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REPORTS

Significance of Levels of Specific Immunoglobulins to DNA in SLE Patients' Sera Detected by Solid Phase Radioimmunoassay

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A solid phase radioimmunoassay was developed for detecting the quantity of double-stranded and single-stranded DNA antibodies in patients with systemic lupus erythematosus and other connective tissue diseases. The assay system employs a solid support 96-well, flex-vinyl microtiter plate to which bovine methyl albumin is layered, followed by denatured or native calf thymus DNA. A 1:80 dilution of patients' sera was added to respective wells followed by tritiated high affinity anti-IgG, -IgA, or IgM.

Denatured DNA (single-stranded DNA) bound to methylated bovine serum albumin had less than 5% reannealment to the double-stranded form and provided a better substrate for Ab binding than double-stranded DNA, producing a linear binding curve.

Of 58 patients diagnosed as having systemic lupus erythematosus (SLE), only 11 having active SLE had IgG antibody levels of > 5.0 ug/ml to single-strand DNA. Renal involvement of some degree was found in all 11 with the high concentrations of IgG antibodies to DNA correlating with severe involvement. Patients with IgM antibodies to DNA alone had more benign types of SLE with little renal involvement.

No abnormal levels of IgA Ab to either single-strand DNA or double-strand DNA were found in SLE patients' sera. Corticosteroid and/or immunosuppressant treatment caused a marked drop in the IgM Ab level to DNA within 10 days while IgG Ab to DNA remained high for up to 30 days.

Quantitation of IgG and IgM Ab to single-strand DNA provides a useful method for diagnosing severe SLE with possible renal involvement and monitoring the course of the disease during therapy.

During the past 17 yr antibodies to DNA from patients with systemic lupus erythematosus (SLE) have been studied by a variety of methods. Disease correlations have progressed by using several tests beginning with gel diffusion [1] and progressing through complement fixation [2], agglutination [3], immunofluorescence [4], and counterimmunoelectrophoresis [5]. Each test has possessed some inherent problem with certain antibodies such as lacking complement (C') fixation, low agglutinating or precipitation ability, or immunoglobulin specificity and quantitation.

Radioimmunoassay (RIA) has an advantage over other methods in that only the binding of the antibody to the DNA is needed, giving the test a greater specificity and an increased sensitivity to antibodies. In the past few years the type of RIA assays that have emerged have been competitive binding and/or a type of solid phase. In using these assays the necessity for determining the type of antibody and the quantity that binds to DNA was realized.

The purpose of this paper is to introduce a solid phase immunoglobulin specific radioimmunoassay for detecting double-stranded and single-stranded DNA antibodies in patients with SLE and other connective tissue diseases. This paper illustrates the need for obtaining immunoglobulin specificity and quantity to provide a useful diagnostic and prognostic test.

MATERIALS AND METHODS

Double-stranded Calf Thymus DNA (ds DNA)

Calf thymus DNA type I (highly polymerized) III, IV, V, and VIII, RNA, and polyuradylic acid were purchased from Sigma.

Single-stranded Calf Thymus DNA (ss DNA)

ss DNA was made from ds DNA by dissolving (1 mg/ml) in distilled water and bringing up to volume (1 mg/10 ml) with radioallergosorbent (RAST) buffer. The solution was heated at 100°C for 12 min, then quickly cooled in ice water.

Tritiated Calf Thymus DNA (^3H DNA)

^3H DNA was made by coupling 100 uCI ^3H actinomycin-D to 5 mg calf thymus DNA type 1 in PBS 7.2 for 24 hr. Excess ^3H actinomycin-D was dialyzed out. 0.01 mg DNA gave 125,000 cpm/well [6].

High Affinity Anti-IgG

Pooled normal serum was fractionated with a 15% final volume Na_2SO_4 solution to precipitate the immunoglobulins. The immunoglobulins were dissolved to original volume with H_2O and chromatographed on a G-200 Sephadex column to obtain the IgG fraction. The IgG fraction was bound to bromoacetyl cellulose (BAC) (approx. 45.0 mg IgG/gram of BAC) according to the method of Robbins, Haimovich, and Sela [7]. After appropriate washing, goat anti-human IgG (Meloy Lab) was incubated with the matrix (9.0 mg Ab/gram matrix) for 4 hr at 35°C using gentle shaking. After washing twice with 0.9% saline, the matrix was treated with pH 4.5 0.01 M acetate-buffer-saline for 10 min, washed once with 0.9% saline, and was then treated with pH 2.5 0.017 M HCl buffered saline to remove high affinity anti-IgG from the matrix which was then neutralized by 0.15 M $NaHCO_3$ [8,9].

Both high affinity anti-IgM and -IgA were isolated by similar methods as high affinity anti-IgG, using goat anti-human IgM or IgA heavy

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Abbreviations:
ANA: antinuclear antibody patterns
BAC: bromoacetyl cellulose
dAT: deoxyadenate and deoxythmidylate
FDNB: ^3H fluorodinitrobenzene
MBSA: methylated bovine serum albumin
PHA: passive hemagglutination assay
RAST: radioallergosorbent
RES: reticuloendothelial system
RIA: radioimmunoassay
SLE: systemic lupus erythematosus

chain specific (Miles Lab). The IgA used as the antigen on the immunoabsorbent matrix was obtained from a pool of 6 IgA myeloma patients' sera.

Radioallergosorbent (RAST) Buffer 10X Solution

30 gm of Dextran T 70 (Pharmacia) were dissolved in 500 ml of 0.9% saline. The remaining reagents were then dissolved in their respective order: 83 gm $NaH_2PO_4 \cdot H_2O$; 85.5 gm NaCl; 3.0 gm NaN_3, 50 ml Tween 20; volume was brought up to 1000 ml with distilled H_2O and pH to 7.2.

Disease Activity

Patients were diagnosed to have SLE if they had at least 4 of American Rheumatism Association preliminary criteria for systemic lupus erythematosus. Those SLE patients were considered to have active SLE if they had evidence of arthralgia (other etiology ruled out), persistent fever and possibly active skin lesions. Increased renal involvement was also indicative of active SLE in patients.

Renal involvement was based on the indices which included the following: lower creatinine clearance, lower C'_3 and C'_4 levels, elevated sedimentation rate and positive renal biopsy.

Serum Specimens

Venous blood was drawn from patients with various connective tissue diseases and from normal controls. These samples, taken at various times during the course of the disease, were received within 6 hr after drawing, after which the serum was separated by centrifugation at 4°C. Serum samples to be tested were stored at −20°C and thawed only once before testing.

Quantitation of working curve for RIA

Quantitation of working curves of patients' anti-DNA antibodies and ^{3}H labeled anti-immunoglobulins were obtained by making dilutions of both components in a grid manner. A series of 2-fold dilutions from known anti-DNA positive patients' serums ranging from 1:20 to 1:640 were used with a series of labeled anti-IgG concentrations ranging from 20 ng to 200 ng/well. Each series was plotted and an empirical decision was made on the best working curve. A 1:80 dilution of patients' serum provided a concentration of anti-DNA antibodies that would not saturate all the antigenic sites of the DNA in the microtiter wells in most serum. The labeled anti-IgG, at 40 ng/well, was found to begin to saturate all the patients' IgG bound to the DNA. Similar titration curves were used to determine the IgM and IgA anti-DNA antibody concentration in patients' sera, and the amount of anti-IgM or -IgA needed.

Solid Phase RIA

The solid-phase microtiter radioimmunoassay of Tan and Epstein [10] was modified by incorporating the RAST technique [11] for immunoglobulin specificity. Flexible, polyvinyl, 96-well microtiter U plates were etched 5 min per well with spectral-grade toluene and washed 3 times with PBS pH 7.2. The plate was coated with 0.1 ml bovine methylated serum albumin (MBSA) per well (1 mg/10 ml H_2O), incubated for 4 hr at room temperature, and washed 3 times with PBS pH 7.2. Double- or single-stranded DNA (0.1 ml/well of 1 mg DNA in 10 ml RAST) was applied to the MBSA-coated wells and incubated for 12 hr at room temperature for double-stranded DNA and at 4°C for single-stranded DNA followed by 6 washings with RAST buffer.

Patients' sera (0.1 ml/well) of a 1:80 dilution in RAST buffer was added to the respective wells in duplicate and incubated at room temperature for 4 hr followed by 6 washings with RAST buffer. High affinity labeled anti-IgG, -IgM or -IgA (0.1 ml/well: 40 ng/ml unlabeled and 40 ng/ml labeled Ab) was added to the respective wells, incubated for 4 hr at room temperature and washed 6 times with RAST buffer. The wells were cut from the plate and placed in scintillation vials. (The anti-immunoglobulins were eluted from the well by adding 1 ml of 0.1 N HCL to each vial.) Scintillation cocktail was added to the vials and they were allowed to stand 1 hr at room temperature before counting for the presence of labeled anti-immunoglobulin. Knowing the concentration of anti-IgG per well and the cpm per ng anti-IgG, the serum sample could be calculated as ug of IgG, IgM or IgA to DNA/well.

^{3}H Labeling of High Affinity Anti-IgG

The general method for labeling high affinity anti-IgG with ^{3}H Fluorodinitrobenezene (FDNB) used was that of Sanger [12]. Generally, 10 molecules of FDNB will label 1 molecule of anti-IgG sufficiently. Excess ^{3}H (FDNB) was dialyzed out and total cpm were calculated per ng of anti-IgG. High affinity ^{3}H anti-IgM and ^{3}H anti-IgA were labeled in a similar method.

Washing of Microtiter Plates

Microtiter plates were washed with either PBS pH 7.2 buffer or RAST buffer using an 8-port (Cooke) micro-drop dispenser and the liquid was aspirated.

RESULTS

The use of a solid support to fix the antigen DNA provides a very reproducible sandwich assay system to detect and quantitate the type of reactive antibody (Fig 1). The MBSA enables DNA to be fixed to a solid matrix, eliminating the need of precipitating a complex and preventing the reannealment of ss DNA to ds DNA. The use of ^{3}H anti-IgG, -IgM or -IgA provides the assay with immunoglobulin type specificity.

The binding capacity of the ss DNA versus ds DNA was determined by using tritiated DNA. Total binding per well was 40 ug ± 0.2 ug and the concentration of bound DNA remained constant after 20 washes with RAST buffer. Consistent binding of DNA to MBSA could only be achieved with calf thymus type I highly polymerized DNA. Types III, IV, V, VII and VIII showed no consistency in the amount bound to each microtiter plate well. Type I DNA, when used as the antigen, gave more consistent results in reacting with human anti-DNA antibodies when tested using RIA, hemagglutination and double immunodiffusion.

A comparison of the binding efficiency of Ab to ds DNA and ss DNA using the solid phase assay was performed (Fig 2). The system employed maintained constant concentrations of MBSA, DNA and ^{3}H anti-IgG and varied only in the concentration of anti-DNA Ab. The ^{3}H anti-IgG (40 ng cold and 40 ng labeled anti-IgG) provided the equilibrium saturation needed for maximum binding in each well; thus the amount of anti-DNA antibody dictated the amount of anti-IgG, -IgM, or -IgA binding to the specific immunoglobulin. The ss DNA system provided the better antigenic binding sites resulting in an assay with an adequate working range, little nonspecific binding of the labeled immunoglobulin to DNA and good equilibrium saturation. The double-stranded system had less binding efficiency as shown by a shallow curve and a slow tendency toward equilibrium saturation (Fig 2).

The concentration of various types of anti-DNA antibodies found in newly diagnosed or very active anti-DNA positive SLE patients are shown in Fig 3. When these anti-DNA positive antibodies sera were evaluated for renal involvement, all the

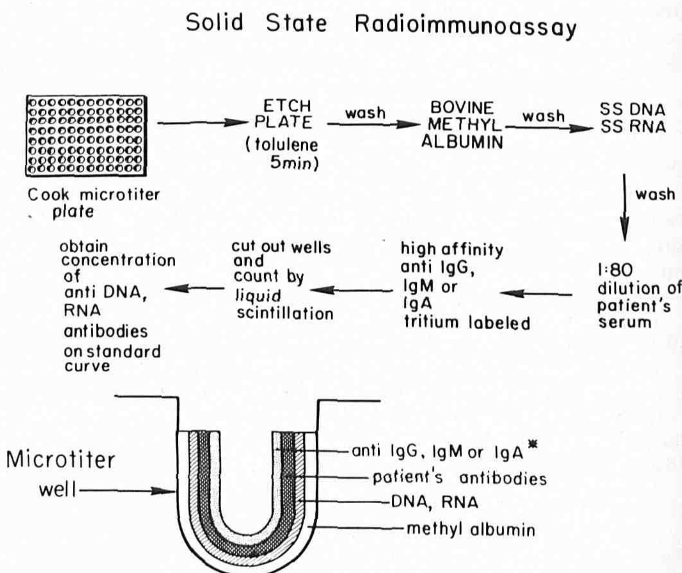

FIG 1. Schematic diagram of assay procedure illustrating the sandwich assay system of the radioimmunoassay.

patients having significant levels of IgG antibodies to DNA possessed several renal involvement. Newly diagnosed SLE patients with low concentrations of IgG antibody to DNA had no clinical signs of renal involvement at the time of assay.

Correlation of IgG antibody levels to renal involvement in patients could only be illustrated if the patient was not on or had just started on immunosuppressive therapy or if the patient was on a low dose of corticosteroids and the disease flared. Patients with low concentrations (< 4 mg/ml) of IgG may possess a slow developing degree of renal involvement and further studies concerning these patients are in progress. Evaluation of IgM anti-body levels to DNA could not be correlated to patients having renal involvement. IgA antibodies to DNA were not found in any significant levels in any of the patients' serums tested.

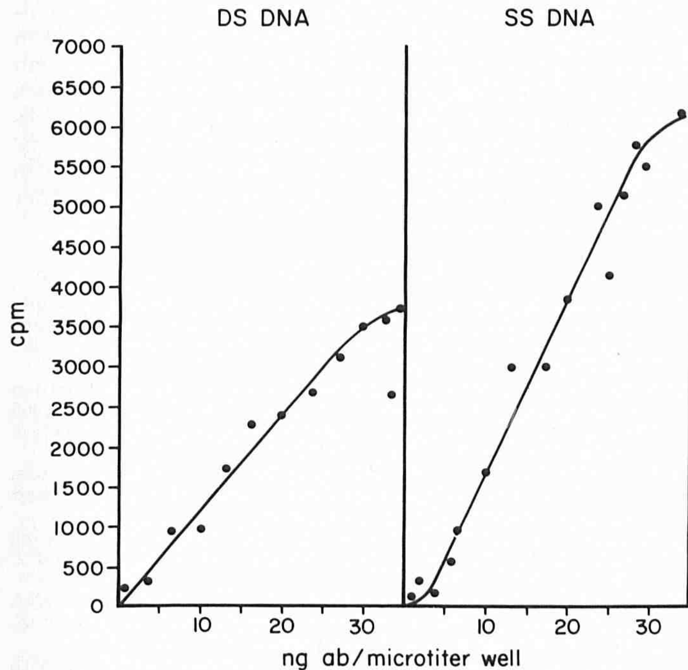

FIG 2. Binding efficiency of antibody to ds DNA and ss DNA determined by tritiated DNA. The relative amount of tritiated antibody binding to DNA is recorded as CPM.

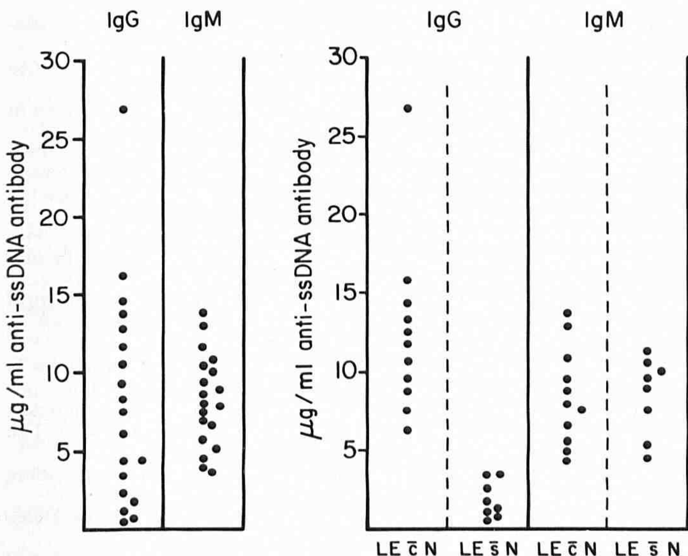

FIG 3. Comparison of the concentration in ug/ml of IgG and IgM antibody to ss DNA in serum of systemic lupus erythematosus patients with active disease (before immunosuppressive therapy). The antibody concentrations are further compared as to whether the patients have renal involvement.

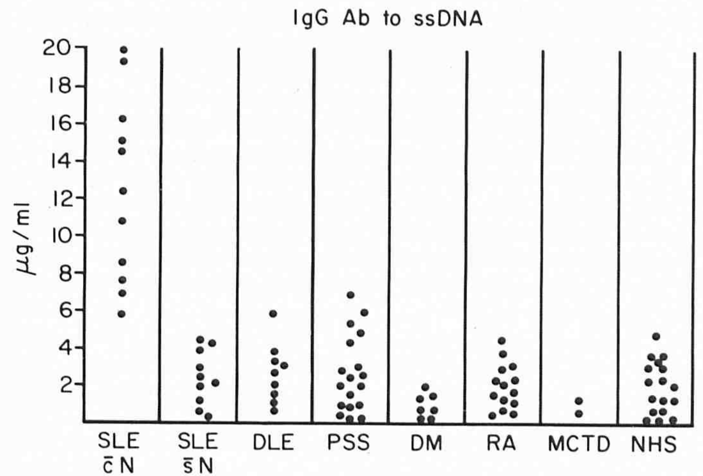

FIG 4. Comparisons of concentration IgG antibody to ss DNA in the various connective tissue diseases. *SLE*, systemic lupus erythematosus with and without nephritis; *DLE*, discoid lupus erythematosus; *PSS*, progressive systemic sclerosis; *DM*, dermatomyositis; *RA*, rheumatoid arthritis; *MCID*, mixed connective tissue disease; *NSH*, normal human serum. The antibody concentration to ss DNA of only the active SLE patients with nephritis were significantly different ($p > .001$) from the other connective tissue disease when comparisons were made.

High levels of IgG Ab to ss DNA were found only in active severe SLE (Fig 4). Lower levels of the IgG Ab to ss DNA were detected in some patients with progressive systemic sclerosis. By using a solid phase RIA for ss RNA and polyuridylic acid, it was found that these Abs were mainly directed toward a mRNA Ag and the positive reaction to ss DNA was actually a cross reaction. IgM Ab to ss DNA produced significant levels in some patients with severe food or gold allergies, patients on renal dialysis or rejecting renal transplants, and some drug induced LE patients. Possibly, these conditions produce a misregulation of the immune system causing the production of such antibodies in these patients.

DISCUSSION

The development of a solid phase RIA for DNA was first initiated by Tan and Epstein [10] who used a "sandwich assay" or solid support (microtiter plates), bound DNA, patient Ab and ^{3}H DNA. Shimizu, Hagura, and Yamamura [13] used a system similar to the IgE RAST assay, using microcellulose as the solid support, to test for specific immunoglobulins to DNA. The assay developed in our laboratory utilizes the best of both assays, namely, the microtiter plate and RAST assay with the microtiter plate as the solid support, which facilitates a better washing procedure and maintains low incidence of nonspecific binding. In addition, the assay has the ability to test for the specific type of immunoglobulin involved.

Studies using ^{3}H DNA in our laboratory have shown that DNA attachment to the vinyl was minimal, with the DNA being removed with consecutive washings. MBSA provided the necessary adhesive needed and maintained 98% binding. MBSA prevented ss DNA from reannealing to a ds DNA form as confirmed by adding ^{3}H ss DNA to the ss DNA already bound to the plate. Less than 5% of ss DNA returned to ds DNA, while as much as 10% of ^{3}H ss DNA bound to ds DNA-MBSA-plate, thus forming a type of triple stranded DNA. It is our contention that ss DNA must be sterically altered by binding to the MBSA, hindering nucleotide annealing but not necessarily altering antigenicity.

A necessary component providing the needed quantitative ability in the anti-DNA is the use of a high affinity anti-immunoglobulin Ab. Studies using various types of regular commercial anti-immunoglobulin antisera gave an irregular binding ratio making quantitation almost impossible. The high

affinity Ab binds very strongly to the patients' Ab and, therefore, may be more rigorously washed to remove excess labeled antisera.

The quantitation of specific types of antibodies to DNA appears to be very important in diagnosing the severity of the disease. IgG antibodies appear to be mostly involved in the immune complexes found in the kidney and possibly in the skin [13]. In our own studies, high concentrations of circulating IgG antibodies to DNA indicate that the lupus patient has severe renal involvement as assayed by kidney biopsy. Talal et al [15] had similar findings in that serum 7s antibodies to DNA correlated with active lupus nephritis in both humans and animals. It is possible that IgG binding to DNA produces immune complexes of a size that can be depositied in the kidney but cannot be cleared readily by the reticuloendothelial system (RES).

Mannik and Arend [16] reported that immune complexes containing 2 or less IgG antibodies per complex circulate in the blood of humans for many hours; whereas, IgM complexes are removed rapidly by the RES. Henson [17] states that aggregated IgG causes neutrophil adherence and release of β glucoronidase enhancing tissue damage whereas aggregated IgM produces neither. It is our conjecture then that the degree of renal involvement in the disease SLE correlates with the amount of IgG free to complex with DNA and the time span between initiation of IgG type DNA antibodies to detection and control.

Our laboratory also evaluated the Farr % binding assay, passive hemagglutination assay (PHA), as well as the solid phase assay and found that only the solid phase assay could actually be quantitative. The % binding assay employs ^3H labeled DNA as the antigenic indicator of the binding of the Ab which may range in molecular weight from 1×10^6 to 2.0×10^7 daltons with multiples of binding sites for the Ab. In the precipitation of the Ag-Ab complex, the assay cannot detect whether one Ab molecule or several have been bound to the same DNA strand. In addition, because the assay is not immunoglobulin specific, the assay cannot reveal whether IgM or IgG is binding to the DNA strand. No correlations cuould be made with the PHA test to SLE disease activity in patients.

Single-stranded DNA or denatured DNA as the antigen provided greater binding efficiency than did double-stranded DNA or natural DNA. This suggested that most of the anti-DNA antibodies assayed for in active SLE patients reacted with purine and pyrimidine bases or a combination of these bases, rather than polydeoxyribose phosphate back bone. In general, results from other studies on the specificity of anti-DNA antibody for DNA determinates showed that ss DNA blocked binding of the antibodies to ds DNA better than ds DNA itself [18]. Picazo and Tan [19] suggest that anti-DNA antibody in many SLE patients may be strongest to repeating adenine and thymidine nucleotides in ds DNA, illustrating that nucleotide bases are the antigenic sites for most DNA antibodies.

A study was performed to test a new substrate; namely, the alternating copolymers of deoxyadenate and deoxythmidylate (dAT). According to the article of Steinman et al [20], high concentrations of antibodies to this dAT, using the % binding assay, correlated to high incidences of active renal lupus in patients. In testing the sera of patients for antibodies to dAT, we found that only sera containing a high concentration of IgG Ab to DNA would bind to dAT. The amount of anti-IgG binding to dAT was lower in concentration, difficult to quantitate, and difficult to distinguish from normal values. The selectivity of the IgG antibodies for the substrate antibodies correlates with our findings that IgG is the main antibody type involved with lupus nephritis.

Corticosteroids and/or other immunosuppressant treatment produced a sharp decrease in the concentration of IgM antibodies to DNA, reducing the concentration to < 1 ug/ml within 10 days in the patients tested. IgG antibodies to DNA decreased to normal levels within 30 days post initiation of treatment. Decreased concentrations of both types of DNA antibody usually correlated with their respective half lives. The parameter which was not measured was the amount of DNA antigen present to complex these antibodies, thus reducing the free DNA antibody concentration. Such information would then enable a more complete measurement of the amount of immunosuppressive therapy needed to control specific DNA immunoglobulin producing B lymphocytes.

High anti-DNA concentrations ($>$ 10 ug/ml) tended to be associated with the peripheral general, peripheral leucocyte specific, homogeneous general and/or homogeneous leucocyte specific antinuclear antibody patterns with or without a particular pattern. [21,22]. Sera having the aforenamed ANA patterns do not necessarily have significant concentrations of DNA antibodies. If one of these ANA patterns is observed, the DNA antibody assay should be performed.

The solid phase assay system used in this investigation quantitates the amount of IgG or IgM antibody to DNA found in patients' sera. Quantities of IgG antibody to DNA greater than 5 ug/ml indicate that the patient has an immune complex disease producing some degree of renal damage. IgM antibodies to DNA of > 4.0 ug/ml are found in some active LE patients' sera and when found alone, the amount of renal disease in minimal. IgM antibodies were also found in various other connective tissue diseases.

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