Avidity of Anti-DNA Antibodies in Serum and IgG Glomerular Eluates from Patients with Systemic Lupus Erythematosus

ASSOCIATION OF HIGH AVIDITY ANTINATIVE DNA ANTIBODY WITH GLOMERULONEPHRITIS

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ABSTRACT Significant differences in both specificity and avidity of anti-DNA antibodies were observed in the sera of groups of patients with active systemic lupus erythematosus glomerulonephritis, active systemic lupus erythematosus without nephritis, and in IgG eluates obtained by DNAase digestion of isolated glomeruli from glomerulonephritic kidneys. With methylated albumin-kieselguhr fractionated ^{3}H-HeLa DNA as a source of native or single-strand DNA antigen in a modified Farr assay, an increased level of antibody to native DNA was associated with active systemic lupus erythematosus, particularly active nephritis. The avidity of antinative DNA estimated from plots of the reciprocals of bound and free antigen according to the Sips distribution formula was significantly lower in active glomerulonephritis sera than in sera from patients with active systemic lupus erythematosus without nephritis. However, antinative DNA of uniformly high avidity was found in the glomerular eluates. Avidity of single-strand DNA antibodies did not differ in the various patient groups. The data strongly support a major role for high avidity antinative-DNA in DNA/antiDNA immune complex-induced glomerular injury in systemic lupus erythematosus.

INTRODUCTION

Evidence from a variety of sources suggests that circulating complexes of DNA antigen and anti-DNA antibody are of primary importance in the immunopathogenesis of renal injury in systemic lupus erythematosus (SLE).[1] Increased titers of both antinative DNA (nDNA) and antisingle strand DNA (sDNA) are correlated with clinical activity and hypocomplementemia (1–3). Both nDNA and sDNA alternate with specific antibody in the circulation (1, 4). DNA antigen has been detected by immunofluorescence techniques in the same distribution along the glomerular basement membrane as gammaglobulin and complement components (5, 6). Polynucleotide immune complexes, chiefly involving antibody to sDNA and nDNA, are highly enriched relative to serum levels in acid buffer or deoxyribonuclease eluates of isolated glomeruli from severely nephritic kidneys of patients with SLE (5, 7–9) and in serum cryoprecipitates (10). Additional evidence for the presence of complexes of DNA and anti-DNA in the cir-

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[1] *Abbreviations used in this paper*: ABC, antigen binding capacity; nDNA, native DNA; sDNA, single-stranded DNA; SLE, systemic lupus erythematosus.

culation derives from the demonstration of increased anti-DNA activity after digestion of serum with deoxyribonuclease (11, 12).

In the present investigation the avidity of anti-DNA antibody in serum, glomerular eluates, and cryoprecipitates of patients with SLE was estimated to define further the qualitative characteristics of anti-DNA having greatest potential for immune complex tissue injury. The data suggest that antinative DNA of high avidity is involved primarily.

METHODS

Patients and sera. Patients studied were receiving medical therapy at the Rockefeller University Hospital (22 patients) or at the University of Virginia Hospital (10 patients). Patient selection was based upon (*a*) fulfillment of the preliminary criteria of the American Rheumatism Association for classification as SLE (13); and (*b*) presence of anti-DNA antibody in their sera. Each patient was placed in one of three clinical groups determined by clinical criteria alone: (*a*) active SLE with active glomerulonephritis; (*b*) active SLE without evidence for glomerulonephritis; (*c*) inactive SLE. Presence of glomerulonephritis was assessed by examination of urinary sediment, blood urea nitrogen and serum creatinine determinations, and, in most cases, renal biopsy. Persistent proteinuria (2+ qualitative, >350 mg/24 h total urinary protein), microscopic hematuria and cylindruria, and progressive decline in creatinine clearance rates were considered to be indicative of active glomerulonephritis. Patients were classified as having active SLE when history and physical examination revealed evidence of at least two of the following signs and symptoms on two consecutive examinations: rash, fever, arthralgia or arthritis, serositis, alopecia, stomatitis, or evidence of central nervous system involvement. Sera were obtained from clotted specimens of peripheral blood; complement was inactivated by heating at 56°C for 30 min before use.

Glomerular eluates. Glomeruli were isolated from autopsy kidneys from patients with severe SLE glomerulonephritis, as described previously (9). Eluates were prepared by treating the glomeruli with deoxyribonuclease, followed by concentration of the eluate immunoglobulin by precipitation with 50% saturated ammonium sulfate. The eluates contained only IgG by gel diffusion analysis with appropriate antisera, and were inactivated by heating at 56°C for 30 min before use.

Cryoprecipitates. Cryoprecipitates were isolated from serum, thoroughly washed, and resolubilized in phosphate buffered saline at 37°C, as described in detail previously (10).

DNA. DNA intrinsically-labeled with [^{3}H]thymidine was prepared from HeLa cells (14). An operationally native DNA preparation was obtained by chromatography of ^{3}H-HeLa DNA on methylated albumin-kieselguhr columns (15–17). 92% of the DNA was eluted from the column with 0.9 M NaCl. Specific activity of the 0.9-M NaCl fraction was ~100,000 cpm/μg. The 260:280 ratio was 1.96. Based upon the following criteria, this material was considered to be nDNA: (*a*) an aliquot was rechromatographed on methylated albumin-kieselguhr by using both linear and stepwise NaCl gradients and eluted exclusively in the 0.9-M NaCl fraction; (*b*) failure to react antigenically with rabbit antiserum to sDNA or with various human disease sera having anti-sDNA but not anti-nDNA. sDNA was prepared before each experiment by boiling an aliquot of the above nDNA for 10 min followed by immediate cooling in an ice bath. This material was reactive with both rabbit and human anti-sDNA antibodies.

Measurement of anti-DNA antibodies. Anti-DNA antibodies were determined by a quantitative ammonium sulfate precipitation radioimmunoassay (Farr Assay) as previously described (17). 25 μl of serum was combined with 75 μl of ^{3}H-HeLa DNA in 0.15 M NaCl, 0.03 M borate buffer, pH 7.85 and incubated for 1 h at 37°C and then for 16 h at 4°C. 100 μl of cold 70% saturated ammonium sulfate was added. After 2 h at 4°C, this mixture was centrifuged for 10 min at 15,000 rpm in a Beckman microfuge. (Beckman Instruments, Inc., Cedar Grove, N.J.) Radioactivity in the supernate was determined with a liquid scintillation counter.

Certain technical modifications of this assay optimized detection of anti-DNA antibody and minimized nonspecific precipitation of DNA. All antibody test samples were heat-inactivated at 56°C before use. This procedure and the use of high ionic strength, alkaline pH, and a low borate concentration largely eliminate Clq and other nonspecific protein-DNA interactions (18). The prolonged 4°C incubation of DNA with test sera was included to maximize DNA binding by less avid antibody not detected at 37°C. All dilutions of test samples were prepared in borate-saline buffer containing 5% bovine serum albumin. This normalized the protein concentration in the samples and prevented the artifactual increase in DNA binding occasionally seen with very dilute serum samples.

By using the conditions detailed above, the mean percent binding of nDNA (10 ng per assay) by undiluted sera from 25 normal individuals was 9.7%±SD of 7.7%; sDNA binding by these sera was 11.6±9.2%. Additional controls included in each assay were several dilutions of a serum from a patient with SLE having high levels of anti-DNA antibody, a normal serum lacking antibody, and diluent alone. The nDNA preparation was examined periodically for development of sDNA determinants by performing assays with sera containing only anti-sDNA antibody. Quantitative data concerning relative anti-DNA antibody levels in the SLE sera were obtained by calculating the 50% antigen-binding capacity (ABC) in μg DNA/ml serum, using a test antigen concentration of 25 ng per assay.

Measurement of anti-DNA avidity. Anti-DNA avidity was estimated by performing Farr assays in duplicate upon appropriately diluted sera, glomerular eluates, or cryoprecipitates by using five different antigen concentrations over a 40-fold range (5 ng–200 ng ^{3}H-HeLa DNA). Dilutions of antibody chosen for the avidity measurements bound approximately 50% of 25 ng of nDNA or sDNA in the asays and were used to minimize the effect of antibody concentration upon avidity estimation by the Farr technique (19). Avidity was calculated from plots of the reciprocals of bound, b, and free, c, antigen according to the Sips distribution formula (20). Radioactivity in cpm was used to quantitate free and bound antigen. The Y-intercept, which is a measure of the total number of binding sites with extrapolation of 1/c to zero ("infinite antigen concentration"), ranged from 0.2 to 0.4 $\times 10^{-3}$) suggesting that the antibody concentrations were appropriately normalized by the dilutions chosen for these measurements. With the antigen concentrations used, the plots of 1/b *vs.* 1/c were generally linear. With several antibody preparations, curvature of the plot 1/b *vs.* $1/c^a$ toward the abscissa at higher antigen concentrations was corrected by choosing a value less than 1 for the exponent, a, (a is the index of heterogeneity).

To establish the precision of the method for estimating avidity, independent assays were performed five times with two sera. The standard deviation was 18% or less of the calculated mean avidity.

TABLE I

Antigen Binding Capacity and Avidity of Anti-DNA in Sera from Patients with Active Glomerulonephritis (Group 1)

Patients	ABC		Avidity	
	nDNA	sDNA	nDNA	sDNA
	(μg/ml)			
Blan	1.4	4.8	0.20	0.45
Tat	9.2	6.4	0.35	0.35
Wms	12.0	13.8	0.37	2.63
San	12.8	9.2	0.21	0.92
Web	10.2	7.7	0.14	0.03
Hen	20.8	30.4	0.16	0.70
Hars	3.7	9.6	0.20	0.56
Gam	4.6	0.8	0.22	0.33
Rus	2.9	1.6	0.25	1.10
Ban	5.6	19.2	0.36	0.27
Esp	10.6	12.2	0.51	0.31
Goz	18.4	12.8	0.53	0.37
Mean±SEM	9.4±1.8	10.7±2.3	0.29±0.04	0.67±0.20

In other experiments, anti-DNA avidity was estimated by calculating the slope between 66 and 33% binding of twofold serial dilutions of serum (21).

RESULTS

Studies on DNA antibodies in serum. Individual data regarding anti-DNA antibody levels in serum and relative avidity are shown in Tables I, II, and III for patients with active glomerulonephritis (Group 1), active SLE without glomerulonephritis (Group 2), and inactive SLE (Group 3), respectively. The antigen binding capacity for nDNA, while variable within the clinical groups, correlated positively with disease activity, particularly active glomerulonephritis. Sera from patients with active glomerulonephritis had the highest ABC for nDNA (mean±SEM = 9.4±1.8) whereas sera from patients with inactive SLE bound less than 0.5 μg nDNA/ml. A similar but less pronounced association between sDNA binding and disease activity also obtained. Low levels of antibody reactive with sDNA could still be detected in the sera of patients with inactive disease, however.

Significant differences in anti-DNA avidity were observed in the three groups (Table I, II, and III). The relative anti-nDNA avidity in patients with active glomerulonephritis was 0.29±0.04. The mean avidity of anti-nDNA antibodies in Group 2 patients without glomerulonephritis was significantly higher, 1.09±0.21 ($P < 0.001$). The avidity of antibody reactive with sDNA antibody did not differ among the three groups.

Studies on SLE glomerular eluates. The anti-DNA activity in IgG eluted from glomeruli isolated from SLE glomerulonephritic kidneys by deoxyribonuclease digestion was characterized in the same fashion as serum. Eluates from kidneys of four patients who died with advanced glomerulonephritis contained sufficient antibody to nDNA for affinity estimations (Table IV). In contrast to serum antibody in patients with active glomerulonephritis, which was of relatively low avidity, the avidity of anti-nDNA antibody in the eluates was more than 10-fold higher (mean±SEM = 3.51±1.21; $P < 0.001$). Note that avidity of anti-nDNA in sera from patients with active SLE without glomerulonephritis was intermediate between that of serum antibody in patients with active glomerulonephritis and that of the glomerular eluates.

TABLE II

Antigen Binding Capacity and Avidity of Anti-DNA in Sera from Patients with Active SLE Without Glomerulonephritis (Group 2)

Patients	ABC		Avidity	
	nDNA	sDNA	nDNA	sDNA
	(μg/ml)			
Ham	1.4	1.6	0.35	0.95
Bro	1.6	5.4	0.69	0.54
Sno	1.2	6.4	1.75	1.43
Wol	2.0	1.9	2.0	1.62
Pear	1.6	12.8	2.1	0.53
Camp	1.0	1.2	1.0	0.71
Ayl	4.6	6.0	0.51	0.17
Fen	2.8	9.6	1.26	0.95
Rich	0.7	3.0	0.57	0.22
Smi	<0.5	3.2	—	0.51
Moo	1.0	4.8	0.67	0.31
Mean±SEM	1.6±0.4	5.1±1.1	1.09±0.21	0.72±0.14

TABLE III

Antigen Binding Capacity and Avidity of Anti-DNA in Sera from Patients with Inactive SLE (Group 3)

Patient	ABC		Avidity	
	nDNA	sDNA	nDNA	sDNA
	(μg/ml)			
Abb	<0.5	3.2	—	0.50
Vra	<0.5	1.6	—	0.18
Dip	<0.5	1.3	—	0.24
Egl	<0.5	5.3	—	0.28
Tit	<0.5	2.6	—	0.53
Ite	<0.5	5.3	—	0.70
Garc	<0.5	1.6	—	0.90
Cart	<0.5	2.1	—	1.19
Bro	<0.5	2.8	—	2.85
Mean±SEM		2.9±0.5		0.81±0.28

TABLE IV
Avidity of Anti-DNA Antibody in IgG Eluted from Glomeruli

SLE glomerular eluate	ABC	Relative anti-nDNA avidity
	(*μg nDNA/ml*)	
Esp	4.0	2.17
Goz	7.6	6.6
Gor	0.5	1.1
Rus	5.6	4.2
Mean±SEM	4.4±1.5	3.5±1.2

Further evidence for a marked difference between avidity of anti-nDNA in serum and glomerular eluates was provided by study of patient Rus. Anti-nDNA avidity in serum drawn during a period of active glomerulonephritis before death was 0.25. Avidity of anti-nDNA in IgG eluted from her kidneys after death was 4.20.

The possibility that nonspecific binding by non-immunoglobulin proteins in serum might account for the relatively higher avidity of antibody in the eluates was examined by comparing the avidity of several sera with that obtained with isolated IgG. Significant differences were not seen. Thus, for patient San, anti-nDNA avidity was 0.21 and 0.23 for whole serum and isolated IgG, respectively; similary, the avidity for anti-nDNA in Ayl serum, 0.51, was virtually identical to that for Ayl IgG, 0.47.

Studies on DNA antibodies in cryoprecipitates. Anti-nDNA avidity was estimated in five cryoprecipitates from patients with active SLE, two of whom had glomerulonephritis (Table V). The mean anti-nDNA avidity of cryoprecipitates was 0.71±0.12, a value not significantly different from that in the supernatant serum, 0.54±0.18. Low avidity anti-nDNA was found in the cryoprecipitate of one patient, Smi, in the absence of detectable antibody in serum.

Anti-DNA binding curve slope as an estimate of avidity. Calculation of binding curve slopes for anti-DNA with nDNA or sDNA as ligands did not provide avidity data which correlated with the values estimated from the Sips distribution formula. The major limitation of the slope method was related primarily to the marked influence on the binding curve slope of differences in antigen-antibody ratio in the assay reaction mixtures. The pronounced effect of antigen concentration on the slope of the ^{3}H-nDNA binding curve is illustrated in Fig. 1. In this experiment, dilutions of a single serum were assayed simultaneously against five concentrations of DNA. The slope for the highest concentration, 52 ng, was 82.5, and for the lowest concentration, 5 ng, was 19.4.

The influence of antigen concentration on the binding slope is further illustrated in Table VI. Four sera containing antibody reactive predominantly with sDNA were titered against nDNA, sDNA, and a 3:1 mixture of nDNA/sDNA. In each case the bind curve slope of the nDNA/sDNA mixture was lower than for either nDNA or sDNA alone.

TABLE V
Avidity of Anti-nDNA Antibodies in SLE Cryoprecipitates Comparison with Serum Avidity

Patient	Clinical status	Relative avidity	
		Cryoprecipitate	Serum
Smi	Active; no nephritis	0.38	—*
Es	Active nephritis	0.46	0.51
Web	Active nephritis	0.85	0.14
Ayl	Active; no nephritis	0.86	0.51
Camp	Active; no nephritis	1.00	1.00
	Mean±SEM	0.71±0.12	0.54±0.18

* Serum $ABC_{nDNA} < 0.5$ μg/ml.

DISCUSSION

In the present study, the low avidity of antinative DNA antibody in the sera of patients with active

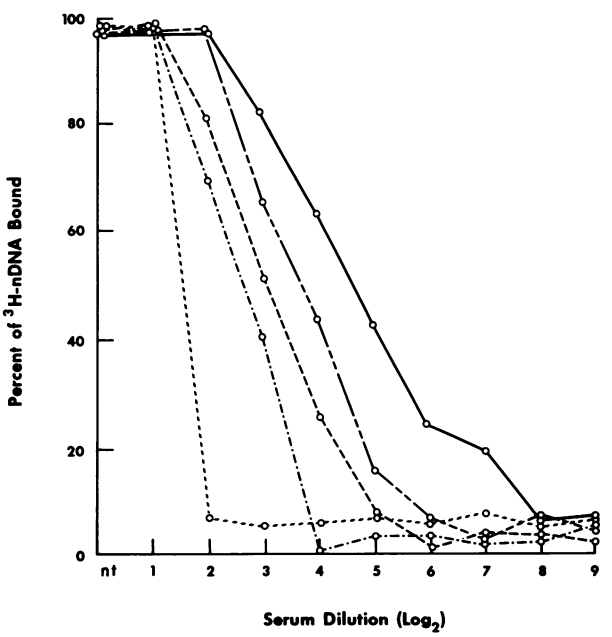

FIGURE 1 Effect of varying antigen concentrations upon the slope of the nDNA binding curve of a serum from a patient with SLE. All assays were performed simultaneously. The binding curve slope became progressively steeper with increasing quantities of antigen in the reactive mixture. ○ · · · · ○, 52 nanograms of nDNA; ○ – · – ○, 25 nanograms; 16 nanograms; ○ – – — – – ○, 9 nanograms; ○——○, 5 nanograms.

TABLE VI

Effect of Minor Contamination of nDNA with sDNA Upon the Binding Curve Slope of Sera Containing Mixtures of Anti-nDNA and Anti-sDNA

Serum	ABC		Binding curve slope		
	nDNA	sDNA	nDNA	sDNA	nDNA/sDNA (3:1)
	(μg/ml)				
Syl	4.6	6.0	41	28	25
Ric	0.7	3.0	25	26	14
Fen	2.8	9.6	28	21	17
Smi	0.5	3.2	—	37	18

glomerulonephritis contrasted sharply with the ~10-fold higher avidity anti-nDNA in DNAase eluates of isolated glomeruli from autopsy kidneys of patients dying with glomerulonephritis. Anti-nDNA of intermediate avidity was found in the sera of patients with active SLE who did not have evidence for active glomerulonephritis. Significant differences in antibody avidity were not observed when sDNA was used as antigen in groups of patients with inactive SLE or with active disease either in the presence or absence of glomerulonephritis.

The levels of anti-nDNA antibodies in the serum correlated particularly well with differences in patient clinical status, the highest levels being seen in patients with active nephritis. These findings are in agreement with earlier data supporting a strong association between increased anti-nDNA antibody in serum and renal injury in SLE (1–3).

The present data are interpreted as implicating high avidity anti-nDNA antibody as the species primarily responsible for DNA-anti-DNA immune complex-induced renal injury. This reasoning is based upon the following considerations. First, the highest avidity anti-DNA was localized to IgG eluted from glomerulonephritic kidneys, i.e. from the actual site of tissue injury. Second, anti-nDNA of lowest avidity was found in the serum of patients with active glomerulonephritis. The most likely explanation for these findings is the removal from the circulation of higher avidity anti-nDNA by DNA.

The intermediate avidity of anti-nDNA in the serum of patients with active disease but not having nephritis can also be interpreted in terms of this conceptual framework by postulating a smaller DNA antigenic load in the circulation of these patients. In this group, increased anti-nDNA of relatively higher avidity was found. If the release of DNA into the circulation was either brief in duration or very low quantitatively, the measureable avidity of detectable antibody would be higher because less of the very high avidity antibody would be removed by antigen. Decreased magnitude of the clinical sequelae of pathogenetically significant DNA-anti-DNA immune complex formation would be expected in the groups, as was in fact observed: less severe hypocomplementemia and absence of clinical nephritis.

Although cryoprecipitates in SLE are highly enriched relative to serum for anti-DNA antibody activity (10), high avidity antibody was not found in most cryoprecipitates. These limited data thus provide additional evidence that high avidity antibodies are removed quickly from the circulation by DNA.

Of considerable interest was the observation that the avidity of serum antibody to sDNA did not differ among the three clinical groups. The explanation for this finding is unclear. As free sDNA has been demonstrated by hemagglutination in the serum of patients with SLE (4), an effect of circulating antigen upon measureable anti-sDNA affinity similar to that postulated for anti-nDNA might be expected. It is possible that binding of sDNA in vivo to nonimmunoglobulin serum proteins, such as Clq, influences anti-sDNA-sDNA interaction in this regard.

Alternatively, the actual anti-sDNA avidity in sera of patients with active nephritis may be in fact relatively lower than in patients without nephritis. Here the higher measured avidity of antibody reactive with sDNA could represent a major contribution of anti-nDNA. A generally greater reactivity of anti-nDNA with sDNA relative to nDNA has been defined clearly in hemagglutination inhibition experiments (3).

The present observations are in general agreement with those in the New Zealand mouse model of immune complex glomerulonephritis and in human SLE in which low avidity anti-DNA antibodies in the serum have been related to the occurrence of nephritis (22, 23). In these studies, time of onset, course, and severity of nephritis were associated with the presence of increasing levels of low avidity anti-DNA in serum. Avidity was estimated by measuring the rate of dissociation of ^{125}I-DNA/anti-DNA complexes in the presence of a large excess of unlabeled DNA. The data were interpreted as indicating that low avidity antibody, which is less efficient in immune elimination of antigen than high avidity antibody (24), favors persistence of immune complexes which can be deposited in tissues. This concept regarding the pathogenicity of low avidity antibody is not consistent with the present data indicating high avidity anti-nDNA as the species deposited along the renal glomerular basement membrane.

The present data do not agree with those of a recent report in which the slope of the DNA binding curve was used to examine anti-DNA avidity and its relationship to glomerulonephritis (21). In this study serum from patients with glomerulonephritis gave steeper DNA binding slopes than serum from patients without glomerulonephritis. The results were interpreted as

indicating an association of high avidity serum anti-DNA with nephritis. Determination of the slopes of sDNA and nDNA binding curves in the present investigation revealed considerable variation among individual patients, but no significant differences between groups. Furthermore, there was no correlation between the DNA binding slope and avidity estimated from the Sips plots either for individual patients or for patient groups. As uncharacterized DNA which had not been subjected to procedures removing sDNA contamination was used in the previous study, it is probable that the avidity of both nDNA and sDNA specific antibody was being measured simultaneously. Variation in the proportion of anti-nDNA and anti-sDNA in the sera relative to a constant mixture of sDNA and nDNA in the assay could give, therefore, apparent differences in avidity. As demonstrated in the present study, the slope of the antigen binding curve is highly dependent upon the amount of available antigen, becoming sharply steeper with increasing antigen: antibody ratios. As patients without nephritis generally have a greater portion of anti-sDNA relative to anti-nDNA, minor contamination of the DNA used as ligand in the binding assay results in a decreased slope.

A number of unanswered questions remain. Given that high avidity antibody to native DNA is the species leading to DNA-anti-DNA complexes with optimum potential for renal injury, what is the nature of the complex itself. This system generally is associated with active glomerulonephritis when anti-DNA antibody exists in excess relative to antigen, a situation opposite to that in serum sickness models of glomerulonephritis with heterologous albumin as the antigen (25). In experimental serum sickness renal injury occurs with albumin in excess. Does Clq play a role in influencing the development and pathogenicity of circulating complexes of DNA and anti-DNA? Clq, a large, complex molecule binds strongly to sDNA in the absence of specific antibody. Finally, to what extent does IgM anti-IgG influence the molecular size or composition of DNA/anti-DNA complexes? In view of the participation, as circulating immune complexes, of these and other elements in tissue injury in SLE, additional investigation of qualitative factors governing their interaction may further clarify this unusual system.

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