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TECHNIQUES

The *Crithidia luciliae* kinetoplast immunofluorescence test in systemic lupus erythematosus

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SUMMARY

An immunofluorescence test for antibodies to native DNA, using the kinetoplast of *Crithidia luciliae* as substrate, has been assessed in comparison with the Farr precipitation technique, on a total of 395 sera from sixty-three patients with systemic lupus erythematosus, 185 other hospital patients and sixty healthy controls. The immunofluorescence test appears to have great specificity as a diagnostic test for SLE, though lacking the sensitivity of the Farr technique. Like the latter, it is altered by immunosuppressive treatment, and in patients with SLE nephritis on immunosuppression it does not show good correlation with activity of renal disease. Its specificity and simplicity nevertheless make it a valuable clinical test.

INTRODUCTION

Probably the most important autoantibodies found in systemic lupus erythematosus (SLE) are those directed against native double-stranded deoxyribonucleic acid (nDNA). Such antibodies in high titre are considered virtually specific for SLE, and by allowing soluble complex formation are responsible for many of the pathological features of the disease. Methods for detecting anti-DNA antibodies include gel diffusion (Seligmann, 1957), complement fixation (Robbins *et al.*, 1957), passive haemagglutination (Jokinen & Julkunen, 1965), immunofluorescence using 'spots' of purified DNA (Casals, Friou & Myers, 1964), counterimmunoelectrophoresis (Davis, 1971) and ammonium sulphate precipitation by the Farr technique (Wold *et al.*, 1968). This last has been found to be reproducible and highly sensitive, and is the method most commonly used in routine tests for anti-DNA antibodies.

In theory, any method involving chemical purification of DNA carries the risk of partially denaturing it with the formation of single-stranded regions. Antibody to single-stranded DNA has been demonstrated in a number of diseases other than SLE (Quismorio & Friou, 1974), so that partial denaturation of substrate DNA could lead to loss of specificity of any test using chemically prepared nDNA.

An elegant method for circumventing this difficulty was proposed by Aarden, de Groot & Feltkamp (1975), who described an immunofluorescent test using the haemoflagellate *C. luciliae* as substrate. This is an organism related to the trypanosomes, which is equipped with an intracellular organelle, the kinetoplast, that contains nDNA in high concentration, while apparently not containing any other recognizable nuclear antigens. Aarden and his colleagues concluded that no antigen other than nDNA played a part in kinetoplast immunofluorescence, and that their test was of the same order of specificity and sensitivity as the Farr test.

We have studied the specificity and sensitivity of the *C. luciliae* kinetoplast immunofluorescent test, and compared it retrospectively with the results of the Farr test, in patients with lupus nephritis and others whose sera had been referred to the Renal Unit or the Department of Medicine for immunological tests.

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MATERIALS AND METHODS

Sera. These were obtained in the majority of cases from the serum file of the Guy's Hospital Renal Unit, which performs all DNA-binding assays for the hospital group. Other sera were obtained from the Department of Medicine, where they had been sent for autoantibody tests; and others from the Rheumatology Department. Sixty control sera were obtained from sperm donors attending an infertility clinic. The distribution of sera according to the referring clinician's diagnosis was as follows:

Diagnosis	No. of patients	No. of sera
SLE with clinical nephritis	44	104
SLE without clinical nephritis	19	23
Discoid LE	5	5
SLE queried (undiagnosed)	17	23
Renal disease other than SLE	54	64
Rheumatological and collagen disease	78	81
Other diagnoses	31	35
Healthy controls	60	60
Total:	308	395

C. luciliae. These were originally obtained as a kind gift from Dr T. E. W. Feltkamp of the Netherlands Red Cross Blood Transfusion Service. They were stored at −70°C in Boné's medium (Bone & Steinert, 1956), pH 7·4 with 20% w/v glycerol, and thawed at 37°C immediately before culturing. They were cultured and applied to slides as described by Aarden *et al.* (1975); briefly, after washing in Boné's medium, an inoculum was introduced into 100 ml of medium and cultured at 25°C for about 48 hr. Organisms were harvested in the logarithmic growth phase, washed three times in phosphate-buffered saline, pH 7·4, at 3000 *g*, and suspended in distilled water at 2×10^7 organisms per ml. Drops of the suspension were applied to glass slides and air-dried at room temperature under a fan, then fixed in 96% ethanol for 10 min. They were stored in closed containers at −20°C until required. Aliquots of cultures were set aside for later use: these were transferred to medium containing 20% w/v glycerol and frozen over liquid nitrogen. Bacterial contamination of cultures were occasionally observed; it could be remedied by 24–48 hr culture in medium containing penicillin 100 u/ml and streptomycin 100 μg/ml, which do not affect *Crithidia* adversely.

Indirect immunofluorescence. This was carried out by standard techniques. Sera were normally diluted 1:10 in Difco FA buffer, pH 7·2, before use. Slides were incubated with serum for 30 min at room temperature, washed in buffer for 30 min, incubated with Wellcome sheep anti-human Ig FITC for 30 min, again washed for 30 min, and mounted in Gurr's Uvinert aqueous mounting medium. Slides were examined on a Leitz Orthoplan microscope equipped with incident ultraviolet illumination from a 200 W mercury vapour lamp, using a BG 38 exciter filter, a K630 barrier filter and a 100/1·20 water-immersion objective. Results were scored on a scale from negative to ++++, and most positive sera were titrated at quadrupling dilutions starting at 1:4.

DNA binding. This was measured using a modification of the method described by Pincus *et al.* (1969), employing internally labelled [^{14}C]nDNA obtained from *E. coli* (Radiochemical Centre, Amersham). Sera were heated at 56°C for 30 min before testing, then diluted 1:10 in borate buffer, pH 8·0. Fifty microlitres of dilute serum were added to 50 μl of [^{14}C]nDNA (2 μg/ml) and incubated for 1 hr at 37°C followed by 24 hr at 4°C. One hundred microlitres of chilled saturated ammonium sulphate was added and the mixture kept at 0°C for 1 hr, then the precipitated material was spun down for 30 min at 1500 *g* and at 4°C. One hundred microlitres of the supernatant (S) was pipetted off, and the remaining supernatant and precipitate redissolved in 1 ml of borate buffer (P). Carbon-14 radioactivity in both P and S was assayed in a LKB-Wallac liquid scintillation counter, using Triton X as scintillant. The DNA binding was calculated as follows:

$$\text{DNA binding (\%)} = \frac{(\text{P}-\text{S})\ \text{d/min}}{(\text{P}+\text{S})\ \text{d/min}} \times 100.$$

Sixty normal sera bound less than 20% in this system.

RESULTS

DNA binding and kinetoplast immunofluorescence

Both DNA binding and kinetoplast immunofluorescence (IF) were measured on 127 SLE sera and ninety-seven sera from patients with other diagnoses.

The results in SLE patients are plotted on Fig. 1. Sera showing high DNA binding but no kinetoplast IF were retested in two ways: by performing the immunofluorescent test without dilution, and with overnight incubation of serum-coated slides at 4°C before washing. Neither technique increased the number of positive tests, though sera positive at 1:10 with standard incubation remained positive under these conditions.

Table 1 compares DNA binding and kinetoplast IF results in patients with and without a diagnosis of SLE. When results from SLE patients with clinical renal involvement are plotted separately, the proportion of sera negative on both tests is lower but otherwise the results are comparable.

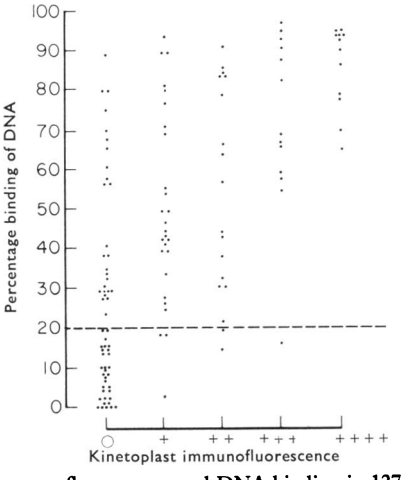

FIG. 1. Kinetoplast immunofluorescence and DNA binding in 127 patients with SLE.

TABLE 1. Kinetoplast IF & DNA binding

		Patients with SLE DNA binding		Patients without SLE DNA binding	
		≥20%	<20%	≥20%	<20%
Kinetoplast IF	+	63 (58)	6 (5)	0	2
	−	26 (20)	32 (21)	17	78

Figures in parentheses show numbers of patients in each group with clinical renal involvement.

All sixty normal controls gave negative results on the IF test; DNA binding was not measured on these sera. All five patients with discoid lupus were negative on both tests. Brief details of the patients in the non-SLE group with positive kinetoplast IF tests and normal DNA binding are given below.

Serial studies

In some cases serial blood samples were available and the variation in the results on the Farr assay and kinetoplast IF test could be studied. Two illustrative cases are shown in Figs 2 and 3.

Clinical state and kinetoplast IF

Eighty-eight sera from thirty-two patients with SLE and clinical nephritis, on whom full clinical data were available, were obtained at a time when clinical activity was scored on a 7-point scale (Cameron *et al.*, 1976) depending mainly on the presence of profuse proteinuria and a falling glomerular filtration rate. All these patients were being maintained on some form of immunosuppressive treatment. The correlation between clinical activity and kinetoplast IF result is shown in Fig. 4.

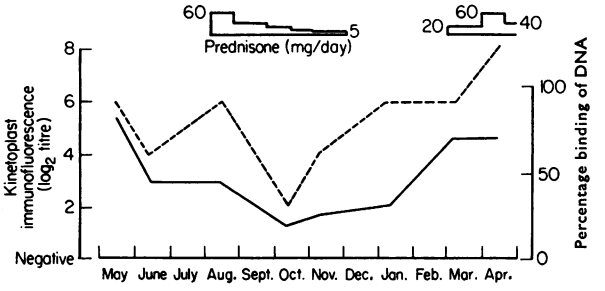

FIG. 2. Drug treatment and anti-DNA antibody in a patient (C.G.) with lupus nephritis. Kinetoplast IF (– – –), DNA binding (——).

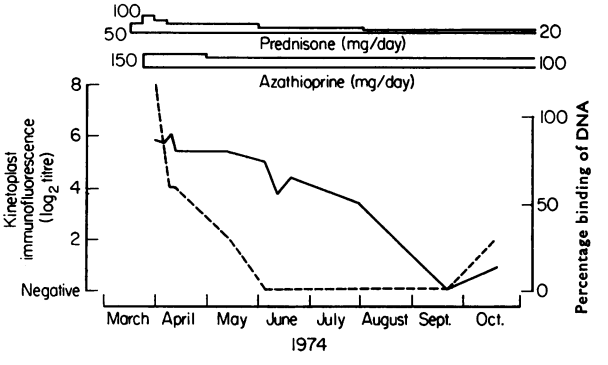

FIG. 3. Drug treatment and anti-DNA antibody in a patient (C.W.) with severe SLE and nephritis. Falling antibody levels were accompanied by clinical improvement (blood urea, glomerular filtration rate) in this case. Kinetoplast IF (– – –), DNA binding (——).

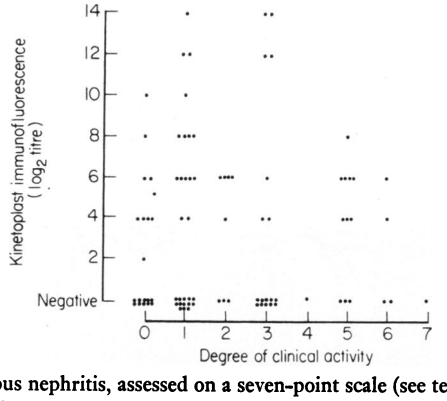

FIG. 4. Clinical activity of lupus nephritis, assessed on a seven-point scale (see text) and kinetoplast immunofluorescence: thirty-three patients (eighty-eight sera).

Antinuclear factor and kinetoplast IF

Antinuclear factor (ANF) results were available for the majority of sera in the study. In no case was a serum found to be negative for ANF and positive for kinetoplast IF.

Altogether sixty-six ANF-positive sera from patients without a diagnosis of SLE were tested. Five of these were positive for kinetoplast IF. Brief details of these five patients (comprising the two appearing in Table 1, one who does not appear because the diagnosis was in doubt, and two on whom DNA binding was not measured) are given here.

A.P., female, aged 41; 2-year history of severe seropositive erosive polyarthritis with nodules. Pleural

effusions; possible myocarditis. One recent epileptic fit. No renal impairment. Treated with corticosteroids. Strongly positive ANF; LE cells present. DNA binding, 19%; C_3, 11%; C_4, 36%. Diagnosis: rheumatoid arthritis with LE cells.

C.T., male, aged 60; 7-year history of seropositive erosive polyarthritis treated with corticosteroids, chloroquine and gold. In 1972 developed a pleural effusion; ANF negative at this time. April 1973, started penicillamine therapy. December 1974, developed haemoptysis and renal failure; biopsy showed proliferative glomerulonephritis. ANF now positive (kinetoplast IF positive at this point). DNA binding: 4%. Diagnosis: rheumatoid arthritis, polyarteritis precipitated by penicillamine. (This patient has been reported in detail by Gibson, Burry & Ogg, 1976).

J.R., female, aged 45; keratoconjunctivitis sicca, fleeting arthralgia, lymphadenopathy, hypergammaglobulinaemia, positive rheumatoid factor and Wassermann tests. Strongly positive ANF; LE cells present. Diagnosis: Sjögren's syndrome.

S.M., female, aged 31; 14-year history of seropositive erosive polyarthritis, with an episode of pleurisy and repeated attacks of pericarditis with effusion, responding only to corticosteroids. She developed hyperlipidaemia which responded to clofibrate and cholestryramine, but suffered two myocardial infarcts, from the second of which she died. ANF strongly positive, LE cells present. DNA binding 14% on this occasion, but 20% and 27% at other times. Diagnosis: rheumatoid arthritis, hyperlipidaemia: probable SLE.

J.C., male, aged 65; 12-year history of severe seropositive nodular erosive polyarthritis. Hepatosplenomegaly, intermittent leucopenia (not drug-induced), cardiomyopathy with right bundle-branch block and atrial fibrillation, pericardial rub. No evidence of renal disease. Pleural effusion, presumed due to congestive failure. ANF and LE cells negative 1968; ANF strongly positive 1975 (kinetoplast IF positive at this point). Diagnosis: Felty's syndrome, rheumatoid cardiomyopathy.

DISCUSSION

The kinetoplast IF test is easy to perform and to read, and there was no difficulty in distinguishing nuclear fluorescence (quite often found with sera with a positive ANF but no evidence of antibodies to nDNA) from kinetoplast fluorescence. A positive test is illustrated in Fig. 5.

The results of the Farr assay and kinetoplast IF test are in moderately good agreement, and this supports the hypothesis that they are measuring the same variable. Unlike the DNA electroprecipitation test described by Edmonds *et al.* (1975), the results in patients with and without clinical renal involvement are similar. As Fig. 1 shows, however, there are quite a number of discrepancies between results on the Farr test and the kinetoplast IF test, and some of these are very great (e.g. DNA binding of 89% with negative

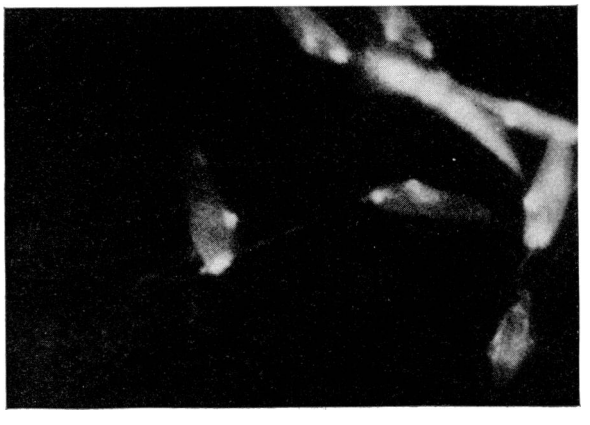

FIG. 5. A strongly positive kinetoplast IF test. The bright patch about one-third of the way along the body is the kinetoplast. The other fluorescent area, at the root of the flagellum, is a non-specific finding and is very common. (Original magnification × 650; further enlarged × 5 on this print.)

kinetoplast IF). The explanation for this is not clear, though it is likely that antibody to single-stranded DNA may give some false positive results in the Farr assay unless every batch of DNA is of a very high standard of purity. The finding of DNA binding above 20% in some patients with diseases other than lupus (compare Table 1) suggests that this may have been occurring with the DNA-binding assay in use at the time. The effect of immunosuppressive therapy on the test results may also be relevant. Figs 2 and 3 both show that nDNA antibody levels may fall when immunosuppression is instituted; Fig. 3 also shows that the fall in kinetoplast IF antibody may be very rapid, with DNA binding results following much more slowly. It is noteworthy that all patients with SLE who had DNA-binding levels over 20% but a negative IF test were on immunosuppression at the time. Furthermore, seven of the sera in this group were from a single patient, on steroids and azathioprine, who regularly showed raised DNA binding and a negative kinetoplast IF test.

The fall in nDNA-antibody levels on treatment, as measured by kinetoplast IF (and by the Farr test) is not necessarily related to the clinical course of the renal disease, and Fig. 4 shows that there is no significant correlation between clinical activity of renal disease and kinetoplast IF results; all these patients were receiving immunosuppression. The kinetoplast test cannot therefore be used as a guide to treatment of lupus nephritis, and in this respect clinical criteria probably remain the most reliable index (Cameron *et al.*, 1976, submitted for publication). We have not examined the ability of the kinetoplast test to assess activity and direct treatment in patients not receiving immunosuppression, and without lupus nephritis.

The relative insensitivity of the kinetoplast IF test also means that it may not be of value as a routine screening test, although in this study no patient with known and untreated SLE has shown a negative IF result.

The area in which the kinetoplast IF test seems likely to be most useful is in primary diagnosis. The difficulty of classifying patients in the no-man's-land between rheumatoid arthritis and SLE is well known. The five cases described, in whom the IF test was positive but SLE had not been diagnosed, all fall in this area. All had severe and complicated disease, and the preference for a diagnosis of rheumatoid arthritis rather than lupus often rested mainly on the erosive and nodular pattern of the arthritis—though this may also be seen in undoubted SLE. The subsequent course of such borderline cases may indicate whether the kinetoplast test is making a clinically useful distinction between them and otherwise similar cases.

Assessing a new serological test by reference to a clinical diagnosis in this uncertain area may be no more valid than the converse procedure; depending on the rigour of one's diagnostic criteria, the same test can be shown to be more or less specific, and more or less sensitive. Unquestioning acceptance of the clinician's own preferred diagnosis was adopted here to avoid bias, and on this basis the kinetoplast IF test appears to be highly specific and of diagnostic value. Its extreme simplicity and cheapness (in marked contrast to the Farr test) contribute to making a strong case for its inclusion in the routine assessment of any patient with a positive antinuclear factor test.

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