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A double antibody solid phase assay for DNA autoantibodies for clinical use

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SUMMARY

DNA autoantibodies in serum will bind to antigen-coated polystyrene tubes and can be detected by radiolabelled anti-immunoglobulin. The method is quantitative, gives information on the antibody class and is not readily subject to interference by factors such as the size of the DNA, minor contamination of double-stranded with single-stranded DNA and the presence of materials which can combine 'non-specifically' with the antigen. Double-stranded DNA gave good discrimination between SLE and rheumatoid arthritis but with single-stranded preparations approximately 40% of the RA patients showed elevated antibody values. Individual results obtained with the two antigens were compared and correlated with the Farr test and ANA titres. Surprisingly, a proportion of the SLE sera gave high background binding to tubes coated with gelatine.

INTRODUCTION

Autoantibodies to several nuclear constituents are regularly present in the sera of the patients with systemic lupus erythematosus (SLE) but those directed against native double-stranded DNA provide the most specific marker for this disease (Pincus *et al.*, 1969; Hughes, 1973). Detection of DNA antibodies is not only of value in the diagnosis of SLE but their level correlates with clinical activity and the presence of immune complex nephritis (Schur & Sandson, 1968; Hughes, Cohen & Christian, 1971; Luciano & Rothfield, 1973).

Of the quantitative methods used for the estimation of these antibodies the most commonly employed is the Farr test in which radiolabelled DNA complexed with antibody is precipitated by ammonium sulphate (Wold *et al.*, 1968; Pincus *et al.*, 1969; Hughes *et al.*, 1971). Alternatively the complexed antigen can be separated from free DNA by filtration on nitrocellulose membranes (Ginsberg & Keiser, 1972; Kredich, Skyler & Foote, 1973) or by precipitation with an anti-immunoglobulin (Glass *et al.*, 1973). A different approach based upon the solid phase radio-immunoassay developed by Catt, Niall & Tregear (1966) and Catt (1970) involves binding of antibody from a patient's serum to a DNA-coated plastic cup and detection of this antibody by addition of radiolabelled nucleic acid (Tan & Epstein, 1973). The method we now describe is similar to this except for the final stage in which the bound anti-DNA is estimated by addition of ^{125}I-labelled purified anti-immunoglobulin. This has the advantage of defining quantitatively the distribution of DNA antibodies between the immunoglobulin classes and subclasses. We have compared the technique with the Farr assay and have used it to study quantitative aspects of the specificities for native and single-stranded DNA.

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MATERIALS AND METHODS

Double-stranded deoxyribonucleic acid (ds-DNA). Calf thymus DNA (Worthington Biochemical Corporation, Freehold, New Jersey) dissolved in normal saline buffered with 0·01 M phosphate, pH 8·0 (PBS) at a concentration of 0·5 mg/ml was stored at 4°C and used for up to 5 days.

Single-stranded DNA (ss-DNA). This was prepared just before coating the plastic by heating ds-DNA at 100°C for 10 min and cooling immediately to 0°C in an ice bath.

Purified antibodies to human immunoglobulins. A globulin fraction was prepared from pooled normal human serum by precipitation with 33% saturated ammonium sulphate. Rabbits were immunized with 5 mg of the protein emulsified in Freund's complete adjuvant, injected intramuscularly at four different sites and boosted with intravenous injections of 1 mg of alum-precipitated protein given 4 times during weeks 3 and 4. Serum was taken 1 week later. The antibodies were adsorbed onto a column prepared by coupling 100 mg human Cohn FII (Miles Laboratory) to 200 mg of Sepharose 4B (Pharmacia, Uppsala) activated with 500 mg of CNBr by the method of Porath, Axen & Ernback (1967). After elution with 0·1 N acetic acid, the antibodies gave a strong anti-IgG and a weaker anti-IgM line on immuno-electrophoretic analysis. The preparation was stored in small aliquots at −20°C until required for iodination.

Radioiodination. This was carried out following a modification (Hay, Nineham & Roitt, 1975) of the method of Hunter & Greenwood (1962). Sixty micrograms of chloramine T and 120 μg of sodium metabisulphite per 1 ml of protein were used. To label 5 mg of protein, 0·5 mCi of ^{125}I (Radiochemical Centre, Amersham, Bucks) was added. Unbound ^{125}I was removed by filtration on Sephadex G-25 (Pharmacia, Uppsala) and the stock solution of ^{125}I-labelled anti-Ig stored frozen in small aliquots.

Double layer solid phase radioimmunoassay for DNA antibodies. Either polystyrene tubes or dispcsable plastic U-plates MRC 96 (Linbro) containing 96 identical cups could be used as the solid adsorbing surface. The present study employed the U-plates. The wells were filled at each step thereby eliminating the need to measure the volume of reagents. First the cups were filled with the ds-DNA or ss-DNA solutions and left to stand at 4°C for 16 hr. After removal of the fluid by suction, the plastic cups were washed with 0·5% gelatine in PBS pH 8·0 and then left with gelatine buffer at 4°C for a further 4 hr to saturate any free binding sites. After three washes with 0·1%gelatine–PBS, test sera diluted 1:10 with the same buffer were added and the tray incubated at 37°C for 1 hr and at 4°C for 18 hr in a humid chamber. The sera were removed and after several washes with 0·1% gelatine-PBS and distilled water, the tray was immersed in 0·1% gelatine-PBS pH 7·4 for 1 hr with agitation. Radiolabelled anti-Ig was then added and allowed to react for 1 hr at 37°C and 30 min at 4°C. After several washes with 0·1% gelatine-PBS pH 7·4 and finally with tap water, the plates were cut into individual cups and the bound radioactivity determined with an autogamma spectrometer (Packard Selectronic). All washings were carried out with cold buffers.

For inhibition tests 0·5 ml of patient's serum diluted 1:10 was mixed with an equal volume of either ds-DNA or ss-DNA (final concentration 0·25 mg/ml), incubated for 1 hr at 37°C and then for 18 hr at 4°C before adding the anti-Ig. Serum diluted only with buffer, and incubated under the same conditions, served as a control.

Determination of DNA antibodies by the Farr technique. The DNA binding test was carried out as described by Pincus (1971). 0·1 μg [^{14}C]DNA (Radiochemical Centre, Amersham, Bucks) diluted in 50 μl of borate–sodium chloride buffer (0·15 M, pH 8·0) was added to 50 μl of a 1:10 dilution of unheated serum. The mixture was incubated for 1 hr at 37°C and 18 hr at 4°C. An equal volume of saturated ammonium sulphate was added and after 1 hr incubation at 4°C, the tubes were centrifuged and the supernatants and redissolved precipitates counted in a liquid scintillation spectrometer (Tricarb Packard) using Bray's scintillation fluid. Under these conditions, binding of more than 36% of the total count was considered abnormal.

Immunofluorescence. Titrations of anti-nuclear antibodies (ANA) were performed using tissue sections of human thyroid and rat liver and kidney with a fluorescein-labelled rabbit anti-human Fabγ.

Sera. The sera examined were selected from those sent to the laboratory for autoantibody tests. They were stored at −20°C in small aliquots and thawed only once. All sera were spun down at 6000 *g* for 5 min before testing. Altogether 113 sera were studied, thirty-nine samples from thirty-one patients with SLE, sera from twenty-nine patients with RA and twenty sera from eighteen patients with other collagenoses. This latter group included discoid lupus erythematosus (eight patients) scleroderma (two patients), Sjögren's syndrome (five serum samples from three patients) and five patients with undefined autoimmune disturbances. The twenty-one control sera were from laboratory staff. To this group were added four myeloma sera (two IgG, one IgA and one IgM).

RESULTS

Initial studies

The most favourable conditions for this double layer radio-immunoassay were established in preliminary studies. The best discrimination between positive and negative sera was obtained by incubation with the test serum for 1 hr at 37°C and 18 hr at 4°C followed by the washing procedure described in the Materials and Methods section. Purification of the anti-immunoglobulin used for the second layer was essential.

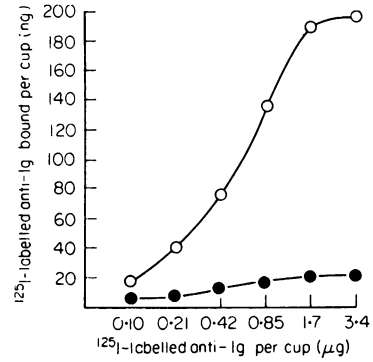

FIG. 1. Titration of radiolabelled anti-Ig. Different amounts of antibody were used in a test employing fixed quantities of a given positive and negative serum. (○) Serum with ds-DNA antibodies; (●) serum negative for DNA antibodies.

The optimal quantity of labelled anti-immunoglobulin required was determined by adding increasing amounts to test cups which had been incubated with a serum known to have high titre DNA antibodies. A virtual plateau value was reached at 3·4 μg of labelled protein (Fig. 1) and this amount was used in all subsequent studies. The results were expressed as nanograms of ^{125}I-labelled anti-Ig bound per cup after initial incubation with 0·15 ml of a 1:10 dilution of test serum, the specific activity of the labelled material being estimated for each experiment. Under these conditions, duplicates did not differ by more than 10% and repeated testing of samples gave coefficients of variation ranging from 9% for a positive serum with DNA antibodies, to 35% for a negative specimen.

The first experiments showed that test sera bound to cups coated with bovine serum albumin (used to saturate non-specific sites on the plastic) in a highly variable manner, in that certain sera gave particularly high values. Substitution of gelatine for bovine serum albumin did not improve the situation (compare Fig. 10) and it became necessary to test each serum against cups coated with gelatine alone, this value being subtracted from the radioactivity obtained with the DNA-coated cup.

The following procedure was finally adopted. Each test serum was added to cups coated with: (1) ds-DNA followed by gelatine; (2) ss-DNA and then gelatine; and (3) gelatine alone. After washing, the labelled anti-Ig was applied and the bound radioactivity measured. A reagent control (4) was included in which buffer alone followed by anti-Ig was added to gelatine-coated cups. The ds-DNA antibody level is given by (cups 1 − 3). The ss-DNA antibody is calculated from (cups 2 − 3). Background binding is given by (cups 3 − 4).

Antibodies to DNA in different patient groups

The double layer technique with sera from control subjects gave binding values for native DNA of not more than 40 ng of ^{125}I-labelled anti-Ig/1·5 ml × 10^{-2} ml of serum after correction for binding to gelatine-coated cups lacking DNA. The majority of SLE sera gave values in excess of this (Fig. 2) but only occasional rheumatoid arthritics and a small proportion of patients with other collagenoses were abnormal in this respect. In general, greater binding was observed with single-stranded DNA and in this case a significant proportion (41%) of the RA patients were positive (i.e. gave values of more than 100 ng bound per cup) (Fig. 3).

The method was compared with results obtained by the Farr technique. There was a good correlation ($r = 0{\cdot}59$; $P < 0{\cdot}001$) between the two tests using ds-DNA as antigen in the double layer technique although there were occasional discrepancies (Fig. 4). A similar correlation ($r = 0{\cdot}55$; $P < 0{\cdot}005$) was seen when the plastic cups were coated with ss-DNA (Fig. 5).

Rather comparable results were seen when the same SLE sera were tested in the double layer technique with both ds-DNA and ss-DNA ($r = 0{\cdot}76$; $P < 0{\cdot}001$), occasional sera being positive with one antigen and not the other (Fig. 6). In most cases the denatured antigen bound more antibody. The ability of ds-DNA

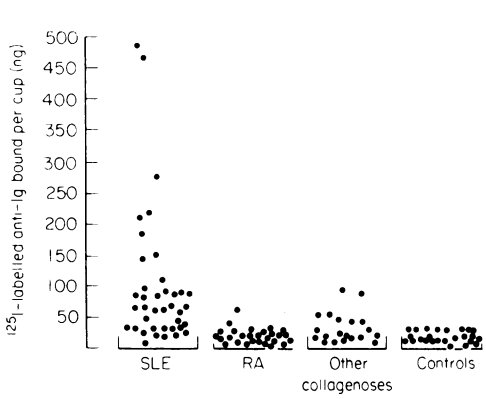

FIG. 2

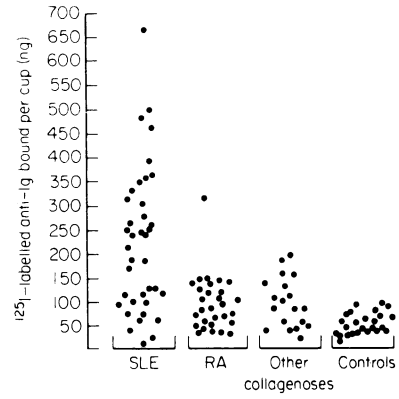

FIG. 3

FIG. 2. Antibodies to ds-DNA in the sera of patients and controls determined by the double layer method. Each point represents the value obtained for each serum.

FIG. 3. Antibodies to ss-DNA in the sera of patients and controls determined by the double layer method. Each point represents the value obtained for each serum.

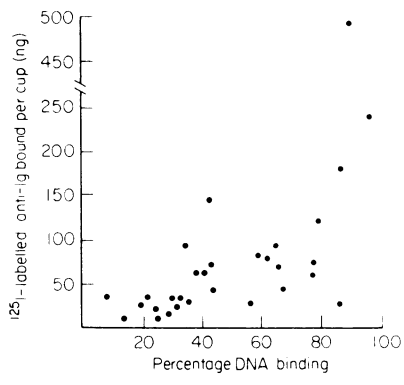

FIG. 4

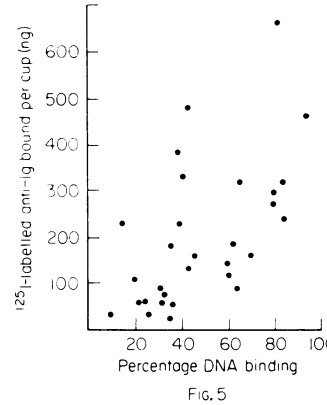

FIG. 5

FIG. 4. Correlation of Farr test with the double layer method for determination of antibody to ds-DNA.

FIG. 5. Correlation of Farr test with the double antibody method for determination of antibody to ss-DNA.

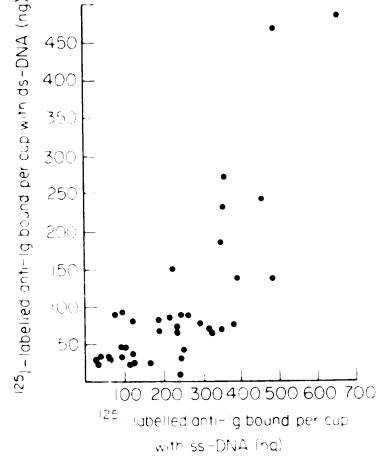

FIG. 6. Correlation between antibodies to ds-DNA and ss-DNA in SLE patients using the double layer method.

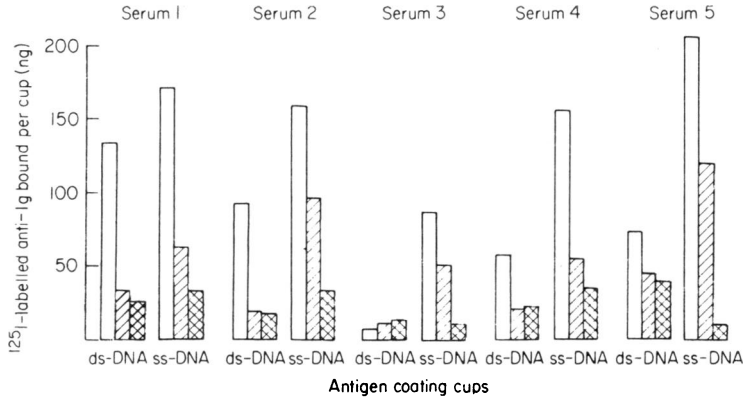

FIG. 7. Inhibition of anti-DNA antibodies by double and single stranded DNA. The binding of five different sera to both ds- and ss-DNA is shown: Open columns, alone; hatched columns, in the presence of ds-DNA; cross-hatched columns, in the presence of ss-DNA.

and ss-DNA to inhibit the binding of five different SLE sera to cups coated with both antigens was investigated. Single-stranded DNA effectively reduced the binding to both ds-DNA and ss-DNA; ds-DNA, as would be expected, almost completely abolished binding to ds-DNA coated cups but only partially blocked the antibodies with specificity for ss-DNA (Fig. 7). There was a fair degree of correlation ($r=0{\cdot}46$; $P<0{\cdot}02$) between the level of antibodies to ss-DNA in SLE patients and the fluorescent anti-nuclear antibody titre (Fig. 8). Whenever the double layer technique was positive, so was the immunofluorescent test. On the other hand, although all those with ANA titres of 1:320 had ss-DNA antibodies, not all sera positive by immunofluorescence gave abnormal ^{125}I-labelled anti-Ig binding values, partly because the sera contained antibodies to nuclear constituents other than DNA and partly because of the greater sensitivity of the fluorescent test.

The correlation between levels of ss-DNA antibodies and the presence or absence of anti-nuclear antibodies in patients with RA and other collagenoses is shown in Fig. 9. Highest levels of ss-DNA antibodies were seen in ANA-positive sera but 54% of these gave values within the normal range.

As mentioned previously, a curious feature emerged when the background binding to cups coated with gelatine only was examined. Most of the SLE patients gave far higher background values than the

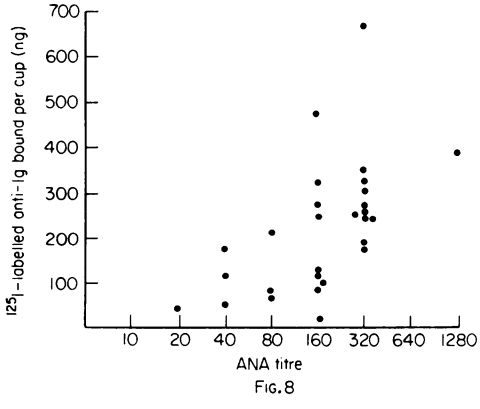

FIG. 8

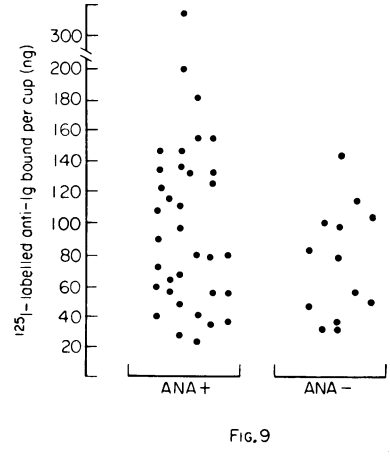

FIG. 9

FIG. 8. Correlation between antibodies to ss-DNA and titre of ANA in SLE patients.

FIG. 9. Correlation of antibodies to ss-DNA with the presence or absence of ANA in patients with RA and other collagenoses.

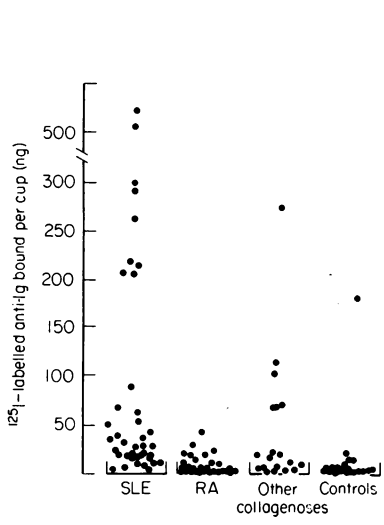

FIG. 10

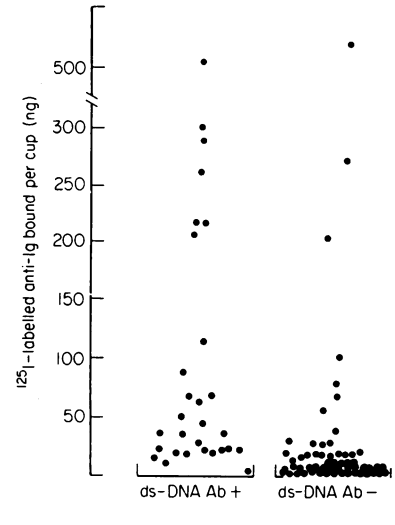

FIG. 11

FIG. 10. Background binding of labelled ^{125}I-labelled anti-Ig after addition of human sera to cups coated with gelatine alone.

FIG. 11. Correlation between background binding to cups coated with gelatine alone with the presence or absence of antibodies to ds-DNA.

majority of controls (Fig. 10). This was seen to a lesser extent with the other collagenoses and was less marked but still evident in the rheumatoid arthritis group. Interestingly, these background values tended to be higher in sera with antibodies to ds-DNA (Fig. 11). Differences were less marked when background binding was compared with the presence or absence of ss-DNA antibodies.

DISCUSSION

The double antibody technique using antigen bound to plastic surfaces as an assay for DNA antibodies has a number of advantages. In the first place, the simplicity of the procedure, particularly the washing stages, makes the method well suited for clinical purposes. The sensitivity is comparable to that of the Farr ammonium sulphate test but unlike the latter, is not dependent on the molecular weight of the DNA. Furthermore, it gives a direct measure of antibody binding to the DNA and with appropriately labelled anti-immunoglobulin reagents, it is possible to define the class, and even the subclass, of the anti-DNA.

Another advantage derives from the need to label the anti-immunoglobulin rather than the DNA as most methods require. Thus the same labelled reagent can be employed for the detection of antibodies, not only to ds- and ss-DNA but also to single- and double-stranded RNA, RNA–DNA hybrids and any other autoantigen which can be obtained in a relatively pure form.

Furthermore, since DNA is negatively charged, it may readily complex with basic proteins such as C1q (Agnello *et al.*, 1969) and give false positive results in certain tests. The solid phase system we have described avoids this problem since the labelled second antibody is directed against immunoglobulin.

The method differs in two important respects from a similar study carried out by Pesce *et al.* (1974) using peroxidase-linked antibody. First, purification of the anti-immunoglobulin required for the final stage improves the discrimination between SLE and normal sera. Secondly, inclusion of a control tube coated with gelatine alone corrects for a variation in binding which does not appear to depend directly on the DNA antibody level. Furthermore, radioactive counting is more convenient than enzyme assay but the latter uses less expensive equipment and is more acceptable from the standpoint of environmental pollution. As Shimizu, Yagura & Yamamura (1975) have shown, microcrystalline cellulose can also be employed as the solid phase for coupling DNA but in this case the washing steps involve centrifugation.

TABLE 1. Summary of tests for DNA antibodies in patients with connective tissue disease and normal controls

Patient group	No. of patients	Percentage positive*		Antibody level†	
		ds-DNA	ss-DNA	ds-DNA	ss-DNA
SLE	39	69	70	100±17	222±24
RA	29	3	41	20·8±2·4	93·8±10·7
Other collagenoses	20	35	42	35·1±5·3	94·5±11·8
Controls	25	0	0	21·7±1·8	51·5±4·5

* Antibody levels > 40 ng anti-Ig bound per well for ds-DNA and > 100 ng for ss-DNA.

† Mean±s.e.: values expressed as nanograms of ^{125}I-labelled anti-Ig bound per well.

With the present technique, antibodies reacting with native and with ss-DNA could be readily distinguished. In agreement with previous studies (e.g. Arana & Seligmann, 1967), SLE sera could be divided into three groups: (I) the most common (22/31) reacting with both ds-DNA and ss-DNA; (II) positive for ds-DNA but giving values in the normal range for ss-DNA (2/31) and (III) reacting with ss-DNA but with normal values for ds-DNA binding (7/31). This is usually interpreted in the following way. Sera containing antibodies against a single sugar–phosphate chain will be found in group I, since they will react with both ss- and ds-DNA. Group II sera probably contain antibodies which react with both sugar–phosphate strands simultaneously while antibodies in group III sera are directed against the nucleotide bases which are 'buried' in the native DNA double helix (Levine & Stollar, 1968).

Inhibition studies on five SLE sera taken from groups I and III were consistent with this interpretation. Both ds- and ss-DNA were effective in adsorbing the antibodies to ds-DNA, whereas ds-DNA only partially inhibited the binding of antibodies to ss-DNA. Serum 3 (Fig. 7) gave a curious result in that binding to ss-DNA was partially blocked by ds-DNA despite the fact that no antibodies to ds-DNA were demonstrable. It is likely that this may be attributed to the presence of small amounts of ss-DNA in the commercial preparation of DNA used in the test. This minor component could inhibit the reaction with ss-DNA since an excess of the antigen was used in the experiment. However, the degree of contamination with ss-DNA was not great enough to affect the solid phase radio-immunoassay, since some sera with high levels of ss-DNA antibody gave normal results with ds-DNA.

A summary of our findings in different patient groups is given in Table 1. The incidence of positive results for ds-DNA antibodies in SLE is comparable to that in other published series (Pincus *et al.*, 1969; Tan & Epstein, 1973; Holian *et al.*, 1975) and this test clearly discriminates between SLE and rheumatoid arthritis. In the latter group only one of twenty-nine sera was marginally positive and this probably represents the known serological overlap with lupus. This was also seen in the significant proportion of patients with other collagen disorders, particularly discoid lupus and Sjögren's syndrome, giving positive results for ds-DNA antibodies.

The majority of sera showed a higher binding to ss-DNA than to the native antigen. Results with the single-stranded form still allowed discrimination from the normal and also from rheumatoid arthritis if one raised the threshold of positivity to 150 ng bound anti-Ig per well. On this basis it might be argued that diagnostic tests should be carried out only with ss-DNA but one would miss a small proportion—of the order of 5% or less—of SLE patients reacting solely with ds-DNA. It would be of interest to make a special study of the clinical status of arthritis patients with raised ss-DNA antibodies in view of the increased incidence of vascular lesions, erosive joint changes and subcutaneous nodules in rheumatoid patients with positive ANA (Ward, Johnson & Holborow, 1964). In the present studies, ss-DNA antibodies in the patients with rheumatoid arthritis and other collagenoses were mostly associated with

positive anti-nuclear antibody tests (Fig. 9), although of course not all sera giving nuclear fluorescence contained anti-DNA. Interestingly, three of thirteen ANA negative sera reacted with ss-DNA presumably through antibodies directed towards the nucleotide bases which would not be freely available in the immunofluorescent test.

There was considerable variation between sera in their ability to bind to wells coated with gelatine alone. This was probably not due to the presence of gelatine antibodies since exactly parallel results were obtained with bovine serum albumin-coated wells. The highest background binding was seen with sera from patients with SLE and other collagenoses, particularly in those containing antibodies to ds-DNA. The level of IgG itself was not a major factor since the highest background given by hyperglobulinaemic IgG myeloma sera (35 ng per well) was lower than that observed with the majority of SLE cases. The phenomenon might be connected with the presence of immune complexes although the mechanism of binding would be obscure.

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