Reaction of Systemic Lupus Erythematosus Antinative DNA Antibodies with Native DNA Fragments from 20 to 1,200 Base Pairs

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ABSTRACT Double-stranded DNA fragments of varying sizes were isolated and tested for binding to systemic lupus erythematosus (SLE) antinative DNA antibodies. Fragments of 20–25, 40–50, 90–110, and 160–180 base pairs (bp), along with intermediate-size pieces were isolated by preparative gel electrophoresis of a limited micrococcal nuclease digest of calf thymus DNA. Larger helical polynucleotides of 160–200, 380, 600–1,000, and 1,200 bp were isolated by preparative gel electrophoresis of DNA from chicken erythrocyte nucleosomes and oligonucleosomes. The fragments behaved as base-paired structures as tested by thermal denaturation, resistance to S1 nuclease, and serological assays with antibodies to native or denatured DNA. At a concentration of 0.27 μM, fragments of 20–25 bp were able to react with two SLE sera in competition with native DNA. With these and two other sera, DNA of 40–50 bp was a much more effective competitor. One serum required DNA >180 bp for competition in the concentration range tested. Denatured fragments were much less effective than native fragments. The results emphasize the heterogeneity of SLE antinative DNA antibodies, confirm that secondary structure of the antigen is important for specific binding to these antibodies, and support the suggestion that bivalent binding to one molecule may be important for high functional affinity.

INTRODUCTION

Of the several kinds of antinucleic acid antibodies that occur in sera of patients with systemic lupus erythematosus (SLE),[1] those that react with native DNA have received most attention. Many studies have detected a close association between the presence of these antibodies and SLE and, in particular, with periods of disease activity (1–4). The use of assays that measure primary binding of antigen has revealed some anti-DNA activity in non-SLE sera (5, 6), but with careful characterization of the test antigen, the assay conditions, and quantitation, high DNA binding activity and SLE remain closely and nearly uniquely correlated (7). Most SLE patients are ill when their sera contain antinative DNA, but some produce DNA-binding antibodies at times of little disease activity (8, 9). Factors such as complement-fixing activity (9–12) or functional affinity (13, 14) may be important in determining the variable pathogenetic potential of the interaction. This may, in turn, depend on how the antibodies bind to DNA, either through intermolecular cross-linking or bivalent binding to repeating determinants on a single molecule as suggested by Aarden (7). It may also depend on the molecular weight of the DNA available for reaction in a given patient. A fuller understanding of the specificities and modes of binding of anti-DNA antibodies may be helpful in evaluating their importance for the diagnosis and management of the disease.

In the case of antibodies that react with single-stranded (denatured) DNA only, specificity has been studied with the use of oligonucleotides as inhibitors of serological reactions of denatured DNA. With rabbit antibodies induced by denatured DNA of high molecular weight and with SLE antidenatured DNA antibodies, maximal inhibition occurred with fragments up to the size of pentanucleotides or hexanucleotides (15, 16), which probably correspond to the largest size of a single antigenic determinant. Similar analyses of polysaccharide and polypeptide antigens provided models for such studies, and indicated that these polymers had maximal antigenic determinants of similar size (17).

Most information on antigenic determinants of helical nucleic acids, on the other hand, has come from

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[1] *Abbreviations used in this paper*: bp, base pairs; SLE, systemic lupus erythematosus.

studies of cross-reactions (18–20), analyses of antigen-antibody precipitates (19, 20), and physical measurements of antigen-antibody interactions (19). From these studies, done mainly with experimentally induced antibodies, it has been learned that a single antigenic determinant of double-helical RNA could encompass the backbones of both strands over a span of 3–4 base pairs (bp) (19, 20). A native DNA determinant for SLE serum antibodies should be of similar size to fit within the dimensions of antibody binding regions.

The use of small fragments has not been tested with helical nucleic acid immune systems because a piece corresponding to one determinant of a few bp would not form a stable helix. Even with somewhat larger fragments, however, this approach can be useful in defining some aspects of the antigenic structure of native DNA, as well as in testing minimal requirements for binding. DNA binding assays such as the Farr procedure, in which antigen-antibody complexes are precipitated with ammonium sulfate, require DNA of very high molecular weight for full efficiency (21, 22). To determine the extent to which the molecular weight dependence of binding assays reflects minimal requirements for binding, we prepared fragments of varying size, from 20 to 1,200 bp, and tested them in a competitive radioimmunoassay in which SLE sera bind labeled native DNA of high molecular weight. Fragments of 20–25 bp or more retained helical structure and did bind to some antinative DNA antibodies, but pieces of 40–50 bp were much more effective competitors. For one serum, a helix of several hundred bp was required for competitive binding at the concentrations tested. The results of these studies emphasize the heterogeneity of SLE anti-DNA antibodies and support the suggestion that bivalent binding to one antigen molecule may be important for high functional affinity.

METHODS

Reagents. Calf thymus DNA and micrococcal nuclease were purchased from Worthington Biochemical Corp. (Freehold, N. J.). Pancreatic DNAse and mononucleotides were from Sigma Chemical Co. (St. Louis, Mo.), and S1 nuclease of *Aspergillus oryzae*, from Miles Laboratories Inc., Ames Div. (Elkhart, Ind.). Polyacrylamide gel electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, Calif.). The HINC II restriction nuclease digest of ØX174 RF DNA was purchased from New England Biolabs (Beverly, Mass.). [^{3}H]Thymidine-labeled DNA was prepared from a mutant *Escherichia coli*, B3, as described previously (23) but with a pulse of 500 μCi of [^{3}H]thymidine rather than 60 μCi of [^{14}C]thymidine during log-phase growth. The DNA was treated with S1 nuclease before being used in radioimmunoassays. SLE sera were obtained through the courtesy of Doctors V. Agnello (Tufts University School of Medicine), M. Benson (Indiana University Medical Center), P. Schur (Harvard Medical School), and, N. Rothfield (University of Connecticut School of Medicine).

Preparation of nucleosomal particles. Nuclei were prepared from washed chicken erythrocytes and digested with micrococcal nuclease, as described previously (24), until 10% of the material (by A_{260}) was acid soluble (in 0.8 M perchloric acid). EDTA was added to a concentration of 0.01 M to stop the reaction, and the mixture was centrifuged at 17,000 *g* for 20 min. The supernate was brought to 0.15 M NaCl, kept at 0°C for 30 min, and centrifuged at 17,000 *g* for 20 min. This supernate contained mainly single nucleosomes. The pellet was redissolved in 10 ml of 10 mM Tris, 0.2 mM EDTA pH 7.2; this fraction, soluble at low ionic strength but insoluble in 0.15 M NaCl, contained mainly oligonucleosomes. The redissolved oligonucleosome-containing fraction was layered on to 30-ml linear 5–20% (wt/vol) sucrose gradients in 10 mM Tris, 0.2 mM EDTA pH 7.2. Gradients, each with 3.5 ml of sample, were centrifuged at 35,000 *g* for 17 h in a SW.27 swinging bucket rotor. Fractions were collected with a peristaltic pump through an ISCO model UA5 monitor (Instrument Specialties Co., Lincoln, Nebr.) and a Gilson microfractionator (Gilson Medical Electronics, Inc., Middleton, Wis.).

Extraction of nucleosomal DNA. DNA was extracted from nucleosomes in 1.2 M NaCl and 0.2% sodium dodecyl sulfate with an equal volume of a 50/50 mixture of chloroform-isoamyl alcohol (24:1) and 80% phenol. The mixture was shaken for 1.5 h at 4°C. The DNA-containing aqueous phase was recovered and dialyzed against 50 mM Tris, 5 mM sodium acetate, 1 mM EDTA pH 7.9.

Preparative digestion of DNA. Commercially obtained calf thymus DNA was purified by chloroform-isoamyl alcohol extraction, ethanol precipitation, and isopropanol precipitation (25). A solution of DNA (2 mg/ml) in 0.1 M NaCl, 6 mM Tris pH 8, 2 mM $CaCl_2$, was digested with micrococcal nuclease (40 U/mg DNA) at 37°C for 4 min; the reaction was stopped by the addition of 1/10 vol of 0.2 M EDTA pH 8. A sample of the digested DNA was 20% acid soluble. The mixture was extracted twice with 10 ml of chloroform-isoamyl alcohol (24:1), and nucleic acid was precipitated with 40 ml of ethanol overnight at −20°C. The precipitate was centrifuged, dried with a nitrogen stream, and redissolved in phosphate-buffered saline (0.14 M NaCl, 0.01 M phosphate pH 7) at a concentration of 4 mg/ml. 5 ml of this material was passed through a 3 × 50-cm column of Bio-Gel A1.5M (Bio-Rad Laboratories, Richmond, Calif.), washed with phosphate-buffered saline; fractions of 3 ml were collected.

Polyacrylamide gel electrophoresis. Gels were prepared with an acrylamide:methylenebisacrylamide ratio of 19:1. The buffer system was 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA pH 7.9. Preparative 10% polyacrylamide gels (11 × 1.4 cm) were prepared with a 1-cm 4% stacking gel. 5 mg (1.25 ml) of DNA digest, mixed with 0.25 ml of 0.025% bromphenol blue in 50% glycerol, was applied to each gel and run at 20 mA/gel for 4.5 h; this amperage avoided heating and denaturation of the DNA fragments. Horizontal sections 4-mm thick were cut from each gel. For elution, the DNA fragments were electrophoresed from the sections through a 1.5-ml plug of 1% agarose in a preparative gel tube into dialysis tubing secured to the bottom of the tube. These eluting gels were run at 10 mA/gel for 6 h.

For analytical electrophoresis, samples of 0.4 μg of DNA fragments in 50 μl of buffer, with 15 μl of tracking dye in glycerol, were run on either 8% polyacrylamide slabs with a 1-cm 4% stacking gel or 5.5% slabs with a 3.5% stacking gel. Electrophoresis was at 50 mA for 2.5 h. Gels were stained with ethidium bromide (1 μg/ml) for 30 min, rinsed with water, and photographed under short ultraviolet light.

Thermal denaturation. DNA fractions (10–20 μg/ml in 0.1 M sodium phosphate pH 7) were heated, with temperature increasing at a rate of 1°C/min from 20°–95°C in the carrier of a Zeiss PM6 spectrophotometer with a Zeiss thermoelectric temperature control unit (Carl Zeiss Inc., New York). Ab-

sorbance was monitored at 5°C intervals, and readings were corrected for the expansion of water.

Digestion with S1 nuclease. The whole micrococcal nuclease digest and the isolated 90–110 bp fragments (native and denatured) were treated with S1 nuclease, which specifically cleaves single-stranded nucleic acids (26). The unfractionated mixture was digested in 10 ml of 0.1 M sodium acetate pH 5.5, 1 mM $ZnCl_2$, with 12,500 U of S1 nuclease (Miles Laboratories Inc.) for 30 min at room temperature. The 90–100 bp fragments (6 μg in 0.5 ml) were digested in 20 mM sodium acetate pH 5, 1.5 mM $ZnCl_2$ with 2,500 U of enzyme for 20 min at room temperature. The reactions were stopped by EDTA (final concentration 5 mM).

Pancreatic DNAse digestion of nucleosomes. Mononucleosomes (100 μg DNA/ml in 10 mM Tris pH 8, 1 mM $MgCl_2$) were digested with 1 U/ml of pancreatic DNAse for 20 min at 37°C. The reaction was stopped with 1/10 vol of 0.1 M EDTA pH 8. The DNA was 82% acid soluble. It was extracted with chloroform-isoamyl alcohol-phenol as described above.

Radioimmunoassay. 100-μl samples of serum diluted in 0.06 M phosphate, 0.03 M EDTA pH 8, were heated at 56°C in 1.5-ml microfuge tubes. Varying amounts of unlabeled polynucleotides in 100 μl of the same buffer (or buffer alone for control tubes) were added, and the mixtures kept for 30 min at room temperature. 50 ng [^{3}H]thymidine-labeled native DNA in 50 μl of the phosphate-EDTA buffer was added, and the incubation continued for another hour. Then 50 μl of the gamma globulin fraction of rabbit antihuman immunoglobulin (Ig)G serum was added, and the mixture was kept at room temperature for an additional hour. The resulting precipitate was centrifuged, washed with phosphate-EDTA, dissolved in 0.2 ml of 0.1 N NaOH, and counted in 3 ml of Beckman Ready-Solv in Beckman microvials (Beckman Instruments). For assays with rabbit antiadenosine and anticytidine antibodies, the labeled DNA was thermally denatured and the second antibody was goat antirabbit IgG.

RESULTS

Preparation of helical DNA fragments. A mixture of oligonucleosomes was separated into fractions of varying sizes by sucrose density gradient centrifugation. DNA extracted from these fractions corresponded to discrete pieces expected for mono- to hexanucleosome particles. The DNA fragments of varying sizes were isolated by preparative polyacrylamide gel electrophoresis, performed with a low enough current to prevent gel heating that could denature the DNA. The purified fractions were then compared with markers of known size in a HINC II restriction nuclease digest of φX174 replicative form DNA. The nucleosome DNA fractions corresponded to 160–200, 380, a mixture of 600, 800–1,000, and 1,200 bp (Fig. 1).

Smaller helical DNA fragments were obtained from a limited micrococcal nuclease digest of calf thymus DNA. Such a digest, when applied to a Bio-Gel A1.5M column, yielded a broad distribution of oligonucleotides (Fig. 2). Analytical polyacrylamide gel electrophoresis indicated that fragments of progressively decreasing size were distributed across the elution profile (Fig. 2 inset). When the limited micrococcal nuclease digest was applied to a preparative 1.4 × 11-cm 10%

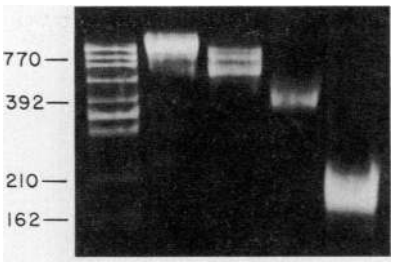

FIGURE 1 Polyacrylamide slab gel electrophoresis of nucleosomal and oligonucleosome DNA fragments. DNA was extracted from the oligonucleosome fraction and separated into fractions by preparative gel electrophoresis. The fractions were analyzed on 5.5% polyacrylamide slab gels run at 50 mA constant for 2.5 h and stained with ethidium bromide (1 μg/ml) for 30 min. A HINC II nuclease digest of φX174 RF DNA was used as a standard size marker.

polyacrylamide gel, a similar broad spreading of polynucleotides, from about 20 to several hundred bp in size, was observed. Sections of this gel were cut, and the DNA fragments were eluted electrophoretically. When rerun on analytical gels, the fractions ran with narrow size distributions. Selected fractions from the preparative gel slices corresponding to 20–25, 40–50, 90–110, and 160–200 bp, were chosen for immunochemical experiments. Because the smallest double-stranded marker in the φX174 DNA digest standard was 79 bp, the first identification of fragments of smaller size depended on a linear extrapolation. Their sizes were confirmed by comparison with oligonucleotides resulting from digestion of nucleosomes by pancreatic DNAse. This digestion yields a series of fragments increasing in size by 10-nucleotide increments (27). The selected oligonucleotides and the standard markers

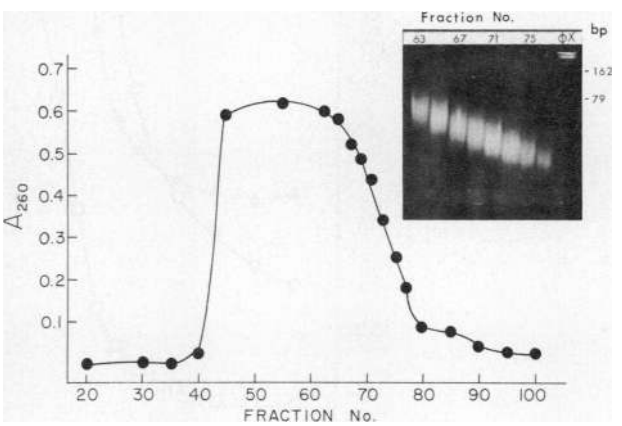

FIGURE 2 Bio-Gel A 1.5 M gel filtration elution profile of calf thymus DNA digested briefly with micrococcal nuclease. 3-ml fractions were collected at a flow rate of 1.5 ml/min. Alternate fractions from 63 to 77 were analyzed on 10% polyacrylamide slab gels along with HINC II digested φX174 RF DNA.

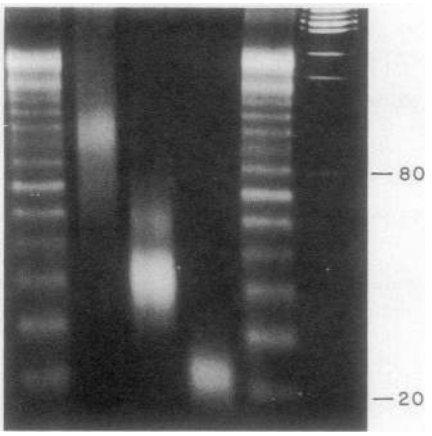

FIGURE 3 10% polyacrylamide slab gel electrophoresis, in the Peacock and Dingman gel systems (28), of 20–25-, 40–50-, and 90–110-bp fractions of the limited micrococcal nuclease digest of calf thymus DNA, and the pancreatic DNase digest of mononucleosomes. Samples were denatured with 0.1 N NaOH and reneutralized, and electrophoresis was run for 2.5 h at 75 mA. The ϕX174 RF HINC II digest was also denatured.

were denatured by the addition of NaOH to a final concentration of 0.1 N, reneutralized, and examined on analytical polyacrylamide gels. The 79-nucleotide marker of ϕX174 DNA identified the 80-nucleotide fragment in the nucleosome digest and the test fragments corresponded, as expected, to the lengths of 20–25, 40–50, and 90–110 nucleotides (Fig. 3). The 40–50 residue fraction showed some trailing material. As this was not present in the gels with native fragments, it may represent some material that was incompletely or only reversibly denatured. The combination of DNA extraction from nucleosomes and oligonucleosomes, along with preparative gel electrophoresis of a limited micrococcal nuclease digest of DNA, thus provided a series of polynucleotide fractions of increasing size, over a range of 20–1,200 bp.

Characterization of helical polynucleotide fractions. The thermal melting behavior of the polynucleotide fractions was examined as a test for their double-helical structure. The nucleosomal fragments and DNA digestion fractions showed a sharp melting transition characteristic of double-helical polynucleotides. Even the 20–25-bp piece showed only a small amount of hyperchromicity before the sharp transition (Fig. 4), and underwent a 40% total hyperchromicity. This behavior contrasted with the gradual increase in absorbance and the lesser hyperchromicity seen on reheating denatured fragments (Fig. 4).

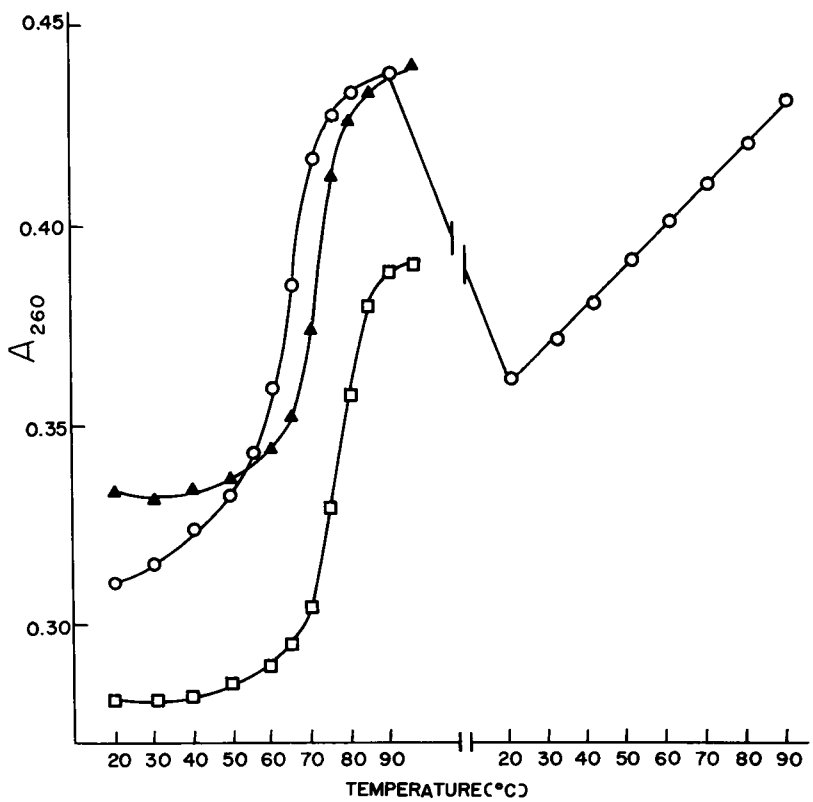

FIGURE 4 Thermal denaturation curves of calf thymus DNA digest fractions: 20–25 bp (○); 40–50 bp (▲); 90–110 bp (□) in 0.1 M phosphate. The temperature was raised at a rate of 1°C/min from 20° to 95°C. The 20–25 bp fragment was cooled slowly back to 20°C and then reheated.

In another test for the presence of single-stranded regions, the polynucleotides were assayed in a competitive radioimmunoassay with antiadenosine and anticytidine antibodies. The isolated fragments from the nucleosomes and the micrococcal nuclease digest of DNA did not compete for these antibodies until they were thermally denatured (Fig. 5). This result, characteristic for helical DNA, indicated that there were few unpaired bases at the ends of the DNA fragments. These experiments also indicated that the helical fragments did not interfere with antigen-antibody reactions in a nonspecific manner.

As a further test of the base-paired structure of the products of this limited digestion, the micrococcal nuclease digest was treated with S1 nuclease to remove single-stranded regions (26) before fractionation on a gel filtration column. The distribution of fragment size was unaltered, and pieces in the range of 20–25 bp had the same serological properties as those prepared without S1 treatment. In addition, exposure of the isolated 90–110-bp fragments to S1 nuclease did not reduce their size, as measured by polyacrylamide gel electrophoresis, or alter their serological activity. When they were heat denatured and then exposed to the same amount of S1 nuclease for the same time, they were completely digested.

Reactions with SLE antinative DNA antibodies. Five SLE sera that reacted with native DNA were selected for study in a double antibody radioimmunoassay. The test antigen was [^{3}H]thymidine-labeled *E. coli* DNA that was treated with S1 nuclease to remove single-stranded regions (26). Binding of the DNA by normal serum or base-specific rabbit antibodies (induced by nucleoside-protein conjugates) was <5%. The binding of native labeled DNA by the five test SLE sera was not inhibited by a 1,000-fold excess of mononucleotides.

In competitive radioimmunoassays with 50 ng of labeled native DNA, unlabeled calf thymus DNA competed in amounts ranging from 10 to 1,000 ng. In tests with the helical fragments, there was heterogeneity among the sera. In two cases all fragments, including the 20–25-bp helix, showed competition, but the 40–50 bp fraction was much more effective (Fig. 6A, Table I). With two other sera, there was no competition by the 20–25-bp fragments at the concentrations tested, whereas the 40–50-bp material did compete (Fig. 6B,

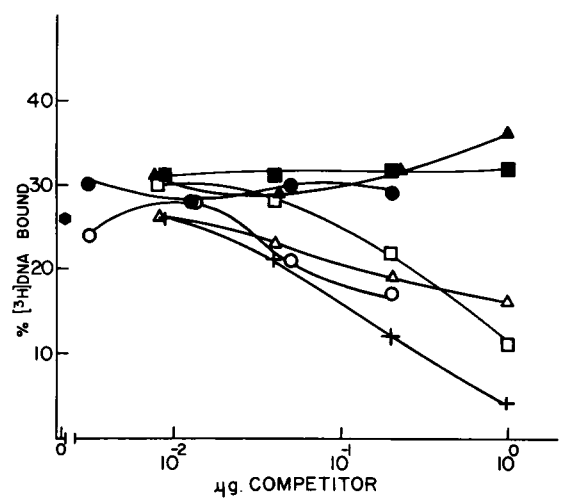

FIGURE 5 Competitive radioimmunoassay of rabbit antiadenosine sera with calf thymus DNA digest fractions: 1,200 bp (●, ○); 160–200 bp (■, □); and 20–25 bp (▲, △). Closed symbols are for native fragments and open symbols for heat-denatured fragments. Denatured calf DNA (+) was used as a positive control.

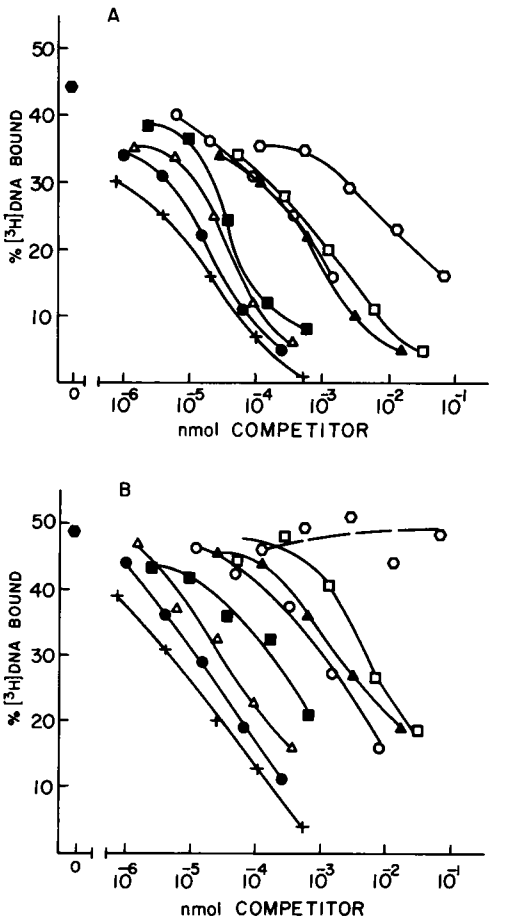

FIGURE 6 Competitive radioimmunoassay with SLE antinative DNA antibodies in sera M1 (A) and M3 (B) with helical DNA fragments: 20–25 bp (○); 40–50 bp (□); 90–110 bp (▲); 160–200 bp (○); 380 bp (■); 600–1,000 bp (△); and 1,200 bp (●). Native calf thymus DNA (+) was used as a positive control. Fractions were reacting in competition with 50 ng of native [^{3}H]thymidine-labeled *E. coli*. DNA treated with S1 nuclease. ●, control binding in the absence of competing polynucleotide.

TABLE I

Competitive Radioimmunoassay of DNA and DNA Fragments Binding to SLE Antinative DNA Antibodies Nanomolar Required for 50% Inhibition

DNA fragment size	Serum				
	M1	M2	M3	M4	M5
bp					
Native DNA 2×10^6 mol wt (3,000 bp)	0.032	0.17	0.048	0.039	0.036
1,200	0.06	0.21	0.12	0.10	0.48
600–1,000	0.12	0.38	0.34	0.31	1.36
380	0.18	0.52	1.88	1.08	3.4
160–200	2.48	6.4	9.6	3.4	600
90–110	2.48	5.6	23.2	6.0	—
40–50	3.68	8.4	40.000	10.8	—
20–25	56	40% at 270	*	*	—

* No inhibition at 270 nM.

Table I). One such serum was tested with fragments of intermediate size; the transition to detectable reactivity occurred at 35 bp (Fig. 7). The fifth serum required chains >180 bp for detectable competition in the concentration range tested (Table I).

The data in Figs. 6 and 7 and Table I are presented in terms of moles of polynucleotide required for competition. There was a progressive increase in the molar concentration required as the molecular weight fell. With a multivalent antigen, this could represent simply the requirement for more moles of smaller pieces to present the same total number of nucleotides or short oligonucleotide regions. Such a linear relationship is shown by the straight line in Fig. 8. The actual increase in the moles of polynucleotide required for competition in the radioimmunoassay was then plotted alongside that line. For sera M1 and M2, the slopes of the two lines were parallel between 400 and 1,200 bp, but there was a sharp deviation between 180 and 400 bp, and another between 25 and 45 bp. Sera M3, M4, and M5 deviated from the theoretical line even at higher molecular weights (Fig. 8).

DISCUSSION

The experiments described in this article were undertaken to increase the precision of our definition of anti-

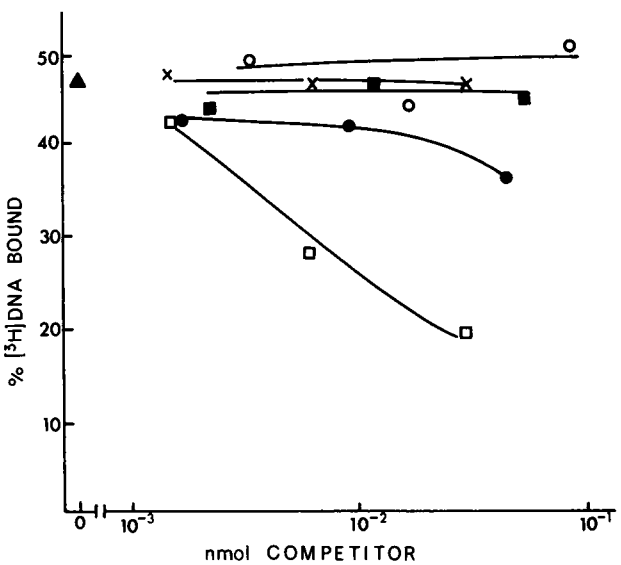

FIGURE 7 Competitive radioimmunoassay of SLE antinative DNA antibodies in serum M3 with native DNA fragments: 20–25 bp (○); 28–31 bp (■); 35–37 bp (●); 40–50 bp (□); and denatured 40–50 bp fragments (×). Fragments were competing with 50 ng of native [^{3}H]thymidine-labeled *E. coli* DNA treated with S1 nuclease. ▲, control binding in the absence of competing polynucleotide.

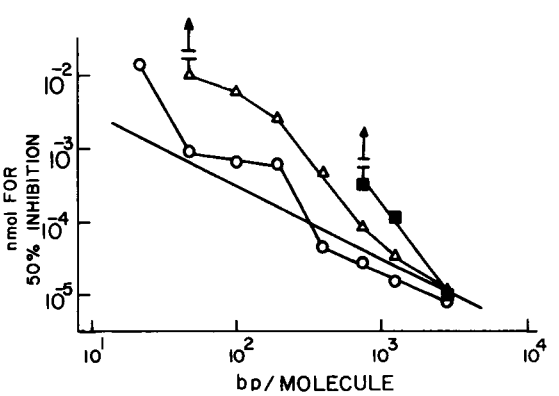

FIGURE 8 Relationship between DNA size and amount required for 50% competition in radioimmunoassay. The straight line describes the linear relationship between the molecular size (base pair/molecule) and the nanomoles of polynucleotide required to provide a constant total number of nucleotide residues. The experimental values are those for native DNA fragments tested with sera: M1 (○), M3 (△), and M5 (■).

genic structure in native DNA for SLE serum antibodies, and to explore the question of how serological reactivity is related to molecular weight of the DNA.

In several assays, the efficiency of detection of anti-DNA antibodies depended on the molecular weight of the test antigen. Complement fixation reactions of denatured DNA were markedly diminished with DNA of $<1 \times 10^6$ mol wt (29), as were native DNA binding assays that used millipore filters (Millipore Corp., Bedford, Mass.) or ammonium sulfate to separate free from bound antigen. Aarden et al (21) found a linear relationship between molecular weight of DNA and binding efficiency; with another binding assay, Geisert et al. (22) also noted a marked dependence on molecular weight, though not a linear relationship. In both cases, little binding of labeled antigen occurred with DNA of 500,000 mol wt (750 bp) or less.

In the complement fixation assays mentioned above, it was possible to measure binding of small oligonucleotides by using inhibition assays; even short segments of one to six nucleotides could inhibit the complement fixation reaction of high molecular weight denatured DNA. We have used a similar approach in these studies and have found that SLE antinative DNA antibodies can indeed bind relatively small helical fragments. Testing fractions of narrow-size distribution, we have found that the smallest reactive fragments were between 35–45 bp in length (30,000 mol wt) for two sera, and that a fragment of 20–25 bp (15,000 mol/wt) reacted with two others. In only one case was a larger segment (400 bp, 2.5×10^5 mol wt) required for competitive binding in these assays. It should be noted that, because of the limited supplies of the fragments of defined size, low concentrations (0.27 μM or less) of each polynucleotide were used in these experiments, so that binding of relatively high affinity was being measured. For example, the 40–50 bp fragments caused 50% competition with four sera when present at a concentration of 1.3 nM.

With two sera that did not react with the 20–25-bp fraction, and with the two sera that did, there was a transition to markedly increased reactivity with fractions between 25 and 45 bp in size. In one case this was studied in detail with fragments of intermediate size, and the transition occurred at about 35 bp. These results do not necessarily establish the size of a single determinant on DNA, which is probably much smaller than 20 or 40 bp. The binding sites of rabbit antibodies to helical RNA, for example, appear to encompass both backbones of a helix over a span of 3–4 bp (19, 20). A determinant of that size would fit within dimensions that are reasonable for a groove crossing the binding surface of the Fab tip, as judged from dimensions of known immunoglobulin structures (30). The requirement for 20–25 or 40–50 bp for detectable binding may mean that a minimal size, larger than the actual determinant, is required to stabilize the base-paired structure that is recognized by the antibody. A second possibility is that, in the range of concentrations tested, the enhancement may depend on a transition from monovalent to bivalent binding, a mode of binding suggested by studies of Aarden (7), and that only two sera (M1 and M2) had a sufficiently high intrinsic affinity to allow detection of monovalent binding with one determinant on the 20–25 bp helix. Because the minimal distance between Fab binding sites on one IgG molecule is >100 Å (30), and the length of a 25-bp fragment of DNA is 85 Å, only monovalent binding could occur with such a fragment. As noted, the transition to detectable binding with one serum occurred at 35 bp, which would provide a rod of 122 Å in length and would be more likely to allow bivalent binding of some IgGs (Fig. 9); such binding would be accommodated even more readily on a 45–50-bp helix (160 Å in length), which was able to compete at low concentrations for antibodies in four sera.

A second transition to increased reactivity occurred between 200 and 400 bp. For one serum, this was a change from no measurable reactivity to detectable reaction at the concentrations tested, and for three other sera it was seen as a deviation from a linear increase of reactivity with increasing molecular weight. This is beyond the size range likely to be involved in determining two-site binding along a simple rod, and it may reflect an additional order of structure, such as the chain folding back on itself to form new determinants or to increase the likelihood of bivalent binding in three dimensions.

The results of these studies also bear on the question of whether reactivity depends on double-stranded structure. In digesting DNA, micrococcal nuclease acts first as an endonuclease, cleaving most rapidly at XpA

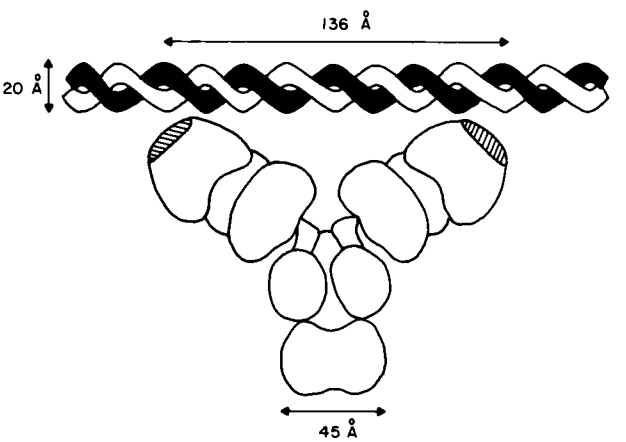

FIGURE 9 Model describing requirements for bivalent binding of one IgG molecule to a DNA fragment. The IgG drawing was based on that of Cathou (31). The angle at the hinge was taken at 115°; it is flexible, but to a minimal angle >80° (31).

and XpT sites (32) where X is any base; it then acts as an exonuclease as well and can finally digest DNA to mono- and dinucleotides (32). With limited digestion, it is possible to obtain mainly helical fragments. Thermal denaturation measurements indicated that the products were mainly base-paired and melted cooperatively, in contrast to their behavior during reheating after denaturation. In tests of whether there were short unpaired regions at the ends of the fragments, treatment with S1 nuclease did not reduce their size or serological activity, and the fragments did not react with base-specific antibodies until they were denatured. Furthermore, whereas denaturation increased their reactivity with base-specific antibodies, it markedly reduced their activity in competition for antinative DNA; backbone secondary structure, rather than the bases, played an important role in determining antigenic specificity in this system.

The five sera described in this study contained complement-fixing antinative DNA antibodies, and the patients had active clinical disease at the time of serum sampling, but sera were not otherwise preselected or screened for inclusion in these experiments. A much broader survey will be required to determine whether there is a relationship between the mode of binding and complement-fixing activity, and whether fine specificity and mode of binding are related to pathogenetic potential. Information from these studies will also help to define minimal requirements for the preparation of hapten-protein conjugates that might be useful in inducing hapten-specific tolerance to nucleic acid antigens important in SLE (33).

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