Measurement of DNA antibodies by double antibody precipitation

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Antibodies to nuclear constituents particularly to native deoxyribonucleic acid (DNA) have been detected in the serum and tissues of patients with systemic lupus erythematosus (SLE) and may be involved in the pathogenesis of the disease when deposited as immune complexes (Tan, Schur, Carr, and Kunkel, 1966). The methods used for the detection in serum included immunoprecipitation (Seligmann, 1957) and complement fixation (Ceppellini, Polli, and Celada, 1957). More recently an isotope-labelled DNA-binding technique (Wold, Young, Tan, and Farr, 1968) has provided an assay which is of considerably increased sensitivity and has become a widely used method of demonstrating DNA antibodies (Pincus, Schur, Rose, Decker, and Talal, 1969; Hughes, Cohen, and Christian, 1971). However, this assay has certain inherent problems in the separation of bound from free antigen which led us to examine the use of immunoprecipitation with an anti-human gamma globulin. This communication describes a method using a double or second antibody.

Material and methods

(1) EXTRACTION OF LABELLED DNA

Thymidine-2-¹⁴C or thymidine-6-³H (Amersham) was incubated with 20 to 30 × 10⁶ HeLa cells cultured in Eagle's MEM for 72 hrs. After the supernatant had been poured off the monolayers were trypsinized (0.25 per cent. bovine pancreatic trypsin in Hanks's basic salt solution), the cells washed twice in Earle's saline, and the DNA extracted by using a modification of the method of Marmur (1961). The cells were disrupted by 1 per cent. sodium lauryl sulphate in 1M sodium perchlorate. The resulting viscous solution was washed five times with chloroform saturated with water (containing 1 per cent. isoamylalcohol) until the white protein precipitate was no longer visible at the interface. The DNA in the final aqueous layer was precipitated with 2 volumes of ethylalcohol, 'spooled' into citrate saline buffer (0.15 M NaCl in 0.015 M sodium citrate (SCS) pH 7.1), and allowed to dissolve. Before the DNA was used it was purified further by two chloroform washes and reprecipitated with 2

volumes of ethylalcohol overnight at -20°C. After centrifugation at 2,000 G for 30 min., the DNA was re-dissolved again in citrate saline buffer and stored at -20°C. The specific activity of the ¹⁴C or ³H-labelled product ranged from 0.005 to 0.14 μc./μg. and was used in the method described. DNA labelled with a gamma emitter could also be obtained by incubation of the thymidine analogue 5-iododeoxyuridine ¹²⁵I with HeLa cells, and provided a satisfactory alternative.

(2) ASSAY FOR DNA ANTIBODIES

50 μl. serum was diluted 10-fold with 0.15 M borate saline buffer pH 7.8 and heated at 56°C. for 30 min. Duplicate 50-μl. aliquots were then incubated with an equal volume of SCS buffer containing labelled DNA equivalent to 1 μg. as estimated by optical density readings at 260 mμ. The mixture was incubated for 1 hr at 37°C. and 16 hrs at 4°C. The bound antigen was then separated from free antigen by one of two following methods:

(a) Double antibody

100 μl. of a goat anti-human gamma globulin* diluted in borate saline buffer and used in excess was added to each tube and incubated at 4°C. The best time course for the double antibody method was determined by experiments in which the bound DNA was separated at intervals over 0 to 96 hrs (Figure, opposite).

Thereafter a 24-hr separation was used routinely. After centrifugation at 2,000 G × 1 hr at 4°C. the supernatant was usually discarded and the precipitate dissolved in 400 μl. distilled water and counted in Bray's scintillant.† A range of antisera used as the second antibody included those specific for the heavy chains of IgG, IgM, and IgA, to determine the class of DNA antibody.

(b) Ammonium sulphate

100 μl. of a saturated solution of ammonium sulphate was added to each tube to give a 50 per cent. saturated mixture which after incubation at 4°C. for 1 hr was centrifuged at 2,000 G × 1 hr at 4°C. Half of the

* Antibodies Inc. Box 1482 Davis, California 95616.

† Bray's scintillant (1960).

FIGURE Time course for addition of second antibody

supernatant (*i.e.* 100 μ l.) was removed and the remainder was dissolved in water and both fractions counted in parallel using Bray's scintillant.

(3) CRITERIA FOR SERUM SAMPLE SELECTION

To estimate the precision of each method, 125 sera from normal individuals and 125 sera from SLE patients were assayed by both methods, each in duplicate pairs. The results were arbitrarily grouped into the following four ranges of percentage DNA binding: 0–25, 25–50, 50–75, and 75–100. Each group contained at least twenty duplicate pairs measured by both assays.

(4) EXTRACTION OF DNA ANTIBODY FROM RENAL TISSUE

DNA antibody was extracted in glycine buffer at pH 2.4 from a homogenate of a kidney obtained at autopsy of a patient with lupus nephritis, using the method described by Koffler, Schur, and Kunkel (1967). The eluate, supplemented with 10 per cent. normal human serum, was examined for the relative concentrations of immunoglobulin classes of DNA antibody in the manner described above for sera, by addition of monospecific antisera to IgG, IgM, and IgA.

(5) CALCULATION AND STATISTICAL SIGNIFICANCE OF RESULTS

The percentage DNA bound was calculated for the ammonium sulphate method of separation by using supernatant and precipitate counts, and for the double antibody assay by counting the precipitate only.

For ammonium sulphate,

$$\text{Percentage DNA bound} = \frac{R - Su}{R + Su} \times 100,$$

where Su = disintegrations per min. (d.p.m.) in half the supernatant and R = d.p.m. in the remaining supernatant and the precipitate.

For the double antibody assay,

$$\text{Percentage DNA bound} = \frac{\text{d.p.m. in precipitate}}{\text{d.p.m. of total DNA}} \times 100.$$

Precision of the results within the five groups for each assay was calculated by the formula derived by Snedecor (1952) for assessing differences between duplicates where S (the standard deviation) is calculated as follows:

$$S = \sqrt{\frac{d^2}{2N}}$$

where d = difference between any two duplicates and N = number of duplicates.

The fiducial limits were obtained using Fisher's formula:

$$\text{Fiducial range} = M \pm \frac{tS}{\sqrt{N}},$$

where M is any value of per cent. DNA binding; t is the appropriate Student's factor obtained from tables of probability; N is the number of replicate values; and S is the standard deviation.

Results

The double antibody and ammonium sulphate separations gave comparable mean values for each of the four ranges of the lupus and 25 normal sera tested (see Table I). The coefficient of correlation between the two methods was $r = 0.91$ ($P < 0.001$).

Table II shows duplicate values of DNA binding in twenty separate samples in the 0 to 25 per cent. range. The double antibody values were in general closer to one another than those obtained with ammonium sulphate. When the differences between the duplicates were evaluated, the 95 per cent. fiducial limits

Table I Mean values of DNA binding of normal and SLE sera

Percentage DNA binding	Ammonium sulphate	Double antibody
Healthy controls*	2	6
SLE†		
sera [range]		
0–25	8	9
25–50	33	38
50–75	63	57
75–100	78	87

* 25 sera.
† 100 sera.

Table II Duplicate DNA-binding percentages estimated by double antibody and ammonium sulphate separation SLE sera: 0 to 25 per cent. range

Method	DNA-binding (duplicate percentages)																			
Double antibody	2.1 2.3	3.4 5.1	4.0 5.3	4.4 5.0	4.6 4.8	4.8 4.8	5.0 5.9	5.3 6.1	5.5 6.6	5.6 7.8	5.7 6.4	6.3 7.7	7.7 8.3	8.3 9.8	9.9 10.7	12.4 12.5	15.3 15.6	15.3 16.5	15.6 17.2	21.9 25.1
Ammonium sulphate	6.0 6.8	-1.5 0.0	3.2 5.5	-2.1 4.9	8.3 10.8	10.8 16.2	1.9 3.6	-1.5 2.1	1.3 -3.4	4.5 10.1	19.4 22.9	0.0 3.8	10.1 11.3	1.1 4.6	0.0 0.0	-2.6 -4.7	11.1 21.6	12.2 13.7	11.5 14.5	13.6 27.6

obtained for both are as shown in Table III. It can be seen that, for any estimate of DNA binding, the double antibody separation gives a more precise value than the ammonium sulphate separation. For example, a serum giving a 60 per cent. binding value would fall between 53.9 and 66.1 per cent. for the double antibody separation and between 47.6 and 72.4 per cent. for the ammonium sulphate separation on 95 out of 100 occasions.

Table III Comparison of 95 per cent. fiducial limits using double antibody or ammonium sulphate separations

Method of separation	Double antibody	Ammonium sulphate
Healthy controls	$M \pm 3.1$	$M \pm 6.6$
SLE sera	$M \pm 2.6$	$M \pm 6.3$
range	$M \pm 5.3$	$M \pm 8.4$
(per cent.)	$M \pm 6.1$	$M \pm 12$
75-100	$M \pm 6.7$	$M \pm 15$

M = Any DNA-binding value within the stated ranges.

The relative DNA binding of the immunoglobulin classes can be detected readily. In the example in Table IV, the patient's serum values are compared with the values obtained with DNA antibodies extracted from her kidney obtained *post mortem*.

Table IV Immunoglobulin in classes of DNA antibodies present in serum and renal tissue of a patient with SLE (per cent. DNA binding)

Double antibody specificity	Serum	Post mortem renal tissue eluate pH 2.4
Anti IgG	31	94
Anti IgM	18.5	13
Anti IgA	25.5	4

Discussion

In our experience the double antibody assay of DNA binding in serum gives a method which has narrower

confidence limits than the ammonium sulphate assay. It is at the same time technically simpler for routine use because the precipitate is easy to separate by simply tipping off the supernatant in contrast to the friable and less stable precipitate obtained with ammonium sulphate which has to be carefully separated from the supernatant. Moreover, the values from counting the precipitate alone were comparable to those obtained by counting both the precipitate and supernatant thus simplifying the procedure further.

The improved precision of the double antibody method gives a more sensitive assay which allows better discrimination between lupus and normal sera. Furthermore, the change in a given value of binding at any range that will be significant will be smaller allowing more meaningful comparisons.

Numerous other methods of separating bound from free antigens are available (Hunter and Ganguli, 1971), and DNA antibody has been assayed by the use of a membrane filter separation (Ginsberg and Keiser, 1972) and a solid phase separation (Epstein, Tan, and Easterbrook, 1971). The double antibody separation has been compared with the ammonium sulphate method in terms of its biological specificity for antibodies to bovine serum albumin and gave corresponding results (Minden, Anthony, and Farr, 1969). In this study a good correlation has also been demonstrated between the two methods. The further advantage of the double antibody assay lies in being able to define the class of DNA antibody by using monospecific antisera and the heterogeneity of DNA antibodies is illustrated in Table IV. Inspection of the Table shows that, whereas in this instance (from a patient with SLE dying of renal failure) the three classes are distributed roughly equally in the serum, renal tissue contained predominantly IgG DNA antibody. Such data derived from the use of this easily-performed double antibody assay are currently being extended and evaluated to assess their full clinical significance.

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