Use of Enzyme-Linked Antibodies to Measure Serum Anti-DNA Antibody in Systemic Lupus Erythematosus

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We describe a solid-phase adsorbent enzyme-linked immunoassay for measuring the amount of antibody in serum. The assay consists of adsorbing an excess of DNA to a polystyrene test-tube support, reacting the anti-DNA serum with the coated tube, following with an additional reaction of an anti-human γ-globulin peroxidase enzyme conjugate with the coated tube, and finally developing a colored reaction product by the action of peroxidase on substrate. In principle, the amount of IgG bound to the tube is proportional to the amount of anti-DNA contained in the serum, and the conjugate also reacts proportionally to the amount of human IgG that has reacted. The assay gives data consistent with that expected for serum from patients with systemic lupus erythematosus, and it is possible to titrate the serum for micrograms of DNA antibody. At present the method still has considerable variability, but offers a new approach to measurement of antibody in autoimmune diseases.

Additional Keyphrases: horse-radish peroxidase–antibody complex • antibody measurement in autoimmune disease • immunologic disorders • immunoassay • solid-phase adsorbent • immunohistologic method compared

Since the discovery that sera of patients with SLE1 contain antibodies to nuclear components (1), more refined methods have been developed to define the antigen involved in the reaction and to measure the amount of antibody. Methods used to establish the nature of the antigen and antibody have included complement fixation (2), hemagglutination (3), precipitation of soluble immune complexes with ammonium sulfate (4), immunoprecipitation by double diffusion in agar (1), and immunohistology (5). The reaction has been quantitated by dilution of the sera for immunohistology, by the extent of binding of DNA by the sera, and by solid-phase radioimmunoassay (6).

The use of antibodies coupled to enzymes has opened the possibility of using such conjugates to replace the radioactive label in immunoassay systems. An attempt was therefore made to measure anti-DNA antibody by using an enzyme-conjugated antibody. Principal reactions of the enzyme-linked antibody method and the quantitation system are outlined in Table 1, and compared with the methods for detecting antibody by using a tissue substrate, a solid-phase immunoassay, and a solid-phase competitive-binding assay.

The technique described herein consists of preparing an antigen on an insoluble support, layering the test serum with anti-DNA, and using this serum as an antigen to react with an anti-human IgG labeled with peroxidase. The steps and reactions are similar to those in the immunohistologic method; there is also a close relationship to radioimmunoassay for antibody (6) in which serum containing anti-DNA is used to bind both to the adsorbed DNA and to the labeled DNA. The major differences in the method presented are: (a) antibody–enzyme conjugate is used; (b) it is a noncompetitive assay procedure; and (c) the substance measured is the antibody itself. This method is described in detail and the results are compared with those obtained by an immunohistologic method.

Materials and Methods

Materials were obtained from the following suppliers: “Diaflo” apparatus, Amicon Corp., Lexington, Mass. 02173; bovine serum albumin, 30 g/dl solution, Miles Pentex, Kankakee, Ill. 60901; calf thymus DNA and horseradish peroxidase, type VI, Sigma Chemical Co., St. Louis, Mo. 63178; 3,3'-dimethoxybenzidine, Eastman Organic Chemicals, Rochester, N. Y. 14650; “Falcon” polystyrene tubes 12 × 75 mm, Matheson Scientific Co., Cincinnati, Ohio 45246; and human γ-globulin (IgG) “Gamulin,” Pitman-Moore Division of Dow Chemical Co., Indianapolis, Ind. 46225. Other chemicals are obtainable from standard sources and should be of the highest possible grade.
Reagents

Buffers

Ammonium acetate, pH 5. Dissolve 7.71 g of ammonium acetate in 800 ml of water, add 2.29 ml of glacial acetic acid and dilute to 1 liter.

Ammonium bicarbonate, 0.1 mol/liter. Dissolve 7.9 g of crystalline ammonium bicarbonate in 800 ml of water; dilute to 1 liter.

Barbital, pH 8.7, 0.07 mol/liter. Dissolve 57.52 g of sodium barbital in 1 liter of water. Adjust the pH to 8.7 with 28 ml of HCl (1 mol/liter) and dilute the solution to 4 liters.

Buffered neutral formalin, pH 7.0. Dissolve 6.5 g of Na₂HPO₄ and 4.0 g of NaH₂PO₄·H₂O in 900 ml of distilled water; add 100 ml of formalin.

Phosphate-albumin, pH 7.0. Dilute 10 ml of bovine serum albumin solution (30 g/dl) with 290 ml of phosphate buffer (0.1 mol/liter, pH 7).

Phosphate-buffered saline (PBS), pH 7.4. Dissolve 1.13 g of Na₂HPO₄, 0.258 g of KH₂PO₄ and 8.5 g of NaCl in 600 ml of water. Dilute to 1 liter.

Potassium phosphate, pH 6.0, 0.1 mol/liter. Dissolve 11.93 g of KH₂PO₄ and 2.14 g of K₂HPO₄ in 800 ml of water. Dilute to 1 liter.

Potassium phosphate, pH 7.0, 1.0 mol/liter. Dissolve 53.0 g of KH₂PO₄ and 106.25 g of K₂HPO₄ in 800 ml of water. Dilute to 1 liter. Dilute to either 0.1 or 0.2 mol/liter buffer as needed.

Potassium phosphate, pH 8.0, 1.0 mol/liter. Dissolve 7.21 g of KH₂PO₄ and 164.9 g of K₂HPO₄ in 800 ml of water. Dilute to 1 liter. Dilute to either 0.01, 0.10, or 0.3 mol/liter buffer, as needed.

Saline citrate, pH 7.0. Dissolve 6.14 g of NaCl in 800 ml of water. Add 3.09 g of Na₃C₆H₅O₇·2H₂O and dilute to 1 liter.

Tris HCl, pH 7.6, 0.05 mol/liter. Dissolve 6.05 g of Tris base in 800 ml of water. Add 38.4 ml of HCl (0.1 mol/liter) and dilute to 1 liter.

Stock solution

DNA solution. Dissolve 30 mg of calf thymus DNA, or preferably calf thymus DNA purified by the method of Marmur (7), in 100 ml of the standard saline-citrate buffer. Adjust the absorbancy at 260 nm to 8.5 if necessary.

Working solutions

Diaminobenzidine stain. Dissolve 7.5 mg of 3,3'-diaminobenzidine in 10 ml of Tris buffer, pH 7.6. Add 0.1 ml of H₂O₂ (1 ml/dl). Make freshly each day.

Dilute goat serum. Add 1 ml of goat serum to 99 ml of phosphate-buffered saline; keep refrigerated.

DNA solution. Add 1 ml of the stock DNA solution to 9 ml of the ammonium acetate buffer.

Enzyme-antibody conjugate. Add 0.1 ml of the concentrated conjugate to 9.9 ml of the phosphate albumin buffer; keep refrigerated.

Enzyme substrate. Add 1 ml of a solution of methanol containing 10 mg of dimethoxybenzidine and 1 ml of hydrogen peroxide (0.3 ml/dl) to 98 ml of 0.01 mol/liter phosphate buffer, pH 6.

Glutaraldehyde solution, 10 ml/liter. Dilute 0.1 ml of the commercial 50% glutaraldehyde solution with 5.0 ml of potassium phosphate buffer (0.1 mol/liter, pH 7).

Human γ-globulin. Dissolve 10 mg of purified human γ-globulin (see Preparations, below) in 10 ml of potassium phosphate buffer (0.1 mol/liter). The stock solution contains 10⁶ ng/ml. Prepare working solutions from this containing 100, 200, 500, 1000, 2000, 5000, and 10 000 ng/ml by dilutions of 10 000-fold, etc., in barbital buffer.

Phosphate-buffered glycerol. Mix 9 ml of glycerol and 1 ml of phosphate-buffered saline.

Saturated ammonium sulfate solution. Add 850 g of (NH₄)₂SO₄ to 1 liter of water, warm to 50 °C and store overnight. Allow to come to room temperature. Excess reagent crystals should be present in the flask; keep covered.

Sodium azide. Add 1 g of sodium azide to 100 ml of water.
Preparations

**Human γ-globulin.** Dialyze 30 ml of human γ-globulin, 165 ± mg/ml, against 0.1 mol/liter ammonium carbonate buffer and add the protein solution to a DEAE-cellulose column (45.5 × 6 cm) previously equilibrated with phosphate buffer (0.1 mol/liter, pH 8.0), and elute with 500 ml of the same buffer, followed by 500 ml of 0.1 mol/liter buffer. Collect the first major protein fraction; concentrate to 30 ml with the “Diaflo” apparatus with an XM 50 filter; dialyze against 0.1 mol/liter NH₄HCO₃ and lyophilize.

**Goat anti-human γ-globulin.** Inject a goat with 10 mg of human γ-globulin in Freund’s adjuvant at two-week intervals and collect the serum (or purchase the anti-serum from commercial sources). Precipitate the γ-globulin fraction by adding 10 ml of water and 10 ml of saturated ammonium sulfate to 10 ml of serum. Collect the precipitate by centrifugation; dialyze against 10 mmol/liter potassium phosphate and isolate the antibody by DEAE-cellulose chromatography, as described above under Human γ-globulin. The major antibody fraction is in the 0.1 mol/liter buffer fraction.

**Antibody–enzyme conjugate.** Prepare the peroxidase antihuman γ-globulin by the method of Avrameas (8). Add 120 mg of horseradish peroxidase to 10 ml of a phosphate buffer (0.1 mol/liter, pH 6) containing 50 mg of goat anti-human γ-globulin. Couple the proteins by adding dropwise, with gentle stirring, 5 ml of the solution of glutaraldehyde. Allow the mixture to stand for 2 h at room temperature, dialyze against phosphate-buffered saline overnight, and centrifuge to remove precipitating protein. Fractionate on a column (35 × 2.5 cm) filled with Sephadex G-200 (Pharmacia Labs., Piscataway, N. J. 08854) and equilibrated with phosphate-buffered saline (9). Pool those fractions corresponding to the void volume (i.e., the first fraction eluted), and concentrate to 2 ml by using an Amicon XM 50 membrane. This is the stock antibody–enzyme conjugate solution.

Procedures

Add 0.2 ml of the DNA solution to each polystyrene tube. Coat the tubes by incubation for 3 h at room temperature, with gentle shaking. Remove the DNA solution by aspiration; add 1 ml of the bovine serum albumin buffer and remove by aspiration. Repeat this latter washing step twice. Coat the additional possible binding sites by adding 0.2 ml of the goat serum solution to the washed tube and incubating the protein solution for 4 h at room temperature, with gentle shaking. Aspirate the goat serum out of the tube and wash the prepared tube with albumin buffer solution as above.

Dilute the sera from patients 25-fold in albumin buffer for the initial assay; use higher dilutions to titrate positive serum samples. Place an aliquot of 0.2 ml of the diluted serum in a tube; allow the solution to react for 4 h at room temperature, with gentle shaking. Remove the serum by aspiration and wash the tubes as described above. Incubate the tubes for 4 h with goat anti-human γ-globulin–peroxidase conjugate. Remove the excess conjugate and wash the tubes with albumin buffer. Develop the color by incubating 0.3 ml of the substrate for 20 min at room temperature; stop the reaction with 20 μl of sodium azide. For titration, repeat the assay using dilutions of serum of 25-, 100-, 400-, and 1600-fold. Read the absorbance of the reaction product at 420 nm.

**Standard γ-globulin curve and quantitation.** Incubate for 4 h, in duplicate, 0.2 ml of the following dilutions of the human γ-globulin solution: 100-, 200-, 500-, 1000-, 2000-, 5000-, and 10 000-fold. Use this assay to calculate the micrograms of human γ-globulin required to obtain a color yield of 0.15, chosen as a standard reference point. The number of micrograms of anti-DNA (IgG) in an unknown serum is obtained by dilution, with reference to the standard absorbancy of 0.15.

**Patients.** Sera from 48 patients with SLE were studied. The diagnosis of SLE was made on the basis of a variety of abnormal clinical and laboratory data (11). Clinical activity was assessed as previously described (12).

Controls consisted of sera from 27 healthy subjects, sera from 51 patients with various renal diseases, and sera from 43 patients hospitalized for a wide range of disorders unrelated to SLE or other so-called collagen diseases. In addition, sera from nine patients with rheumatoid arthritis were studied.

Most sera from patients with SLE and with renal diseases had been stored for periods of months to a few years at −75 °C or, if used within one to two weeks, at 4 °C.

**Titration of antinuclear antibody on tissue.** Mount rat-liver tissue (from male Sprague-Dawley rats) in the center of the choke filled with tragacanth, freeze in liquid nitrogen for several minutes, place in a plastic bag, and store at −75°C. Cut the tissue (4–6 μm thickness) in a cryostat, and place the sections on gelatinized slides.

Fix the slides in phosphate-buffered formalin (pH 7.0) for 6 min, and wash in phosphate-buffered saline for 6 min. Mop the slides dry around the tissue and cover with a drop of serum. Incubate for 30 min at room temperature under inverted Petri dishes lined with moist filter paper. Wash the slides in phosphate-buffered saline for 30 min, mop them dry around the tissue, and layer with peroxidase-conjugated goat anti-human γ-globulin serum, incubate for 30 min, and repeat wash for another 30 min. Then wash the slides briefly with distilled water, immerse them in the freshly prepared diaminobenzidine solution for 5 to 7 min, wash again with distilled water, and mount in buffered glycerol (10).

Examine the slides for the presence of a brown reaction product in nucleus and cytoplasm, by using a Leitz-Ortholux microscope. Scan the tissue under low power (125X); if positive, grade the pattern of
staining (peripheral, speckled, diffuse, nucleolar) and degree of positivity at 300X in the following manner:

\[ \pm = 1-5 \text{ nuclei in at least 2-3 fields} \]
\[ + = 6-15 \text{ nuclei in at least 2-3 fields} \]
\[ ++ = 16-30 \text{ nuclei in at least 2-3 fields} \]
\[ +++ = \text{more than 30 nuclei in at least 2-3 fields, or all nuclei stained.} \]

Test all sera undiluted and up to an eightfold dilution. Do the tests in duplicate on the same or different days. If the test scores as + or greater, dilute the sera with phosphate-buffered saline and repeat. The highest serum dilution with which specific nuclei staining is demonstrated is called the titer of antinuclear antibody in that serum.

Results

Binding of γ-globulin to polystyrene tubes was studied first. In dilute solutions, 90% of the human γ-globulin was adsorbed to 12 × 75 mm polystyrene tubes, up to about 5 μg/tube (13). When tubes coated in this manner with various amounts of human IgG were reacted with peroxidase-conjugated goat anti-human γ-globulin, a proportional relationship was observed between color yield and the logarithm of the protein adsorbed to the tube. A typical assay curve is shown in Figure 1. In general, the assay worked in the range of 125 to 5000 ng/ml of protein. The assay curves were sigmoidal, and leveled off at an absorbance of about 0.25. This leveling off was associated with exhaustion of the enzyme-antibody conjugate, for the final absorbance values were higher if the conjugate was used in greater concentration. Both the maximal color yield and the slope of the curve varied from day to day. When the assays resulted in curves with low color yield and shallow slope, the color yield and slope were increased by adding more antibody enzyme conjugate. This assay was used as the standard to calculate micrograms of antibody attached to the DNA in the anti-DNA system.

To show that tubes coated with DNA could be used to measure the anti-DNA content of serum, we chose a serum from a patient with SLE and high DNA-binding capacity for assay (Figure 2). The slope of the curve was similar to that formed by incubation of γ-globulin; it leveled off at an absorbance of about 0.25, the maximum color yield of the system. This serum was then used as the reference standard for anti-DNA. Sera from healthy subjects yielded little color.

In initial experiments it was found that undiluted control sera yielded absorbance values >0.05; at a dilution of 10-fold, many yielded an absorbance >0.05. At a dilution of 25-fold, the absorbance values of most controls were <0.05, and those of many sera from patients with SLE were >0.05. All sera was therefore studied at this dilution. To minimize any effects of day-to-day variation in the assay, the results were plotted as a percentage of the absorbance of the standard positive serum run on that day (Figure 3). On each of six separate occasions, four different samples were run. The average coefficient of variation was 35%. All 39 sera from healthy controls yielded values below 30%; all but three were below 20%. Of 42 sera from patients with SLE, values above 20% were obtained on 26, and values above 30% on 18. Of 38 sera from patients with renal diseases, two had values above 30%; two of eight sera from patients with rheumatoid arthritis gave values over 30%.

All but three of the sera from SLE patients with values over 30% were titrated by dilution, and micrograms of antibody was calculated. Sample titration curves are presented in Figure 4. The slopes for sera with high antibody titers were similar to that of the standard lupus serum; a leveling off of color yield was observed at higher serum concentrations. With few exceptions, sera with lower antibody titer showed increasing color yield that did not level off at the 25-fold dilution. A few sera (Figure 4, open circles) gave a different pattern, with leveling off at an intermediate value.
The titrations of all sera with high values were converted to micrograms of antibody per milliliter (Figure 5). Of the 15 sera presumed to be positive, 14 gave anti-DNA antibody concentrations of 10 μg/ml.

Figure 6 compares results of the titration of DNA antibody with those for antinuclear antibody obtained on tissue. As the tissue test for anti-nuclear antibody is positive if the serum contains antibody to DNA or to several other antinuclear antibodies, it might be expected to be positive considerably more often than a test for only one of these antibodies, the anti-DNA antibody, and this was observed. We found a high anti-DNA titer only when a high titer of anti-nuclear antibody titers yielded low color values. This was to be expected, because the tissue test has broad specificity.

When sera with color yields greater than 20% were titered and plotted vs. the antinuclear antibody titer, a good relationship between the two assays was observed (Figure 7). Those sera with high values in the test tube system for anti-DNA antibody had high titers by the tissue assay for antinuclear antibody. No sera were found with high anti-DNA and low antinuclear antibody titers; six sera gave high antinuclear antibody but a relatively low anti-DNA titer.

Discussion

In this study, we propose that a solid-phase non-competitive binding assay, with use of a second antibody conjugated to an enzyme as the quantitative or detector system, is a valid way to measure antibody to DNA in serum. From Figure 1 it is evident that a proportional quantitative relationship exists between the amount of human γ-globulin bound to a polystyrene tube and the color yield obtained when the
bound γ-globulin is incubated in the presence of an excess of anti-human γ-globulin coupled to the enzyme peroxidase. Similar data have been obtained by Perlmann and Engvall, who used an antibody to bovine serum albumin conjugated to the enzyme alkaline phosphatase (14).

The titration data of sera containing antibody to DNA (Figures 2 and 4) yielded curves similar to that in Figure 1, thus indicating that the system was measuring proportionately greater amounts of antibody. The observations of Tan and Epstein (6) show that on the polystyrene substrate coated with DNA, binding of antibody is proportional to the amount used. Thus, in a given range the amount of antibody bound in this system is proportional to the amount added.

At this point it is worthwhile to consider the assumptions underlying the four techniques used for the detection and quantitation of antinuclear anti-DNA antibodies, along with their respective advantages and disadvantages (Table 2). In the immunohistologic method the antiserum is taken up stoichiometrically and detected by an excess of antibody labeled with a fluorescent or enzyme molecule. The actual reaction measured is that of the immunoglobulin; its distinct advantage is that the reaction can be seen with a broad range of antigens. However, a specific antigen is often difficult to identify, and quantitation is by dilution.

The solid-phase colorimetric assay measures the antibody to DNA by its reaction with the coated surface and then quantitates that immunoglobulin molecule. Its disadvantages are high background, variability of the method, and difficulty in preparing the conjugate. Careful titration partially obviates the variability.

The method of Tan and Epstein measures the bifunctional binding component in the serum to DNA and is highly specific for the antigen. Its disadvantages are theoretical: (a) the antibody is presumptive; i.e., it is assumed that it is immunoglobulin rather than a binding protein for DNA; and (b) it is not quantitative for a specific protein. Technically, it is often difficult to make or obtain labeled antigen.

Finally, the Farr technique has some advantages in being broadly specific for serum components binding to DNA. It has the same theoretical disadvantage as the method of Tan and Epstein (6) in that the antibody is presumptive and it is not possible to

![Graph showing comparison of anti-DNA and antinuclear antibody titers in all sera from patients with SLE](image)

**Fig. 7.** Comparison of anti-DNA and antinuclear antibody titers in all sera from patients with SLE

<table>
<thead>
<tr>
<th>Presumed substance measured</th>
<th>Solid-phase enzyme-linked</th>
<th>Immunohistology (12 &amp; 17)</th>
<th>Solid-phase binding (9)</th>
<th>Ammonium sulfate (5 &amp; 15)</th>
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<tbody>
<tr>
<td>Anti-DNA</td>
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<td>Anti-DNA</td>
<td>Anti-DNA</td>
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<tr>
<td>Ig Ab to DNA</td>
<td></td>
<td>Ig Ab to tissue nuclear Ag</td>
<td>Bifunctional binding component in serum to DNA</td>
<td>Ppt. protein, which binds to DNA</td>
</tr>
<tr>
<td>ng Ig</td>
<td></td>
<td>Serial dilution with known + anti-serum</td>
<td>ng DNA bound</td>
<td>ng DNA bound</td>
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**Table 2. Comparison of Assumptions, Advantages, and Disadvantages of Methods for Detection of Antinuclear (Anti-DNA) Ab**

- **Advantages**: specific for Ag or for Ab; ng Ab protein measured; no labeling of Ag, patient Ab
- **Disadvantages**: high background and variability
- **Application to detection of other AbAg systems**: easy, provided Ag adheres to support
quantitate it as a specific immunoglobulin. In addition, experience has shown that results by the Farr technique are rather variable (15).

Another major difference between the method described herein and those of Tan and Epstein and of Farr is that the two latter methods quantify the amount of antigen bound, whereas the first technique quantifies the antibody itself. This is apparent when the difference in the number of antigenic sites on single and double-stranded DNA is considered. If quantitation is by radioactive counting of the DNA, then it is likely that there is a difference in the amount of DNA bound by two antibodies, one to single- and one to double-stranded DNA, and the number of counts bound do not necessarily have the same relationship in both cases. In the technique presented here, color yield is proportional to antibody bound. Because the number of DNA antigenic sites, either single or double-stranded on the tube, are in excess, this factor does not influence the assay.

The method of solid-phase immunoassay described has a unique characteristic in that there is no competition for either antigen or antibody. Every reagent is in stoichiometric excess except the antibody in the test serum. Thus, all the antibody binds quantitatively to the DNA on the tube. There is no competition for the labeled antibody–enzyme system, because this is present in excess.

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