Evaluation of a standardised assay for the measurement of antibodies to double-stranded (native) DNA

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SUMMARY A standardised commercially available radioimmunoassay kit for the detection of antibodies to native DNA (N-DNA) has been evaluated in clinical practice. This test system is shown to be a reliably reproducible method of detecting these antibodies. In addition, evaluation of the purity of the radiolabelled test antigen in this assay has shown it to be almost entirely double stranded (native) DNA with virtually no contamination with single stranded (denatured) DNA, and with few areas of single stranded breaks or ends in the duplex molecule. The inclusion of known standards and precise characterisation of the DNA has partially overcome variability in results and provides for interlaboratory standardisation which is lacking in the techniques used at present.

It has previously been shown that antibodies to double-stranded (ds) native DNA have a high degree of specificity for systemic lupus erythematosus (SLE) (Pincus *et al.*, 1969; Hughes, 1971). In addition, serial studies of these antibodies during the course of the disease have shown that in most patients a good degree of correlation exists between antibody levels and evidence of disease remission or relapse (Lightfoot *et al.*, 1973; Davis *et al.*, 1977a). Several problems with radioimmunoassays for the detection of DNA antibodies remain to be resolved. The purity of DNA used in each assay has to be clearly defined, as sera from patients with systemic lupus erythematosus and many other diseases also contain antibodies to other polynucleotides, especially single-stranded (ss) DNA. Single-stranded DNA present as an impurity in test-DNA will cause spuriously high levels of antibody binding to be recorded (Winfield and Davis, 1974). The validity of DNA assay results, therefore, can be interpreted only with the knowledge of the specific nature of the DNA.

A further problem in quantitation of antibody to native DNA is standardisation, not only between laboratories but also in the same laboratory from year to year. This problem is compounded by the number of different assay systems and different

forms of DNA which are used in the different laboratories for detecting antibodies to ds DNA.

The development of a standardised commercially available assay for quantitation of DNA antibodies, suitable for routine clinical application, would potentially be of value. We have had the opportunity to test one such commercially available assay provided by Amersham/Searle¹ and have demonstrated (Davis *et al.*, 1977b) that a good degree of correlation occurs between the presence of antibodies to double-stranded DNA as detected by this method, and antibodies to double-stranded DNA as detected by the Millipore filter assay with the ³H-DNA procedure now used routinely in our laboratory.

The aim of the present study was to evaluate the use of this standardised assay in routine clinical practice after careful assessment of the purity of the radiolabelled DNA.

Methods

The Amersham/Searle kit assay is based on the Farr ammonium sulphate precipitation technique. All essential chemicals and apparatus are provided to perform this technique on a microassay basis; 42 sera can be tested with each kit, which is supplied

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¹Available from Radiochemical Centre, Amersham, Bucks, UK or Amersham/Searle, 2636 S Clearbrook Drive, Arlington Heights, Illinois 60005, USA and 400 Iroquois Shore Road, Oakville, Ontario, Canada.

with detailed background information and technical instructions.

Each kit contains four known SLE standard sera whose DNA binding activity is expressed in arbitrary units of DNA bound. The ^{125}I iodine-labelled double-stranded DNA is extracted from HeLa cells with a final concentration of $1\text{ }\mu\text{g DNA/ml}$, specific activity $200\text{ mCi}/\mu\text{g}$ ($\text{MW} \approx 10^7$). Analysis on a caesium chloride gradient shows a single peak of radioactive DNA and no radioactivity in the RNA fraction. Selective absorption chromatography on hydroxy apatite shows $<1\%$ single stranded DNA (Holian *et al.*, 1975; Holian, personal communication, 1976).

This degree of purity of radioactive antigen has been confirmed in our own laboratory. The DNA was not found to contain significant contamination with ss DNA by the following criteria.

ETHIDIUM BROMIDE TECHNIQUE (Morgan and Pulleybank, 1974)

Ethidium intercalates into the DNA duplex with, at low ionic strengths, a binding constant of around 10^7 M^{-1} and with a 50-fold increase in the fluorescence of ethidium. The high binding constant and the intrinsic sensitivity of fluorescence allows DNA to be very sensitively assayed, $0.1\text{ }\mu\text{g}$ being quantitated to $\pm 1\%$. In order to differentiate between native and denatured DNA, the much lower stability of the short intramolecular base-pairing that occurs in denatured DNA relative to the fully duplex native DNA is exploited. Heat denatured DNA can be made completely single-stranded by raising the pH to 11.8, while under the same conditions native DNA remains intact. Thus at pH 8, owing to the short intramolecular base-pairing of denatured DNA, it shows a fluorescence about 50% that of native DNA at pH 8. However, at pH 11.8 no fluorescence is observed for denatured DNA at all owing to destabilisation of intramolecular base-paired regions. The fluorescence of native DNA at pH 11.8 is about 70% that of native DNA at pH 8 owing to the higher ionic strength necessary for buffering the system. For an unknown sample of DNA containing both denatured and native DNA equal volumes containing approximately $0.1\text{ }\mu\text{g}$ samples are added to the pH 8 and pH 11.8 ethidium assay mixtures. As a worked example, a mixture of DNAs might give a fluorescence reading in arbitrary units of 100 at pH 8 and of 40 at pH 11.8. The latter is due only to native DNA, and therefore of the 100 fluorescent units at pH 8, $\frac{100}{70} \times 40 = 57$

units are due to native DNA and therefore $100 - 57 = 43$ units are due to denatured DNA. Since the

latter has half the specific fluorescence of native DNA, the actual ratio of native to denatured DNA

on a nucleotide basis is $\frac{57}{2 \times 43} = 1:1.51$.

Experimental

DNA at approximately 1 A_{260} ($50\text{ }\mu\text{g/ml}$), $2\text{--}20\text{ }\mu\text{l}$, is added to 2 ml of pH 8 or pH 11.8 ethidium assay mixture, the former being $5\text{ mM tris HCl pH } 8$, 0.1 mM EDTA , $0.5\text{ }\mu\text{g/ml}$ ethidium bromide; the latter is $20\text{ mM potassium phosphate pH } 11.8$, 0.1 mM EDTA , $0.5\text{ }\mu\text{g/ml}$ ethidium bromide. The solutions are then read for their fluorescence against the appropriate blank ethidium assay mixture with excitation at 525 nm and emission at 600 nm. In our experience, the Turner 430 spectrofluorometer is an excellent machine with good long-term stability. An argon laser now in use can be used to give much higher sensitivity but the expense would not make it a generally useful instrument. Great care must be taken to use scrupulously clean glassware. We recycle cuvettes and test-tubes, rinsing with glass-distilled water. Marking pens should be avoided as the ink gives a very intense interfering fluorescence which, when the cuvettes or test-tubes are washed, can contaminate the solution in trace amounts. The fluorometer is thermostated at room temperature as the assay is sensitive to temperature. By this method the ^{125}I HeLa cell DNA was found to contain 15% single-stranded areas of the DNA molecule. As this technique will detect duplex DNA with significant amounts of denatured regions, this represents a substantially pure DNA preparation. Analysis on hydroxyapatite columns confirms the manufacturer's finding of no significant contamination with predominantly ss DNA.

OPERATIONAL CRITERIA

The specificity of the test antigen for n DNA in practice can be confirmed by either testing sera known to contain antibody to ss DNA alone or by inhibition of n DNA binding by preincubation of test sera with excess quantities of other polynucleotides including ss DNA.

Rabbit antisera were raised to ss DNA by immunising with ss DNA inked to MBSA in complete Freund's adjuvant. The specificity of this antibody was confirmed by demonstrating binding of antiserum to heat-denatured ^3H labelled DNA but not to n DNA. Rabbit antisera bound 67% of ^3H labelled ss DNA (normal rabbits bound $<10\%$).

On agar immunodiffusion plates sera from three sensitised rabbits failed to show precipitation lines against the Amersham/Searle ^{125}I HeLa cell DNA but did show precipitation lines against the same antigen after heat denaturation. In addition, n

significant binding of the HeLa cell DNA was demonstrated when tested with rabbit antisera using both a Millipore or Farr radioimmunoassay. Ten sera from patients with high levels of binding to the HeLa cell DNA were preincubated with a 20-fold excess of both unlabelled ss DNA and n DNA. Significant inhibition of DNA binding of up to 95% was demonstrated using excess n DNA but no sera showed inhibition of more than 9% when preincubated with ss DNA.

The procedure is performed according to the detailed instructions, and a curve of counts (^{125}I counts) is plotted against the anti-DNA antibody concentration in units per ml using the standards included. Anti-DNA antibody concentration in the same units per ml in the unknown serum can then be extracted from the standard curve as drawn. In most cases, where anti-DNA antibody activity is greater than the maximum standard serum provided, these antibody activities can be calculated by repeating the test using dilutions of the unknown serum.

Using this technique we have tested 78 sera from 54 patients with SLE fulfilling the ARA criteria (Cohen *et al.*, 1971), 50 sera from patients with classical rheumatoid arthritis (RA) fulfilling the ARA criteria (Ropes *et al.*, 1959), 33 sera from patients with progressive systemic sclerosis (PSS), and 57 sera from patients with definite ankylosing spondylitis (AS) fulfilling the New York criteria (Bennett and Burch, 1967). Fifty sera from normal subjects have been evaluated for the presence of DNA antibodies and used as controls.

Results

Results of DNA antibody concentration in the serum tested are expressed in the Table. Using this kit, levels of DNA binding greater than 50 units were

seen only in the sera from patients with SLE. No sera from the other disease groups tested had more than 50 units. Five sera from patients with SLE had 25-50 units of DNA binding, but levels in the same range were seen also in one serum from a patient with PSS and in six sera from patients with RA. All sera from patients with AS and the majority of sera from patients with RA and PSS bound less than 25 units of DNA.

Some patients with SLE whose disease was in remission also bound levels less than 25 units.

These results suggested that the upper limit of normal of 25 units of DNA bound per ml, as expressed by the manufacturers, may be too low for the most effective differentiation between patients with SLE and other connective tissue diseases, particularly rheumatoid arthritis.

Discussion

Our results show that the Amersham/Searle assay kit is a convenient, reliable, and reproducible, method for detecting antibodies to double-stranded DNA. In particular, the kit has clinical value and does appear to differentiate between those sera containing significant amounts of n-DNA antibodies and those which do not, as has previously been reported (Holian *et al.*, 1975). The few false positive results could be eliminated if the upper limit of normal were designated as 50 units per ml.

The inclusion of known standards is an important factor as it allows for interlaboratory standardisation. In the past, however, most workers in this field have included known standards of their own when routinely testing for double-stranded DNA antibodies in their own laboratory using their own specific technique and DNA. This approach has led to day-to-day standardisation within one laboratory although it has not improved interlaboratory reference. While the inclusion of standards with the assay kit will overcome this to some extent it is perhaps unfortunate that another expression of results has had to be introduced (ie, units DNA bound/ml).

The main advantage of this assay system is the nature of the DNA used as test antigen. Recent reports have clearly shown that the differing antigens used in different centres do not necessarily react similarly in given test conditions. In particular, variations in molecular weight and contamination with ss DNA can significantly alter results (Aarden *et al.*, 1975; Picazo and Tan, 1975).

This problem has previously been emphasised in other studies using differing assay systems and differing DNA (Winfield and Davis, 1974). Evaluation of the nature of the test antigen used is there-

Table Number of sera with elevated DNA binding as detected by kit assay in patients with SLE, PSS, AS, and RA

Disease	No. of sera tested				
		Total	< 25 units	25-50 units	> 50 units
Controls	50	50	50	0	0
Systemic lupus erythematosus	78	11	5	62	
Progressive systemic sclerosis	33	32	1	0	
Ankylosing spondylitis	57	57	0	0	
Rheumatoid arthritis	50	44	6	0	

fore critical as impure antigen will lead to falsely high results which will reduce the sensitivity and specificity of the assay. Our assessment of the purity of the ^{125}I HeLa cell DNA used in this kit, with the low levels of ss DNA, suggests a high degree of specificity of the antibody being measured. Although this DNA has a high MW (10^7) relative to other DNAs, variations in MW due to age and batch with differing chain lengths will again be overcome by the inclusion of the known standards.

It is our opinion that this standardised assay and others like it will be of clinical value in measuring anti-DNA antibodies in the routine service laboratory and will improve the reliability with which DNA antibody results can be interpreted.

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