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A SENSITIVE SOLID PHASE MICRORADIOIMMUNOASSAY FOR ANTI-DOUBLE STRANDED DNA ANTIBODIES

FALK FISH and MORRIS ZIFF

A sensitive solid phase microradioimmunoassay has been developed for measurement of anti-double stranded DNA (dsDNA) antibodies. In this procedure, advantage has been taken of the capacity of poly-L-lysine (PLL) to facilitate the binding of pure dsDNA to plastic surfaces. In the absence of PLL, binding did not occur. Diluted sera were incubated in PLL-treated dsDNA-coated microtitration trays and anti-dsDNA Ig was measured using affinity purified ^{125}I-anti-Ig of high specific activity. The synthetic DNA, poly dA-dT, was used as a model for dsDNA. In initial experiments, specific anti-DNA binding could not be demonstrated because of high background binding of patient Ig to PLL-treated surfaces. This was reduced by diluting test sera and anti-Ig in buffer containing 2% BGG and 1% BSA. Specificity of the assay for DNA was demonstrated by absorbing the anti-DNA activity on DNA-coated plastic. The binding of systemic lupus erythematosus (SLE) patient serum Ig to poly dA-dT coated trays did not diminish after digestion with nuclease S_1, suggesting that the synthetic polymer is an appropriate model for dsDNA. Patient and normal sera were screened for anti-dsDNA activity using poly dA-dT as antigen. None of the 38 normal sera, 23 of 35 active SLE sera, 1 of 25 treated SLE, 4 of 35 rheumatoid arthritis, 3 of 35 scleroderma, and 1 of 13 polymyositis sera demonstrated positive anti-dsDNA activity. The anti-dsDNA values obtained in the radioimmunoassay correlated significantly with those obtained in the *Crithidia luciliae* assay.

Antibodies to double-stranded DNA (dsDNA) are generally considered to be specific for systemic lupus erythematosus (SLE) (1–3) and the SLE-like syndrome of some autoimmune mouse strains (4). These antibodies have been considered the principal factor in the pathogenesis of lupus nephritis (5,6) and are of importance in assessing the state of the disease (7–11). The occurrence of anti-dsDNA in other disease states reported by some workers has been attributed to the molecular form of the DNA preparations used in the different assays (12,13).

The current techniques commonly used for measuring anti-dsDNA, namely, the membrane filter binding assay (14) and the ammonium sulfate precipitation assay (15) have their inherent deficiencies: they may detect proteins other than immunoglobulins interacting with DNA (16,17); the radioactively labeled DNA may be subjected to radiation damage (12); and the methods may not be sensitive enough to measure low concentrations of antibody in dilute fluids, such as tissue culture supernatants, without concentration before assay (18). The solid phase type of immunoassay offers high sensitivity because of the amplification of the measurements by the isotope or enzyme-labeled secondary anti-Ig antibody utilized in this type of procedure. In addition, it is possible to determine the Ig class of the anti-DNA antibody by this technique.

In spite of these theoretical advantages of solid

From the Department of Internal Medicine, Rheumatic Diseases Unit, University of Texas Health Science Center at Dallas, Dallas, Texas 75235.

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Falk Fish, PhD: Faculty Associate; Morris Ziff, MD, PhD: Professor and Chief, Rheumatic Diseases Unit, Department of Internal Medicine, University of Texas Health Science Center at Dallas.

Address reprint requests to Morris Ziff, MD, Department of Internal Medicine, University of Texas Health Science Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235.

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phase immune assays, there are only a few reports of attempts to adapt this type of method, using DNA coated plastic tubes as the solid absorbent, to the measurement of anti-dsDNA antibodies (19–22). Pesce et al (20) have used the ELISA version of the solid phase immunoassay and reported poor reproducibility. Lange et al (21) have used radioimmunoassay to measure anti-DNA and detected increased binding of nonspecific Ig from autoimmune sera to the gelatin coated plastic. These reports have led Pesce et al (23) to conclude that technical and biologic problems interfere with the application of solid phase assays to the measurement of anti-DNA antibodies.

A reason for technical difficulty has been recently identified. Pure double-stranded DNA does not bind to plastic unless the surface is first treated with poly-L-lysine (PLL), a positively charged polymer (22). At the same time there is binding of high affinity Ig in autoimmune sera to the solid phase (21). Therefore solid phase immunoassays attempting to demonstrate specific anti-dsDNA must include a basic specificity control for each sample tested, namely, the binding of Ig to a surface not coated with DNA, but otherwise treated identically with the DNA-coated surface.

The present communication describes a method for the measurement of specific anti-native DNA antibody in which the high surface binding of immunoglobulin from autoimmune sera has been reduced. Under the conditions employed, this assay is specific and highly sensitive. Sensitivity has been increased by the use of affinity purified anti-Ig iodinated to high specific activity by a simple and reproducible radioiodination method.

MATERIALS AND METHODS

Sera. Sera was obtained from 35 patients with active systemic lupus erythematosus (SLE) and 25 patients with treated SLE. All patients met the American Rheumatism Association (ARA) criteria for systemic lupus erythematosus (24). The criteria for the activity of the SLE patient have been previously reported (25). Sera from 35 patients with definite or classic rheumatoid arthritis, 35 with scleroderma, 13 with polymyositis or dermatomyositis, and 38 normal individuals were also examined. Most of the sera of patients with connective tissue disease were obtained from the inpatient services of Parkland Memorial Hospital and were previously assayed for anti-DNA antibodies by Chubick et al (25) employing the *Crithidia luciliae* method (26). Some sera were provided by Dr. S. Cohen from patients of the St. Paul Hospital inpatient and outpatient services. The normal sera were collected from laboratory staff.

Antisera. Goat anti-human IgG directed against heavy and light chains was produced by Miles Yeda, Rehovot, Israel. As determined by immunoelectrophoresis, this antiserum demonstrated activity against IgG and also some activity against IgM.

IgG. Crude human IgG was precipitated from human Cohn fraction II (Sigma, St. Louis, Missouri) by ammonium sulfate (27).

Immunoabsorbents. Sepharose 4B (Pharmacia, Uppsala, Sweden) was activated with cyanogen bromide by the method of March et al (28). The freshly activated resin was then reacted with a 10 mg/ml solution of human IgG. After incubation for 16 hours at 5°C, the resins were washed and the remaining active groups were neutralized with 1.0*M* glycine.

Affinity purification of anti-IgG antisera. One milliliter of heat inactivated anti-human IgG (56°C, 30 minutes) was incubated for 2 hours at room temperature with 2–4 ml of appropriate immunoabsorbent. The resin was then washed with phosphate-buffered saline (PBS) until the optical density of the wash at 280 mμ was less than 0.01. The bound antibody was eluted with 0.5*N* acetic acid (29) at room temperature. The eluate was neutralized and dialyzed overnight at 5°C against PBS. After centrifugation at 10,000 *g* for 10 minutes, the purified antibody was concentrated by ultrafiltration to 1 mg protein/ml. Aliquots, 0.1 ml, of this preparation were stored at −20°C.

Radioiodination of purified anti-IgG. This was performed by the chloramine T method (30) using one atom of carrier iodide per molecule of protein (31). To 100 μl of the purified antibody solution (1 mg/ml PBS) reagents were added in the following order: 1) 10 μl potassium iodide ($6.25 \times 10^{-5}M$), 2)10 μl of solution containing 1 mCi ^{125}I-sodium iodide (13–17 mCi/μg I, Amersham Co., Arlington Heights, Illinois), and 3) 10 μl of freshly prepared chloramine T solution (10 mg/ml) (Eastman, Rochester, New York). The mixture was agitated for 30 seconds at room temperature. The iodination reaction was terminated by the successive addition of 0.1 ml of a freshly prepared solution of sodium meta bisulfite (2 mg/ml), 0.1 ml fetal calf serum, and 0.7 ml PBS.

The iodination process was monitored with potassium iodide-starch indicator paper after the addition of the chloramine T for the existence of excess of chloramine T and absence of unreduced chloramine T after the addition of sodium meta bisulfite. In addition, a 2 μl sample was removed after completion of the reaction and added to 1 ml of 2% bovine serum albumin (BSA) and precipitated with 1 ml 10% TCA. The suspension was spun at 4,000*g* and the incorporation of the radioactive isotope into protein then determined. The incorporation values were consistently about 90%. Unreacted iodide was then removed by incubating the mixture with 0.2 ml of washed Dowex 1-x10 beads for 10 minutes at room temperature. After this treatment the amount of TCA nonprecipitable radioactivity was reduced to less than 2% of the total. The radiolabeled anti-IgG preparation was stored at −20°C in 50 μl aliquots.

Nucleic acids. The synthetic double stranded copolymer of deoxyadenosine and deoxythymidine (poly dA-dT) and the synthetic homopolymers of deoxyadenosine (poly dA) and deoxycytidine (poly dC) were purchased from Miles Laboratories (Elkhart, Indiana). They were dissolved in PBS containing 25% ethanol to a concentration of 5 A_{260} units/ml (approximately 250 μg/ml) and stored at −20°C. Denatured calf thymus DNA was prepared by heating a 0.1 mg/ml solution

Table 1. Binding of ^{3}H-poly dA-dT to polyvinyl microtitration trays

^{3}H poly dA-dT added (μg/ml)*	% bound to	
	Untreated wells	PLL coated wells
2.5	0.2	19.7
5	0.1	17.5
10	0.04	10.8

* 50 μl/well in Tris-HCl (pH 7.3).

of calf thymus DNA (Sigma) in water for 15 minutes at 100°C followed by rapid cooling in an ice bath. ^{3}H-poly dA-dT (0.05 μCi/mg) was obtained from Miles Laboratories.

DNA coated microtitration trays. A 50 μg/ml solution of poly-L-lysine (PLL type VII-B, Sigma) in 0.1*M* Tris-HCl buffer, pH 7.3 (32), was prepared immediately before use. Twenty-five microliter aliquots of the PLL solution were introduced into each well of a V-shaped polyvinyl microtitration tray (Dynatech, Alexandria, Virginia). Special care was given to spread the solution over the bottom of the well by lightly tapping the plate. After 45 minutes of incubation at room temperature the trays were washed three times in normal saline and 25 μl of poly dA-dT solution, 10 μg/ml, in Tris buffer (optical density at 260 mμ = 0.2) were introduced into each well of alternate rows in the tray. These wells were designated DNA+ wells. The wells in the other rows were incubated with Tris buffer only (DNA− wells). After 60 minutes' incubation at room temperature, the trays were washed three times in normal saline and coated with 2% BSA (Fraction V, Sigma) in Tris buffer for 30 minutes at room temperature as a means of blocking protein binding sites on the plastic surface ("blocking coat").

Single stranded DNA coated trays. A mixture of poly-dA (5 μg/ml) and poly-dC (5 μg/ml) in Tris buffer was introduced into PLL-coated microtitration trays as described previously. Heat denatured calf thymus DNA was suspended in Tris buffer to an optical density at 260 mμ of 0.2 and similarly introduced into the microtitration trays.

Assay for anti-DNA antibodies in human serum. The serum to be tested was heat inactivated at 56°C for 30 minutes and serially diluted in the reaction buffer solution; the starting dilution for screening human sera was 1:100. The buffer solution consisted of 0.1*M* Tris buffer, pH 7.2, containing 2% w/v bovine gamma globulin (BGG, Cohn's Fraction II, Sigma) and 1% w/v BSA (Cohn's fraction V, Sigma). The BSA solution was then removed from the DNA coated tray previously prepared and the tray was washed once with normal saline. Twenty-five microliter aliquots of the diluted serum were delivered into the DNA+ and DNA− wells. After 3 hours' incubation at room temperature, the tray was washed five times in normal saline. Twenty-five microliters of the ^{125}I-anti Ig, diluted 1:100 in 2% BGG, 1% BSA were then added to all wells. The tray was covered tightly with Parafilm (American Can Co, Greenwich, Connecticut) or with wet paper towel and incubated overnight at room temperature. The assay was terminated by washing the trays five times in normal saline and drying over a warm hotplate.

The bottoms of the wells were cut from the dried trays with scissors and the bound radioactivity was measured in a Packard Autogamma Spectrometer model 5230. The specific anti-DNA activity was calculated by subtracting the radioactivity bound to DNA− wells from that bound to DNA+ wells. Negative values were considered equal to zero. The results were expressed either as counts per minute or as the amount of ^{125}I-anti Ig specifically fixed to DNA.

Nuclease S_1 treatment of DNA coated trays. Nuclease S_1 (from *Aspergillus oryzae*, Type III, Sigma) was diluted in 0.1*M* NaCl, 0.05*M* acetate pH 4.6 to a final concentration of 2600 units/ml. Fifty microliters of the enzyme solution were introduced into DNA+ and DNA− wells immediately after the DNA coating phase of the microtitration tray preparation. After 20 minutes incubation at 37°C, the tray was washed in saline 3 times and covered with 2% BSA as usual. The enzyme nontreated wells were incubated in the same buffer solution (NaCl, acetate pH 4.6) containing 5% glycerol (to compensate for the glycerol present in the nuclease preparation).

RESULTS

The effect of PLL treatment on DNA surface binding. To examine the effect of PLL on DNA binding, the wells of microtitration trays were incubated with 50 μl aliquots of a solution of PLL (50 μg/ml) in Tris buffer. After 45 minutes at room temperature the PLL solution was removed and the trays were washed three times in normal saline. Fifty microliter aliquots of solutions of ^{3}H-labeled poly dA-dT at various concentrations in Tris buffer were pipetted into both PLL-treated and PLL-untreated wells in the microtitration tray. After 60 minutes at room temperature, the solution was removed and the tray was again washed and the wells counted. The percent of added ^{3}H-poly dA-dT bound to the wells was calculated. The results are summarized in Table 1. It is seen that the synthetic DNA, poly dA-dT, did not bind to the polyvinyl surface unless this surface was first treated with PLL. This confirms the observations of Aotsuka et al (22) and extends them to the binding of poly dA-dT to polyvinyl surfaces.

Inability to demonstrate specificity for DNA in SLE serum diluted in buffer alone. As an initial approach to the assay of anti-dsDNA antibodies in human serum, tested sera were diluted in Tris buffer. Twenty-five microliter aliquots of the serum dilutions were introduced into the wells of DNA-coated microtitration trays containing alternate rows of DNA-coated and DNA-uncoated wells. After 3 hours' incubation at room temperature, the incubation mixtures were removed and the trays washed. Twenty-five microliters of ^{125}I-anti-Ig diluted 1:100 in Tris buffer containing either 2% BSA or 2% BGG plus 1% BSA were then introduced into all the wells of the trays, which were then incubated overnight at room temperature. Thereafter, the trays were washed and counted. The results, presented in Table 2, show that under the conditions employed in this experi-

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