNAs, including stability and folding. These findings hold true for other preparations of antibodies with RNA hydrolyzing Mg^{2+} stimulated activities purified from various sera of SLE or hepatitis B patients and tested on the same couples of substrates (data not shown). Therefore, these new RNase activities may become novel tools for investigation of RNA structures in solution. However, since the specificities of each antibody sample appear to be multiple, adequate application will require amplification of monoclonal antibodies.

Biological considerations

The discovery of RNA hydrolyzing activities of human antibodies from patients leaves a number of unanswered biological questions. For example, it is of particular interest to understand the correlations between specific activities of antibodies and particular diseases and to know whether there is a relationship with the autoimmune character of some of the diseases. Also, a possible correlation between RNA cleaving autoantibodies and antibodies directed against RNA-recognizing proteins, e.g. aminoacyl-tRNA synthetases, as found in patients with autoimmune diseases (26,27), has to be considered. In any case, the biological significance of these activities remains unclear. Interestingly, all samples tested in the present work possess not only RNase, but also DNase activities (not shown), which opens the question of a double enzymatic activity for the same IgG molecule or for individual activities of different molecules. A fine understanding of the molecular mechanisms of RNase activities of human antibodies will require further investigation.

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