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DNA-Hydrolyzing Autoantibodies

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ABSTRACT

Catalysis by antibodies could be a frequent phenomenon if the immune system generates a sufficiently diverse number of antibody-active sites, some of which may possess catalytic activity. A catalytic antibody can be expected to do more damage than one that simply binds antigen. The best biochemical marker of systemic lupus erythematosus (SLE) is presence of autoantibodies to DNA. In the present article, we describe the DNA-hydrolyzing activity of DNA-binding autoantibodies purified from SLE patients. The substrates employed were supercoiled plasmid, radiolabeled plasmid fragments, and oligonucleotides. Hydrolysis of DNA by the antibodies was indicated by the appearance of fragments visualized by ethidium bromide staining of agarose gels or autoradiography of polyacrylamide gels. Changes in linear dichroism values were also indicative of DNA hydrolysis. The antibody activity was purified by protein A-sepharose chromatography, high-performance liquid chromatography gel filtration, and DNA-affinity chromatography. Scrupulous control studies were done to demonstrate that DNA-hydrolyzing activity really belongs to the antibodies. Purified Fab fragments showed hydrolyzing activity, whereas the Fc fragment was inactive. The specificity of DNA cleavage was investigated, and the rate parameters of hydrolysis by antibodies and conventional nucleases were compared.

Index Entries: DNA antibodies; antibody catalysis; DNA hydrolysis.

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INTRODUCTION

The concept of existence of natural catalytic antibodies has received support recently. Peptidolytic (*1*) and DNA-hydrolytic activities of antibodies (*2,3*) were detected in the sera of patients with asthma and SLE, respectively, but the reason for appearance of a catalytic function in antibodies remains unknown. The physiological role of catalytic antibodies is also unclear.

There are several possible explanations for the catalytic function of autoantibodies. The first possibility is based on the recently described induced-fit mechanism for antigen–antibody complex formation (*4*). Such a mechanism might lead to a conformational rearrangement in the antigen and introduce electronic strain in the scissile bond. A second possible explanation for formation of the natural abzymes is that anti-idiotypic antibodies to enzymes are potential biocatalysts (*2*). This idea is now supported by the experimental evidence presented by Friboulet et al. in this symposium. This group has successfully raised an anti-idiotypic antibody to acetylcholinesterase that displays catalytic function (*5*).

The possible problem of contaminations with conventional enzymes is of considerable concern in the investigation of catalytic antibodies. We have previously presented formal evidence for natural antibody catalysis (*1–3*), i.e.:

1. Homogeneity of catalytic antibody preparations;
2. Retention of catalytic activity after separation of antibodies at acid pH;
3. Removal of the catalytic activity by immobilized protein A and antihuman IgG; and
4. Detection of catalytic activity in Fab fragment preparations and the lack of such an activity in the Fc fragment.

We note that extension of our work to detect catalytic activities in monoclonal antibody preparations and antibody cDNA expression products would provide additional evidence for the existence of antibody catalysis.

The examination of the substrate specificity and catalytic efficiency of the natural DNA abzymes is a subject of special interest. According to data on their binding activity, anti-DNA antibodies can be divided into groups displaying the following specificity: antibodies to single-stranded DNA, antibodies to double-stranded DNA, antibodies to z-DNA, and polyreactive antibodies capable of binding several types of antigens in addition to DNA. Limited preference for base composition has been reported for the antisingle-stranded and antidouble-stranded DNA antibodies (*6*). Recognition of the 5′-phosphate group has been demonstrated as a determinant for binding of $d(T)_3$ by antisingle-stranded DNA antibodies. The antibody-combining site, as shown in (*7*), accommodates trinucleotide single-stranded DNA fragments.

Like peptide-specific catalytic antibodies (*8*), DNA-hydrolyzing antibodies have not revealed unique specificity for individual bonds in the substrate (*2*). A further characterization of the specificity of antibody-mediated DNA hydrolysis will require examination of:

1. Influence of length and base composition of substrate on cleavage patterns and rate of the catalysis;
2. Identification of preferred cleavage sites in asymmetric segments of the substrate;
3. Comparison of oligonucleotide degradation patterns for DNA-hydrolyzing antibodies and known nucleases, including restriction enzymes; and
4. Localization of the substrate domain recognized by the active site of antibodies.

In the present article, we describe data on the patterns and rates of cleavage observed with double-stranded DNA, single-stranded DNA of varying lengths and compositions, and the comparative kinetics of DNA hydrolysis by antibodies and conventional nucleases.

METHODS

Purification of IgG

This was done according to following scheme:

1. Three precipitations with ammonium sulfate (40%);
2. Molecular-sieving chromatography (TSK 3000 SW column; elution buffer: 50 m*M* Tris-HCl, pH 7.5, containing 200 m*M* NaCl), using an Altex (Beckman) high-performance liquid chromatography system (flow rate, 0.5 mL/min). At this step, the IgG and IgM fraction were separated, and further detailed analysis was performed with the IgG fraction;
3. Affinity chromatography on a protein A Sepharose column (flow rate 0.5 mL/min). The samples were injected in 50 m*M* Tris-HCl, pH 7.5, containing 100 m*M* NaCl, washing was performed with 50 m*M* Tris-HCl (pH 7.5) containing 1*M* NaCl, and IgG fraction was eluted with 100 m*M* glycine-HCl, pH 3.0. This procedure permitted purification of the IgG fraction, free of blood DNAse activity; and
4. Affinity chromatography on DNA-cellulose (flow rate 0.5 mL/min). IgG in 50 m*M* Tris-HCl, pH 7.5, containing 100 m*M* NaCl was injected on the column, washing was performed with 50 m*M* Tris-HCl, pH 7.5, containing 1*M* NaCl, and elution was with 1*M* potassium phosphate, pH 7.5. Because the DNA-hydrolyzing activity was inhibited by potassium phosphate, a

desalting step was done on the protein A-Sepharose column. A final purification step was performed on the TSK300SW column. Fab fragment was obtained by papain hydrolysis of IgG fraction, followed by chromatography on the protein G column and the molecular sieve chromatography step on TSK300SW (*9*).

Determination of DNA-Hydrolyzing Activity

IgG or Fab (1–8 μL) was incubated overnight with 1 μg of supercoiled DNA (pUC19) at 37°C. The reaction products were analyzed by electrophoresis in 1% agarose gel in 40 m*M* Tris, 20 m*M* sodium acetate, and 1 m*M* EDTA, buffer (pH 7.4) (7 V/cm, 30 min). Oligonucleotide cleavage was determined by incubation of various 5′-[^{32}P]-labeled oligonucleotides (0.1 p*M*) with DNA-hydrolyzing autoantibodies (1–8 μg) in 20 m*M* Tris-HCl, pH 8.0, at 37°C overnight in the case of IgG, and for 45 min in the case of Fab. The reaction products were separated in 20% PAAG containing 7*M* urea in 50 m*M* Tris, 50 m*M* boric acid, 1 m*M* EDTA, pH 8.3 (70 V/cm, 6 h), and analyzed by autoradiography.

Kinetics of DNA Hydrolysis

The kinetic parameters were obtained by activity determination as described above as a function of incubation time, substrate concentration, or antibody concentration. Reactions products identified in agarose gels and autoradiograms were quantitated by computerized densitometry (Molecular Dynamics 300A densitometer).

The kinetic parameters were also determined by the linear dichroism (LD) method. Supercoiled plasmid pUC 19 (0.01 mg) was digested with DNase I (0.01 ng), *Eco*RI (50 U), or the Fab-fragment prepared from DNA-hydrolyzing antibodies (0.1 μg) in 20 m*M* Tris-HCl, pH 8.0, buffer containing 5 m*M* $MgCl_2$ in 10% glycerol at 37°C. The LD signal was measured using a JASCO spectrophotometer (J-500C) at 260 nm. The DNA was oriented by stream in a 200-μL cuvet. The flow gradient mean value was 3000 cm^{-1}. The LD signal was measured as a function of time. Parallel determinations were done using the agarose electrophoresis technique. The time dependence of the reaction was obtained by measuring samples at 5-min intervals. Gels were photographed, the film scanned (LKB Ultroscan), and values of K_m and k_{cat} obtained from Lineweaver-Burk plots (*10*).

RESULTS AND DISCUSSION

As was demonstrated in our previous studies (*2*), the IgG and IgM fractions of autoantibodies isolated from sera of patients with SLE displayed DNA-hydrolyzing activity. This activity was proven to belong to

the antibody fraction. In order to study the properties of DNA-hydrolyzing autoantibodies, we purified the fraction of interest from autoimmune serum using the following protocol:

1. Precipitation of IgG with ammonium sulfate;
2. Molecular sieving chromatography;
3. Affinity chromatography on Protein A-Sepharose;
4. Affinity chromatography on DNA-cellulose in absence of metal ions; and
5. A second molecular sieving chromatography step.

The antibody preparation obtained from the second molecular sieving chromatography step retained the DNA-hydrolyzing activity. We demonstrated the activity of antibodies by the following methods:

1. Measurement of supercoiled DNA transformation by agarose gel electrophoresis;
2. Measurement of supercoiled DNA transformation by the linear dichroism method (LD) (*2*);
3. Comparison of degradation patterns formed by antibody cleavage of 100–300 bp DNA fragments with those obtained by using different enzymes (*2*);
4. Quantitative autoradiographic analysis of products of oligonucleotide cleavage separated by electrophoresis; and
5. The analysis of the DNA degradation catalyzed by abzyme using coupled reaction with DNA-polymerase I (*2*).

Studies using single-stranded oligonucleotides composed of different sequences (Fig. 1) did not reveal a striking sequence specificity associated with single-stranded hydrolytic activity of the antibodies. Essentially, similar degradation patterns were demonstrated using homopolymeric and heteropolymeric substrates. Hydrolysis of diverse oligonucleotides was evident, and in each case, the reaction occurred at multiple sites in the substrate. Since the different bands observed using various substrates were not equivalent in their intensities, the antibodies appear to display some preference for individual cleavage sites. Whether these preferences arise from antibody interaction with the 3′-, 5′-, or both termini of the oligonucleotides is not clear at this time.

To investigate length requirement for substrate activity, we constructed a group of oligonucleotides by random addition of bases to the 3′ end of a tetranucleotide. Antibody hydrolytic activity was detected for substrates six nucleotides or greater in length (Fig. 2). The velocity of the reaction increased rapidly for substrates from six to eight nucleotides in length. Further increases in oligonucleotide length were essentially without effect on the rate of hydrolysis. The patterns of hydrolysis were not identical for different oligonucleotide substrates. Since the 5′-tetranucleotide TTGC was conserved in these substrates, the 5′ terminus may be excluded as the specificity determining region. The observed differences in

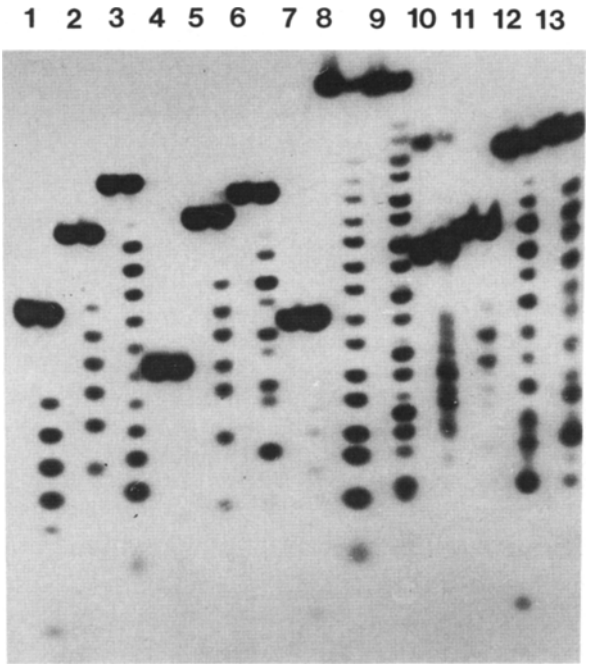

Fig. 1. Influence of oligonucleotide sequence on the degree and specificity of hydrolysis by DNA-specific antibodies. 5′-[^{32}P]-labeled oligonucleotides (0.5 p*M* each) were digested overnight at 37°C with 5 μg IgG in 20 m*M* Tris-HCl buffer, pH 8.0, and 10 m*M* $MgCl_2$. The reaction products were separated in 20% polyacrylamide gels (6 h, 75 V/cm) in 50 m*M* Tris-HCl, 50 m*M* boric acid, and 1 m*M* EDTA, pH 7.4, containing 7*M* urea, and detected by autoradiography. The left lane under each numeral shows the background reaction, and the right lane, the antibody-catalyzed reaction. 1, d(C9); 2, d(T10); 3, d(A13); 4, d(GGTT); 5, d(GT)6; 6, d(CA)6; 7, d(CA)3; 8, d(AC)10; 9, d(TC)10; 10, d(TGA)3; 11, d(TTC)3; 12, d(CAA)5; 13, d(TCA)5.

cleavage patterns may arise, therefore, from the sequence differences at the 3′ ends of these oligonucleotides.

Using 5′-^{32}P-labeled double-stranded DNA as substrate, radioactive products with a broad range of mass distribution were observed (Fig. 3). This suggests that the antibodies hydrolyzed this substrate without a strong preference for sites located at the 3′ and 5′ termini. As reported previously (*2*), the cleavage patterns observed by Fab- and DNase I-catalyzed hydrolysis of double-stranded DNA were not identical.

To determine the kinetics of antibody-mediated DNA hydrolysis, we studied $d(A)_{10}$ and pUC19 as substrates for purified Fab fragments. A comparison of K_m values for $d(A)_{10}$ oligonucleotide and for pUC19 plasmid DNA (Table 1) revealed that pUC19 formed more stable complexes with the Fab. Similarly, the turnover of pUC19 was more rapid than that of the $d(A)_{10}$. The k_{cat}/K_m value for double-stranded DNA was 430-fold greater

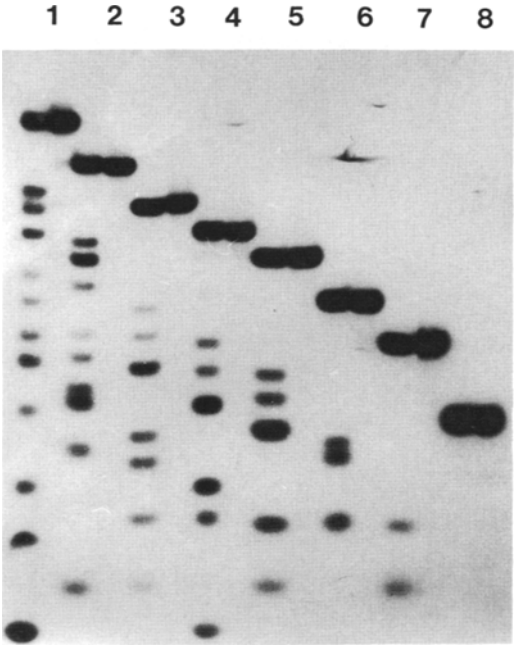

Fig. 2. Influence of oligonucleotide length on the degree and specificity of hydrolysis by DNA-specific antibodies. 5′-[^{32}P]-labeled deoxyoligonucleotides (0.5 m*M* each) were digested with 5 μg IgG and the reaction products analyzed as in Fig. 1. The right lane under each numeral shows the background reaction, and the left lane, the antibody-catalyzed reaction. 1, TTGCAGCACTGACC: 2, TTGCAGCACTGA; 3, TTCCACCACT; 4, TTCCAGCAC; 5, TTGCAGCA; 6, TTCCAGC; 7, TTGCAG; 8, TTGC.

than for single-stranded $d(A)_{10}$, demonstrating that the former is the preferred substrate. Nonphosphorylated $d(A)_{10}$ served as a competitive inhibitor of 5′-phosphorylated $d(A)_{10}$. The K_i for nonphosphorylated $d(A)_{10}$ was $3.2 \times 10^{-5}M$ compared to a K_m of $1.5 \times 10^{-7}M$ for the phosphorylated substrate. We concluded that the antibodies must bind the phosphorylated substrate more strongly than the nonphosphorylated inhibitor.

We compared next the efficiency of the double-stranded DNA hydrolysis by antibodies and conventional nucleases using pUC19 as substrate. Close agreement was observed between the kinetic parameters determined by product quantitation by the LD or agarose gel electrophoresis method (Table 1). Efficient hydrolysis by the Fab was observed. The kinetic efficiency (k_{cat}/K_m) of the Fab fragments was only about 10-fold smaller than that observed for DNase I and *Eco*RI. Whereas DNase I displayed very high k_{cat} and a low K_m compared to Fab, the kinetic constant values for Fab and *Eco*RI were in the same range. The similarity of these constants may derive from similar recognition requirements for these catalysts.

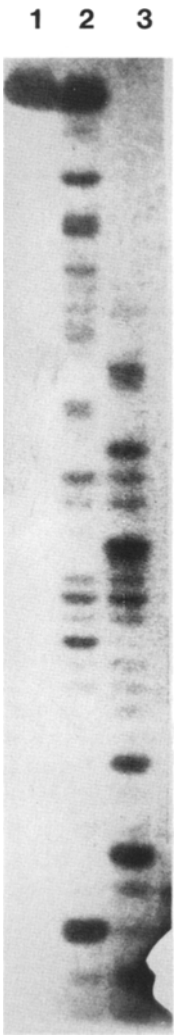

Fig. 3. Comparison of antibody- and DNase I-catalyzed pUC19 cleavage patterns. pUC19 DNA was treated with *Eco*RI, end-labeled with ^{32}P using polynucleotide kinase, and treated with *Pvu*II. The purified *Eco*RI-*Pvu*II fragment of the plasmid DNA was digested with Fab fragment of DNA-hydrolyzing antibodies or DNase I in 20 m*M* Tris-HCl buffer, pH 8.0, containing 10 m*M* $MgCl_2$. Lane 1, background reaction; lane 2, DNase I, 0.01 ng (10 min, 37°C); lane 3, Fab fragment, 0.5 μg (4 h, 37°C).

Based on these data, we concluded that the observed DNA-hydrolyzing activity really belongs to autoantibodies. The efficiency of the antibody-catalyzed DNA hydrolysis is within the range observed for conventional nucleases. This observation supports the possibility of a physiological role for the newly identified DNA-hydrolyzing antibodies. The chemical basis for antibody-mediated DNA hydrolysis remains to be studies. An

Table 1
Comparison of DNA Cleavage by DNase I, *Eco*RI, and Human Fab Fragments

		Linear dichroism method[1]			Electrophoresis method[1]			Published values[2]	
Substrate	Enzyme	K_M^{obs}, M	k_{cat}, min^{-1}	k_{cat}/K_m^{obs}, $\times 10^9\ min^{-1}/M$	K_M^{obs}, M	k_{cat}, min^{-1}	k_{cat}/K_m^{obs}, $\times 10^7\ min^{-1}/M$	K_M^{obs}, M	k_{cat}, min^{-1}
pUC 19	DNAse I	$5.8(0.3) \times 10^{-5}$	$3.2(0.2) \times 10^5$	5	$4.6(0.9) \times 10^5$	$2.5(0.5) \times 10^{-5}$	540	2.5×10^{-5}	10^5
	*Eco*RI	$3.9(0.2) \times 10^{-9}$	10(0.6)	2.6	$1.7(0.3) \times 10^{-9}$	2.3(0.5)	130	4×10^{19}	6.5
	Fab	$4.3(0.6) \times 10^{-8}$	14(1.8)	0.32	$9.2(3.2) \times 10^{-8}$	40(14)	43	–	–
$(dA)_{10}$	Fab	–	–	–	1.5×10^{-7}	0.2	0.1	–	–

[1] *See text* for methods; values in parentheses are standard deviations.
[2] References *9,10.*

induced-fit mechanism described for binding of single-stranded DNA by an antibody (4) permits consideration of possible changes in the double-helical structure of DNA on binding of the hydrolytic antibodies. The binding may induce local conformational changes in the sugar-phosphate backbone, and the new conformation of the DNA could be more sensitive to hydrolysis because of introduction of strain in the molecule. The flexibility of an intact double-stranded DNA structure may be important in the interaction with antibodies. A lack of stringent sequence specificity in the binding step may permit formation of several types of antibody–DNA complexes and possibly underlie the cleavage at multiple sites in the substrate.

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DISCUSSION

A. Gabibov

Wu: Do you care to comment on what is the best substrate, the supercoiled, double-stranded, or single-stranded DNA. You mentioned that the 5′ phosphate is important. If you have a supercoiled structure, there is no 5′ end.

Gabibov: Comparable velocities of hydrolysis were observed using the supercoiled DNA and linear double-stranded DNA as substrate. The role of 5′ phosphate was demonstrated using oligonucleotide substrates. I think that it is difficult to compare the kinetics of the reaction when radically different types of DNA substrates are used.

Polanovsky: Are there any correlations between the abzyme concentration in blood and the level of severity of some pathological conditions. Can the abzyme levels be useful in diagnosis of a disease?

Gabibov: There is some correlation between the activity of antibodies and the stage of certain disease conditions. We have studied this correlation for SLE and AIDS. Dr. Gololobov will present this data in his talk. In general, the activity of antibodies increases with the severity of disease. We are not prepared to use this test in practical medicine yet because of lack of statistically enabling data.