Enzyme-linked Immunosorbent Assay for Detection of Antibodies to Extractable Nuclear Antigens in Systemic Lupus Erythematosus, with Nylon as Solid Phase

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In this enzyme-linked immunosorbent assay (ELISA) for detection of antibodies against extractable nuclear antigens (ENA) in sera of patients with systemic lupus erythematosus (SLE), nylon is used as solid phase for antigen binding instead of the commonly used polystyrene surface. Optimal conditions for activation of the nylon beads, antigen coating, and other relevant factors have been investigated. We compared the incidence of anti-ENA antibodies in SLE, using chromogenic and fluorogenic enzyme substrates. Of SLE patients, 54% were positive for anti-ENA antibodies when chromogenic substrate was used as compared with 68% for fluorogenic substrate. Antibody activity against Sm and RNP antigens was distinguished on the basis of ribonuclease sensitivity of the RNP antigen. The method described offers advantages such as decreased background activity, increased surface area, facility for prolonged storage of antigen-coated solid phase, and miniaturization of the assay.

Additional Keyphrases: autoantibodies • extractable nuclear antigens • autoimmune disease

Antibodies against saline-soluble antigens of mammalian tissues called "extractable nuclear antigens" (ENA) are characteristic of certain autoimmune diseases (1). Among the highly conserved cell components included in ENA are the so-called Sm and RNP antigens (2). Antibodies to Sm are present predominantly, if not exclusively, in systemic lupus erythematosus (3), whereas antibodies against RNP are seen in high concentration in mixed connective-tissue disease (4).

Several techniques have been used to detect anti-ENA antibodies (5). Recently, an enzyme-linked immunosorbent assay (ELISA) was described for their detection in which polystyrene microtiter plates are used for antigen binding (6). The peroxidase (EC 1.11.1.7) activity of the conjugate was measured with a chromogenic substrate. The sensitivity and reproducibility of an ELISA can be limited by such factors as the type of solid phase used for antigen binding and the method used for determining the enzyme activity. Seeking to achieve the full potential of such assays, workers have made several modifications: (a) use of various materials as solid support, to increase the surface area and decrease background activity (7, 8); (b) use of fluorogenic enzyme substrates in monitoring the enzyme activity (9); and (c) modifications in the assay procedure and in data expression (10).

Nylen has been used for the covalent immobilization of antibodies for use in enzyme immunoassays (11). Brief treatment of nylon with HCl exposes free amino and carboxyl groups and makes it highly reactive.

Here we describe an enzyme-linked immunosorbent assay for detection of anti-ENA antibodies in cases of systemic lupus erythematosus (SLE), with activated nylon used as the solid support for antigen binding and alkaline phosphatase (EC 3.1.3.1) conjugated goat anti-human IgG for the detection of bound immune complex. We compared the incidence of such antibodies in SLE, using both chromogenic and fluorogenic substrates. Antibodies against Sm and RNP were distinguished on the basis of RNase (EC 3.1.4.22) sensitivity of the latter.

Materials and Methods

Extractable nuclear antigens. A powdered acetone extract of buffalo thymus prepared by the method of Horvicker (12) and extracted according to Kurata and Tan (13) was the source of ENA. Proteins, RNA, and DNA, estimated in the extract as described elsewhere (14), were in the ratio of 8:1:0.06 by weight.

RNase digestion of buffalo thymus extract. Buffalo thymus extract was treated with RNase A for 1 h at 37 °C. The ratio of enzyme to substrate was 1:10 by weight.

Sera. Normal sera were obtained from ostensibly healthy subjects. Sera of patients with SLE (meeting the preliminary criteria of the American Rheumatism Association) were obtained as described (14).

Nylen. Nylen 66 (BDH Chemicals Ltd., Poole, England) was used as solid phase for the coating of ENA. These nylon beads (3 × 2 mm) were treated with 3.5 mol/L HCl for 36 h at 37 °C, then washed with distilled water until they were free of acid. Activation of the beads, in terms of exposure of amino groups, was checked qualitatively by treatment with picryl sulfonic acid, a reagent that reacts with amino groups (15). Alkaline-phosphatase-conjugated goat anti-human IgG, ribonuclease A, 4-methylumbelliferyl phosphate (4 MU-P), 2-amino-2-methyl-l-propanol, bovine serum albumin (BSA), and p-nitrophenyl phosphate (PNP-P) were from Sigma Chemical Co., St Louis, MO 63178.

Procedure for ENA-ELISA. Activated nylon beads were incubated separately with untreated and RNase-treated buffalo thymus extract at a protein concentration of 150 mg/L in McIlvaine's buffer, pH 5.0 (20 mmol of Na2HPO4, 150 mmol of NaCl, pH adjusted with 100 mmol/L citric acid, and diluted to 1 L) for 12 h at 4 °C, with occasional shaking. The beads were freed of unreacted antigen by washing four times with saline-Tween 20 (per liter, 150 mmol of NaCl and 0.5 mL of Tween-20 surfactant). Unoccupied sites were filled by exposure to 145 μmol/L BSA in saline–Tween for 12 h at 4 °C. The beads were washed four times with saline–Tween to remove excess BSA. Beads treated with BSA alone were washed again and treated with 20 μmol/L enolase in saline–Tween for 12 h at 4 °C. The washed beads were dried and stored at below –20 °C. After washing, unreacted antigen was extracted from the beads by incubation of 10 mg of beads in 1 mL of McIlvaine's buffer, pH 5.0, containing 0.4 mg of ribonuclease A for 1 h at 4 °C, followed by centrifugation at 10,000 rpm for 15 min. The supernatant containing antigen was stored at below –20 °C. All reagents used were of highest purity and were obtained from Sigma Chemical Co., St Louis, MO 63178.

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without prior antigen coating were used as control. Antigen-coated and BSA-saturated beads were dried and stored at 
-20 °C until use.
Sera were tested for anti-ENA antibodies by use of five 
antigen-coated beads. All the sera were assayed at 100-fold 
dilution in saline–Tween.
In a typical procedure, five antigen-coated, BSA-saturated 
beads were transferred to a 7.5 × 1 cm test tube and 
incubated with 0.5 mL of diluted serum for 1 h at 37 °C, with 
occasional mixing. Unreacted serum was washed away by 
four 2- to 3-min washings with saline–Tween. Next, the 
beads were incubated with 0.5 mL of 1000-fold diluted 
conjugate (dilution made in saline–Tween) for 2 h at 37 °C, 
with occasional shaking. Unreacted conjugate was washed 
away by four washings as above, plus one with distilled 
water. The beads were dried on Whatman filter paper and 
transferred to a clean tube containing 1 mL of substrate 
(PNP-P, 2.7 mmol/L in 1 mol/L diethanolamine buffer, pH 
9.8, or 4 MU-P, 2 mmol/L in 150 mmol/L 2-amino-2-methyl- 
1-propanol buffer containing 3 mmol of MgCl2 per liter, pH 
10.3).

The tubes were incubated for 1 h at 37 °C, with occasional 
shaking. The reaction was stopped by adding 1.0 mL of 3 
moL/L NaOH (for PNP) or 1.0 mL of 1 mol/L K2HPO4–KOH 
buffer, pH 10.4 (for 4 MU-P). The colorimetric reaction was 
monitored at 420 nm with a Spekol-10 spectrophotometer 
(Veb Carl Zeiss, Jena, F.R.G.). Fluorescence intensity (FI) 
was recorded with an Amino-Bowman spectrophotofluo-
rometer (American Instrument Co., Inc, Silver Spring, MD 
20910). Excitation and emission wavelengths were 365 nm 
and 455 nm, respectively. Each serum sample was run 
simultaneously with antigen-coated and control beads. All 
the sera positive for ENA antibodies were tested with 
RNase-treated antigen-coated beads, to distinguish Sm and 
RNP antibodies. Results were expressed as A (or FI)test – A 
(or FI)control.

Results

Nylon proved satisfactory as solid phase owing to the 
increased surface area available for binding, low back-
ground noise, and ease of washing. A series of preliminary 
experiments was carried out with positive SLE sera to 
define the experimental conditions for optimum results. 
These experiments were carried out in triplicate and results 
expressed as the means.

Activation of nylon. Treatment of nylon beads with 3.5 
moL/L HCl exposed reactive amino and carboxyl groups 
necessary for antigen adsorption. Figure 1 shows the abso-
bance values obtained in ELISA with nylon beads activated 
for different time periods. We chose a 36-h activation, 
because with longer times the beads tend to crack.

Antigen coating. Coating was at 4 °C for 12 h because of 
the relative lability of antigens, especially RNP, at room 
temperature.

To select the appropriate pH at which the coating was to 
be performed, we determined the effect of pH on antigen 
coating as follows. A series of activated nylon beads was 
treated for 12 h with antigen prepared in buffers of various 
pH (3–10). The sensitized beads were processed for ELISA as 
described. Antigen coating was maximum at pH 5.0 (Figure 
2). Coating was therefore performed routinely at this pH.

Figure 3 compares the dose–response curve obtained 
when ELISA was carried out with beads coated with various 
concentrations of antigen (1–300 mg of protein per liter), 
with PNP-P and 4 MU-P as substrates. Our purpose was to 
find the optimum antigen concentration for coating and to 
compare the minimum concentration of antigen that could 
be detected with either PNP-P or 4 MU-P as substrates.

Apparentl y, the lowest concentration of antigen detected 
with 4 MU-P as substrate is 4 mg/L, whereas with PNP-P it 
is 20 mg/L; hence, sensitivity is increased fivefold with 
4 MU-P as substrate. The optimum antigen concentration 
was 150 mg/L. The dose–response curve was highly repro-
clicable, indicating the reproducibility of the antigen-coating 
procedure. Under identical conditions for antigen adsorption 
such as pH, protein concentration, and temperature, the 
test assay (n = 7) and intra-assay coefficients of variation 
of these curves were 8.3% and 4.6%, respectively.

Experiments were also performed to find the optimal 
time for coating. Beads were coated with use of 150 mg 
of antigen per liter for different intervals from 1 to 36 h. After 
each interval the beads were processed for ELISA. Although 
coating attained equilibrium in 6 h (Figure 4), we routinely 
coated the beads for 12 h.

Experiments similar to the above were also done to 
optimize the incubation interval for serum, conjugate, and 
substrates. These experiments showed that a 1-h incubation
of serum, 2-h incubation with conjugate, and 1-h incubation with substrates are appropriate.

Number of beads. When ELISA was done with use of various numbers of antigen-sensitized beads, there was a nearly linear relation with absorbance values up to seven beads; thereafter, the values remained constant (Figure 5). For technical reasons, such as ease in washing and low background activity, we chose to use five beads for anti-ENA determination.

Storage of antigen-coated beads. ELISA, when carried out with antigen-coated beads that had been stored dry at 4 or −20 °C, showed almost no change in absorbance values with beads stored up to three weeks, but a 20% decrease in these values was observed for those stored more than 30 days. Decreased absorbance values were also seen if the beads were not stored dry. We suggest that antigen-coated beads, stored dry at 4 or −20 °C, can be used for ELISA for no longer than three weeks without appreciable loss in antigenicity.

Inhibition ELISA. The specificity of the ELISA was checked by carrying out an inhibition experiment as follows. Anti-

gen in various concentrations was mixed with positive antiSm and RNP sera so that the final concentration of antigen protein in the mixture varied from 10 to 50 mg/L in 100-fold diluted serum. The solutions were kept at 4 °C for 2 h, then used for testing inhibition of ELISA. The results of such an experiment are shown in Figure 6.

ENA-ELISA. We tested normal human sera and sera from patients with SLE for anti-ENA antibodies as described, using chromogenic (PNP-P) and fluorogenic (4 MU-P) enzyme substrates. Figure 7 gives the results of these experiments.

Twelve of 22 (54%) SLE patients were positive for antibodies to ENA when PNP-P was used as enzyme substrate; with 4 MU-P as substrate, 15 of 22 (68%) were positive. All of the normal human sera were negative for anti-ENA antibodies. Antibodies against ribonuclease-sensitive anti-
Fig. 7. Incidence of anti-ENA antibodies in SLE as measured by ELISA with chromogenic and fluorogenic substrates

Sera from normal human subjects (NHS) and SLE patients were processed for ENA-ELISA as described, with chromogenic (PNP-P) and fluorogenic (4 MU-P) substrates. The mean (± standard deviation) values for absorbance and fluorescence intensity so obtained are indicated at the bottom of the figure.

Table 1. ENA-ELISA with RNase-Treated Antigen (15 Patients)

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<th>Fluorescence intensity x 10</th>
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Sera positive for anti-ENA antibodies with untreated antigen-coated nylon were retested along with beads coated with RNase-treated antigen. The enzyme activity was measured with use of fluorogenic substrate. Sera showing fluorescence intensity of greater than 5 were considered positive for anti-ENA antibodies.

Discussion

We report the successful use of nylon as a solid support for binding of ENA for use in ELISA in the detection of anti-ENA antibodies. The assay developed is simple, accurate, and conveniently performed, and is highly sensitive as compared with such commonly used techniques as immunodiffusion (15), counterimmunoelectrophoresis (13, 14), and hemagglutination (16). The sensitivity of the assay is further increased by using fluorogenic substrate.

The major constraint in the sensitivity of enzyme immunoassays is the background activity caused by nonspecific binding of enzyme conjugate on the solid phase. For perfection of such assays, nonspecific binding must be decreased. Our experiments with ENA-ELISA indicate that background activity is substantially removed when nylon is used as solid phase instead of the conventional polystyrene surface, which reportedly gives increased nonspecific reaction and variation in binding capacity from one product to another (8). Even different wells in the same microtiter plate have been shown to give different results (17).

We found that by simple modifications in the assay procedure the background reaction could be decreased considerably. Thus by performing the final reaction in separate tubes, the high background activity generally obtained because of nonspecific binding of conjugate onto the walls of the container is greatly eliminated. Moreover, by introducing the last washing step with distilled water and carrying out the color reaction with dried beads a further decrease in the background reaction was obtained (Table 3). By these modifications we have been able to increase the specific signal/background ratio by almost three-fold.

The method described for anti-ENA ELISA combines the advantage of high sensitivity obtained by the use of fluorogenic substrate and facility of prolonged storage of antigen-coated nylon. The latter is of considerable importance, because the daily time-consuming antigen coating procedure is avoided. These advantages make the present assay suitable and convenient for adaptation in clinical laboratories where many samples are to be handled for anti-ENA screening. Moreover, the antibody type (Sm or RNP) can simultaneously be identified by performing ELISA with RNase-treated antigen-coated beads. Besides its use in the routine anti-ENA antibody determination, the assay, especially in the miniaturized form, offers potential for use in conditions where only small amounts of antigen or antibody are available, with savings in reagents. Such a modification...
can be particularly useful in structural studies of purified Sm and RNP antigens.

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References