Low avidity antibodies to dsDNA as a diagnostic tool

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SUMMARY An evaluation of the diagnostic value of low avidity antibodies to double stranded DNA (dsDNA) measured by the polyethylene glycol (PEG) assay was undertaken. By routine screening low avidity anti-dsDNA were detected in the serum samples of 106 hitherto unknown patients. Clinical data of these patients were collected and when only low avidity anti-dsDNA was present (n=92) a varied disease spectrum was observed. A diagnosis of systemic lupus erythematosus (SLE) was established in 48/92 (52%), lupus-like disease in 21/92 (23%), autoimmune hepatitis in 9/92 (10%), rheumatoid arthritis in 8/92 (9%), and mixed connective tissue disease in 2/92 (2%) of all patients. Patients with definite SLE were all older than 45 years and predominantly female (46/48, 96%). They showed a remarkably low incidence of renal disease (2/69, 3%). When high avidity antibodies to dsDNA as measured by the Farr assay were present as well (n=14) a diagnosis of SLE could be established in 12/14 (86%) of all patients indicating the secondary importance of low avidity anti-dsDNA in these patients.

Antibodies to double stranded DNA (dsDNA) are thought to play a part in the pathogenesis of systemic lupus erythematosus (SLE).¹⁻³ The precise mechanism by which they contribute to tissue damage is a subject of discussion. Deposits of circulating immune complexes⁴⁻⁶ and in situ formation of dsDNA/anti-dsDNA⁷⁻¹⁰ immune complexes always seemed valid explanations, but anti-dsDNA antibodies are heterogeneous and differ with respect to (sub)class, specificity, complement fixing ability, and avidity.^{11,12}

Nowadays, there are several methods of detecting antibodies to dsDNA.^{13,14} The main difference between such assays lies in their ability to detect antibodies to dsDNA with varying degrees of avidity.¹⁵

A clear relation has repeatedly been shown between high avidity antibodies detected by the Farr assay and disease activity.¹⁶⁻¹⁸ The role of low avidity antibodies has remained relatively uncertain,¹⁹ some reports showing a relation with a less fulminant form of SLE.²⁰ Therefore we undertook a study to evaluate the diagnostic significance of low avidity antibodies to dsDNA.

Patients and methods

SERUM SAMPLES/PATIENTS

From 1 January 1986 until 31 December 1987, every serum sample sent to the central laboratory for routine testing of anti-dsDNA that showed positive fluorescence by the *Crithidia luciliae* test was routinely tested in the polyethylene glycol (PEG) and Farr assays. When a positive test was observed in the PEG assay the attending physician was asked to provide clinical information about the patient by means of a questionnaire containing specific questions pertinent to symptoms of rheumatic diseases whenever possible the diagnosis was confirmed by clinical record investigation. During this period 376 serum samples of new patients were found positive on immunofluorescence test screening; 106 of these samples were positive on subsequent testing in the PEG assay, and this group was the subject of our study.

We divided patients into two groups: group I (n=92), patients whose serum samples showed anti-dsDNA antibodies only in the PEG assay (>10 U/ml) and group II (n=14), patients whose serum samples were positive (>10 U/ml) in both PEG and Farr assays.

Patients fulfilling four or more American Rheumatism Association (ARA) criteria for SLE³ were considered to have definite SLE, while patients

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fulfilling three ARA criteria for SLE were diagnosed as having lupus-like disease. Rheumatoid arthritis was considered present in patients with five or more ARA criteria²¹ for rheumatoid arthritis (definite rheumatoid arthritis), whereas autoimmune hepatitis was diagnosed on evidence of hepatitis without viral cause and the presence of antibodies to smooth muscle or mitochondria, or both.

CRITHIDIA LUCILIAE
IMMUNOFLUORESCENCE TEST

Details of this immunofluorescence test have been published.²² *Crithidia luciliae* were grown, harvested, and used as described. Serum samples were diluted 1:10 in phosphate buffered saline (PBS; 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.4) and 20 µl of such a dilution was incubated for 30 minutes at room temperature on a spot of *C. luciliae*. After washing the slide for 30 minutes in PBS 0.5 ml of fluorescein isothiocyanate conjugated anti-immunoglobulin (batch No SH 17-1-F9; dilution 1:50) was layered over the slide, followed by incubation for 30 minutes at room temperature. The slide was again washed with PBS and mounted, using a solution of 65% (w/v) sucrose in PBS pH 8.0, in which 0.5 µg/ml propidium iodide (Calbiochem) was dissolved. Fluorescence of the kinetoplasts was considered to indicate anti-dsDNA activity. The propidium iodide acts as a counterstain enabling localisation of the kinetoplasts. A KP-560 barrier filter was used to separate propidium iodide fluorescence from that of fluorescein isothiocyanate.

PEG ASSAY

Details of the PEG assay have been described.¹³ To 50 µl of a dilution of serum, 50 µl of a solution of 1.6 mg/ml normal human gammaglobulin, 50 µl of a solution of 0.2 mg/ml dextran sulphate (Pharmacia Fine Chemicals AB, Uppsala, Sweden, lot No 5259), 200 µl of 7% (w/v) PEG (mol. wt 6000; Koch-Light Laboratories, Colnbrook, UK), and 50 µl of a solution of 2 µg/ml [³H]-PM2-DNA (pseudomonal bacteriophage; specific activity 40–60×10³ dpm/µg DNA) were added, forming an incubation volume of 400 µl. All components were dissolved or diluted in PBS.

The incubation was carried out at 37°C for one hour, followed by a two hour incubation at 4°C. The mixture was then centrifuged for 15 minutes at 4000 rpm in a Hettich Rotanta/K centrifuge (2500 g). The radioactivity in 200 µl of the supernatant was measured after addition of 10 ml of NE-260_{SP} (New England Nuclear Corp, USA). A '0% binding' control, in which serum was omitted, was included in each experiment. Anti-DNA activity was expressed in U/ml instead of percentage binding. A sample

contained one unit of anti-DNA if it bound 30% of 100 ng PM2-DNA under the conditions described above.

FARR ASSAY

The Farr assay was performed as described by Aarden.¹⁴ To 100 µl of the appropriate serum dilution was added 50 µl of a solution of 16 mg/ml normal human gammaglobulin and 50 µl of a solution of 2 µg/ml [³H]-PM2-DNA. All components were dissolved or diluted in PBS.

After incubation of the mixture for one hour at 37°C 5 ml of a cold 50% saturated ammonium sulphate solution was added. The precipitate was allowed to form for 30 minutes at 4°C, after which it was pelleted by centrifugation for 15 minutes at 3000 g. The precipitate was washed twice with 50% saturated ammonium sulphate and, finally, dissolved in 1 ml of Soluene-100 (Packard). After addition of 10 ml scintillation fluid (Instafluor 11, Packard) the radioactivity was measured. The mean (SD) binding of 150 normal control sera was 5 (3)%. Units are defined as stated under the PEG assay.

ANTI-DNA AVIDITY

As antibodies to ds-DNA vary widely in their avidity towards DNA we used two assays to discriminate between patients on the basis of their anti-DNA avidity. As has been shown before the Farr assay is strictly selective for high avidity anti-DNA, whereas the PEG assay allows for the simultaneous measurement of low and high avidity anti-DNA.^{23 24} In fact, antibodies of the highest avidity are missed by the PEG assay because such antibodies tend to form rather small DNA/anti-DNA complexes, which are not precipitated by 3.5% PEG.¹⁵

STATISTICS

Statistical testing of data was performed with Student's *t* test for mean values and with χ^2 testing (with Yates's correction) for absolute numbers; a resulting *p* value <0.05 was considered significant. Values are expressed as means (SD).

Table 1 Number of patients whose serum samples were positive on anti-dsDNA testing by various assays

Assay	No (%) positive	
Positive on immunofluorescence testing	376	(100)
Positive in Farr assay only	270	(72)
Positive in PEG* assay only	92	(24)
Positive in Farr and PEG assays	14	(4)

*PEG=polyethylene glycol.

Table 2 *Diagnosis and demographic features of patients in groups I and II**

	Group I (n=92)		Group II (n=14)	
	No of female patients (%)	No of male patients (%)	No of female patients (%)	No of male patients (%)
Definite SLE†‡	46 (50)	2 (2)	7 (50)	5 (36)
Mean (SD) age at diagnosis (years)	46.9 (16.3)	75.5 (1.5)	30.7 (13.9)	35.7 (20.4)
Lupus-like disease†	20 (22)	1 (1)	0	0
Mean (SD) age at diagnosis (years)	52.9 (21.1)	34.0		
Autoimmune hepatitis‡	9 (10)	0	0	0
Mean (SD) age at diagnosis (years)	61.8 (19.5)			
Rheumatoid arthritis†	8 (9)	0	0	0
Mean (SD) age at diagnosis (years)	48.7 (14.8)			
MCTD‡	2 (2)	0	0	0
Mean (SD) age at diagnosis (years)	51.5 (2.5)			
Miscellaneous	3 (3)	1 (1)	1 (7)	1 (7)
Mean (SD) age at diagnosis (years)	54.6 (29.6)	69.0	35.0	37.0
Total No of patients	88 (96)	4 (4)	8 (57)	6 (43)

Group I=patients whose serum samples showed anti-dsDNA antibodies only in the PEG assay (>10 U/ml); group II=patients whose serum samples were positive (>10 U/ml) in both PEG and Farr assays.

† $p<0.01$ for number of patients in group I and group II.

‡SLE=systemic lupus erythematosus; MCTD=mixed connective tissue disease.

Results

SERUM SAMPLES

In the two year study period we found 376 serum samples positive on immunofluorescence testing for anti-dsDNA; these samples were all sent for diagnostic testing (Table 1). Upon subsequent testing 106 serum samples (28%) were positive in the PEG assay; 92 of these samples were positive in the PEG assay only, while 14 gave positive results in both Farr and PEG assays. Two hundred and seventy (72%) serum samples gave positive results in the Farr assay only; they were not studied further as our interest lay with the PEG positive sera, and the significance of Farr positive sera is well established.¹⁶⁻¹⁹

DIAGNOSIS AND DEMOGRAPHIC FEATURES

Table 2 shows diagnosis and demographic features of patients in both groups. A definite diagnosis of SLE was made in 12/14 (86%) of group II patients and in only 48/92 (52%) of group I patients ($\chi^2=3.12$; $p<0.002$). No other definite diagnosis was established in group II patients, while the remaining group I patients showed a varied pattern of autoimmune diseases, including lupus-like syndrome in 21/92 (23%), autoimmune hepatitis in 9/92

(10%), and rheumatoid arthritis in 8/92 (9%). Sex differences were striking, with a female preponderance (88/96, 96%) in group I patients compared with group II patients (8/14, 57%) ($\chi^2=19.75$; $p<0.001$). Age at the moment of diagnosis was similar for male and female patients with SLE in group II, whereas male group I patients with SLE were older than the women in this group ($p<0.001$). Group I patients with SLE (male and female) were significantly older than group II patients with SLE ($p<0.05$).

DISEASE MANIFESTATIONS

When only patients with definite SLE and lupus-like disease were considered (81/106, 76.4% of all patients) a very low rate of renal disease was observed in group I patients ($p<0.001$), while other disease manifestations did not differ between the two groups (Table 3).

ANTI-dsDNA TITRES

Figure 1 shows anti-dsDNA values by PEG assay for group I patients. Lower titres were found for patients with lupus-like disease (mean (SD) 41.1 (23.2) U/ml) and mixed connective tissue disease (mean (SD) 13.0 (19.2) U/ml) than for patients with SLE, whereas patients with other diseases had similar titres. Levels of anti-dsDNA by PEG assay

Table 3 Disease manifestations in patients with definite systemic lupus erythematosus and lupus-like disease

Disease	Group I† (n=69) No (%)	Group II‡ (n=12) No (%)
Arthritis	52 (75)	7 (58) NS
Skin abnormalities	29 (42)	4 (33) NS
Serositis	13 (19)	4 (33) NS
Nephritis	2 (3)	5 (42) *
CNS† manifestations	11 (16)	1 (8) NS
Haemolytic anaemia	4 (6)	1 (8) NS
Leucocytopenia	14 (20)	3 (25) NS
Thrombocytopenia	12 (17)	3 (25) NS

*p<0.001.
†CNS=central nervous system.
‡For description of groups see Table 2.

Fig. 1 Anti-dsDNA levels in the polyethylene glycol assay divided by the various diagnoses in group I; p value indicates difference from patients with definite systemic lupus erythematosus (bars indicate group mean). SLE=systemic lupus erythematosus; MCTD=mixed connective tissue disease.

in group II patients with SLE (mean (SD) 111.2 (105) U/ml) did not differ from levels in group I patients with SLE (mean (SD) 85.4 (117) U/ml), whereas anti-dsDNA levels by Farr assay in group II patients with SLE were higher (1053 (1985) U/ml) than those in the PEG assay of these patients (0.05<p<0.1).

Discussion

Previous experience has taught us that a significant number of patients' serum samples on testing for anti-dsDNA show a positive fluorescence in the *Crithidia* test but are negative upon subsequent analysis in the Farr assay. This discrepancy is explained by the fact that these patients have antibodies to dsDNA of low avidity, which are dissociated from

DNA by the high salt concentration used in the Farr assay to precipitate DNA/anti-DNA complexes.²³ Such antibodies can, however, be detected by the PEG assay, which is in fact a modified Farr assay, in which ammonium sulphate precipitation is replaced by polyethylene glycol precipitation.

High avidity antibodies, which are not dissociated in the Farr assay, have been shown to be specific for SLE¹⁶⁻¹⁹ and are now one of the criteria on which the diagnosis SLE can be based.³ The significance of low avidity antibodies to dsDNA is less clear.

In this study the diagnostic value of low density antibodies to dsDNA was analysed. When patients' serum samples contained high and low avidity antibodies (group II) a diagnosis of SLE could be made in 12/14 (86%) of the patients. This confirms the value of the Farr assay for diagnosing SLE, and thus the PEG assay has little additional value in these patients. When only low avidity antibodies were present, however, a much more diverse spectrum of diseases was observed: definite SLE in 48/92 (52%) and lupus-like disease (three ARA criteria) in 21/92 (23%) of all patients; autoimmune hepatitis in 9/92 (10%); rheumatoid arthritis in 8/92 (9%); and mixed connective tissue disease in 2/92 (2%). From this we conclude that there is an intermediate specificity of low avidity anti-dsDNA for SLE. When patients with SLE from both groups were compared, however, it was clear that patients with only low avidity anti-dsDNA had significantly less signs of renal involvement. Although early reports correlated the presence of low avidity anti-dsDNA with earlier onset and increased severity of lupus nephritis in mice and humans,²⁵ our results support the later reports that lupus nephritis in man is mainly associated with high avidity antibodies to dsDNA.^{20 26 27}

We found little correlation between titres of low avidity antibodies to dsDNA and the different diseases. Patients with lupus-like disease (a prodrome or a mitigated form of SLE?) and mixed connective tissue disease had lower values, and although no definite conclusion can be drawn from these data, it seems that the low avidity character of anti-dsDNA, rather than its level, is important in the diagnosis.

In conclusion, we studied the diagnostic value of low avidity antibodies to dsDNA and our results indicate that when only low avidity antibodies to dsDNA are found a spectrum of autoimmune disease is present and SLE can be diagnosed in about 50% of these patients. These patients which SLE are all elderly (>45 years) and predominantly female (95%), and they have a remarkably low incidence of renal disease. When high avidity antibodies are present as well a diagnosis of SLE can

be made in 86% of the cases, leaving only a minor role for low avidity antibodies to dsDNA in this setting.

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