

DNA-anti-DNA Immune Complexes Antibody Protection of a Discrete DNA Fragment from DNase Digestion In Vitro

W. Emlen, R. Ansari, and G. Burdick

Department of Medicine, Division of Rheumatology,
Pacific Medical Center, University of Washington,
Seattle, Washington 98144

Abstract. We examined the ability of DNase I to digest DNA that was contained with DNA-anti-DNA immune complexes. IgG isolated from the sera of 20 patients with systemic lupus erythematosus (SLE) and containing antibodies to DNA was incubated with double-stranded DNA to form immune complexes. Excess DNase was added, and digestion of DNA was monitored by the conversion of DNA to TCA soluble products. IgG from 8 of the 20 SLE patients protected DNA from degradation by DNase in direct proportion to the amount of DNA bound to IgG as measured in the Farr binding assay. Using IgG from these sera, we showed that the DNA protected from degradation remained bound to IgG during digestion and was 35-45 base pairs in size. The size of this fragment is the same as that which has been proposed to be the minimal size necessary for monogamous bivalent binding of IgG to DNA. We therefore compared the ability of F(ab')₂ and Fab' to protect DNA from DNase digestion and demonstrated that the bivalent F(ab')₂ fragments were protective, but that the univalent Fab' fragments were not.

These results suggest that some antibodies to DNA that bind to DNA via monogamous bivalent binding can protect a 35-45-base pair DNA fragment from DNase digestion. The implications of this finding are discussed with regard to the in vivo behavior and potential pathogenicity of small DNA-anti-DNA immune complexes.

Introduction

DNA-anti-DNA immune complexes are thought to play an important role in the pathogenesis of systemic lupus erythematosus (SLE)¹ (1, 2). While numerous studies have demonstrated DNA-anti-DNA complexes in the circulation of SLE patients (3, 4), the mechanism of formation, clearance, and tissue deposition of these complexes remains unknown. DNA itself was cleared rapidly from the circulation by digestion with circulating nucleases and by hepatic binding (5, 6, 7). Immune complexes containing large DNA were cleared from circulation at the same rate and with the same distribution as DNA alone (8). Studies by Harbeck et al. (9) suggested that DNase is able to digest DNA away from the DNA-anti-DNA complex, and can thereby release free antibody. With such efficient degradation and clearance of DNA, survival of large DNA-anti-DNA complexes in the circulation would be extremely short, and their deposition in tissues unlikely.

Liebling and Barnett (10) have shown that antibodies to DNA can compete with DNase for binding to single-stranded DNA (ss DNA), and that DNA antibody can therefore retard DNase digestion of ss DNA. Furthermore, studies in microbial systems have shown that certain DNA binding proteins are able to restrict the accessibility of DNA polymerases and nucleases to DNA, and thus can "protect" DNA from digestion (11, 12). In this study, we examined the ability of DNase I to digest double-stranded DNA (ds DNA) bound to DNA antibody. We have demonstrated that a 35-45-base pair (bp) DNA fragment is protected from DNase digestion and remains bound to antibody, thereby forming a small, DNase resistant DNA-anti-DNA immune complex.

Methods

Preparation of DNA. Calf thymus DNA (Worthington Biochemical Corp., Freehold, NJ) was dissolved in phosphate-buffered saline (PBS) (0.01 M phosphate, 0.15 M NaCl, pH 7.4) at 0.5 mg/ml, sonicated for 5 min, and passed over a Sepharose C14B column (Pharmacia Fine Chemicals, Piscataway, NJ). Fractions from the included peak were pooled, rechromatographed, and passed over a benzoylated naphthoylated DEAE cellulose column (13). The 1.0 M NaCl eluate ds DNA was radiolabeled using T₄ polymerase (Bethesda Research Laboratories, Bethesda, MD) to incorporate ¹²⁵I-iododeoxycytidine triphosphate (Amersham Corp.,

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1. *Abbreviations used in this paper:* bp, base pairs; CF II, Cohn Fraction II; ds DNA, double-stranded DNA; PAGE, polyacrylamide gel electrophoresis; SAS, saturated ammonium sulfate; ss DNA, single-stranded DNA; SPA, staphylococcal protein A; SLE, systemic lupus erythematosus.

Arlington Heights, IL) (14). DNA was dialyzed and ethanol was precipitated to remove triphosphates, yielding a final specific activity of $5\text{--}10 \times 10^6$ cpm/ μg . Digestion of this DNA with S1 nuclease showed $<10\%$ TCA solubility at 2 h, which confirmed that it was $>90\%$ double stranded. Analysis of the DNA on polyacrylamide gel electrophoresis (PAGE, see below) showed a mean size of 350 bp with a range of 250–400 bp. DNA molecular weight standards were obtained by end labeling a Hpa II digest of pBR322 DNA (Bethesda Research Laboratories) with ^{125}I -iododeoxyctidine triphosphates using T_4 polymerase.

Preparation of IgG. Sera from 20 patients with classical SLE and with high DNA binding were precipitated in 50% saturated ammonium sulphate (SAS). The pellets were resuspended and dialyzed into borate buffer (0.1 M borate, 0.15 M NaCl, pH 8.0), applied to a staphylococcal protein A (SPA) column (Pharmacia Fine Chemicals), and, after thorough washing, IgG was eluted with 1.0 M acetic acid. After dialysis into PBS, this IgG migrated as a single band on PAGE and maintained its high DNA binding.

F(ab)₂ fragments were obtained by pepsin digestion of IgG at pH 4.2 for 16 h at 23°C (15). Intact IgG was removed by passage over a SPA column and amino acids and pFc' were removed by gel filtration over Sephadex G50 (Pharmacia Fine Chemicals). Fab' fragments were obtained by reduction and alkylation of F(ab)₂ using 4.5 mM dithiothreitol for 20 min followed by 9 mM iodoacetamide for 1 h (15). Analysis of the F(ab)₂ and Fab' fragments in a neutral PAGE system showed no intact IgG in either preparation, and it confirmed the reduction and alkylation of Fab'.

Measurement of DNA binding. DNA binding was measured using a modified Farr assay as previously described (16). Varying amounts of IgG that was isolated from sera of patients with SLE was brought to a volume of 150 μl with PBS, added to 50 μl of a standard DNA preparation (350 bp), and were incubated at 37°C for 30 min. 200 μl cold SAS was added, and after 30 min at 4°C, samples were spun in a microfuge and the percentage of binding was calculated as the cpm in pellet per cpm total. Controls using Cohn Fraction II (CF II) as a protein source were subtracted as background ($<5\%$).

Since ammonium sulfate did not effectively precipitate IgG fragments, binding of DNA by Fab' and F(ab)₂ was measured by precipitation of complexes with goat anti-human IgG (Cappel Laboratories, Cochranville, PA). Initially, the amount of anti-IgG necessary for optimum precipitation of each IgG fragment was determined using radiolabeled Fab' and F(ab)₂. DNA binding was measured by incubating ^{125}I -DNA with Fab' or F(ab)₂ at 37°C for 30 min to form complexes, followed by the addition of the appropriate amount of anti-IgG. Incubation was continued at 4°C for 16 h to allow a precipitate to form, samples were spun in a microfuge, and binding was calculated as described above.

Production and digestion of immune complexes. Varying amounts of IgG or IgG fragments (10–300 μg) were incubated with DNA (10–100 μg) at 37°C for 30 min to form immune complexes, and an aliquot was removed to measure DNA binding. Pancreatic DNase I (Miles Laboratories Inc., Elkhart, IN) was added to the remaining complexes to a final concentration of 0.5 mg/ml, MgSO_4 was added (final concentration 10 mM), and incubation was continued at 37°C for an additional 30 min. 50% TCA was added to give a final concentration of 10% and samples were incubated at 4°C for 30 min, spun in a microfuge, and TCA precipitability was calculated as the cpm in pellet per cpm total. Controls using DNA alone or DNA incubated with CF II before the addition of DNase were run in parallel to obtain background values and to assure the complete digestion of DNA ($<5\%$ remaining TCA precipitable). The percentage of DNA "protected" from digestion by a given IgG preparation was calculated as the percentage DNA remaining

precipitable when incubated with IgG minus the percentage DNA remaining precipitable when incubated with an equivalent amount of CF II.

Characterization of digested DNA. For electrophoretic analysis, DNase digestion of immune complexes was terminated with $10 \times$ Tris-borate EDTA buffer to yield a final concentration of 0.1 M Tris, 0.1 M borate, and 10 mM EDTA, pH 8.0. The digested immune complexes were immediately applied to an SPA column; DNA not bound to IgG emerged in the fall through peak, while DNA remaining bound to IgG adsorbed to the SPA column and was eluted with 1.0 M acetic acid. The fall through (free DNA) and acetic acid eluate (IgG-bound DNA) were extensively phenol extracted to remove protein, and phenol was removed by ether extraction. Phenol-extracted DNA samples were analyzed on 8% polyacrylamide disc or slab gels using a radiolabeled Hpa II digest of pBR322 DNA (Bethesda Research Laboratories) as molecular weight standards. Disc gels were cut into 1-mm slices, counted in a gamma counter, and plotted as cpm in each slice vs. distance migrated. Slab gels were dried onto filter paper and autoradiographed using Cronex intensifying screens (DuPont Instruments, Wilmington, DE) and Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY). Curves relating the logarithm of DNA size vs. distance migrated were linear from 10–300 bp and therefore allowed accurate determination of the size of DNA samples.

Results

Protection of DNA from DNase Digestion by DNA antibodies. IgG from 8 of the 20 SLE sera that were tested significantly blocked DNase digestion of DNA. For those antibodies which blocked DNase digestion (protecting antibodies), the amount of DNA remaining TCA precipitable after DNase digestion (protected DNA) increased as increasing amounts of DNA were bound to antibody, as measured in the Farr assay (Fig. 1, patients A and B). Antibodies from other sera (Fig. 1, patients C and

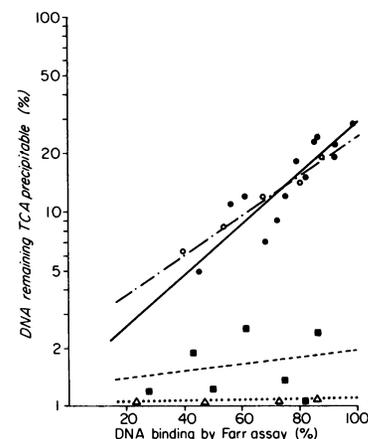


Figure 1. Relationship between DNA binding and DNA protection. Increasing amounts of IgG from SLE patients were incubated with DNA to form immune complexes, and DNA binding was determined by Farr assay. In parallel experiments, immune complexes were digested with excess DNase, and TCA precipitability of DNA was measured. When DNA was incubated with CF II or with buffer and then digested, (controls)

$<5\%$ of the DNA remained TCA precipitable. Data from four representative patients are shown here. IgG from patients A (●) and B (○) resulted in increased protection of DNA with increased DNA binding (protecting antibodies); IgG from patients C (Δ) and D (■) did not protect DNA despite high binding (nonprotecting antibodies). Curves were obtained by regression analysis of data points obtained in at least three separate experiments; background values are subtracted from each data point.

D) showed no DNA protection, even at high Farr binding levels (nonprotecting antibodies).

Characterization of the protected DNA fragment. DNA could appear TCA precipitable either by virtue of its size, i.e. >15 bp (17), or as a result of binding to protein. From the data presented above, it was not possible to determine if DNA protection represented: (a) binding of anti-DNA to a completely digested DNA fragment, (b) binding of anti-DNA to a larger DNA fragment, and thereby limiting access of DNase to the bound DNA, or (c) inhibition of DNase by anti-DNA independent of binding to DNA. To test the first possibility, DNA was completely digested with DNase, and IgG was then added to the oligonucleotide breakdown products and incubated at 37°C for 30 min. The initial DNase digest was $5 \pm 2\%$ TCA precipitable. The addition of IgG did not increase the TCA precipitability of DNA ($6 \pm 3\%$), which indicated that the protection demonstrated in Fig. 1 could not be accounted for by DNA breakdown products binding to anti-DNA. To distinguish between the remaining two possibilities, immune complexes were formed (70% Farr binding, 10% protection), digested as above, and the DNA that remained bound to IgG after digestion (bound DNA) was separated from the DNA released from IgG (free DNA) by passage over a SPA column. Greater than 90% of the acetate eluted DNA was SAS precipitable, which confirmed that it was bound to IgG, whereas the SPA column fall through DNA was <5% SAS precipitable. Each of these DNA preparations was phenol extracted, and removal of IgG was monitored by SAS precipitation. After phenol extraction, <5% of both preparations were precipitable in SAS, which indicated that IgG had been completely removed.

Fig. 2 shows the electrophoretic analysis of these two DNA preparations on a polyacrylamide disc gel (Fig. 2 A) and on a slab gel (Fig. 2 B). The DNA not bound to IgG after DNase digestion had been degraded to oligonucleotides <15 bp in length (Fig. 2 B, lane 3). This breakdown product was the same size as that which was produced by DNase digestion of DNA alone or of DNA incubated with CF II. In contrast, the DNA that remained bound to IgG during DNase digestion was 35–45 bp in length (Fig. 2 B, lane 2). The size of this protected DNA fragment was the same for all protecting antibodies tested. To exclude a gel artifact, the size of both DNA preparations was tested by TCA precipitation before electrophoresis (after phenol extraction). The free DNA was 20% TCA precipitable, whereas the bound DNA was >95% precipitable. This confirmed that the IgG-bound DNA was larger than the free DNA. To exclude the possibility that the IgG bound DNA was contained in a subset of immune complexes that were simply not available to DNase, i.e., due to precipitation of complexes or due to insufficient DNase, we redigested the SPA acetate eluate (IgG-bound DNA) with excess DNase at 37°C for an additional 30 min. After redigestion, no decrease in ammonium sulfate or TCA precipitability of DNA was observed.

Protection of DNA by IgG fragments. TCA precipitability of DNA after DNase digestion was studied after incubation of

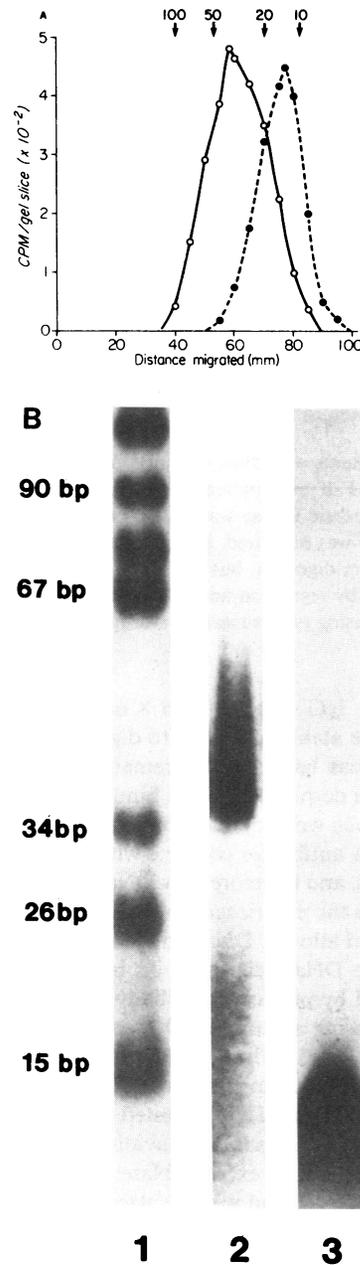


Figure 2. Electrophoresis of phenol-extracted DNA. DNA-anti-DNA immune were digested with DNase, and the DNA released from IgG was separated from the DNA which remained bound to IgG by passage over SPA. Both preparations were phenol extracted and analyzed on 8% polyacrylamide gels using Hpa II fragments of pBR322 as size standards. (A) Disc gel: DNA released from IgG (●) showed a peak at <15 bp, which was identical to the peak seen with DNase digestion of DNA alone. DNA that remained bound to IgG during digestion showed a peak at 35–45 bp (○). Arrows show the migration of DNA of varying sizes (bp) based on standard curves. (B) Slab gel: lane 1-molecular weight standards (size indicated in base pairs); lane 2-DNA that remained bound to IgG during digestion; lane 3-DNA released from IgG during digestion.

DNA with varying amounts of $F(ab)_2$ and Fab' from the IgG isolated from patient A. Both Fab' and $F(ab)_2$ were equally effective in binding DNA as measured in an immunoprecipitation assay. Despite equivalent binding, however, increasing amounts of $F(ab)_2$ protected increasing amounts of DNA, but Fab' was without effect (Fig. 3).

Discussion

In this study we have shown that IgG antibodies to DNA can be divided into two groups, based on their ability to protect

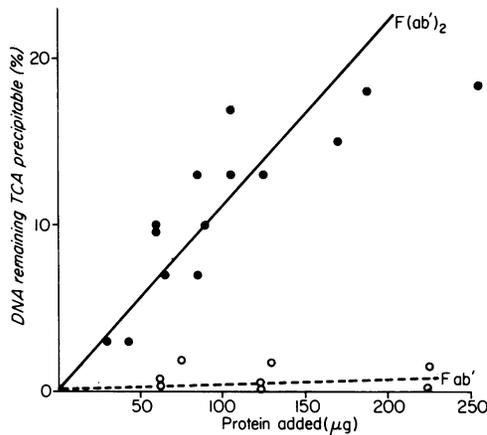


Figure 3. The effect of IgG fragments on DNase digestion of DNA. Increasing amounts of F(ab')₂ or Fab' from patient A were incubated with DNA at 37°C for 30 min; excess DNase was added for 30 min, and TCA precipitability of DNA was measured. Increasing amounts of F(ab')₂ (●) protected DNA from digestion, but Fab' (○) was without effect. Curves were obtained by regression analysis of data points from four separate experiments using two preparations of IgG fragments.

DNA from DNase digestion. IgG isolated from 8 of 20 SLE sera significantly inhibited the ability of DNase to digest DNA (protecting antibodies), whereas IgG from the remaining sera did not affect DNase digestion despite high DNA binding (non-protecting antibodies). Previous work by Liebling and Barnett demonstrated that some DNA antibodies compete with DNase for binding to ss DNA (10, 18), and therefore slow DNase digestion of ss DNA. In contrast to the experiments of Liebling and Barnett, we used ds DNA, and allowed DNA-anti-DNA complexes to form *in vitro* before DNase digestion. As increasing amounts of DNA were bound by protecting antibody, as measured in the Farr assay, increasing amounts of DNA were protected from DNase digestion (Fig. 1). The protected DNA represented DNA that remained bound to IgG during digestion despite the presence of excess DNase. This suggested that protecting antibodies bound to DNA with sufficient avidity to prevent their displacement from DNA by excess DNase; nonprotecting antibodies bound less tightly, and were displaced by the DNase, thereby allowing digestion of DNA. Differences in binding between protecting and nonprotecting antibodies may be due to higher intrinsic avidity of protecting antibodies for DNA, or due to the ability of protecting antibodies to bind DNA by monogamous bivalent binding, as discussed below. Direct measurements of the avidity of protecting and nonprotecting antibodies, as well as studies on their epitope specificities and correlations with clinical disease, are currently underway.

A major finding of this study was that the DNA that was protected during DNase digestion was a discreet fragment 35–45 bp in length. The size of this DNA was determined directly (Fig. 2), but could also be independently calculated from the

data shown in Fig. 1, as explained below. Assuming that IgG molecules bind to DNA randomly, then the number of IgG molecules on each DNA molecule will follow a Poisson distribution (19). As shown by Aarden et al. (19), at any given value of DNA binding in the Farr assay, the number of DNA molecules with 1, 2 . . . *n* IgG molecules bound can be calculated. If each bound IgG prevents DNase digestion of a DNA fragment representing a certain percentage (*x*) of the starting DNA size, then the total percentage of the starting DNA that is protected will equal the percentage of DNA with one IgG molecule bound times *x*, plus the percentage of DNA with two IgG molecules bound times 2*x* plus . . . the percentage of DNA with *n* IgG bound times *n**x*. These calculations assume that IgG binds randomly to DNA, and that two bound IgG molecules will protect twice the amount of DNA as one IgG. From these calculations, a family of curves that express DNA protection as a function of DNA binding for different values of *x* can be generated (Fig. 4). It should be noted that if *x* is small, as a result either of large starting DNA or of a small protected fragment, DNA protection will be difficult to detect. This is the reason why we used small molecular weight DNA (350 bp) as our starting preparation.

If we compare the measured data in Fig. 1 with the curves in Fig. 4, the observed curve closely approximates the curve predicted if *x* (size of the protected fragment divided by the size of the initial DNA) is 10%. Our starting DNA was 350 bp; 10% of 350 is 35 bp, which agrees with the measured size of the DNA fragment shown in Fig. 2.

The specific size of the protected DNA fragment is of interest for several reasons. In competitive binding experiments, Papanian et al. (20) showed that DNA fragments >40 bp are effective inhibitors of DNA-anti-DNA binding, but that fragments <40 bp are not. They postulated that a 40-bp DNA fragment is the minimal DNA size necessary to span between binding sites on a single IgG molecule, and therefore is the minimal size necessary

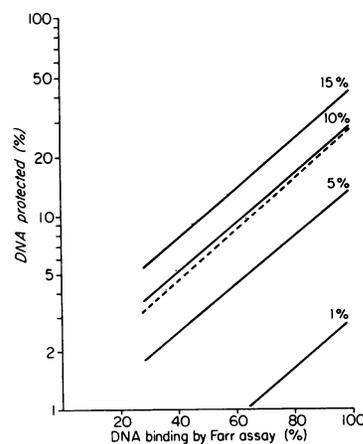


Figure 4. Relationship between DNA binding and DNA protection. Theoretically derived curves of DNA protection as a function of DNA binding at different values of *x*, where *x* represents the size of the DNA fragment protected by one IgG molecule divided by the size of the starting DNA (expressed as percent). The observed data from Fig. 1 (---) closely approximate the curve for *x* = 10%; if the starting DNA is 350 bp, 10% of

350 is 35 bp, a figure which is in agreement with the measured size of the DNA fragment shown in Fig. 2.

for monogamous bivalent binding. Monogamous bivalent binding results in an extremely high avidity bond, and has been postulated as a mechanism for the marked stability of some DNA-anti-DNA immune complexes (21, 22). The DNA fragment that we have isolated by DNase digestion of DNA-anti-DNA immune complexes is almost identical with Papalian's minimal size fragment. This suggests that protection of the DNA fragment may be a result of monogamous bivalent binding of IgG to DNA. Such a high avidity bond would be expected to be resistant to displacement by excess DNase. To test this hypothesis, the ability of bivalent F(ab)₂ and monovalent Fab' to protect DNA from DNase was examined. As shown in Fig. 3, the bivalent F(ab)₂ fragments did protect DNA from DNase digestion, but the univalent Fab' fragments had no effect.

We postulate, therefore, that when an IgG molecule binds to DNA via monogamous bivalent binding, a stable complex is formed that prevents access of DNase to the DNA between IgG binding sites. As a result, a small immune complex is formed which contains one IgG bound to a piece of DNA 35-45 bp in length. We have shown that DNA-anti-DNA immune complexes containing large pieces of DNA are cleared rapidly from the circulation (8). This clearance is mediated by the antigen in the complex (DNA), and occurs at the same rate as the clearance of DNA alone. Therefore, if the DNA within a DNA-anti-DNA immune complex is protected or inaccessible, one can speculate that such a complex might escape the ordinary clearance mechanisms of DNA-anti-DNA immune complexes in vivo. Recently, Sano and Morimoto (23) have isolated immune complexes from SLE sera, and have shown that the DNA contained within these complexes was of two small molecular weight species, either in the range of 30-40 or 150-200 bp. Furthermore, clinical disease activity appeared to correlate with the amount of these DNA fragments that could be isolated (24). Protection from DNase digestion of DNA contained within small, circulating immune complexes could account for these observations. Such small lattice immune complexes would be unlikely to fix complement or deposit in tissues (25), and therefore may not be the critical pathogenic complex in SLE. It should be pointed out, however, that in our experiments to isolate the protected DNA fragment, we did not form immune complexes in antibody excess. With increasing antibody concentration, multiple IgG molecules bind to each DNA molecule. With increased antibody packing, the chances of IgG molecules binding next to each other increases. If adjacent IgG molecules protect a larger DNA fragment, larger immune complexes resistant to DNase digestion could be formed. Studies examining the effect of increasing antibody excess on DNA protection, as well as studies on the in vivo behavior of protected immune complexes, are currently underway.

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