CLINICAL SIGNIFICANCE OF
ANTI–DOUBLE-STRANDED DNA ANTIBODIES
DETECTED BY A
SOLID PHASE ENZYME IMMUNOASSAY

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A solid phase enzyme immunoassay (EIA) detected anti–double-stranded (ds) DNA antibodies in 88%
of sera from patients classified clinically as having active systemic lupus erythematosus (SLE) without renal
symptoms and 93% with renal disease. Fifty-six percent of sera from patients with inactive SLE were EIA posi-
tive for anti-dsDNA antibodies. The EIA had a sensitivity and specificity comparable to radioimmunoassay
(RIA) and hemagglutination for patients with active SLE with or without renal disease, but it detected anti-
dsDNA antibodies more frequently in patients with inactive SLE than the latter procedures. Precipitating
antibodies detected by counterimmunoelectrophoresis (CIE) were less common in patients with renal disease
(23% incidence) than clinically active patients without renal disease (79% incidence). Twenty-four SLE sera
with elevated levels of C1q binding showed a 96% concordance for a positive EIA for anti-dsDNA antibodies
in contrast to 66% concordance by RIA or hemagglutination. These findings suggest that the EIA is a sensi-
tive and specific method for detection and measurement of anti-dsDNA antibodies. Several clinical applica-
tions of the EIA are discussed.

Antibodies reactive with double-stranded DNA (dsDNA) are of primary importance for the diagnosis of
systemic lupus erythematosus (SLE), appear to play a central role in the pathogenesis of tissue injury, and are
closely correlated with clinical activity (1,2). The demon-
stration of precipitating antibodies in SLE sera reactive with DNA by using agar gel diffusion (3) initiated a
series of investigations to define optimal serologic assays for the detection, characterization, and quantitation
of these antibodies. The intrinsic properties of both the an-
tigen and the antibody have complicated the serologic
evaluation of anti-DNA antibodies. The antigen, DNA,
is a high molecular weight double-stranded molecule
susceptible to the formation of single-stranded regions.
It is strongly anionic, manifesting nonimmunologic in-
teractions which may interfere with antibody binding
(4). The anti-dsDNA antibody population is hetero-
genous with respect to class (5–7), complement fixing
properties (8,9), avidity (10–16), and specificity (17,18).
The concurrence of antibodies to single-stranded DNA
(19), frequently in high titer, in sera of patients with
SLE may be responsible for false positive or spuriously elevated titers of anti-dsDNA antibodies unless the conditions of the assay maintain the double-stranded structure of DNA (20).

In recent years, solid phase immunoassays have been developed which measure the interaction of antibody with insoluble DNA bound to a support medium (21–27) or incorporated within the kinetoplast of *Cricetidina luciliae* (28,29), using labeled antibody to human gamma globulin. These tests appear to offer advantages over the conventional assays for anti-dsDNA antibodies. If the solid phase assays manifest comparable sensitivity and specificity for the detection of anti-dsDNA antibodies, they merit consideration for routine clinical application. In the present study, a solid phase enzyme immunoassay (EIA) has been compared to three standard assays—radioimmunoassay (RIA), hemagglutination, and counterimmunoelectrophoresis (CIE)—in order to determine the clinical utility of the EIA procedure for the detection of anti-dsDNA antibody in subpopulations of patients with clinically active and inactive SLE.

**MATERIALS AND METHODS**

**Clinical data**

The following sera were obtained: 88 samples from 81 patients with SLE; 33 from patients receiving procainamide who developed positive antinuclear antibody titers of 1:10 or greater after 3 to 6 months of procainamide administration; 62 from adult patients with rheumatoid arthritis; and 33 from age- and sex-matched normal individuals. Patients with rheumatic disease satisfied the American Rheumatism Association criteria for the classification of either SLE (30) or rheumatoid arthritis (31). All patients with SLE had either antibodies to double-stranded DNA and/or Sm antigens at some time during the clinical course of their disease.

Patients with SLE were classified as having active disease 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. Patients were classified as having active renal disease if they had at least one of the previous signs on two consecutive examinations accompanied by persistent and increasing proteinuria (2+ qualitative and at least 350 mg total urinary protein per 24 hours) and abnormal urinary sediment consisting of microscopic hematuria and cylindruria, an increasing serum creatinine, or decreasing creatinine clearance. C3 levels were depressed in greater than 80% of these patients, although this was not considered a criterion for renal activity. Renal biopsies were performed in more than half of the patients and uniformly showed evidence of glomerulonephritis.

Patients classified as having inactive disease did not show any of the previously cited signs of active or active renal SLE for at least 6 months prior to the date of testing, with two exceptions: 2 patients with renal disease considered to be inactive had persistent proteinuria of 1–2 gm per 24 hours or a 2+ qualitative test which persisted without change for a period of 6 months and was not associated with hematuria, cylindruria, or extrarenal manifestations of disease. They also had normal serum creatinine and creatinine clearances.

The clinical criteria for each patient were reviewed by three of the authors (DK, RL, and VZ). Patients with incomplete clinical data were excluded prior to the performance of the present study. The classification of SLE patients was formulated independent of any serologic tests and was based on the clinical and laboratory criteria listed above. The presence of antibodies to nDNA, ssDNA, or Sm antigen was not considered for the purpose of classification.

**Reagents**

**Goat anti-human immunoglobulin conjugate.** Goat antiserum reacting only with immunoglobulins as tested by immunoelectrophoresis and agar gel diffusion was subjected to ammonium sulfate precipitation and DEAE chromatography for purification of the immunoglobulin fraction. F(ab')2 fragments were prepared by pepsin digestion and isolated by using Sephacryl 200 gel filtration (32). Fractions containing intact IgG as determined by SDS electrophoresis were discarded, and F(ab')2 fractions were conjugated with horseradish peroxidase type VIII (Sigma Chemical Corp., St. Louis, MO) (33). The molar peroxidase to antibody ratio was 1.9:1.0. Conjugates were tested as appropriate reagents for use in the enzyme immunoassay by using tubes coated with purified IgG and IgM (22). The F(ab')2 conjugate used in this study was shown to give color reactions to dilutions of 1.0 µg/ml of conjugate reacting with IgG and 2 µg/ml for IgM, and negative reactions with Clq globulin and human serum albumin coated tubes.

**3H dsDNA.** 3H dsDNA prepared from HeLa cells and unlabeled calf thymus dsDNA (Worthington Biochemical Co., Freehold, NJ) were both purified by methylated albumin kieselguhr (MAK) chromatography (10). The MAK chromatographed DNA was "operationally" defined as double-stranded in fluid and solid phase on the basis of the following criteria: 1) failure of soluble dsDNA to inhibit the reactivity of rabbit anti-adenosine (A), anti-thymidine (T), and high titered anti-human single-stranded DNA (ssDNA) antibodies in an RIA procedure using 3H ssDNA (10); 2) inhibition of the reaction of anti-dsDNA antibodies with solid phase dsDNA by soluble dsDNA (Table 1). Equivalent inhibition was obtained with aliquots of dsDNA treated with S1 nuclease (24); 3) failure of sera containing high titered anti-ssDNA antibodies (human and rabbit) to react with solid phase dsDNA (Table 2); 4) failure of S1 nuclease treatment to alter the antigenic properties of solid phase dsDNA. Six SLE sera containing anti-dsDNA antibodies showed equivalent reactivity with untreated MAK dsDNA and S1 nuclease treated MAK dsDNA (24), when these preparations were coated on polystyrene tubes.

The molecular weight of both the unlabeled and labeled DNA preparations was determined to be approximately 1.0 × 106 daltons by sucrose density gradient centrifugation following MAK chromatography. The chromatographed preparation showed homogeneity of molecular weight. Unlabeled ssDNA or 3H ssDNA was prepared by heating MAK
chromatographed dsDNA for 10 minutes at 100°C followed by immersion in an ice bath.

Rabbit anti-A and anti-T were prepared as previously described (17).

Procedures

Enzyme immunoassay (EIA)

Coating of antigen. A solution of dsDNA at 1.0 µg/ml diluted in 0.1M ammonium acetate pH 5.0 was coated on 75 × 12 mm polystyrene tubes (Evergreen Scientific Company, Los Angeles, CA) by incubation at room temperature for 2 hours and then at 4°C for 16 hours. The DNA solution was removed, the tubes were washed with distilled water, then incubated with 1% human serum albumin (HSA) in Tris KCl buffer (10 mM Tris HCl, pH 7.8, 0.1M KCl) for 1 hour at 37°C, and washed with a 0.1% solution of DEAE chromatographed normal goat IgG diluted in Tris KCl buffer.

Test procedure. The test sera were diluted 1:20 with a solution containing 1% HSA, 0.01% normal goat IgG, and 0.05% Tween 20 in Tris KCl buffer, incubated for 1 hour at 37°C, and washed with distilled H₂O containing 0.05% Tween 20. The goat anti-human gamma globulin peroxidase conjugate was used at a protein concentration of 1.0 µg/ml diluted with 1% HSA in Tris KCl buffer and incubated for 1 hour at 37°C. The tubes were washed with distilled water and the substrate added. 5-Aminosalicylic acid was used as a substrate for color development (34). After incubation at room temperature for 20 minutes, the reaction was stopped with 0.1 ml of 0.1N NaOH. The absorbance of the solution was measured at 449 nm in a Gilford spectrophotometer. Each set of assays was run in duplicate with appropriate controls including 2 positive sera for each experiment, 10 normal sera, a conjugate control (incubation with buffer instead of serum), and substrate control (incubation without conjugate).

The mean EIA value of 0.4 optical density (OD) units was established as the upper limit for normal sera. This was determined prospectively by testing a group of normal sera which manifested a mean EIA value of 0.26 ± 0.07 OD units. Three moderately to strongly positive SLE sera were used as standard positive controls. The assay was reproducible within ±10% by using peroxidase antibody conjugates of comparable titer and enzyme-antibody-molecular ratio over the course of this study (approximately 6 months).

Inhibition procedures. dsDNA or ssDNA was added to sera diluted with 1% HSA, 0.01% normal goat IgG, and 0.05% Tween 20 in Tris KCl buffer. The mixture was incubated for 1 hour at 37°C (25), and the absorbed sera were assayed for anti-dsDNA or anti-ssDNA antibodies by EIA or RIA.

Solid phase dsDNA poly-l-lysine (PLL). The method of Aotsuka et al (25) for solid phase RIA was adapted for EIA. The binding of solid phase DNA directly to polystyrene was compared to dsDNA bound to polystyrene coated with PLL. SDS and heparin removed greater than 75% of DNA bound with PLL tubes but did not remove detectable DNA directly bound to polystyrene. Fluid phase dsDNA incubated with ³H DNA in solid phase did not enter into equilibrium reactions with the labeled DNA bound directly to polystyrene tubes or PLL coated tubes.

Farr radioimmunoassay. This assay was performed as previously described by using ³H dsDNA (10).

Hemagglutination. The hemagglutination tests for antibodies to dsDNA were determined by a previously described method (17) utilizing formalinized human erythrocytes coated with dsDNA.

Counterimmunoelectrophoresis. A two-stage electrophoresis system was utilized by using a modified method of Davis and Winfield (35) with dsDNA. The DNA at 8 µg/ml was added to the cathode well of agar gel plates following
Table 3. Serologic tests for anti-dsDNA antibodies

<table>
<thead>
<tr>
<th></th>
<th>No. sera tested</th>
<th>EIA*</th>
<th>RIA*</th>
<th>Hem*</th>
<th>CIE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE active</td>
<td>33</td>
<td>88</td>
<td>70</td>
<td>76</td>
<td>79+</td>
</tr>
<tr>
<td>SLE active renal</td>
<td>30</td>
<td>98+</td>
<td>77+</td>
<td>80+</td>
<td>238</td>
</tr>
<tr>
<td>SLE inactive</td>
<td>25</td>
<td>56</td>
<td>28g</td>
<td>28g</td>
<td>20g</td>
</tr>
<tr>
<td>Drug LE</td>
<td>33</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>62</td>
<td>3</td>
<td>5</td>
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</tr>
<tr>
<td>Normal</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* % sera positive. EIA = enzyme immunoassay; RIA = radioimmunoassay; Hem = hemagglutination; CIE = counterimmunoelectrophoresis.
+ P < 0.05 compared to CIE for SLE active renal and SLE inactive.
§ P < 0.05 compared to SLE inactive.

The specificity of the system was also evaluated in antigen excess by using sera at a constant dilution of 1:100 and increasing dilution of soluble DNA.

RESULTS

Sensitivity of EIA. Serial dilutions were performed on three sera, two with high and one with lower OD binding values for anti-dsDNA antibody. Sera with high OD readings did not show a linear decrease in OD readings with increasing dilution until a dilution of 1:640 to 1:1,280 had been reached, in contrast to a serum with a lower OD reading (Figure 1). These sera when diluted for the RIA procedure showed a sharp decrease in titer with negative results obtained at a 1:40 dilution. The specificity of the system was also evaluated in antigen excess by using sera at a constant dilution of 1:100 and increasing dilution of soluble DNA.

Figure 1. Serial titration of three positive SLE sera tested for anti-dsDNA antibodies by EIA to assess the sensitivity of the test.

Figure 2. Sensitivity of the EIA assessed by antigen inhibition. Sera from two patients with anti-dsDNA antibodies (1:100 dilution) incubated with dilutions of dsDNA antigen.
Figure 3. SLE sera assayed by EIA (OD units) compared with % binding of the RIA test. A. Active SLE without renal involvement. B. Active SLE with renal involvement. C. Inactive SLE. All groups of SLE sera showed a significant correlation between the titers of anti-dsDNA antibodies determined by the two tests. Precipitating antibodies demonstrated by CIE (○) were found in both low and high titered sera. Sera with non-precipitating antibodies (●) were most prominent in patients with renal disease. The absence of precipitating antibodies was also observed in both low and high titered sera.
antigen which was detectable as inhibitor in the range of 0.1 to 0.01 μg/μl serum (Figure 2).

Comparison of EIA using DNA-polystyrene coated and DNA-PLL-polystyrene tubes. A close correlation was observed between OD values for 16 sera assayed by both methods (r = 0.966).

Incidence of anti-dsDNA antibodies detected by EIA, RIA, hemagglutination, and CIE (Tables 3 and 4). The EIA technique had comparable sensitivity to RIA and hemagglutination for SLE patients with active disease, but it detected a significantly higher incidence of anti-dsDNA antibodies in patients with inactive disease. A marked decrease in the occurrence of precipitating antibodies as detected by CIE was observed in patients with active renal disease. Significant titers of anti-dsDNA antibodies were not found in the sera of patients with drug-induced lupus or rheumatoid arthritis, despite the high incidence of antibodies to ssDNA by RIA in these patients (Table 2). Anti-ssDNA antibodies were detected in 26% of drug-induced lupus sera (mean percent binding 38.7%; range 16.5–100%) and 37% of rheumatoid arthritis sera (mean percent binding 36.8%; range 18.7–100%). Of the 14 EIA positive sera in patients with inactive SLE, 43% of these sera contained no measurable anti-dsDNA antibodies by any other assay.

Quantitation of anti-dsDNA antibodies by EIA, RIA, and hemagglutination. No differences were observed for the quantitation of anti-dsDNA antibodies in patients with active or active renal disease (Table 5). Lower titers of anti-dsDNA antibodies were found by all assays in patients with inactive disease. A significant correlation for the titer of anti-dsDNA antibodies was found between EIA and RIA in all patient groups. The figures compare the titers of antibody of EIA-RIA: Figure 3A shows active disease; Figure 3B, active renal disease; and Figure 3C, inactive disease. A similar correlation was found for titers of anti-dsDNA antibodies with the hemagglutination test compared with EIA for patients with active disease (r = 0.67, P < 0.001) and active renal disease (r = 0.47, P < 0.005). The hemagglutination test showed no significant correlation for patients with inactive disease (r = 0.27, P > 0.05). A tabulation of all SLE sera tested by RIA and hemagglutination indicated an overall correlation between the two tests for quantitation of anti-dsDNA antibodies (r = 0.74, P < 0.001).

Correlation of EIA with C1q binding assay. The C1q binding assay was performed on 42 of the 88 sera. Twenty-four of these sera were positive for circulating immune complexes. Of these 24, only 1 was negative for anti-dsDNA antibodies by EIA, whereas 8 sera were negative by RIA, 8 by hemagglutination, and 6 by CIE.

Twenty-one sera from the active group were studied by both C1q binding and EIA. The mean OD value for EIA determination of anti-dsDNA antibodies in C1q positive sera was 0.78, and for the C1q negative sera, the mean OD value was 0.54. The difference between the two groups was significant (P < 0.02).

**DISCUSSION**

Anti-dsDNA antibodies are detectable by a solid phase enzyme immunoassay (EIA) in over 90% of patients with symptoms of active SLE. The presence of renal disease did not influence the incidence or the quantity of antibodies detectable by EIA, whereas patients with inactive SLE manifested a lower incidence and decreased titer of anti-dsDNA antibody. The incidence of anti-dsDNA antibody detected by EIA, RIA, and hemagglutination was comparable for patients with active SLE with and without renal disease. In contrast, patients with inactive SLE manifested an increased incidence of anti-dsDNA antibodies detectable by EIA. There was a significant quantitative correlation for anti-dsDNA antibodies measured by EIA, hemagglutination, and RIA in the sera of both groups of patients with active SLE. Although no attempt was made to determine the titer of precipitating antibodies to dsDNA detected by CIE, there was a striking decrease in the prevalence of these antibodies for SLE patients with active renal disease, a phenomenon observed in several previous studies (38,39).

From the clinical standpoint, the combined use of the EIA and CIE procedures may give information...
about the incidence and qualitative characteristics of anti-dsDNA antibody. Although the basis for the low incidence of precipitating antibodies by CIE in renal disease patients has not been established, avidity, restricted heterogeneity, and the formation of soluble complexes are known factors which may affect precipitation of antibody. The simultaneous use of these tests may offer some prognostic indication concerning the clinical course of disease with reference to renal involvement. Several studies have indicated that clinically inapparent renal injury may occur and, therefore, patients with high EIA values and absent precipitins by CIE may be candidates for renal biopsy in order to evaluate immune complex deposition and consequent glomerular damage. Data concerning immune complex levels in patients with active disease indicate that combined Clq binding and EIA testing may also be closely correlated with clinical activity. Studies are in progress to determine the ability of the latter tests for evaluating patients with clinical evidence of renal disease.

The high incidence of anti-dsDNA antibodies detectable in patients with inactive SLE suggests that EIA is potentially useful for diagnostic screening of asymptomatic individuals and patients with syndromes suggestive of SLE. Less than 5% of patients with rheumatoid arthritis and drug-induced lupus were positive for anti-dsDNA antibodies by the EIA test, despite a significant incidence of antibodies to ssDNA in these patient populations. The selective demonstration of anti-dsDNA antibodies in SLE sera, particularly in patients with inactive disease, suggests that both cytotoxic and "benign" anti-dsDNA antibodies may be detectable by this assay.

One important consideration concerning the EIA procedure relates to whether the putative increase in sensitivity of the test is a property of the assay or the result of the cumulative detection of other antibodies, e.g., anti-ssDNA antibodies which interact with solid phase DNA. Convincing evidence has been presented that DNA contained within crithidia organisms is double-stranded and does not react with antibodies to ssDNA. In contrast, previous studies utilizing in vitro prepared DNA placed on a solid phase have not analyzed the nativity of the solid phase antigen. The data obtained in the present study indicate that the DNA bound to polystyrene is double-stranded and is not reactive with antibodies to ssDNA of either human or rabbit origin. The EIA is also a sensitive method for the detection of anti-ssDNA antibodies, and the solid phase may prove to be an optimal substrate for preventing renaturation of ssDNA.

The EIA test circumvents several problems inherent to the fluid phase radioimmunoassay procedure. Molecular weight should not influence the reaction of antibodies with solid phase DNA. For the RIA only DNA molecules of molecular weight of 1.0 x 10^6 or less are precipitated by a single antibody molecule, and therefore high molecular weight DNA requires a molar antibody-antigen ratio of greater than one for precipitation by ammonium sulfate (40). The interaction of antibody molecules with fluid phase DNA molecules follow a Poisson distribution and, therefore, a linear plot of percent binding does not express the quantity of antibody bound. Therefore, a threefold increase in percent binding in the Farr assay may require greater than a tenfold quantity of antibody (41). In contrast, the EIA assay when expressed as OD units may have a linear relationship to the amount of antibody present. This assumption is theoretically correct if there is an excess number of epitopes available for antibody binding. The results of several antibody titration experiments indicate that certain high titered sera have a plateau before a linear decrease in EIA color reaction is observed. Therefore, the EIA is potentially capable of accurately quantitating antibody if the system is adjusted to a state of excess of solid phase antigen. This is illustrated by the fact that lower titered sera show a linear decrease in OD units with doubling dilution, whereas sera with higher OD units manifest a linear decrease only after dilution to the range of 1:640 to 1:1280. Sera with heterogeneous populations of anti-dsDNA antibodies binding to a larger number of different epitopes may have higher OD values but a lesser quantity of anti-dsDNA antibody.

Antibody binding avidity is another factor which may account for differences between the EIA test and radioimmunoassay. Several studies have suggested that the lowest avidity anti-polysaccharide (42) and anti-DNP (43) antibodies are not detectable by solid phase EIA. Recent studies have also suggested that Farr radioimmunoassay detects primarily higher avidity anti-dsDNA antibodies (11). No information concerning direct measurement of the avidity requirements of the solid phase assay for anti-dsDNA is presently available, although the results of this study suggest that the EIA is capable of detecting lower avidity anti-dsDNA antibodies than RIA, hemagglutination, or CIE. Further investigation will be needed to clarify whether the low avidity antibodies detected by the PEG assay are measurable by EIA (13).

The EIA directly measures gamma globulin, thereby excluding interference by non-immunoglobulin interactions with DNA such as Clq globulin and DNA binding proteins. In addition, the test system may be
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utilized to assay a variety of properties of the antibody such as immunoglobulin class, complement fixing properties, and relative avidity. Inhibition studies suggest that the EIA is a sensitive method for detecting low concentrations of serum DNA. The ability to objectively quantitate antibody as well as the potential for automation indicates that the solid phase immunoassay is a potentially useful procedure for the study of a variety of other antibodies occurring in the sera of patients with SLE.

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REFERENCES
