

Anti-ENA Antibody in Serum Determined by ELISA-Technique

Description of Method and Recommended Procedure

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An enzyme linked immune sorbent assay, ELISA, for determination of serum antibodies against two different extractable nuclear antigens, the RNase sensitive ribonucleoprotein (RNP), and the RNase resistant Sm-antigen is described. The technical variables are systematically evaluated and a recommended procedure selected and described.

Key words: Connective tissue diseases; extractable nuclear antigen; mixed connective tissue disease; ribonucleoprotein; RNA-antibody.

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CLINICAL ASPECTS

High serum titers of antibody against ribonucleoprotein (RNP), the RNase sensitive part of RNA, is a decisive feature of mixed connective tissue disease. Antibodies against the RNase resistant part of RNA, anti-Sm antibodies, are mainly seen in systemic lupus erythematosus. A sensitive, reproducible and comparatively simple technique for detection of anti-RNP antibodies and anti-Sm antibodies is highly needed in clinical routine. This paper describes a handy technique for detection of these antibodies, which can be established in most routine hospital laboratories.

Since the recognition of MCTD (mixed connective tissue disease) as a separate clinical entity (16) in the group of rheumatic diseases several studies have been performed with the purpose of revealing the serological and clinical characteristics of MCTD patients. Clinically, the syndrome is characterized by features similar to those of systemic lupus erythematosus (SLE), scleroderma and polymyositis (12, 16).

Since the clinical symptoms and signs do not selectively allow any clear distinction between MCTD and other rheumatic syndromes, attempts to accomplish a serological separation of the syndromes have been the object of several works (1, 5, 6, 8–14, 18) classifying autoantibodies directed against extractable nuclear antigens (ENA).

Various methods for detection of anti-ENA antibody have been used: counter immune

electrophoreses (1), hemagglutination inhibition (5, 6, 8, 9, 10, 12, 15, 16, 17) and indirect immunofluorescence (11).

We have adapted the enzyme linked immune sorbent assay (ELISA) technique according to the principle originally described by Engvall & Perlmann (2, 3, 4) as a 1-day microanalysis with a simple colorimetric endpoint. The purpose was to apply a simple, sensitive, non-radioactive, and specific procedure for anti-ENA-antibody detection.

The ELISA principle is based upon two assumptions:

a) that an antigen or antibody can be linked to an insoluble carrier surface and will retain activity.

b) that an enzyme marker can be attached to an antibody/antigen complex with preservation of both immunological and enzyme activity.

Both assumptions have previously been proven true (2, 3, 4).

Anti-ENA antibodies may be directed against the ribonucleoprotein (RNP) antigen, the RNase sensitive part of RNA or against the RNase resistant part of RNA called Sm-antigen.

This study describes in detail a recommended experimental procedure for anti-ENA antibody determination and presents some technical variables which, if uncontrolled, may influence the results of the assay.

MATERIAL AND METHODS

Normal material and patients

Eighty sera from blood donors were examined to find a normal range reference value.

The sera were grouped according to age of donor with 10 persons in each age group: 10–19 years, 20–29 years, 30–39 years, 60–69 years, and 20 patients in each age group: 40–49 years, 50–59 years. The female/male ratio was 1:1.

Five sera were derived from patients, all women, with clinical evidence of MCTD. These sera were also examined for anti-ENA antibodies in the laboratory of Prof. G. D. Sharp, USA and were all found RNP positive with titers above 1:100,000.

None of these sera were found positive for rheumatoid factor. The sera were collected during admission to the ward and had in some cases been stored at -20°C for different periods of time.

ENA-antigen. The ENA antigen was derived from calf thymus and prepared according to the principles described by Holman (7). The antigen preparation was performed at the State Serum Institute and kindly provided by M. Høier-Madsen. The antigen was delivered in freeze-dried state and stored at -20°C until use. No ENA antigen used was more than 120 days old.

Buffer solution. As routine a standard 0.1 M PBS buffer pH 7.2 was used. (NaH_2PO_4 , H_2O 0.0250

M, Na_2HPO_4 , $12\text{H}_2\text{O}$ 0.0748 M, NaCl 1.4581 M. H_2O ad 1 liter). The buffer was diluted 10 times with distilled water giving a final concentration of 0.01 M. In some experiments the buffer composition was altered (see below).

Tween buffer solution. To 100 ml 0.1 M PBS buffer pH 7.2 was added 0.5 ml Tween 20. This volume was diluted to 1000 ml with distilled water. The final concentration was 0.01 M.

Solid phase material. Polystyrene with the capability to unspecifically absorb proteins was used as solid phase material. Several different microtiter plates were tested including V-bottom, U-bottom and flat bottom varieties. As standard procedure (see results) we use the Croke microtiter flat bottom system, Dynatech[®], (M129A).

Albumin. 1% bovine albumin (Sigma No. A 4503) in 0.01 M PBS, pH 7.2 was used. Experimentally altered concentrations are described.

Colorimetric reagents. 1) Ortho phenylendiamin (Flutea 78440) suspended in citrate phosphate buffer pH 5 (243 ml 0.1 M citric acid + 257 ml 0.2 M Na_2HPO_4 + 580 ml H_2O), 2) H_2O_2 30% and 3) 0.1 N H_2SO_4 .

Peroxidase conjugated rabbit anti-human immunoglobulin. As routine we used a horse radish peroxidase conjugated rabbit anti-serum to kappa, lambda and heavy chains of pooled human immunoglobulins (DAKO code No. P222, P214, P215, P216). The solution was stored at 4°C .

RNase. Ribonuclease-A from bovine pancreas (Sigma A 4875). Activity 60 Kunitz-units/mg.

Spectrophotometer. The colorimetric reaction was registered in a Titertek[®] multiscan spectrophotometer which measures absorption directly in each microtiter well.

Pipettes. Titertek multipipettes semiautomatic 100 and 200 μl were employed.

STANDARD PROCEDURE

A) *Antigen-coating of the microtiter plate*

1) ENA-antigen is dissolved in PBS buffer 0.01 M, pH 7.2 with an antigen concentration of 0.1 mg/ml. 100 μ l antigen solution is placed in each microtiter well and incubated for 60 min at 24°C. The plate is emptied by shaking it upside down and repeated blotting on suction-paper.

2) The plate is washed with Tween buffer solution 0.01 M, pH 7.2., 200 μ l/well with manual multipipette, repeated 3 times with 3 min intervals.

3) Unoccupied polystyrene surface sites are covered by coating the tubes with a 1% bovine albumin PBS solution. 200 μ l is added to each well, and incubated for 60 min at 24°C.

4) Excess unbound albumin is removed by three manual, multipipette washings with PBS-Tween. Same washing procedure as A2.

B) *Incubating the antigen-coated plates with serum and enzyme marker*

1) Patient serum diluted 1:100 with PBS 0.01 M, pH 7.2 is placed in each well. The sera are simultaneously applied in four microtiter wells allowing quadruple measurements. After incubation for 60 min at 24°C the plate is emptied. A normal and a known pathological serum are included in the analysis along with suspected positive sera.

2) Washing procedure as described in A2.

3) Peroxidase conjugated rabbit anti-human immunoglobulin is diluted 1:500 with PBS 0.01 M, pH 7.2. 100 μ l suspension is added to each microtiter well and incubated for 60 min at 24°C.

4) Washing procedure as described in A2.

C) *Colorimetric reaction*

100 μ l freshly prepared ortho-phenylendiamin solution in citrate buffer pH 5.0 and H₂O₂ 30% is added to each well. The enzymatic reaction is allowed to persue for 10 min and then stopped by adding 0.1 N H₂SO₄, 100 μ l/well. The colorimetric value is quantitated by recording ex-

tinction in a spectrophotometer at 450 nm within 2 h after the reaction has been stopped.

RNAse treatment of antigen

It is well documented that ENA can be separated into two antigenic groups by treating it with RNAse.

a) A RNAse resistant component designated Sm-antigen.

b) A RNAse sensitive component designated RNP-antigen.

If a test serum is proven positive by the initial ELISA procedure, an enzymatic bovine RNAse treatment of the antigen prior to the anti-ENA antibody determination can show whether the antibody detected shall be classified as either anti-Sm (no extinction fall) or anti-RNP (extinction fall) -antibody.

Procedure. 0.5 ml ENA-antigen solution (7 mg/ml) is added to 35 ml PBS, 0.01 M, pH: 7.4 buffer solution with Ca⁺⁺ (0.9 mM/l) and Mg⁺⁺ (0.4 mM/l) ions. Final Ag concentration 0.1 mg/ml. 14 mg bovine RNAse (Sigma A 4875) is added to a glass test-tube. 2 ml Ag-suspension prepared according to the previous description is added. Final enzyme concentration 7 mg/ml. The ENA-antigen RNAse suspension is incubated for 60 min at 37°C allowing enzymatic digestion of the antigen. The altered antigen is thereafter incubated on the plate, following the same procedure as previously described.

RESULTS

Examination of technical variables

When one of the variables in the following results is changed, all other variables are kept constant and remain as described.

1) *Solid phase materials.* The microtiter plate we found most suitable was a flat bottom plate, Croke microtiter system Dynatech, which gave the highest extinction values for positive sera with corresponding low values for negative controls.

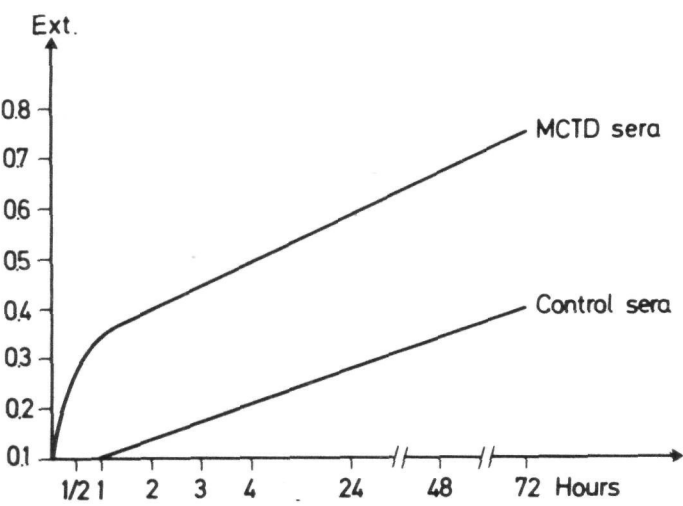


Fig. 1. Anti-ENA antibodies as function of incubation time in sera of two patients with mixed connective tissue disease, and controls.

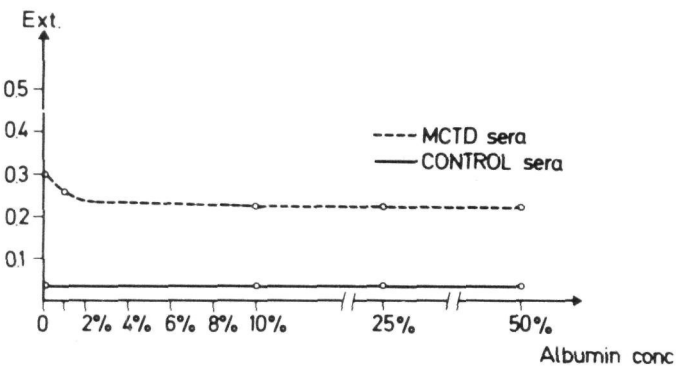


Fig. 3. Anti-ENA antibodies in sera as function of various albumin concentrations during incubation.

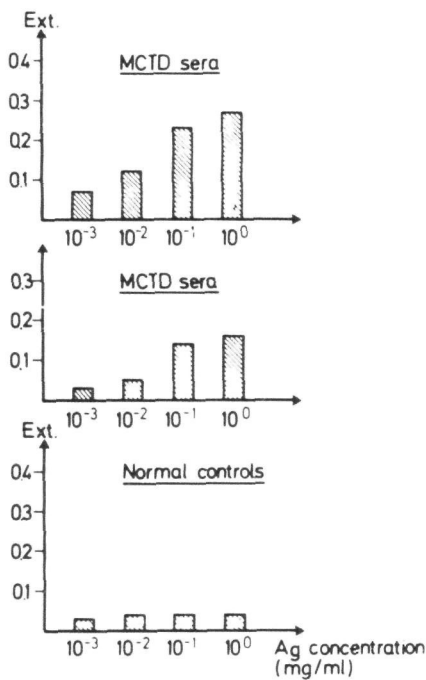


Fig. 2. Anti-ENA antibodies in sera as function of various ENA concentrations during antigen coating of plates.

2) *Effect of pH.* The effect of pH-variation under antigen incubation was tested with different buffer systems composed of carbonate- and phosphate-buffers with a pH variation from 5.0–10.0. Within these limits we found no dependence on pH variation, and a standard 0.01 M PBS buffer with pH value of 7.2 was chosen.

3) *Incubation time.* The incubation time was varied from 15 min – 72 h for each ELISA-step. The extinction values recorded were increasing

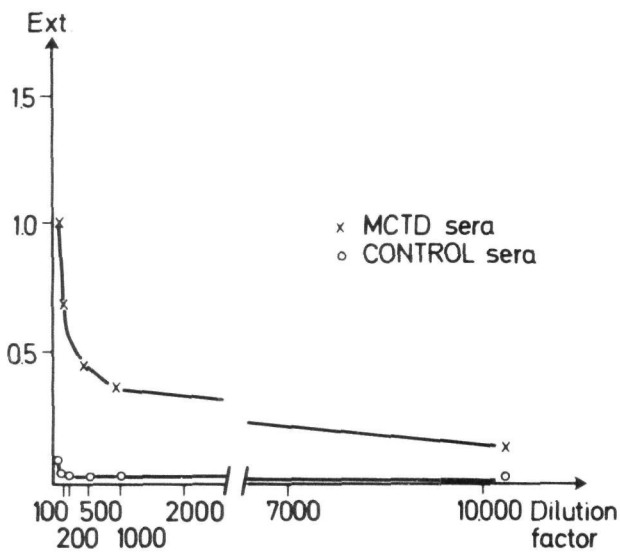


Fig. 4. Anti-ENA antibodies in sera as function of various serum dilutions.

within the first hour after which there was only a slight increase with concordant increase in negative controls, thus keeping the ratio positive/control constant. Therefore 1 h was selected as the incubation time for each step in the analysis (Fig. 1).

Effect of antigen concentration

Increasing the antigen concentration in the PBS solution for antigen-coating within the limits of 0.01 mg/ml to 1 mg/ml gave extinction values as shown in Fig. 2. It appears that an antigen concentration of 0.1 mg/ml gives the highest extinction values for positive sera as compared to a constant low value for controls. 0.1 mg Ag/ml was selected.

Table 1
Anti-ENA antibodies in sera at different incubation temperatures.

Ip	4°	20°	37°
MCTD sera	170	205	375
MCTD sera	185	225	365
CONTROL sera	32	30	50
CONTROL sera	25	30	60

Effect of bovine albumin concentration

The bovine albumin concentration was varied within the limits of 0% – 50%. As shown in Fig. 3 extinction values were high at concentrations below 1%. Above 1%, extinction values remain constant for positive as well as negative sera. There was no difference in this respect whether the antigen was RNase digested or not. 1% albumin concetration was therefore selected.

Effect of serum dilution

The effect of serum dilution is illustrated in Fig. 4. The dilution curve shows falling extinction values with increasing dilution factors for both positive MCTD sera and normal controls. The dilution factor 100 gives a clear distinction between positive and negative sera, and was chosen routinely.

Effect of incubation temperature on antigen-coating

The effect of different incubation temperatures on two controls and MCTD sera gave extinction values as shown in Table 1.

It appears that rise of temperature causes an increasing extinction value for MCTD patients but with a concordant rise for controls. However, control values remained low at 4°C and 20°C. Above this temperature control values increased and 20°C was therefore selected as incubation temperature.

Effect of RNase incubation outside microtiter plates

MCTD patients are known to have high titers of RNP-antibodies and would therefore be ex-

pected to have their high anti-ENA titers reduced to nearly normal levels after RNase treatment of the antigen. Initially, we incubated the ENA-antigen with pancreas RNase, at 37°C, directly in the microtiter wells. Interestingly, we found no titer fall in MCTD patients. This was even the case when the RNase incubation time was extended from 1 to 24 h, and when varying the concentrations of Ag and enzyme. Varying the incubation time for Ag alone and the joined reaction time of antigen and RNase likewise had no effect. Changing the Mg²⁺ and Ca²⁺ ion concentrations was also without significant effect.

However, by incubating the antigen with pancreas RNase in a test-tube for 1 h at 37°C, we obtained enzymatic separation of the ENA, and on microtiter plates coated with this enzyme-digested antigen product, it was possible to demonstrate a clear fall in extinction values for the MCTD patients.

Table 2

The effect on values of anti-ENA antibodies after preincubation ENA with RNase (7mg/ml) outside the microtiter plates

	RNase + Ag incubated on plate	RNase + Ag preincubated outside
MCTD patient	400	100
Normal control	100	100

A typical example is given in Table 2. As seen the RNase preincubation has reduced the extinction values for a MCTD patient to normal values. In other patients with positive ENA-titer and no clinical evidence of MCTD we found no such reduction.

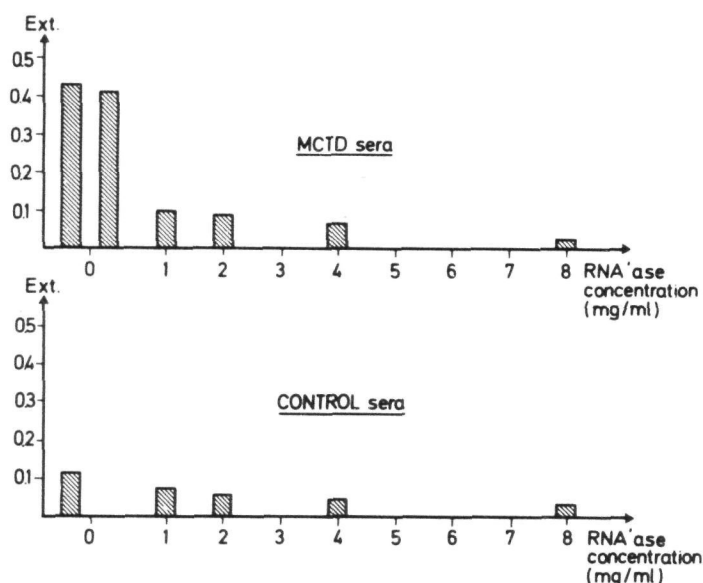


Fig. 5. Anti-ENA antibodies in sera after digestion of antigen with various RNase concentrations.

Effect of varying RNase concentration during incubation outside microtiter plates

During preincubation in a separate test-tube of ENA and RNase the effect of increasing RNase concentration was recorded. The values are given in Fig. 5.

A RNase concentration which clearly reduces anti-RNP antibodies to normal levels must be chosen. In our hands and with the reactants used this was obtained with a RNase concentration of 7 mg/ml.

Effect of repeated freezing and thawing

No demonstrable effect on titers was observed even though sera were frozen and thawed 20 times.

Alteration of other factors

No alteration of either MCTD sera or normal sera extinction values were recorded associated with changing well positions on the microtiter plates.

Coefficient of variation

Within 1 day repeated determinations of the serum and day-to-day determinations on the same sample gave an overall coefficient of varia-

tion of about 10%. This included plate-to-plate variation.

Normal material

Of all 80 blood donors tested, three sera were found positive (more than mean + 2SD). Also at repeated testing they were found positive. In two cases the antibody was mostly directed against the Sm antigen, and in one case the RNP antigen.

The five sera from patients with MCTD were all strongly positive and had antibodies mainly directed against the RNP antigen.

DISCUSSION

The clinical diagnosis of MCTD can be extremely difficult since it is actually a syndrome with symptoms resembling SLE, Sjögren's syndrome, scleroderma and polymyositis. Several studies (10, 12) have emphasized that patients with MCTD have a better prognosis regarding renal involvement and skin changes. Also a better response to corticosteroid treatment has been observed. It is therefore of both prognostic and therapeutic importance to separate the MCTD patient from the SLE patient, which is often the crucial differential diagnosis.

Thus, an assay that can provide serological separation between Sm-antibody, as found in patients with SLE, and high titers of RNase-sensitive RNP-antibody in MCTD patients is highly needed.

Existing methods, e.g. hemagglutination inhibition, counter immune electrophoreses and immunofluorescents and RIA have some disadvantages. In the hemagglutination inhibition technique a high Sm-antibody titer may mask a low RNase-sensitive RNP-antibody titer (8, 9). The immunofluorescence test characteristically gives a speckled nuclear pattern with MCTD sera, but this is non-specific since a speckled pattern may also be found in sera with antibodies to ScL-1 and SS-B (13), which may be found in patients suffering from scleroderma and Sjögren's syndrome. A radio-immunoassay

has the disadvantage of employing radioactive agents which furthermore are unstable.

The ELISA principle offers some advantages: a) rapid test results, b) simple colorimetric endpoint, c) employs stable reagents, d) non-radioactive, e) inexpensive.

In our experience the most important individual factors in this analysis are the antigen concentration, the serum dilution and the RNase incubation.

Of special interest is the fact that the enzymatic RNase digestion of the antigen was obtained only when incubation was undertaken outside the microtiter plates. Probably the antigen is absorbed to the polystyrene surface in a way which renders it unavailable for the enzymatic digestion, or the RNase enzyme may be absorbed to the polystyrene surface and thereby made inactive.

The finding of positive reaction in 4% of the blood donors is not unexpected. In a study of 466 randomly selected blood donors positive reactions to granulocyte specific antinuclear antibodies were found in approx. 10% of the cases where IgM GS-ANA dominated (19).

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