EVALUATION OF AN ELISA METHOD FOR THE MEASUREMENT OF ANTIBODIES TO dsDNA

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ABSTRACT

Anti-dsDNA is found in 60-70% of patients with active, untreated systemic lupus erythematosus (SLE) and its detection serves as an important tool in the diagnosis and monitoring of these patients. This study evaluates the use of an Enzyme Linked Immunosorbent Assay (Elisa) to detect these antibodies. Its performance is also compared to the older, but established, method of detecting anti-dsDNA using Crithidia luciliae.

The sera of the 56 normal healthy blood donors revealed a mean anti-dsDNA titre of 0.93mg% with a standard deviation of 0.23mg%. All 14 patients found to be negative by the Elisa method and 10 of the 11 patients found to have borderline anti-dsDNA Elisa titres were negative by immunofluorescence. 35 patients were found to harbour raised titres of anti-dsDNA by the Elisa method. All patients found to have anti-dsDNA titres exceeding 2mg% by the Elisa test were also positive by immunofluorescence. In fact, those with very high titres by the Elisa test were also strongly (titre > 1:100) positive by immunofluorescence.

As a measurement of the kit's accuracy, the percentage of recovery of the activity of known amounts of antibody in a specimen fell within the range of about 89-104%. As a measurement of the kit's reproducibility, the coefficient of variation in the assayed titres of sample replicates was found to be 7.5% for within-batch assays and 9.7% for between-batch assays.

The Elisa assay compared favourably to the immunofluorescence test in terms of enhanced sensitivity, quantitative approach with an objective end-point and the large number of samples that may be assayed simultaneously.

Key Words: Anti-dsDNA, Systemic Lupus Erythematosus, Elisa, Crithidia luciliae.

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INTRODUCTION

Natural DNA appears in at least 3 different forms:

i. right-handed double stranded
ii. left-handed double stranded
iii. single-stranded or 'denatured' DNA

Anti-single-stranded DNA antibodies may be present in several rheumatic diseases, in certain malignancies and also in various types of other illnesses(1-2). In contrast, antibodies to double-stranded DNA (anti-dsDNA) are more specific in their occurrence pattern. Anti-dsDNA is found in 60-70% of patients with active, untreated systemic lupus erythematosus (SLE) and its detection serves as an important tool in the diagnosis and monitoring of these patients (2-7).

To detect these antibodies, several methods have come into routine laboratory use, each with its relative merits and demerits (8, 9).

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The purpose of this study is to evaluate the use of an Enzyme Linked Immunosorbent Assay (ELISA) in the detection and measurement of anti-dsDNA. Its performance is also compared to the older, but established, method of detecting anti-dsDNA using Crithidia Luciliae (10-11).

MATERIALS & METHOD

THE ELISA TEST:

96-well microplate kits coated with highly purified, S1-nuclease treated ds-DNA and standard controls with known high, medium and low titres of anti-dsDNA were obtained from BioHyTech, Israel. Alkaline phosphatase conjugated to trivalent-anti-human globulin was used with p-nitrophenyl phosphate as the substrate.

Blood samples were obtained from:

1. 56 healthy blood donors
2. 60 patients being investigated for autoimmune diseases, including some from patients known to have active systemic lupus erythematosus.

100ul of each person's serum (diluted to 1:200), known controls (High, Medium and Low) and blank (assay diluent) was transfered into the antigen-coated microwells. The microplate was incubated at 37°C for 30 minutes. After washing, 100ul aliquots of diluted trivalent-antibody-alkaline-phosphatase conjugates were transfered into the wells. The plate was re-incubated and following another rinse-dry cycle, substrate solution (PNPP) was added: 100ul per well. After a final incubation at 37°C for an hour, the optical densities (O.D.s) of the reactants in the wells were recorded using an automatic EIA reader at 405nm.

Optical density may be seen as a linear function of the anti-dsDNA titre. The O.D.s recorded for the 3 standard controls and their known titres permit one to define this linear function using linear regression analysis. From this, the titres of all the samples assayed were calculated.

The ELISA test, as described above, was performed for the 56 blood donors and the 60 patients. To analyse the variability and reproducibility, the assay for the healthy blood donors was:

a) Done in duplicate within each plate and
b) Repeated 1 week later.

Immunoflourescence

Crithidia luciliae impregnated slides were obtained from Kallestad Laboratories.

A 20ul volume of each test serum (at a screening dilution of 1:2) was layered onto the Crithidia substrate spots. The slides was incubated in a humid chamber for 30 minutes at 37°C. After a 10 minute immersion in phosphate-buffered saline (PBS), the substrate-spots were layered with FITC-conjugated anti-human immunoglobulin and re-incubated in a moist-chamber at 37°C for another 30 minutes. Following a 10 minute PBS rinse the slides were mounted and examined for kinetoplast fluorescence. Sera positive by this test were diluted further to assess the strength of the antibody titres. Sera from the 60 patients were classified as follows:

a) negative
b) low positive (titre: 1:2 to 1:100)
c) strong positive (titre > 1:100)

RESULTS

The sera of the 56 normal healthy blood donors revealed a mean anti-dsDNA titre of 0.93mg% with a standard deviation of 0.23mg%. As auto-anti-dsDNA do occur in normal human sera, cut-off values must be defined. Positive sera are those with titres 3 or more standard deviations greater than the mean. Negative sera are those with titres within 2 standard deviations of the mean. Sera with titres between 2 and 3 standard deviations above the mean are considered borderline (i.e. 1.386-1.614mg%).

The accuracy of the kit was measured by its ability to recover the activity of known amounts of antibody in a specimen added to a sample with an established titre (Table 1). The percentage of recovery fell within the range of about 89-104%.

The reproducibility of the kit with respect to the antibody titres was assessed and the results are presented in Table 2. The coefficient of variation (%) was found to be:

a) 7.5% for within-batch assays
b) 9.7% for between-batch assays.

The ELISA and immunofluorescence test results of the 60 patients are presented in Table 3. All 14 patients found to be negative by the ELISA method were also negative for anti-dsDNA by immunofluorescence. 10 of the 11 patients found to have borderline anti-dsDNA titres by the ELISA method were negative by immunofluorescence.

35 patients were found to harbour raised titres of anti-dsDNA by the ELISA method; Of the subset of these who had titres not exceeding 2mg%, 50% were negative by immunofluorescence, with the remainder being low positives. All patients found to have anti-dsDNA titres exceeding 2mg% by the ELISA test were also positive by immunofluorescence. In fact, those with very high titres by the ELISA test (see Fig. 1) were also strongly (titre > 1:100) positive by immunofluorescence.

Table 1.
A recovery experiment to assess the accuracy of the ELISA in detecting anti-dsDNA.

WELL NO.	CONTENTS ADDED TO WELLS: POSITIVE SERUM (2.11 MG%)	BLANK (0 MG%)	OPTICAL DENSITY: EXPECTED	OBSERVED	% RECOV
1	100 ul	0 ul	–	2.33	–
2	50 ul	50 ul	1.165	1.034	88.8%
3	33 ul	67 ul	0.776	0.73	94.1%
4	25 ul	75 ul	0.583	0.604	103.7%
5	20 ul	80 ul	0.466	0.466	100%

Table 2.
The intra-batch and inter-batch coefficient of variation (% C.V.) in anti-dsDNA titres.

EXPERIMENT NUMBER	NUMBER OF BLOOD DONORS	COEFFICIENT OF VARIATION (%): ** INTRA-TEST	* INTER-TEST
1	10	10.9	12.1
2	26	6.1	9
3	10	8.6	10
4	10	6.5	8.7
WEIGHTED AVERAGE	56	7.5	9.7

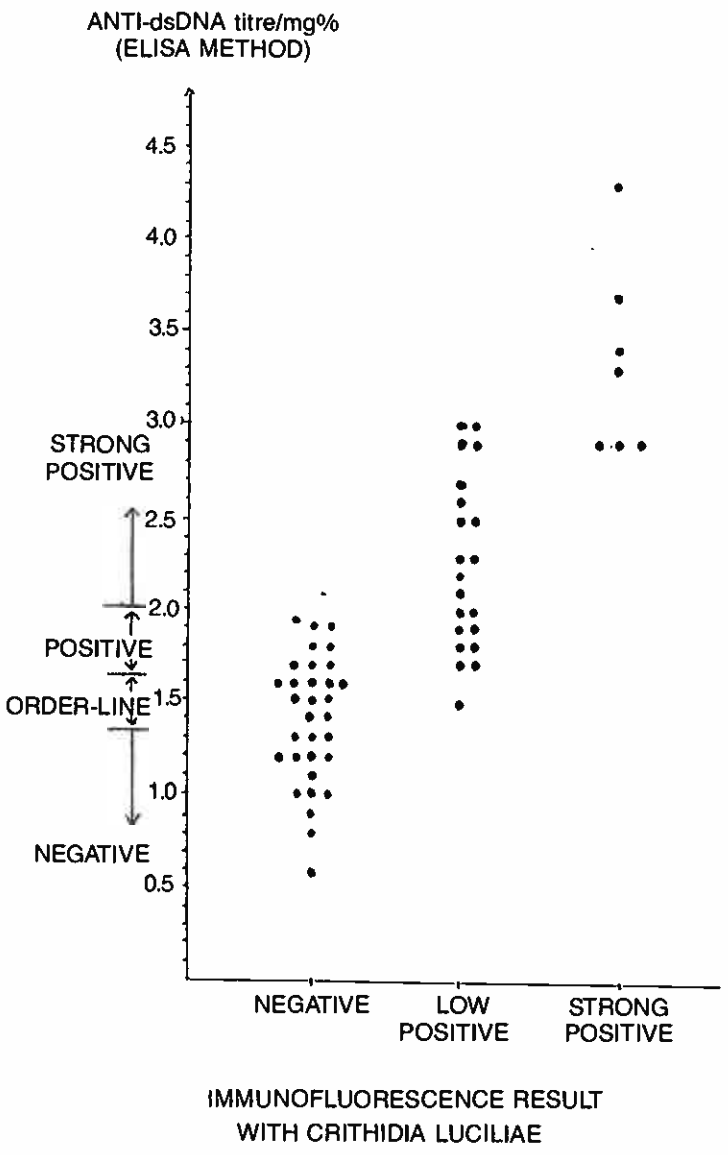
* Each of the 4 experiments with the 4 different lots of healthy blood donors was repeated on a second kit to derive the inter-test coefficient of variation.

** In running each test, each donor's serum was tested in duplicate to obtain the intra-test coefficient of variation

Table 3.
Correlation of anti-dsDNA titres by ELISA with the corresponding immunofluorescence results.

ELISA TEST (MG %)	IMMUNOFLUORESCENCE BY CRITHIDIA NEGATIVE	LOW POSITIVE (1:2 – 1:100)	STRONG POSITIVE (>1:100)	TOTAL
NEGATIVE	14	0	0	14
BORDER-LINE POSITIVE	10	1	0	11
(but up to 2 mg%) STRONG POSITIVE	8	8	0	16
(>2 mg %)	0	12	7	19
TOTAL	32	21	7	60

Fig. 1: Correlation of Immunofluorescence results with anti-dsDNA titres as determined by the ELISA test for 60 patients.



DISCUSSION

Immunofluorescence using Crithidia luciliae is essentially a qualitative assay and at most can be rendered semi-quantitative. The prime advantage of the Crithidia luciliae assay was the belief that the kinetoplast was devoid of other nuclear antigens. However this assumption is challenged by the demonstration of histones in this organelle (12) which means that this assay may score anti-histones in addition to anti-dsDNA. Besides, the experience required of the observer to distinguish dubious positives from negatives may inject an element of subjectivity into this method. While still a valuable clinical test, the Crithidia luciliae assay is a poor tool for the study of anti-dsDNA immunochemistry.

In contrast, the ELISA method has the prime advantage of increased sensitivity compared to the Crithidia luciliae assay, hemagglutination, counterimmunoelectrophoresis and fluid-phase radioimmunoassay in detecting low concentrations of anti-dsDNA(13,14). The test is also quantitative. The assay end-point is objective unlike as in immunofluorescence. The full assay for a large batch of patients can be performed simultaneously and can be completed within two and a half hours.

We have noted 8 patients in this study (Table 3) who were positive by the ELISA test but negative by immunofluorescence. The possibilities for this are:

1. False positive ELISA tests
2. False negative immunofluorescence results

Which of the above 2 reasons is the more pertinent is difficult to say, but the performance of an additional confirmatory test, such as radioimmunoassay, may illuminate the situation.

The radioimmunoassay of Farr(15) has proven to be a valuable research tool. In addition to being more sensitive and quantitative than the Crithidia luciliae assay, it has the additional advantage in that the native DNA substrate can be varied. Thus the Farr assay can evaluate binding to dsDNA of known molecular weights, as well as to a variety of synthetic polyneuclotides. Unfortunately the specificity of the Farr assay or anti-dsDNA is in doubt: as little as 0.1% denaturation of the dsDNA substrate can cause spurious DNA precipitation by anti-dsDNA. Obtaining radio-labelled dsDNA without even this small degree of denaturation is difficult. Furthermore the Farr assay measures only those anti-dsDNA which stay bound to dsDNA during 50% saturated ammonium sulphate precipitation, i.e. high affinity anti-dsDNA(16). Consequently the entire spectrum of naturally occuring anti-dsDNA cannot be studied. In handling the materials for radioimmunoassay, finally, one has to pay attention to the hazards associated with the storage and disposal issues.

By comparison, the reagents used in the ELISA test are more stable and safer than those used in radioimmunoassay (17). This simple assay also introduces versatility into the routine laboratory as different antigen coated wells can be employed (in the same run, if necessary) to detect antibodies to different specificities without much increase in cost or assay materials.

Finally the ELISA method enables the investigator to identify the isotype of the antibody by using monovalent conjugates: This may be important as, for example, the anti-dsDNA antibodies in lupus nephrities have been shown to be more likely to be complement-fixing and precipitating antibodies of the IgG class(18). In view of these multiple advantages, the use of the ELISA assay for detecting anti-dsDNA is certainly welcomed.

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