Secondary Structure in Denatured DNA is Responsible for Its Reaction with Antinative DNA Antibodies of Systemic Lupus Erythematosus Sera

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ABSTRACT Experiments were designed to determine the basis for the strong competitive reaction of denatured DNA with systemic lupus erythematosus (SLE) antinative DNA antibodies. Secondary structure in denatured DNA was reflected in hyperchromicity upon heating and in multiphase kinetics of its digestion by S1 nuclease. Partial digestion by S1 nuclease completely eliminated the ability of denatured DNA to react with antidenatured DNA antibodies, but not its ability to react with SLE sera. S1 nuclease-resistant cores were isolated from extensively digested denatured DNA. These cores had secondary structure, including some stable fold-back helical regions. The cores, from 20 to several hundred base pairs in size, competed with native DNA for binding by SLE sera. Other experiments measured reactions of denatured DNA under conditions that affected its secondary structure content. Its competitive activity decreased as temperature was increased from 0° to 37°C, whereas the activity of native DNA was not altered in this temperature range. With DNA pieces of 90–110 base pairs, native fragments were much more effective than the denatured fragments, in which stable helical structure is less likely to occur than in high molecular weight denatured DNA. Competitive assays with mononucleotides, oligonucleotides, homopolymers, and RNA-DNA hybrids also indicated that two strands of polydeoxyribonucleotide were required for optimal reactions with these SLE serum antibodies. The antibodies can measure stable helical regions in denatured DNA; they may also stabilize short helical regions that occur in an equilibrium of conformational forms.

INTRODUCTION

Sera of patients with systemic lupus erythematosus (SLE)[1] contain a variety of antinucleic acid antibodies. There has been considerable interest in defining the specificities of these antibodies, since questions have arisen whether anti-DNA assays are reliable for diagnosis and monitoring of disease activity, and whether antibodies with a particular kind of specificity may be especially significant for pathogenesis and diagnosis (1–7).

Anti-DNA antibody specificity has been divided on the basis of reactivity with either denatured DNA or native DNA. One class of antibody clearly reacts with denatured DNA only, and recognizes the purine or pyrimidine bases or base sequences, which are not available for reaction in the helical native structure. The binding of radioactive denatured DNA by these antibodies is competed for by unlabeled denatured DNA but not at all by native DNA (8). Similar antibodies can be induced in experimental animals by immunization with denatured DNA-methylated bovine serum albumin complexes (9), or with nucleoside- or nucleotide-protein conjugates (10).

A second type of antibody in SLE serum reacts with native DNA. These antibodies bind closed circular bacteriophage DNA (11) or completely helical poly(dAT) (12). They react with circular helical DNA *in situ* in immunofluorescence assays with *Crithidia luciliae* (13), and they bind DNA treated with S1 nuclease to remove single-stranded regions (14). Interestingly, however, the binding of native DNA by these antibodies is usually inhibited by denatured DNA (8, 15), and this inhibition may be seen with denatured DNA concentrations that are nearly the same as those required for native DNA itself. Similarly, denatured DNA inhibits the precipitation of native DNA (16) or passive hemagglutination of native DNA-coated erythrocytes (17) by most SLE sera.

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[1] *Abbreviations used in this paper*: PBS, phosphate-buffered saline (0.14 M NaCl, 0.01 M phosphate, pH, 7.2); SLE, systemic lupus erythematosus.

The finding that a number of SLE sera react with determinants that are present on both native and denatured DNA raises the question whether the antibodies truly recognize the secondary helical structure of DNA. Three possibilities raised by these observations are, first, that the reaction is due to exposure of the bases in local single-stranded regions of the DNA; second, that the reaction is with the sugar-phosphate backbone of one polydeoxyribonucleotide strand and that such a structure is accessible in both the native and denatured DNA (it should be noted that antibodies to such a determinant, even in combining with denatured DNA, would differ from those that recognize bases or base sequences; and third, that the antibodies react with a determinant that does involve the backbone of both strands, and that denatured DNA competes for binding because there is in fact a significant amount of helical secondary structure (either stable or transient) in denatured DNA (18). Such antibodies would resemble the experimentally induced antibodies to double-stranded RNA or RNA-DNA hybrids, which recognize specific helical shape (19).

Experiments described in this article were designed to test these possibilities. The results indicate that optimal reaction with antinative DNA antibody does require secondary structure, and that the antibodies measure localized helical regions in denatured DNA rather than localized denatured regions in native DNA.

METHODS

Antigens. Calf thymus DNA, obtained from Worthington Biochemicals Corp., Freehold, N. J., was purified further as described (20). For denaturation, DNA was heated at 100°C for 10 min and quickly chilled in an ice-water bath. Poly(dA), poly(dT), poly(dI), and poly(A)·poly(dT) were purchased from P-L Biochemicals Inc., Milwaukee, Wis. [^{3}H]Thymidine-labeled DNA was prepared from a partially thymine-requiring mutant, B3, as described (21), but with a pulse of 500 μCi of [^{3}H]thymidine, rather than 60 μCi of [^{14}C]thymidine, during log-phase growth. The labeled DNA was treated with S1 nuclease (Miles Laboratories, Inc., Elkhart, Ind.) in a reaction mixture containing 48 μg of DNA in 1.5 ml of 0.03 M sodium acetate, pH 5, 0.01 mM $ZnCl_2$, and 15 μl of enzyme (4,000 U). The mixture was incubated at 37°C for 20 min, and the reaction was then stopped by the addition of 2.5 ml of 0.06 M sodium phosphate and 0.03 M EDTA, pH 8. The treated DNA was diluted further to 1 μg/ml for addition to the radioimmunoassay mixtures.

Helical DNA fragments of 90–110 base pairs were prepared as described previously (20). Oligonucleotides averaging 20 residues in length were prepared from a pancreatic DNase digest of calf thymus DNA. 50 μg of DNase was added to 35 ml of DNA (1.3 mg/ml with 5 mM $MgCl_2$). The reaction proceeded until 25% hyperchromicity was reached, and was stopped with EDTA (10 mM final concentration). Digestion products were applied to a 15-ml DEAE-cellulose column, which was then washed extensively with 0.25 M NaCl in 0.05 M cacodylate buffer, pH 6.8. Larger oligonucleotides were then eluted with 0.6 M NaCl in the cacodylate buffer. Average chain length was determined from total and terminal phosphorus measurements (22).

Analytical S1 nuclease digestion of denatured DNA. 10 μl of S1 nuclease (2,700 U) was added to 2 ml of a solution of 100 μg/ml of denatured DNA in 0.03 M acetate, pH 5, with 0.015 mM $ZnCl_2$; and the change in absorbance was monitored at 260 nm at room temperature. When the rate of increase slowed markedly, the temperature was raised to 37°C and the absorbance monitored during further incubation.

Preparative S1 nuclease digestion of denatured DNA. 50 μl (13,500 U) of S1 nuclease was added to 10 ml of denatured calf thymus DNA (1.8 mg/ml) in 0.03 M sodium acetate, pH 5, with 0.05 mM $ZnCl_2$. The mixture was incubated at room temperature. When the rate of increase in absorbance at 260 nm slowed, 10–25-μl increments of enzyme were added until hyperchromicity reached 27%; a total of 110 μl of enzyme was used over a 5-h period. The reaction was stopped by addition of EDTA to 10 mM. The digestion products were applied to a 2.5 × 40-cm Bio-Gel P-60 column (Bio-Rad Laboratories, Richmond, Calif.) and washed through with phosphate-buffered saline (PBS). The material emerging at the void volume was diluted threefold with water and applied to a 1.0-ml column of DEAE-cellulose equilibrated with PBS. The column was washed with PBS and then with solutions of 0.1, 0.2, 0.3, 0.5, 0.75, and 1.0 M NaCl in PBS. Eluted nucleic acids were dialyzed against a 1:2 dilution of PBS in water.

Polyacrylamide gel electrophoresis. Samples of 1–2 μg of DNA fragment were loaded onto 10% polyacrylamide slab gels (12 × 16 × 0.16 cm) with a 1-cm, 4% stacking gel. The acrylamide:bisacrylamide ratio was 19:1. After electrophoresis at 50 mA for 3.5 h, gels were stained with 1 μg/ml ethidium bromide for 30 min, rinsed with water, and photographed under short wave UV light.

Thermal denaturation curves. Calf thymus DNA or DNA fragments were heated in stoppered l-ml silica cuvettes in a Zeiss PM6 spectrophotometer with a thermo-electric temperature control unit (Carl Zeiss, Inc., New York, N. Y.). Absorbance at 260 nm was recorded after equilibration.

Double-antibody radioimmunoassays. Double-antibody radioimmunoassays (20) or ammonium sulfate radioimmunoassays (21) were performed as described previously. SLE sera were those used in a previous study (20). Rabbit antinucleoside antibodies were prepared as described by Erlanger and Beiser (10). Rabbit antibodies to double-stranded RNA and RNA-DNA hybrids were described previously (19).

RESULTS

DNA binding and competition. Initial experiments were performed to ensure that the antibodies being measured were comparable to those studied previously (8, 15, 16). The DNA used for binding was [^{3}H]thymidine-labeled *Escherichia coli* DNA, prepared from a partial thymine-requiring mutant by the procedure of Marmur (23). Without further treatment, this DNA was still bound by base-specific antibodies to denatured DNA as well as by SLE sera (Fig. 1*a*). After treatment of the DNA with S1 nuclease, which specifically digests single-stranded regions (24), most of the binding by SLE serum was retained (Fig. 1*b*). The reduction that did occur in binding by SLE serum may have resulted from a decrease in molecular weight of the DNA if the S1 nuclease cleaved nicked regions

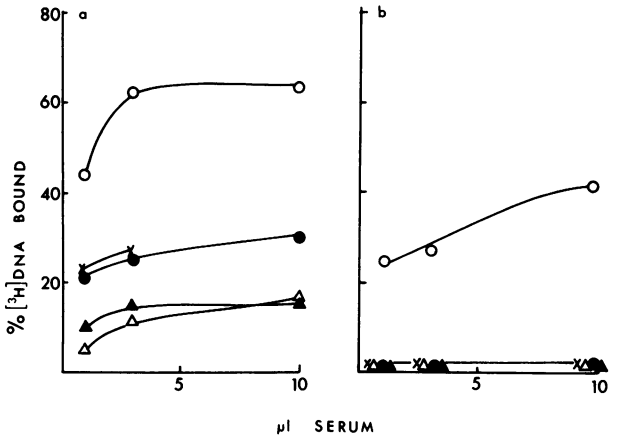

FIGURE 1 Binding of [^{3}H]thymidine-labeled native *E. coli* DNA. (*a*) Before treatment of the DNA with S1 nuclease. (*b*) After treatment of the DNA with S1 nuclease. Binding of 50 ng of labeled DNA was tested with varying amounts of SLE serum M2 (○), rabbit antiadenosine (×), antiguanosine (△), anticytidine (●), and antithymidine (▲) antisera. All assays were double-antibody assays carried out at room temperature.

within the molecules; alternatively, it may have resulted from elimination of binding by antidenatured DNA antibodies in the serum. The reaction with all base-specific sera was reduced to the background level of 5% binding or less (Fig. 1*b*). Normal sera also consistently bound <5% of the labeled DNA. In all further experiments, S1 nuclease-treated native DNA was used as the labeled antigen.

Also as noted previously (8, 15), unlabeled native or denatured DNA compared for the binding of native DNA. Six SLE sera were tested in competitive radioimmunoassays carried out at 4°C. Unlabeled denatured DNA competed at concentrations that varied from one to eight times those required for competition by cold native DNA. Similar ratios of inhibitory concentrations were observed both when the labeled and unlabeled antigens were added to serum simultaneously and when the unlabeled DNA was preincubated with serum; in the latter case, lower concentrations of both native and denatured DNA were required.

The competition by denatured DNA was a specific action, inasmuch as the same preparation did not inhibit the binding of double-stranded RNA or RNA-DNA hybrids by corresponding rabbit antibodies even when the denatured DNA was tested at 500-fold higher concentrations than those effective for homologous antigen.

Competition by mono- and oligonucleotides. As a test of the possibility that exposed bases or short base sequences were still accessible and formed determinants in the native DNA, mononucleotides and oligonucleotides were used as competitors. Whereas 10–50 ng of native or denatured DNA inhibited native DNA binding by 50%, 3,000-fold more (160 µg) of a mixture of monodeoxyribonucleotides inhibited binding by only 15–20%. With oligonucleotides of mixed-base composition (averaging 20 nucleotides per chain), 2,000–20,000 ng was required for 50% inhibition (Table I). As expected, the 20-nucleotide oligomer inhibited the binding of denatured DNA by SLE antidenatured DNA antibodies, and only 100 ng was required for 50% inhibition in that case. These findings negate the possibility that the binding of native DNA by the SLE sera is due simply to local denaturation and base exposure. When such exposure does occur, base-specific antibodies are able to detect it (Fig. 1*a*) (25).

With DNA fragments of 100 base pairs, denaturation

TABLE I
Competitive Radioimmunoassay of Native DNA and Native and Denatured DNA Fragments with SLE Antinative DNA Antibodies

		Mass required for stated inhibition			
			90–110 Base-pair fragment		
Serum	Inhibition	Native DNA	Native	Denatured	Oligonucleotide
	%		ng		
M1	50	10	80	2,100	4,000
M2	50	28	90	2,300	2,350
M3	50	30	30	700	2,800
M4	50	30	350		22,000
	15		10	1,000	
M5	50	10			
	15		20	1,000	10,000

Competing polynucleotides and SLE sera were preincubated at room temperature for 30 min before addition of 50 ng of labeled S1 nuclease-treated native DNA. After another hour at room temperature, complexes were precipitated by goat antihuman immunoglobulin.

reduced reactivity with all sera: 25–80 times as much denatured polynucleotide was required for competition equal to that caused by a given amount of native DNA fragment (Table I). These denatured fragments did have some secondary structure, as revealed by hyperchromicity on heating (20). From experiments with mononucleotides, oligonucleotides, 100-nucleotide fragments, and high molecular weight DNA, it appeared that the longer the chain length, the closer the single-stranded DNA approached the competitive activity of double-stranded DNA. This may have reflected the increased possibilities for secondary structure in the longer chains.

Tests for the role of a single polydeoxyribose phosphate backbone. If only a single chain were required for recognition by antibody, then homopolydeoxyribonucleotides should be reactive. Poly(dI), poly(dA), and poly(dT), however, showed little or no competition at low concentrations for antibody binding of native DNA (Fig. 2). When the same poly(dA) and poly(dT) samples were annealed together, a much more reactive product was formed (Fig. 2*b*). Interestingly, neither this helix nor poly(dG)·poly(dC) was as effective as poly(dAT)·poly(dAT) or native DNA, suggesting that the antibodies can discriminate among different helical shapes, as can experimentally induced antidouble-stranded RNA antibodies (26). The effectiveness of one polydeoxyribonucleotide backbone as part of a non-DNA helix was also tested. Poly(I)·poly(dC) (Fig. 2*a*) and poly(A)·poly(dT) (not shown) were both unable to compete at low concentration for binding by antinative DNA antibodies.

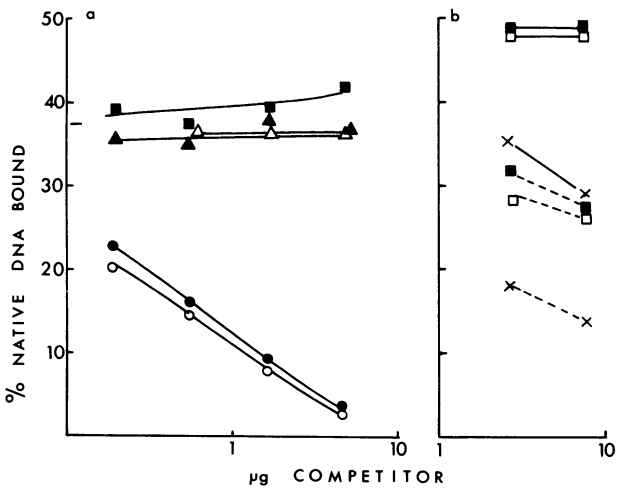

FIGURE 2 (*a*) Competitive radioimmunoassay with SLE serum M2. Binding of 50 ng of S1 nuclease-treated native DNA competed with varying amounts of native DNA (○), denatured DNA (●), poly(dA) (■), poly(dI) (▲), and poly(I)·poly(dC) (△). Antigen-antibody complexes were precipitated by ammonium sulfate (21). In the absence of competitor, 35% of the DNA was bound. (*b*) Competitive radioimmunoassay with SLE sera M2 (dashed lines) and M5 (solid lines). Competing polynucleotides were poly(dA) (■), poly(dT) (□), and an annealed mixture of the same poly(dA) and poly(dT) (×). Complexes were precipitated by goat anti-human IgG. In the absence of competitor, serum M2 bound 33% and M5 bound 50% of the DNA.

The effect of S1 nuclease digestion of denatured DNA. One of the lines of evidence for the existence of secondary structure in denatured DNA is the relative resistance of a portion of the DNA to digestion by S1 nuclease (18). When digestion of denatured DNA by S1 nuclease was monitored at room temperature, a rapid early rate of digestion was followed by a second, slower phase (Fig. 3). This slowing did not depend on exhaustion of the enzyme, inasmuch as the more rapid rate returned when the temperature was raised to 37°C. A sample taken during the second phase retained 35–70% of its ability to inhibit the binding of labeled native DNA by the three SLE sera tested, even though it has lost completely its ability to inhibit the binding of denatured DNA by rabbit antidenatured DNA antibody (Fig. 4). For this experiment, an anti-denatured DNA methylated bovine serum albumin antibody was chosen, because the determinant for such antibodies is about the size of a pentanucleotide (27), and inhibition by mono- or dinucleotides released from DNA would be minimized. These experiments showed that a major portion of the denatured DNA structure that inhibited native DNA binding by SLE serum was resistant to S1 nuclease under conditions in which accessible single-stranded regions were virtually completely digested, and suggested that secondary structure was involved in reactivity with the SLE sera.

When the S1-digested denatured DNA was reboiled and quickly chilled, it was able again to inhibit the

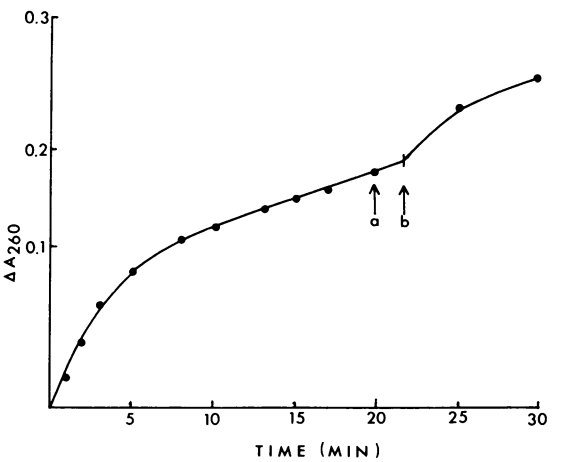

FIGURE 3 Digestion of denatured calf thymus DNA by S1 nuclease, monitored by hyperchromicity. After 20 min at room temperature (arrow a), a sample was taken for serological assay. 2 min later (arrow b), the temperature was raised to 37°C.

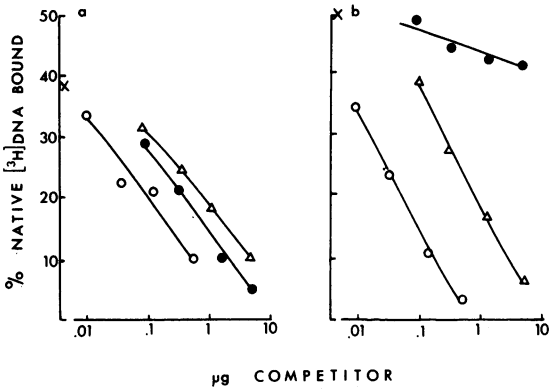

FIGURE 4 Competitive radioimmunoassays of (*a*) SLE serum M2 with labeled native DNA, and (*b*) rabbit antidenatured DNA serum with labeled denatured DNA. Binding of 50 ng of labeled DNA was tested in competition with denatured DNA (○), denatured DNA digested for 20 min at room temperature by S1 nuclease (see Fig. 3, arrow a) (●), and the S1-digested denatured DNA after it was boiled for 10 min and quickly chilled (△). Control values in the absence of competitor are shown (×). Results with SLE sera M1 and M4 were similar to those obtained with M2.

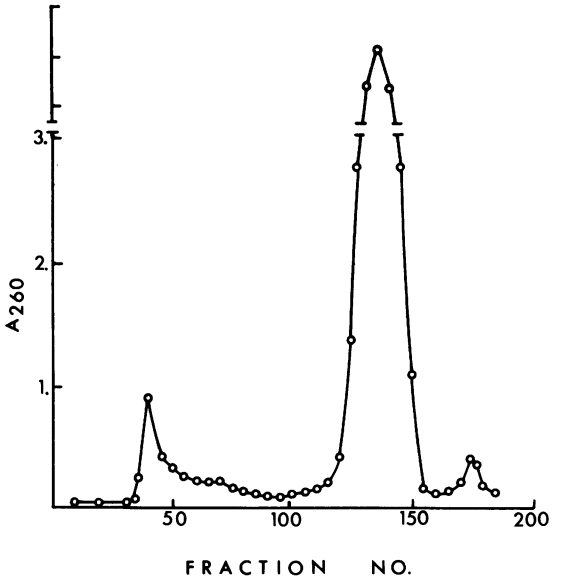

FIGURE 5 Bio-Gel P-60 gel filtration of denatured DNA extensively digested by S1 nuclease. Digestion of the denatured DNA (1.8 mg/ml), at room temperature in 0.03-M acetate buffer, was continued for 5 h, until 27% hyperchromicity was reached.

antidenatured DNA-methylated bovine serum albumin antibodies (Fig. 4*b*). The boiling, which restored reactivity with antidenatured DNA, reduced further the reactivity with SLE antinative DNA (Fig. 4*a*). A residual 10–20% of the reactivity of denatured DNA was left, as measured with the SLE antisera. This corresponded to 3–8% of the original activity of native DNA for these sera.

Isolation of helical cores from denatured DNA. Experiments were performed to isolate and characterize the S1 nuclease-resistant and serologically reactive material in the denatured DNA. A sample of 18 mg of DNA in 0.03 M acetate, pH 5, was denatured and then digested with S1 nuclease for 5 h at room temperature, until it reached a hyperchromicity of 27% at 260 nm. The digested material was passed through a column of Bio-Gel P-60. A small fraction was recovered at the void volume, whereas most material appeared as small oligonucleotides just before the end of the retained volume (Fig. 5). The high molecular weight material competed for antinative DNA antibodies, whereas the included oligonucleotides did not.

The void volume material was then applied to a 1-ml column of DEAE-cellulose in 0.05 M NaCl, 0.01 M phosphate, pH 7.2, and was eluted stepwise, with increasing concentrations of NaCl (Table II). 37 μg was eluted by 0.5 M NaCl and 190 μg by 0.75 M NaCl. These fractions, resistant to S1 nuclease even at the relatively low ionic strength of 0.03 M acetate, corresponded to about 1.25% of the total DNA. Upon analytical polyacrylamide gel electrophoresis, the 0.5-M NaCl fraction was found to consist of material that was uniformly smaller than an 80 base-pair marker; the 0.75 M NaCl fraction was more heterogeneous, ranging from about 20 to several hundred base pairs (Fig. 6). Measurements of thermal denaturation revealed that the 0.75-M NaCl fraction was mainly a double-stranded structure, since it reached a 38% hyperchromicity and showed little melting below 50°C (Fig. 7). The 0.5-M NaCl fraction showed only 21% hyperchromicity, but it also had a cooperative melting profile, with little hyperchromicity below

TABLE II
DEAE-Cellulose Chromatography of S1 Nuclease-resistant Cores of Denatured DNA

Eluant	Polynucleotide eluted
	μg
PBS alone	1
With	
0.1 M NaCl	1
0.2 M NaCl	1
0.3 M NaCl	1.2
0.5 M NaCl	37
0.75 M NaCl	190
1.0 M NaCl	5

The void volume material from the Bio-Gel P-60 column (Fig. 5) was applied to a 1.0-ml column DEAE-cellulose in 0.05 M NaCl, 0.003 M phosphate, pH 7.2. Eluants were applied in 1.5-ml portions until it was clear that no polynucleotide was being eluted or until absorbance returned to base-line levels.

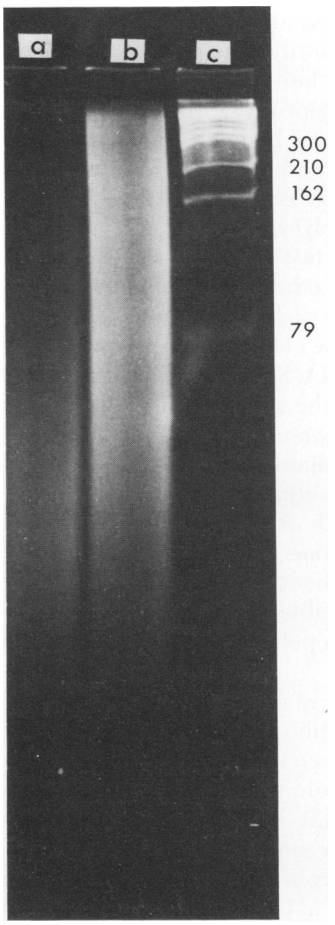

FIGURE 6 Polyacrylamide gel electrophoresis of S1 nuclease-resistant cores from denatured DNA. The void volume peak from the Bio-Gel P-60 column (see Fig. 5) was applied to DEAE-cellulose. Material eluted by 0.5 M NaCl (*a*) and 0.75 M NaCl (*b*) were analyzed on 10% polyacrylamide gels. A marker of HINC II digest of ϕx 174 replicative form DNA was included (*c*).

50°C. When cooled, the samples did not return to starting absorbance levels, again reflecting the secondary structure content of the original material. Interestingly, however, when the samples were reheated, there was still cooperative melting, with little change below 50°C. This contrasted with the behavior of total denatured DNA (Fig. 7) or of helical fragments isolated from a micrococcal nuclease digest of DNA (20), and indicated that part of the original secondary structure of the S1 nuclease-resistant fragments had renatured during the cooling.

Both the 0.5-M and the 0.75-M fractions competed with labeled native DNA for binding by SLE serum antibodies (Fig. 8). As observed for completely helical fragments described previously (20), the larger polynucleotides (0.75-M NaCl fraction) were more effective competitors than the smaller fragments. In comparison,

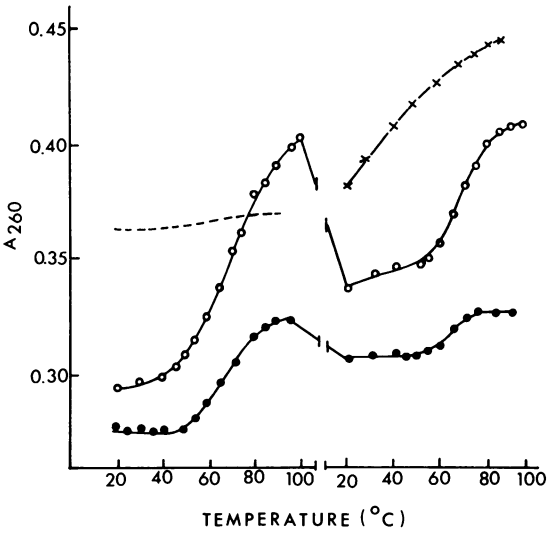

FIGURE 7 Thermal denaturation of the S1 nuclease-resistant cores of denatured DNA. The 0.5-M NaCl (●) and 0.75-M NaCl (○) eluates from DEAE-cellulose (Table II) were heated, cooled to 20°C, and reheated. The mono- and oligonucleotides near the included volume of the Bio-Gel column (-----) and total denatured DNA (×) were also tested. Native DNA heated under the same conditions showed a sharp transition, with a maximum temperature of 85°C and a hyperchromicity of 40%.

a denatured 110 base-pair fragment from a micrococcal nuclease digest did not compete for the antinative DNA binding unless higher concentrations were added (Table I).

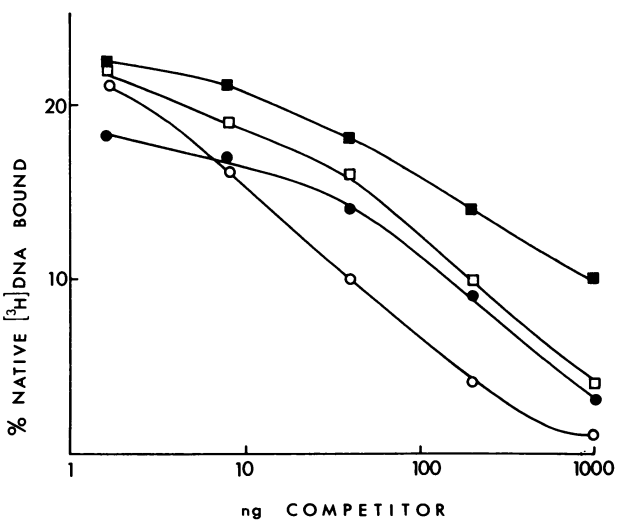

FIGURE 8 Competitive radioimmunoassay of S1 nuclease-resistant cores with SLE serum M1. Binding of 50 ng of labeled native DNA was measured in competition with native DNA (○), denatured DNA (●), the 0.75-M NaCl eluate from DEAE-cellulose (□), and the 0.5-M NaCl eluate from DEAE-cellulose (■). The assay was at room temperature; complexes were precipitated by goat anti-human IgG.

The effect of temperature. Secondary structure, which is known to occur in denatured DNA, is unfolded gradually with increasing temperature in the range of 0°–37°C in 0.15 M NaCl. Completely helical DNA is not altered in this temperature range, but melts sharply at higher temperatures. In view of this property, the ability of denatured DNA to compete with labeled native DNA for antibody was tested at various temperatures. In preliminary experiments it was found that the labeled native DNA was bound in the assay when all steps were carried out at 4°, 20°, or 37°C, and that the same amount of serum could be used at each temperature. In typical experiments, control binding values at 4° and 37°C were respectively, 32 and 30%, with 1 μl of serum M5, and 35 and 44% with 2 μl of serum M2.

With four sera, competition by denatured DNA was decreased at the higher temperatures (Table III). A three- to sixfold higher concentration was required at 37°C than at 4°C. Native DNA was equally effective at both temperatures. With one serum (M4), the same amount of denatured DNA was effective at 4° and 37°C, but at both temperatures this was eight times the amount of native DNA required for identical competition. In all cases, therefore, denatured DNA had only 10–30% of the reactivity of native DNA at 37°C; at this temperature about 30–40% of the secondary structure in denatured DNA was melted (Fig. 7).

DISCUSSION

The reaction of SLE serum antibodies with native DNA has had a puzzling aspect, for the reaction can be inhibited by denatured DNA. This has raised questions whether the antibodies do in fact recognize secondary structure of helical DNA or, alternatively, detect denatured regions in apparently native DNA. Several approaches have been taken to support the conclusion that the antibodies react with native DNA. These include the use of closed circular DNA (11), synthetic poly(dAT)·poly(dAT) (12), or intracellular circular DNA (13) as test antigens that should be completely helical. With other DNA samples, treatments such as absorption by various column materials (6) or digestion by S1 nuclease (14) are used to remove single-stranded regions of DNA. Still, it may be argued that subsequent exposure to the test serum itself may create single-stranded regions by action of serum nucleases. Also, hydrogen-exchange experiments indicate that DNA is in a dynamic equilibrium, in which bases are locally unpaired and exposed to solvent for a significant fraction of time (with an equilibrium constant for opening of about 0.001–0.01 at neutral pH and 0°C) (28). It is possible, therefore, that the reaction of native DNA could depend on local denaturation and exposure of bases.

The ability of denatured DNA to react with antinative DNA antibodies, however, could depend on the presence of secondary structure in the denatured DNA. This could include base-paired helical regions of varying length and stability. Such structures have been isolated from single-stranded DNA of small viruses (29–32). Hairpin loops of 44 and 60 base pairs were isolated from M13 virus DNA (30); similar helical segments have been identified and sequenced in phage G4 viral DNA (31). These structures were isolated first as cores of single-stranded DNA that resist digestion by S1 nuclease or other enzymes specific for unpaired nucleic acids. S1 nuclease-resistant cores with fold-back helical structure also occur in more complex samples such as bacterial and mammalian DNA (18, 33, 34). In a situation that may parallel the ability of antibodies to recognize secondary structure in denatured DNA, duplex structure within single-stranded φX 174 DNA appears to be responsible for recognition and cleavage by restriction endonucleases (35). In addition to these stable cores, many other regions with less stable secondary structure would be present in high molecular weight DNA at low temperature.

In view of the above considerations, it may not be possible to identify conclusively the reactive structure simply by characterizing the test antigen as native or denatured. The experiments reported in this article, therefore, were designed to determine whether there is a relationship between changes in the amount of secondary structure present and the degree of reactivity. Additional experiments were done to isolate the S1 nuclease-resistant cores from calf DNA and to deter-

TABLE III

Temperature Dependence of the Competition of Denatured DNA for Antinative DNA Antibodies

Serum	DNA	DNA required for 50% competition			37°C / 4°C
		4°C	20°C	37°C	
		ng			
M1	Denatured	60	100	250	4.1
	Native	65		70	1.1
M2	Denatured	70	170	450	6.4
	Native	55		75	1.4
M3	Denatured	35	85	210	6.0
	Native	50		50	1.0
M4	Denatured	1,000	800	800	0.8
	Native	120		100	0.8
M5	Denatured	120		330	2.8
	Native	25		37	1.5

Competing and labeled DNA were added simultaneously to heated diluted serum. Control binding without competing DNA was 30–60% at all temperatures.

mine whether they contained base-paired structure and could react with antinative DNA antibodies.

The failure of mononucleotides to compete, even at very high concentrations, indicates that the antibodies do not recognize single bases; even with oligonucleotides averaging 20 residues in length, competitive binding required concentrations some 400-fold higher than those effective with helical DNA. Most significant in this regard, helical fragments of 100 base pairs were effective competitors, whereas the same fragments, when denatured, were much less effective (Table I). There was some residual binding activity of the denatured fragments. This could mean that a single polydeoxyribonucleotide backbone can bind to these antibodies. SLE sera are known to contain heterogeneous antibody populations (17), and some may in fact bind preferentially to the single backbone structure. In competition for antibodies that bind labeled native DNA, however, the single-chain DNA fragments were always much weaker than the helical forms. This was true also for poly(dA) or poly(dT) in comparison with poly(dA)·poly(dT). Hybrid helices containing one polydeoxyribonucleotide strand, such as poly(A)·poly(dT) or poly(I)·poly(dC), also showed no reactivity when tested at concentrations at which poly(dA)·poly(dT) was reactive. These results support the conclusion that, although these populations of antinative DNA antibodies may bind one chain with low energy, they show much higher binding energy with (i.e., specificity for) helical structures.

Even in the denatured 100-base-pair fragments, secondary structure may be responsible for reactivity with these antibodies. The denatured fragments do show hyperchromicity upon heating (20) and therefore do have secondary structure, but they are unlikely to have long stable helical segments. There may be an equilibrium in which portions of the fragments spend a small but finite fraction of time in a helical form, which is then stabilized by interaction with antibody. The competition by higher concentrations of the denatured fragments may reflect, in part, such a conformational equilibrium. A similar analysis has been applied in greater detail for the reactions of native and unfolded protein with conformation-dependent antiprotein antibodies (36).

The effect of incubation temperature on reactivity also indicated that secondary structure in denatured DNA was important for reaction with the SLE antinative DNA antibodies. Native DNA was equally reactive at 4° and 37°C, over which range its secondary structure was not appreciably altered. In contrast, as denatured DNA was gradually unfolded between 4° and 37°C, its reactivity decreased three- to sixfold with four sera; with the fifth serum, it was only one-eighth as reactive as native DNA at either temperature. At 37°C, native DNA was from 3.5–9 times as effective as denatured DNA with these five sera. Residual activity at 37°C may have reflected the weak binding of a single polydeoxyribonucleotide backbone or a conformational equilibrium, as discussed above. In this case, however, there was still a possibility that stable helical structure was present within the remaining secondary structure evident in the thermal denaturation profile (Fig. 7). Experiments with S1 nuclease digestion demonstrated that at least part of the serological reactivity of denatured DNA depended on relatively stable helical secondary structure. With this enzyme, an early rapid rate of digestion probably reflected the hydrolysis of easily accessible, unpaired single strands; more highly folded regions were digested more slowly. When the enzyme had eliminated virtually all regions recognized by antidenatured DNA antibodies, the products still competed for SLE antinative DNA antibodies. Boiling the products restored their reactivity with the antidenatured DNA and reduced further their reaction with antinative DNA. The latter was not eliminated completely, however: residual activity reflected either the single backbone contribution to binding energy or the presence of readily renaturable structures in the DNA. In further exploration of these possibilities, a larger sample of DNA was digested more extensively by S1 nuclease and resistant cores were isolated by gel filtration. This preparative scale digestion was done at relatively low ionic strength, so that only regions of relatively stable secondary structure should remain resistant to prolonged digestion. The resistant cores were heterogeneous in size, ranging from about 20 to several hundred base pairs in size. Thermal denaturation experiments indicated they had helical structure that melted cooperatively. Some of the denatured material renatured readily upon cooling, as reflected in the cooperative melting observed during a second heating cycle (Fig. 7); this was unlike the curve seen with total denatured DNA (Fig. 7) or of fragments that were not selected on the basis of resistance to S1 nuclease (20). The renaturable material corresponds to snap-back hairpin structures. The remainder of the S1 nuclease-resistant structure corresponds to base-paired segments that are separable at high temperature and do not renature rapidly because they do not have a connecting loop. Identical products of S1 nuclease digestion of mammalian DNA have been reported by Lin and Lee (34), who used aqueous dioxane to select the more stable helical regions. The isolated fold-back helical structures competed effectively for antinative DNA antibodies.

The experiments described in this article support the conclusion that optimal reactions of SLE serum antibodies that bind native DNA depend on helical structure, and that the antibodies detect secondary

structure in denatured DNA rather than denatured regions in native DNA. The conclusions are based on tests of competition by mono- and oligonucleotides, on measurements of quantitative changes in reactivity in relation to changes in secondary structure, and on the isolation of reactive helical structures from denatured DNA. These antibodies are clearly distinct from base-specific antidenatured DNA antibodies of SLE sera. Exploration of the question of whether antibodies of different specificities have different pathogenetic importance or diagnostic value does have a valid immunochemical basis.

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REFERENCES

1. Pincus, T., P. H. Schur, J. A. Rose, J. L. Decker, and N. Talal. 1969. Measurement of serum DNA binding activity in lupus erythematosus. *N. Engl. J. Med.* **281:** 701–705.
2. Hughes, G. R. V., S. A. Cohen, and C. L. Christian. 1971. Anti-DNA activity in systemic lupus erythematosus: a diagnostic and therapeutic guide. *Ann. Rheum. Dis.* **30:** 259–264.
3. Hasselbacher, P., and E. C. Leroy. 1974. Serum DNA binding activity in healthy subjects and in rheumatic disease. *Arthritis Rheum.* **17:** 63–71.
4. Bell, C., N. Talal, and P. Schur. 1975. Antibodies to DNA in patients with rheumatoid arthritis and juvenile rheumatoid arthritis. *Arthritis Rheum.* **18:** 535–540.
5. Adler, M. K., A. Baumgarten, and N. J. Siegel. 1975. Prognostic significance of DNA binding capacity in patients with lupus nephritis. *Ann. Rheum. Dis.* **34:** 444–450.
6. Locker, J. D., M. E. Medof, R. M. Bennett, and S. Sukhupunyaraska. 1977. Characterization of DNA used to assay sera for anti-DNA antibodies; determination of the specificities of anti-DNA antibodies in SLE and non-SLE rheumatic disease states. *J. Immunol.* **118:** 694–701.
7. Lightfoot, R. W., Jr., and G. R. V. Hughes. 1976. Significance of persisting serological abnormalities in SLE. *Arthritis Rheum.* **5:** 836–843.
8. Stollar, B. D. 1975. The detection of anti-DNA and DNA/anti-DNA complexes. *Scand. J. Rheumatol. Supp.* **11:** 7–11.
9. Plescia, O. J., W. Braun, and N. C. Palczuk. 1964. Production of antibodies to denatured deoxyribonucleic acid (DNA). *Proc. Natl. Acad. Sci. U. S. A.* **52:** 279–285.
10. Erlanger, B. F., and S. M. Beiser. 1964. Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. *Proc. Natl. Acad. Sci. U. S. A.* **52:** 68–74.
11. Aarden, L. A., F. Lakmaker, and E. R. deGroot. 1976. Immunology of DNA. IV. Quantitative aspects of the Farr assay. *J. Immunol. Methods.* **11:** 153–163.
12. Steinman, C. R., U. Deesomchok, and H. Spiera. 1976. Detection of anti-DNA antibody using synthetic antigens. *J. Clin. Invest.* **57:** 1330–1341.
13. Aarden, L. A., E. R. deGroot, and T. E. W. Feltkamp. 1975. Immunology of DNA. III. *Crithidia luciliae*, a simple substrate for the determination of anti-ds DNA with the immunofluorescent technique. *Ann. N. Y. Acad. Sci.* **254:** 505–515.
14. Izui, S., P. H. Lambert, and P. A. Miescher. 1976. Determination of anti-DNA antibodies by a modified ^{125}I-labelled DNA-binding test. *Clin. Exp. Immunol.* **26:** 425–430.
15. Picazo, J. J., and E. M. Tan. 1975. Specificities of antibodies to native DNA. *Scand. J. Rheumatol. Supp.* **11:** 35–41.
16. Arana, R., and M. Seligmann. 1967. Antibodies to native and denatured deoxyribonucleic acid in systemic lupus erythematosus. *J. Clin. Invest.* **46:** 1867–1882.
17. Koffler, D., R. Carr, V. Agnello, R. Thoburn, and H. G. Kunkel. 1971. Antibodies to polynucleotides in human sera: antigenic specificity and relation to disease. *J. Exp. Med.* **134:** 294–312.
18. Bagchi, B., D. N. Misra, S. Basu, and N. N. Das Gupta. 1969. Conformation of denatured and renatured DNA. *Biochem. Biophys. Acta.* **182:** 551–561.
19. Stollar, B. D. 1975. The specificity and application of antibodies to helical nucleic acids. *Crit. Rev. Biochem.* **3:** 45–69.
20. Papalian, M., E. Lafer, R. Wong, and B. D. Stollar. 1980. The reaction of SLE antinative DNA antibodies with native DNA fragments from 20 to 1,200 base pairs. *J. Clin. Invest.* **65:** 469–477.
21. Stollar, B. D. 1977. Quantitative microcomplement fixation and radioactive antigen binding assays for measuring anti-DNA antibodies. *Ann. Rheum. Dis.* **36**(Suppl.): 102–107.
22. Seaman, E. 1968. Chain length determination by end to total phosphorus. *Methods Enzymol.* **12**(Pt. B): 218–220.
23. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3:** 208–218.
24. Ando, T. 1966. A nuclease specific for heat-denatured DNA isolated from a product of *Aspergillus oryzae*. *Biochim. Biophys. Acta.* **114:** 158–168.
25. Seaman, E., L. Levine, and H. Van Vanukis. 1966. Antibodies to the methylene blue sensitized photooxidation product in deoxyribonucleic acid. *Biochemistry.* **5:** 1216–1223.
26. Johnston, M. I., and B. D. Stollar. 1978. Antigenic structure of double-stranded RNA analogues having varying activity in interferon induction. *Biochemistry.* **17:** 1959–1964.
27. Wakizaka, A., and E. Okuhara. 1975. Immunological studies on nucleic acids: an investigation of the antigenic determinants of denatured deoxyribonucleic acid (DNA) reactive with rabbit anti-DNA antisera by a radioimmunoassay technique. *Immunochemistry.* **12:** 843–845.
28. Teitelbaum, H., and S. W. Englander. 1975. Open states in native polynucleotides. II. Hydrogen exchange study of cytosine-containing double helices. *J. Mol. Biol.* **92:** 79–92.
29. Schaller, H., H. Voss, and S. Gucker. 1969. Structure of the DNA of bacteriophage fd. II. Isolation and characterization of a DNA fraction with double strand-like properties. *J. Mol. Biol.* **44:** 445–458.
30. Niyogi, S. K., and S. Mitra. 1977. Isolation and characterization of naturally occurring hairpin structures in single-stranded DNA of coliphage M13. *Biochem. Biophys. Res. Commun.* **79:** 1037–1044.
31. Fiddes, J. C., B. G. Barrell, and G. N. Godson. 1978.

Nucleotide sequences of the separate origins of synthesis of bacteriophage G4 viral and complementary DNA strands. *Proc. Natl. Acad. Sci. U. S. A.* **75:** 1081–1085.
32. Shishido, K., and Y. Ikeda. 1971. Isolation of double-helical regions rich in guanine-cytosine base pairing from bacteriophage f1 DNA. *Biochem. Biophys. Res. Commun.* **42:** 482–489.
33. Shishido, K., and T. Ando. 1972. Estimation of the double-helical content in various single-stranded nucleic acids by treatment with a single strand-specific nuclease. *Biochim. Biophys. Acta.* **287:** 477–484.
34. Lin, H. J., and C. L. H. Lee. 1979. Isolation of foldback DNA utilizing nuclease S1 digestion in aqueous dioxane. *Anal. Biochem.* **96:** 144–151.
35. Blakesley, R. W., J. B. Dodgson, I. F. Nes, and R. D. Wells. 1977. Duplex regions in "single-stranded" ϕx 174 DNA are cleaved by a restriction endonuclease from *Haemophilus aegyptius*. *J. Biol. Chem.* **252:** 7300–7306.
36. Furie, B., A. N. Schechter, D. H. Sachs, and C. B. Anfinsen. 1975. An immunological approach to the conformational equilibrium of staphylococcal nuclease. *J. Mol. Biol.* **92:** 497–506.