

Anti-DNA Antibody in Serum Measured by Radioimmunoassay (Farr Technique)

Description of Method and Recommended Procedure

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Anti-DNA antibody determination in serum is increasingly used because it supports the diagnosis of systemic lupus erythematosus (SLE) with high selectivity. The present work evaluates several of the technical variables of the Farr radioimmunoassay for anti-DNA antibody determination and describes a recommendable procedure, emphasizes the most important sources of error and gives the range of normal blood donors and a group of hospital patients without SLE.

Key words: anti-DNA antibody; DNA; Farr technique; SLE (systemic lupus erythematosus).

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Since the observations in 1957 (2, 13, 17, 18) that anti-DNA antibodies could be found in serum of patients with systemic lupus erythematosus (SLE), several studies have confirmed that the occurrence of anti-DNA antibodies is selectively associated with SLE, although now and then reported in a few other conditions (cf. 9). Estimation of the antibody level is used clinically in monitoring SLE patients (5, 12, 14, 19). The principle of the Farr assay (6) lies behind most analytical methods, although other techniques are advocated and used. The Farr assay utilizes the fact that half-saturated ammoniumsulphate solution precipitates proteins, including DNA-specific antibody molecules which have had the opportunity to react with and bind to DNA antigen, whereas native DNA is not precipitated. With the proper *in vitro* conditions antibody-bound DNA will thus be sedimented with the precipitate, whereas free DNA will remain in solution. Isotope labelling makes it possible

to trace and quantitate the bound or non-bound DNA and thereby elicit the amount of DNA-binding antibody in biological materials, e.g. a serum sample. As this analysis is being increasingly used, it seems of interest to evaluate all relevant methodological aspects that may be of importance for variation and reproducibility of the assay, and if possible to find and recommend an optimal and standardized procedure. So far, the best methodological descriptions are given by Pincus (15) and Aarden et al. (1), whereas other publications have not attempted a similar systematic evaluation of the technique and its later modifications. This study describes in detail a modification of the anti-DNA antibody determination based on the principle of the Farr assay, and presents experimental observations that illustrate the influence of some technical variables which, if uncontrolled, may influence the results of the assay.

MATERIAL AND METHODS

Normal material

Sera from male and female normal healthy blood donors were collected. The normal material comprised 10 samples from males and 10 from females in each of the age groups of 20–29, 30–39, 40–49, 50–59 and 60–69 years.

Patients

Sera from 33 patients with SLE, who all satisfied the American Rheumatoid Association (ARA) criteria for SLE (3), were either collected during admission in the ward or had been stored at -20°C for different periods of time, maximally 60 months. Sera from 30 patients with other medical disorders than SLE (neoplastic diseases, rheumatoid arthritis, polymyositis and other rheumatological diseases) were collected and stored similarly.

Blood sampling technique

Blood was collected from a vein into a dry tube without any additive. Cells and clot were removed and serum isolated by centrifugation for 30 min, 1 to 3 days after vein puncture.

DNA-antigen

^{14}C -DNA from *E. coli* with internal label (^{14}C -thymine) was employed. According to the manufacturer (Amersham, code CFB 170) the contents of single-stranded DNA was 1–5%.

^{12}C -DNA. A highly purified DNA preparation from calf thymus (DNA Sigma type I, code D-1501) was used as routine. In some experiments other DNA preparations were examined (DNA Sigma type V, calf thymus, code D-1751, and DNA Sigma type VIII, *E. coli*, code D-2001).

Buffer solution. As routine, a borate buffer (55 mM sodium borate, 100 mM NaCl) at pH 8.0 was used. In some experiments

another composition of the buffer was assessed (see below).

Liquid scintillation counting. Liquid scintillation counting was performed with a Beckmann type LS-100 C with a commercial scintillation liquid (Packard).

Tubes and pipettes. Sera were stored in soft polyethylene tubes (Nunc No. 1050, Roskilde, Denmark). Polystyrene tubes with conical bottom (Nunc No. 1060) were used for the analysis, and hard polyethylene tubes with flat bottom (Scintitex, Humlebaek, Denmark) for the scintillation counting. Samples and analytical reagents were handled with semiautomatic or Carlsberg pipettes.

Procedure

10 μl serum is mixed with 100 μl borate buffer solution containing 50 ng ^{14}C -DNA and 150 ng ^{12}C -DNA. After slight shaking, the mixture is incubated at 37°C for 40 min and thereafter at 4°C for 20 h. 100 μl 4°C full-saturated ammoniumsulphate solution is added, the mixture is immediately shaken for a few seconds with a mechanical vibrator and placed at 4°C for 20 min. After centrifugation at 4°C for 15 min the supernatant is carefully removed by aspiration, and the ^{14}C -activity (ct/min) of the sediment is measured in liquid scintillation counter by counting 2 min. The total ^{14}C -activity was about 2,300 ct/min.

A blind sample, a 0.7% albumin sample, a normal serum, and a known pathological serum sample are run in parallel in each course of analysis. All samples are examined in duplicates and the mean ct/min is used for the calculations. The ct/min value of the blind sample is subtracted from the ct/min value of each sample, and the ammoniumsulphate-precipitated ^{14}C -value, which is the final measure of DNA-binding capacity, is expressed as per cent of the total ^{14}C added.

When one of the variables is changed (see Results) all other variables remain as described.

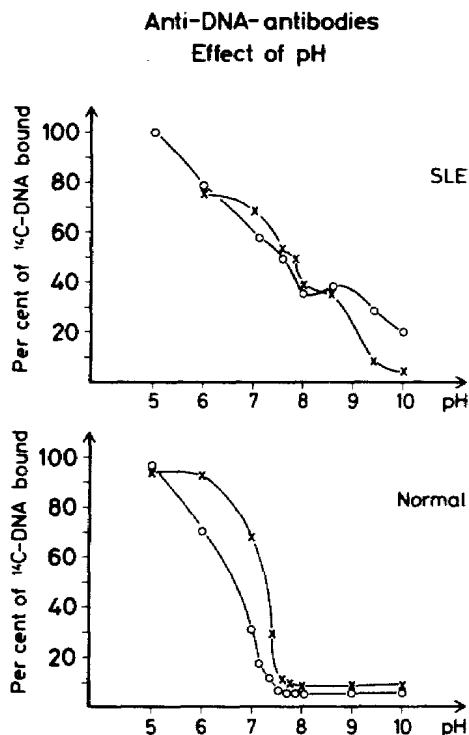


Fig. 1. Effect of pH on the result of anti-DNA antibody analysis in sera of normal persons and patients with SLE.

x or O indicates mean of duplicate determinations.

RESULTS

Effect of pH. The effect of pH variations in normal and SLE sera is shown in Fig. 1. Even minor variations of pH can clearly cause considerable alterations in the anti-DNA antibody determination. For instance, a pH below 7.4–7.6 will as a rule lead to falsely raised values in normal control sera.

Effect of DNA-antigen concentration. The effect of variations in the amount of antigen added is shown in Fig. 2. In SLE sera with increased concentration of anti-DNA antibody or in sera with antibody concentrations in the upper end of the normal range, the amount of DNA antigen added is of great im-

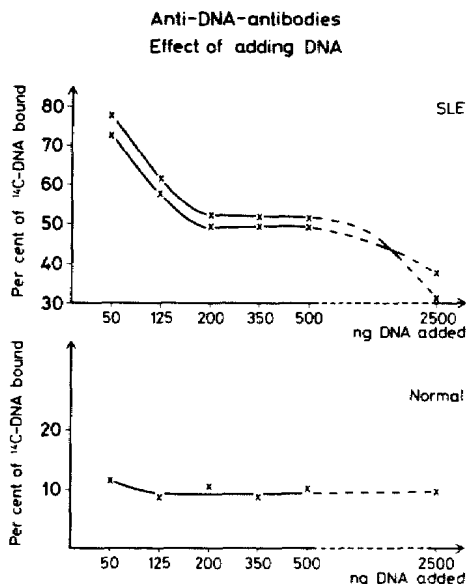


Fig. 2. Effect of amount of antigen (DNA) on the result of anti-DNA antibody analysis in a normal person and patients with SLE.

x indicates mean of duplicate determinations.

portance. Falsely raised values are found if less than 200 ng DNA is added per 10 μ l serum. None of the tested highly positive SLE samples had a plateau interval different from those shown in Fig. 2. Error due to inhibition of antigen-antibody complex formation is observed only with a very large antigen excess (2,000 ng).

Kinetics of the initial incubation at 37°C. The period of incubation at 37°C following the addition of DNA antigen to the samples can vary without affecting the result (Fig. 3). The incubation should last at least 15–20 min, but can be protracted considerably longer.

Kinetics of incubation at 4°C. The period of incubation at 4°C is far more critical than the preceding period of incubation at 37°C (Fig. 4). In SLE sera the amount of DNA antibody which binds to the antigen may readily increase by 50% during overnight incubation.

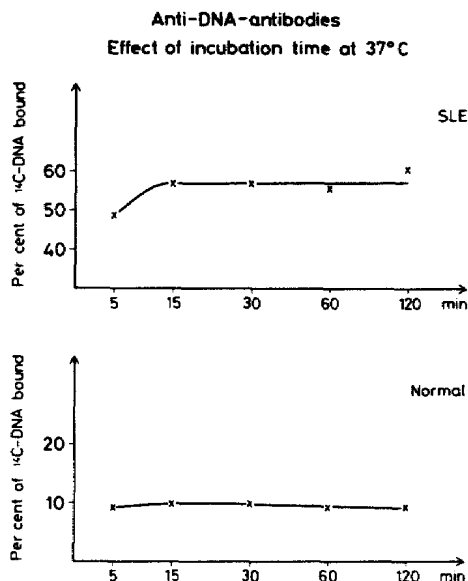


Fig. 3. Kinetic of time of incubation at 37°C on the result of anti-DNA antibody analysis in serum from a normal person and patient with SLE.

x indicates mean of duplicate determinations.

Effect of ammoniumsulphate concentration. The effect of different concentrations of $(\text{NH}_4)_2\text{SO}_4$ added for precipitation of the proteins is seen in Fig. 5. The analytical reagent must be fully saturated $(\text{NH}_4)_2\text{SO}_4$. If a less saturated solution is used, the antigen-antibody complexes are not completely precipitated, and the consequence is falsely negative values in sera with pathologically increased anti-DNA antibody.

Effect of different borate buffer molarity. Twelve SLE sera were analysed in parallel with: 1.

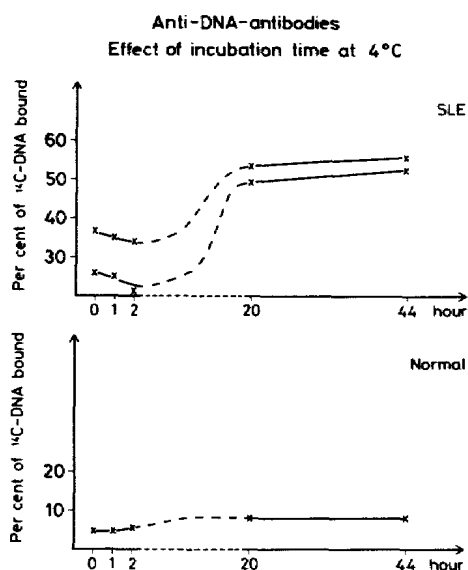


Fig. 4. Kinetic of time of incubation at 4°C on the result of anti-DNA antibody analysis in serum from a normal person and patients with SLE.

x indicates mean of duplicate determinations.

the borate buffer used in the routine procedure described above (55 mM sodium borate, 100 mM NaCl), 2. a borate buffer of 25 mM borate, 130 mM NaCl. Table 1 shows that a low borate concentration leads to falsely pathologically raised values.

Effect of repeated freezing and thawing. Four normal sera and four pathological SLE sera were examined for DNA-antigen binding capacity and thereafter frozen and thawed repeatedly 10 to 20 times. Table 2 shows that the anti-DNA antibody determination was

Table 1
Anti-DNA antibody determination in 12 SLE sera with two different borate buffer concentrations

	Borate 25 mM NaCl 130 mM		Borate 55 mM NaCl 100 mM		P Wilcoxon
Per cent of ^{14}C -DNA bound	50%	(14-80) (range)	38%	(16-65) (range)	< 0.001
	mean		mean		

Values given: Per cent of ^{14}C -DNA bound. Mean of duplicate determinations. The coefficient of variation in this experiment was 6%.

Anti-DNA-antibodies

Effect of concentration of $(\text{NH}_4)_2\text{SO}_4$

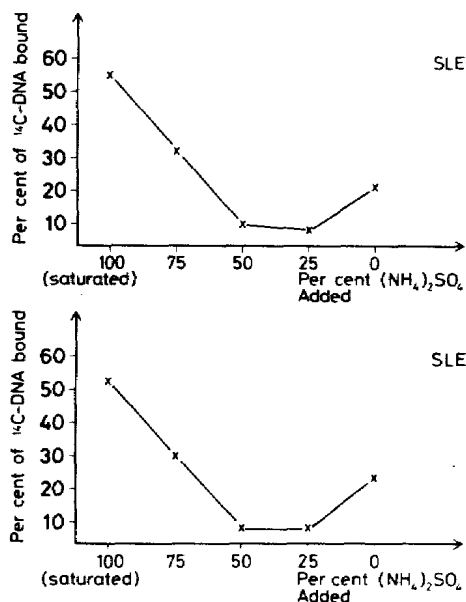


Fig. 5. Effect of ammoniumsulphate $[(\text{NH}_4)_2\text{SO}_4]$ concentration on the result of anti-DNA antibody analysis in serum from two SLE patients. In serum of a normal person less than 10% of added ^{14}C -DNA was bound. x indicates mean of duplicate determinations.

unaltered, even after freezing and thawing 20 times.

Effect of transport. Transport and storing of blood and sera for 5 days at room temperature did not alter the anti-DNA antibody values. After about 14 days at $25\text{--}30^\circ\text{C}$, a slight increase was seen.

Effect of dilution of SLE serum with normal serum.

Three SLE sera with pathological anti-DNA antibody values were diluted with a normal serum pool at different ratios (Fig. 6). Three different shapes of curves are seen. It appears that it is impossible to predict the dilution needed to obtain the 50% value of the original anti-DNA antibody value in the pathological serum.

Anti-DNA-antibodies

Effect of dilution of 3 different SLE sera with normal serum

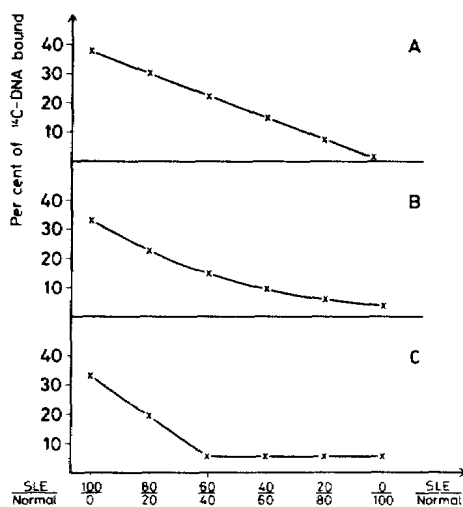


Fig. 6. Anti-DNA antibody level in three different SLE sera after dilution with a normal serum pool. x indicates mean of duplicate determinations.

Alteration of other factors. Prolonged storing at 4°C after addition of saturated $(\text{NH}_4)_2\text{SO}_4$ and centrifugation for more than 15 min did not alter the values in either normal sera or pathological SLE sera.

Effect of DNA antigen from different sources.

Three different pathological SLE sera and one normal serum sample were examined (Table 3) with the use of two different ^{12}C -

Table 2

Effect of repeated freezing and thawing on the result of anti-DNA antibody analysis in serum from a normal person and two patients with SLE

	0	10 times	20 times
SLE	56	61	60
SLE	50	55	54
Normal	10	9	11

Values given: Per cent of ^{14}C -DNA bound. Mean of duplicate determinations. The coefficient of variation in this experiment was 9%.

Table 3

Effect of three different DNA antigen sources on the result of anti-DNA antibody analysis in serum from SLE patients

	Type I From calf thymus	Type V	<i>Eschericia coli</i>
SLE	56	54	53
SLE	29	26	30
SLE	36	38	35
Normal	8	8	7

Values given: Per cent of ^{14}C -DNA bound. Mean of duplicate determinations. The coefficient of variation in this experiment was 9%.

DNA antigens from calf thymus and one ^{12}C -DNA antigen from *E. coli*. Comparable and constant values were found with all three types of antigen.

Normal values. The anti-DNA antibody value measured in the normal control persons was $9\% \pm 5$ (mean ± 2 s.d.). The corresponding value in several patients of all categories except SLE in the medical ward was $13\% \pm 8$. On this basis the borderline between normal and pathological values was chosen at 21%. The albumin standard value varied from day to day between 4% and 6%. If the albumin standard value is subtracted as a background, the normal value is decreased by 5%. There was no difference between male and female normal control persons and patients.

Coefficient of variation. Repeated determinations on the same day on the same sample, and day-to-day determinations on the same sample both gave a coefficient of variation of about 10%. This was true with normal as well as highly positive SLE sera.

DISCUSSION

The clinical diagnosis of SLE can be extremely difficult. Thus different criteria are used to classify this clinical entity and delineate it from similar and associated

clinical disorders. The SLE criteria of the American Rheumatoid Association (ARA) (4) are at present those most commonly used, and patients fulfilling at least four of 14 different criteria are supposed to have SLE. These criteria, however, have been increasingly criticized, since they do not sufficiently cover all cases. For instance, a patient with the following seven symptoms and signs does not fulfil a single ARA criterion: 1. young female, 2. arthralgiae, 3. microscopic haematuria, 4. lupus nephritis verified by biopsy, 5. low serum complement, 6. fever, 7. raised anti-DNA antibody in serum.

A main point in the discussion on SLE criteria is the question of anti-DNA antibodies in serum. Determination of these antibodies is thus of great importance. One possible source of error, which may tend to decrease the specificity of the analysis, may be variations of the standardized procedure for anti-DNA antibody determination in different laboratories. Information about reproducibility and coefficient of variation is usually lacking in publications on this assay, and comparison from one material to another is thus usually of limited value.

The three most important causes of false positive anti-DNA antibody values are: 1. pH below 7.4–7.6, 2. too low molarity of the borate buffer, 3. addition to samples of a too small amount of DNA antigen.

1. It is extremely important to keep pH at a constant level, about 8.0. Even a slight decrease of pH to less than 7.6 may lead to increasing anti-DNA antibody values, which is most pronounced in the analysis of sera with normal and low anti-DNA antibody content. This observation is not sufficiently emphasized in previous publications on this assay, although Pincus (15) and Aarden et al. (1) both found that at pH 6.4 the analysis led to a considerable increase of anti-DNA antibody values. Aarden et al. prefer pH 7.4, but it appears from Fig. 4 in their work (1) and from Fig. 1 in this paper that it is safer to keep pH constantly at 8.0.

Alteration of pH to the more alkalic side influences only SLE-sera with pathological

values. Increase of pH leads to lower anti-DNA antibody values and thus in some cases to false negative results.

2. The composition of the borate buffer may be of decisive importance for the result of the anti-DNA antibody test (Table 1) (1, 15). By too low concentration of the borate buffer the anti-DNA antibody values are determined too high. Some normal sera, therefore, may be found to contain pathological amounts of anti-DNA antibody. Differences in borate buffer concentration may explain the discrepancies between analytical results in different laboratories (3).

3. Fig. 2 shows that the amount of DNA antigen added is critical for the outcome of the analysis, if less than 200 ng DNA is added to 10 μ l serum. From the diagram it appears that it would be optimal to add, for instance, about 500 ng DNA per 10 μ l serum. However, to obtain results comparable to those of other laboratories our standard procedure was to add 200 ng DNA to each 10 μ l serum sample, which is the amount of antigen used by several laboratories after the method was introduced by Pincus et al. (16). In the method described and recommended in this paper, 50 ng DNA is added to 10 μ l serum as ^{14}C -DNA. The rest is added as ^{12}C -DNA. In patients with a variety of internal medical and especially rheumatological disorders, false positive values of anti-DNA antibody may be found if an insufficient amount of DNA antigen is added (3, 8, 10, 20). The use of a sufficiently large amount of DNA antigen thus improves the diagnostic selectivity of the test, and the use of ^{12}C -DNA as part of the antigen improves the economy of the assay.

This study confirms that it is of decisive importance that the DNA antigen and the anti-DNA antibody are allowed a period of at least 15 h at 4°C to react and form antigen-antibody complexes. The cause of this is unknown (15). If this period of interaction is shortened, false negative results can be expected on examination of SLE sera. This stage of the analysis is time-consuming, but obviously necessary. It seems reasonable

therefore to assume that determination of anti-DNA antibody values by the glassfiber absorption method (11) may give too low values with samples from patients with active SLE.

$(\text{NH}_4)_2\text{SO}_4$ must be added to the sample in a way which ensures half-saturation of the final sample solution. This is especially important as far as pathological SLE sera are concerned. In some modifications of this technique, a lower final concentration of $(\text{NH}_4)_2\text{SO}_4$ is described (20).

In our hands the use of an internal standard as daily reference was not necessary, since the day-to-day coefficient of variation was low and the value of the daily pathological serum sample fell within this limit.

It has previously been observed that the dilution curve of anti-DNA antibody of SLE sera is different in different patients (8). It has been suggested (7) that this phenomenon is due to differences of avidity of the antibody, with the implication that a more abrupt inclination is due to a comparatively larger avidity. A vertically shaped curve usually indicates renal involvement. This assumption is of clinical interest and should be tried out in future investigations.

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