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STUDIES ON DNA ANTIBODIES USING DNA LABELLED WITH ACTINOMYCIN-D (^{3}H) OR DIMETHYL (^{3}H) SULPHATE

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SUMMARY

Native calf thymus DNA was labelled *in vitro* with tritiated actinomycin-D or tritiated dimethyl sulphate. These chemical methods yield DNA of high specific activity, without degradation or detectable alteration in reactivity with antibody to nucleic acid. The labelling procedures are simple, rapid and capable of producing relatively large amounts of radioactive DNA. Furthermore, they are applicable to a wide range of molecules of interest in the study of nucleic acid antibodies.

The labelled DNA was used in the ammonium sulphate precipitation technique to demonstrate DNA antibodies in the sera of patients with systemic lupus erythematosus. Whereas only nineteen of thirty-seven sera studied had demonstrable precipitins, thirty-three had significant binding ability for native DNA with this more sensitive assay. Serial studies on sera from a patient with systemic lupus erythematosus illustrate the value of this assay system for following the course of the disease.

INTRODUCTION

Antibodies specific for DNA have been implicated in the pathogenesis of systemic lupus erythematosus (SLE) (Tan *et al.*, 1966; Koffler, Schur & Kunkel, 1967). These immunoglobulins were first demonstrated by complement fixation (Robbins *et al.*, 1957; Seligmann & Milgrom, 1957) and precipitin reactions (Seligmann, 1957; Deicher, Holman & Kunkel, 1959) and subsequently have been studied by passive cutaneous anaphylaxis (Deicher *et al.*, 1960) haemagglutination (Jokinen & Julkunen, 1965) immunofluorescence (Casals, Friou & Myers, 1964) bentonite flocculation (Bozicevich, Nasou & Kayhoe, 1960) and ammonium sulphate precipitation (Wold *et al.*, 1968). Since certain populations of antibodies do not fix complement and may have low precipitating and haemagglutinating ability (Minden, Reid & Farr, 1966) the ammonium sulphate method, which measures the binding of labelled antigen to γ-globulin, has the advantage of detecting a wider spectrum of antibodies. Its main limitation is the requirement for radioactive DNA. The present report concerns the

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use of DNA labelled *in vitro* with tritiated actinomycin-D or tritiated dimethyl sulphate in the ammonium sulphate precipitation test.

MATERIALS AND METHODS

Antigen and reagents

Highly polymerized calf thymus deoxyribonucleic acid (DNA) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Tritiated actinomycin-D (Schwarz Bioresearch Inc., Orangeburg, New York) at a specific activity of 2·7 c/m-mole, and tritiated dimethyl sulphate (New England Nuclear, Boston, Massachusetts) at a specific activity of 100 mc/m-mole, were used for labelling.

Labelling procedures

Actinomycin-D DNA (AM-DNA) was prepared by adding 100 μc of actinomycin-D (^{3}H) to 10 mg of native DNA in 20 ml of 0·02 M-phosphate buffered saline (PBS) pH 7·4. The solution was incubated in the dark at 37°C for 16 hr and then extensively dialysed against pH 7·4 PBS until the dialysate was without significant counts. The specific activity of the AM-DNA was 15,000–20,000 counts/min/μg of DNA (counting efficiency 35%). Higher specific activities could be obtained by increasing the amount of actinomycin-D (^{3}H) added.

Methylated DNA (CH_3-DNA) was prepared by the method of Pochon & Michelson (1967) using tritiated dimethyl sulphate. The specific activity of the product was 400–600 counts/min/μg of DNA.

Scintillation counting

The scintillation fluor was made up by dissolving 7 g PPO (2,5-diphenyloxazole), and 0·36 g POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene) (New England Nuclear, Boston, Massachusetts) in 1 litre of spectroquality toluene (Matheson, Coleman, and Bell, East Rutherford, New Jersey). NCS (Amersham-Searle, Chicago, Illinois) was utilized to solubilize the precipitates and a Packard Tri Carb series 3000 liquid scintillation spectrometer with external standardization was used for counting. Counting efficiency for tritium was 35–40%.

Antibody assay

Precipitins to native DNA were demonstrated by double diffusion in 0·6% agarose (Tan *et al.*, 1966). The ammonium sulphate technique (Minden & Farr, 1967) was used with the following modifications: 0·1 ml of serum was added to AM-DNA or CH_3DNA and diluted to 1·0 ml with PBS, pH 7·4. The samples were incubated at 37°C for 1 hr, and at 4°C for 70–72 hr. An equal volume of saturated ammonium sulphate was added and stirred vigorously for 30 min. The resultant precipitate was centrifuged at 2000 rev/min for 1 hr, and washed once with half saturated ammonium sulphate. It was dissolved in 1–2 ml of NCS and added to 14 ml of fluor. Counts were corrected for quenching. Antibody assay was carried out with the AM-DNA complex diluted 1:10 with cold carrier DNA. The CH_3DNA was used as prepared, without carrier dilution.

Chemical determination of DNA in the precipitates

A modified diphenylamine test for determining DNA in the presence of serum proteins was used (Tan *et al.*, 1966).

Specificity of binding

The specificity of binding to AM-DNA and CH_3DNA was tested by pre-incubating the serum with 100, 250 and 500 μg of native DNA for 1 hr at 37°C, and then overnight at 4°C.

RESULTS

Development of method

Precipitin tests

Quantitative precipitin tests (Kabat & Mayer, 1961) performed with three SLE sera containing precipitating antibodies to DNA revealed no differences between the reactivity of native DNA and AM-DNA (Fig. 1) or CH_3DNA. Normal serum showed no reactivity with either the native or labelled preparations.

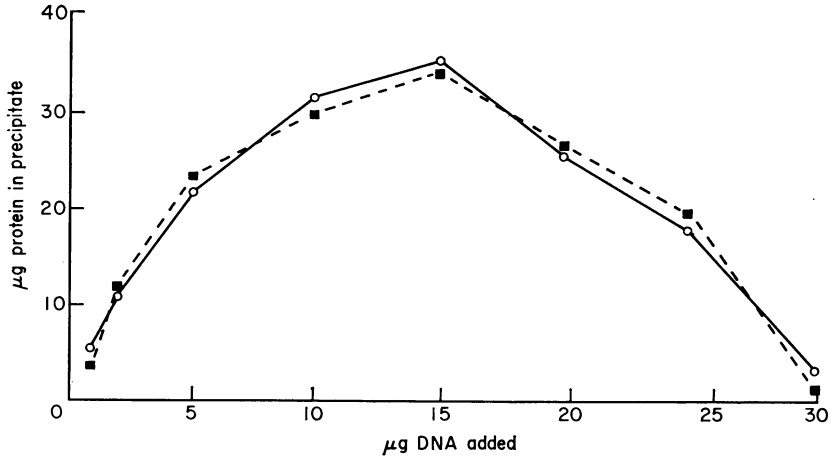

FIG. 1. Comparison of the quantitative precipitin curves obtained with native DNA (○) and AM-DNA (■).

Comparison of chemical and radioactive quantitation of DNA in precipitate

The amount of DNA in the ammonium sulphate precipitate, measured by the diphenylamine test or calculated from the per cent of radioactivity precipitated, was the same for the three sera studied (Table 1).

Determination of optimum amount of antigen and incubation time

Sera from twenty patients with SLE and twenty normal subjects were incubated with four concentrations of AM-DNA (Table 2). The best differentiation between the SLE and normal sera was obtained with 50 μg of AM-DNA and this amount was used in all further studies.

The optimal incubation time was determined by adding AM-DNA to two SLE and two normal sera. The amount of nucleic acid bound after incubation at 4°C for 19, 41, 72 and 110 hr was determined (Fig. 2). A 72-hr incubation period was used for all subsequent studies.

TABLE 1. Comparison of chemical and radioactive assays for DNA bound to γ-globulin of three reactive SLE sera

Serum	μg AM-DNA added	Counts/min added	AM-DNA in precipitate			Ratio* DPA/counts/min
			Diphenylamine (μg AM-DNA)	Radioactivity		
				Counts/min in precipitate	μg AM-DNA	
1	44·5	381,500	23·5	199,000	23·5	1·00
2	44·5	381,500	9·0	73,800	8·7	1·03
3	44·5	381,500	12·5	106,100	12·7	0·99

* Expressed as μg AM-DNA in precipitate measured by diphenylamine reaction (DPA) divided by μg AM-DNA calculated from per cent of counts/min precipitated.

TABLE 2. Per cent AM-DNA in precipitate when different amounts of antigen were incubated with 0·1 ml of serum

Sera	No. tested	μg AM-DNA added			
		25	37·5	50	75
SLE sera with precipitins for DNA	10	*67·3	58·6	59·3	51·5
SLE sera without detectable DNA precipitins	10	18·2	15·9	15·3	13·2
Normal sera	20	9·3	8·1	6·1	5·8

* Per cent of AM-DNA precipitated (mean value).

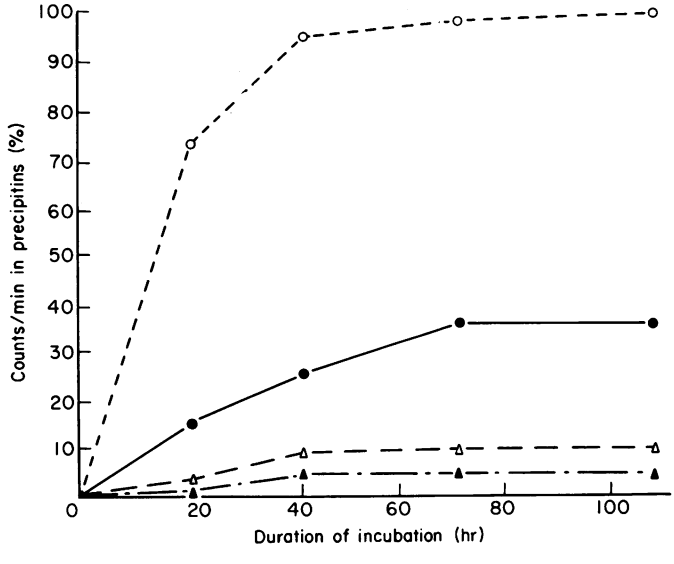

FIG. 2. Time course of binding of AM-DNA to γ-globulin assayed by ammonium sulphate precipitation technique. Circles, SLE sera; triangles, normal sera.

Comparison of Reactivity of AM-DNA and CH_3DNA

The reactivity of thirty sera with AM-DNA and CH_3DNA was compared (Table 3). There was no significant difference in affinity for the two types of labelled native DNA. Of the SLE sera, the ten with precipitins bound a mean of 37·8% of the added AM-DNA, and 35·6% of the CH_3DNA. The mean binding shown by the ten SLE sera without demonstrable precipitins was 12·5% for AM-DNA and 11·6% for CH_3DNA. Less than 5% of either

TABLE 3. Comparison of binding of AM-DNA and CH_3DNA with SLE and normal sera

		% of labelled DNA detectable in the half saturated ammonium sulphate precipitate	
		AM-DNA	CH_3DNA
SLE sera with precipitins to DNA	1	31·8	26·9
	2	61·3	64·1
	3	47·1	41·2
	4	15·2	11·2
	5	18·6	18·7
	6	41·0	36·2
	7	57·4	53·2
	8	30·9	25·4
	9	42·1	45·3
	10	33·0	33·6
Mean		37·8	35·6
SLE sera with no detectable precipitins to DNA	1	8·8	5·6
	2	10·8	9·8
	3	4·8	6·3
	4	13·4	14·4
	5	9·1	8·4
	6	20·9	18·9
	7	18·3	15·1
	8	18·9	18·2
	9	5·1	4·5
	10	14·5	15·1
Mean		12·5	11·6
Normal sera	1	4·9	4·0
	2	4·3	4·1
	3	3·2	1·6
	4	4·6	2·7
	5	3·9	3·1
	6	3·5	3·3
	7	3·8	1·9
	8	4·1	3·4
	9	6·2	5·7
	10	5·3	4·7
Mean		4·4	3·9

antigen was found in the ammonium sulphate precipitates of normal sera (AM-DNA 4·4%, CH_3DNA 3·9%).

Inhibition

Pre-incubation of reactive SLE sera with 100 μg of native DNA reduced the binding capacity for AM-DNA and CH_3-DNA by 40–60%. Pre-incubation with 500 μg of DNA inhibited the binding of labelled DNA 65–80%.

Sensitivity

The sensivity of the ammonium sulphate precipitation technique for the demonstration of antibody to DNA was shown by the fact that selected reactive sera could be diluted 1:10,000 or more before their capacity to bind DNA was reduced to the level of normal sera.

Applications

The sera of thirty-seven patients with systemic lupus erythematosus were examined (Table 4). Nineteen sera contained precipitating antibodies to native DNA and all of these showed significant binding capacity for DNA. Fifteen to 90% of the added antigen was found in the γ-globulin precipitates. Of the eighteen sera without detectable precipitins for native DNA, fourteen showed ability to bind AM-DNA. In general their binding capacity was lower than those sera with demonstrable precipitins.

TABLE 4. AM-DNA binding by systemic lupus erythematosus sera, isolated myeloma proteins, rheumatoid arthritis sera, normal human sera and isolated human Fraction II

% AM-DNA in Pcpt.	SLE sera with DNA precipitins	SLE sera without DNA precipitins	Isolated myeloma proteins (20 mg/ml)	Rheumatoid arthritis sera	Normal sera	Isolated human Fraction II (20 mg/ml)
<10		4	10	9	40	4
10–25	5	12		1		
25–50	8	1				
50–75	6	1				
75–100	1					
Totals	19	18	10	10	40	4

With the sera of forty normal subjects, ten isolated myeloma proteins (including eight IgG, one IgA and one IgM proteins), and four different preparations of isolated human Fraction II, less than 10% of the AM-DNA was found in the precipitate. Of ten rheumatoid arthritis sera studied, one bound more than 10%.

Fig. 3 illustrates serial studies on sera from a patient without demonstrable precipitating antibody to DNA. These sera, however, showed significant reactivity using the ammonium sulphate technique, and there was a correlation between the DNA binding of the serum and

the activity of the disease. The peak of DNA binding occurred concomitantly with maximal proteinuria, depressed serum complement levels, and depressed creatinine clearance. When steroids were increased there was a rapid drop in anti-DNA activity which was paralleled by a rise in serum complement and a decrease in proteinuria.

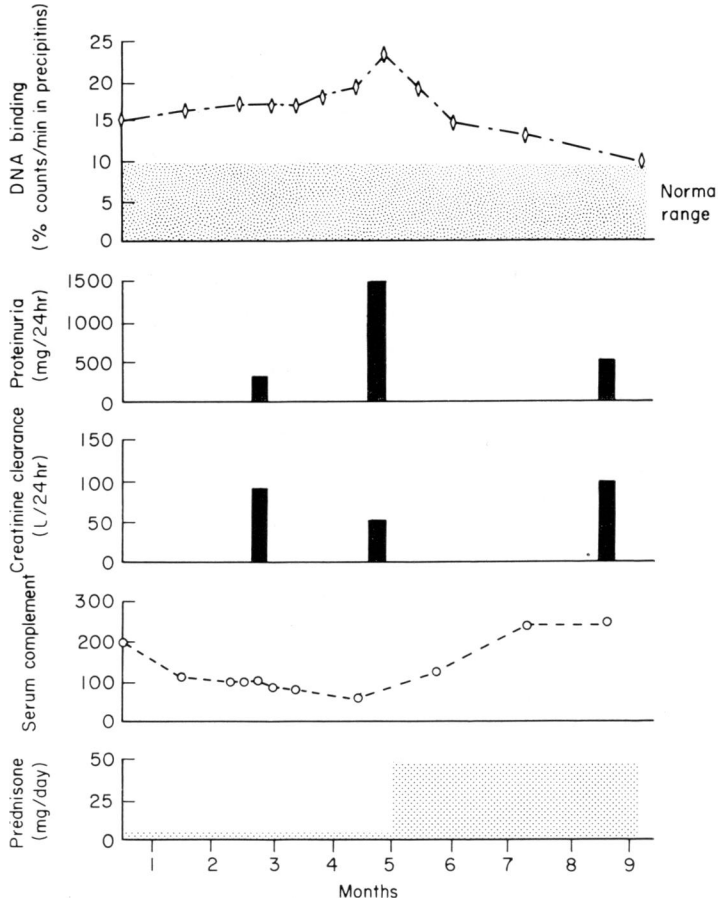

FIG. 3. Presence of DNA antibodies in consecutive serum samples of a patient with SLE during the acute phase of her illness. No precipitins to DNA were demonstrable.

DISCUSSION

Recently (Wold *et al.*, 1968) it was reported that DNA is soluble in half saturated ammonium sulphate, but DNA bound to γ-globulin is precipitated. Thus the ammonium sulphate precipitation technique could be used for the demonstration of DNA antibodies. This method detects a broad spectrum of antibodies whereas precipitin tests, complement fixation, and haemagglutination measure select populations of antibody (Minden *et al.*, 1966). The ammonium sulphate technique requires the use of radioactive antigen of high specific activity and several methods have been used to prepare radioactive DNA. The growth

of micro-organisms in a medium containing radioactive nucleic acid precursors is commonly employed (Wold *et al.*, 1968) but can only be used to obtain labelled DNA from bacteria and viruses and may require elaborate culture procedures. Mammalian DNA has been labelled by culturing tumour cells (Trowell, 1966) in the presence of radioactive nucleotides or by injection of partially hepatectomized rats with ^{32}P (Niehaus & Barnum, 1964) but the yields or specific activities are relatively low. Since the study of anti-DNA antibodies should include the examination of isologous and homologous, as well as heterologous nucleic acids, a labelling method which would yield radioactive DNA of high specific activity from any species is desirable. Several techniques for the labelling of DNA *in vitro* have been suggested, but the specific activity is low, or degradation of the DNA occurs (Searcy, 1968).

Tritiated actinomycin-D and tritiated dimethyl sulphate were utilized in this study to introduce radioactivity into DNA. The labelling procedure with both compounds is simple, rapid, and does not result in detectable alteration of the precipitability of the DNA by antibody. Although the binding of actinomycin-D to DNA is dependent on hydrogen bonding, it is resistant to changes in pH, temperature, and to high salt concentration (Reich & Goldberg, 1964). In the ammonium sulphate assay for antibody, the AM-DNA complex remains intact and the radioactivity found in the precipitate is a reliable indicator of the amount of nucleic acid. This is shown by the comparison between the quantity of DNA in the precipitate measured chemically and as calculated from the per cent of radioactivity precipitated. Both methods yield the same results. Furthermore, with sera containing DNA antibodies, the AM-DNA complex exhibited reactivity similar to the covalently labelled CH_3DNA.

In the assay of anti-DNA antibodies in the sera of thirty-seven patients with active systemic lupus erythematosus, 92% were positive with the ammonium sulphate technique, whereas only 51% gave precipitin lines in agarose. Certain sera at dilutions of 1:10,000 to 1:25,000 showed significant reaction with AM-DNA. In contrast precipitins were not demonstrable with sera diluted 1:100 and haemagglutination with the same sera gave titres of less than 1:512 (data to be published). The maximum titre assayed by complement fixation was 1:1000 and several sera with significant AM-DNA binding showed no complement fixation (unpublished observations).

The value of the ammonium sulphate technique for following the fluctuations in native DNA antibody levels is shown by the serial studies on patient R.J. Although no anti-DNA precipitins were demonstrable, the AM-DNA binding capacity increased during an exacerbation of the disease, reaching a maximum level when the creatinine clearance and serum complement levels were most depressed, and proteinuria was 1·5 g/day. When steroids were increased there was a rapid fall in reactivity with AM-DNA, paralleled by a decrease in activity of the disease. Thus the test may be a useful guide for therapy.

Actinomycin-D also binds to single strand DNA although the complex is not as stable (Reich & Goldberg, 1964). When the ammonium sulphate technique was used to measure antibody activity against actinomycin-D labelled single strand DNA (AM-SSDNA) it was found that normal sera showed considerable reactivity (unpublished observations). This reactivity could be abolished in twenty-four of twenty-six sera examined by heating them at 56°C for 30 min prior to addition of the AM-SSDNA. Further investigations as to the nature of this heat labile reactivity are being carried out. The heating procedure had no effect on the antibodies in SLE sera and the test system appeared feasible.

Interest in the possible existence of soluble DNA–anti-DNA complexes *in vivo* has been

stimulated by the discovery of native DNA circulating in the sera of certain SLE patients with active renal disease (Tan *et al.*, 1966). A selective localization of anti-DNA antibody and DNA antigen in the glomeruli of some patients has provided further support for the antigen–antibody complex hypothesis for renal injury in SLE (Koffler *et al.*, 1967). The concentration of DNA and specific antibody in the glomerulus suggests that soluble circulating complexes are present in the sera, although no direct evidence is as yet available. Actinomycin-D binds to both free DNA and to DNA combined with antibody in soluble complexes (unpublished observations) and we are examining its use for an assay to demonstrate and measure free and bound DNA in biological fluids.

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