Clinical significance of antibodies to ribonucleoprotein

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SUMMARY Antibodies to ribonucleoprotein (RNP) were detected by an immunofluorescence technique based on the sensitivity of speckled antinuclear antibodies to ribonuclease. These antibodies were found to identify a group of patients with a consistent set of clinical features, especially arthritis, swollen hands, Raynaud's phenomenon, and myositis. The presence of anti-RNP antibodies in sera from patients with polymyositis, systemic lupus erythematosus, and systemic sclerosis was also associated with these clinical features. Other studies of the clinical significance of these antibodies support the concept that they appear to identify a group of patients with a distinct clinical condition.

Although various antinuclear antibodies (ANAs) have been recognised for years, the clinical significance of each variety is poorly understood. Antinuclear DNA antibodies detected by multiple techniques are of undoubted value in the recognition and assessment of systemic lupus erythematosus (SLE). Of the patterns delineated by immunofluorescence, nucleolar antinuclear antibodies have been associated with SLE and progressive systemic sclerosis (PSS) and speckled ANAs with SLE, Sjögren's syndrome, and the more recently described syndrome of mixed connective tissue disease (MCTD). The 'overlap' syndrome shares clinical features of SLE, PSS, and polymyositis and has been characterised serologically by the presence in high titre of antibodies to the ribonucleoprotein component of a saline-soluble extractable nuclear antigen (ENA) prepared from thymus (Sharp *et al.*, 1972).

An important development has been the demonstration that different antibodies may be responsible for the speckled pattern of nuclear fluorescence. These include antibodies to ribonucleoprotein (RNP) (Northway and Tan, 1972), to the Sm antigen (Tan and Kunkel, 1966), and to another antigen identified in Sjögren's syndrome (Alspaugh and Tan, 1975; Akizuki *et al.*, 1977a). Methods for detecting antibodies to RNP depend on treatment

of the antigen with ribonuclease (RNase) and include haemagglutination (Sharp *et al.*, 1971), counter-immunoelectrophoresis (Kurata and Tan, 1976) and double diffusion (Akizuki *et al.*, 1977b). We have further developed an immunofluorescence method which relies on the abolition of the speckled pattern of nuclear fluorescence after treatment of the acetone-fixed substrate with RNase (Tan and Kunkel, 1966).

In a previous study (Cohen *et al.*, 1977) we examined 46 sera with speckled ANA by this immunofluorescent technique. Two groups of sera were identified: those with titres greater than 1:1000 which fell by a mean 100-fold after enzyme treatment of the substrate, and those with titres less than 1:1000. The clinical features of the 2 groups of patients thus defined were compared. The high titre group had a high prevalence of arthritis, Raynaud's phenomenon, swollen hands, sclerodermatous skin changes, and myositis, and the clinical spectrum resembled that described for MCTD. As it appeared that the immunofluorescent technique was identifying a group of patients similar to those with the antibodies detected by haemagglutination, the present study was undertaken to investigate more precisely the clinical significance of these antibodies.

Methods

DETECTION OF ANTI RNP BY FITC

The substrate (rat kidney, snap-frozen and cut to 4 μ m thickness) was fixed in acetone (Tan, 1967) at room temperature for 10 minutes to minimise the

Accepted for publication 21 March 1978

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elution effect of saline in buffers. After air drying, substrates for enzyme treatment were immersed for 60 minutes at 37°C in a solution of ribonuclease A (Worthington Biochemical Corporation) diluted to 4 mg per 100 ml in 0.01 M PBS (pH 7.2) containing 0.4 mM magnesium chloride. The control substrate was similarly fixed, dried, and immersed in the above buffer without RNase. Sera of the appropriate dilutions were added to both control and enzyme treated substrates. After incubation in a moist chamber for 45 minutes the sections were washed twice for 5 minutes in PBS at pH 7.2 before addition of fluorescein-conjugated polyvalent antihuman immunoglobulin (Wellcome) at normal working dilutions. After a further 45 minutes' incubation 2 20-minutes washes in PBS at pH 7.2 were performed before the sections were mounted in buffered glycerol at pH 8.2. The fluorescence of speckled ANA was read to extinction on an Ortholux epifluorescence microscope using the 1, 5 ultraviolet light filter system.

OTHER IMMUNOLOGICAL METHODS

Rheumatoid factors were detected by Latex (Hyland) and Rheumaton (Denver) kits. Serum IgG, IgM, IgA, C3, and C4 were measured by radial immunodiffusion (Oxford). Cryoglobulins were sought by collection and separation of sera at 37°C, before 96-hour storage at 4°C. Anti-DNA binding was measured by the Farr assay (normal less than 26%).

IMMUNODIFFUSION

To detect precipitating antibodies a modification of the Ouchterlony agar gel diffusion was used and essentially followed the method of Tan and Kunkel (1966). Plastic petri dishes (50 × 10 mm) were filled with 5 ml of 0.4% agarose (Sigma Chemical Co.) in phosphate buffered saline at pH 7.2, 0.01M, with 0.1% sodium azide. The well pattern consisted of 6 outside wells 4 mm in diameter and a central well 9 mm in diameter. The reacting diffusion dishes were incubated in a moist chamber at 4°C and observed daily for 5 days and then every second day until day 14.

The basic antigen used throughout this series was rabbit thymus (Pel-Freeze Biologicals) extracted overnight at 4°C by mixing with PBS at pH 7.2, 0.01 M, and standardised at 10 mg/ml of protein.

Prototype sera which fulfilled criteria according to Akizuki *et al.* (1977b) of anti-RNP and anti-Sm, that is, reactivity or nonreactivity after antigen treatment with various enzymes and temperatures, and which appeared to have no other detectable antibody to ENA by immunodiffusion, were selected as 'reference' sera to establish identity or nonidentity

of precipitin lines with test sera. All test sera were screened at various dilutions and exposed both to treated and non treated antigens.

SERA AND PATIENTS

Two groups of sera were studied. The first (group A) was identified from the routine laboratory screen for autoantibodies over a 2-year period. Twenty-one such sera were found to have speckled antinuclear antibodies in a titre of 1:1000 or greater. The records of patients from whom these sera were taken were studied to extract clinical features and other laboratory data for correlation with the serum findings. Particular attention was paid to the presence of arthritis, Raynaud's phenomenon, myositis, swollen hands, lymphadenopathy, and hepatosplenomegaly. Abnormal oesophageal motility and diminished pulmonary diffusing capacity were noted. Myositis was considered to be present if a proximal weakness was accompanied by at least one abnormal investigation, namely, raised serum creatine kinase or aldolase, abnormal electromyograph, or abnormal muscle biopsy. Renal assessment was based on protein and white cell excretion and biopsy where this was performed.

The second group of sera (group B) was obtained from (1) 35 patients with polymyositis according to the criteria of Dawkins (1977); (2) 14 patients with progressive systemic sclerosis; and (3) 26 patients with SLE according to the American Rheumatism Association's preliminary criteria. A similar correlation of serum findings and clinical features was undertaken. The application of the IF technique to sera from patients with SLE is complicated by the different patterns of nuclear fluorescence which may occur, the homogenous and peripheral patterns tending to obscure speckling especially at low titre. The double diffusion technique was therefore used for qualitative detection of anti-RNP antibodies.

Results

GROUP A SERA

Titres of the 21 patients with high-titre speckled antinuclear antibodies were found to be markedly sensitive to RNase. The results of differential titring are shown in Table 1. The reciprocal of titre on control substrate over reciprocal of titre on enzyme-treated substrate ranged from 20 000/400 to 1200/5. The results of the same sera subsequently examined by the double diffusion method are also shown as the reciprocal of titre of antibodies to RNP. Non RNase-sensitive antibodies to ENA are shown when detected.

The clinical and laboratory features of these patients are shown in Table 2 and are compared with results from other series of patients whose sera

contain a high titre of antibodies to RNP. In the present study there were 7 males and 14 females, their ages ranging from 14 to 80, with a mean of 40 years. There was a predominance of arthritis,

Table 1 *Serological findings in 21 patients with high-titre speckled antinuclear antibodies*

Patient no.	Differential titre of speckled ANA	Anti-RNP by double diffusion	Other anti-ENA detected
1	20 000:400	64	—
2	10 000: 20	256	—
3	8000: 5	128	—
4	8000: 60	64	Sm
5	6000: 5	64	NI
6	> 5120: 5	128	—
7	5000: 20	32	—
8	4000:300	32	Sm
9	3000: 10	—	NI
10	3000: 10	8	—
11	3000: 20	4	—
12	2500:300	16	NI
13	2000: 5	16	—
14	2000: 5	ND	—
15	2000: 40	ND	—
16	1200: 5	—	NI
17	1200: 5	—	NI
18	1200: 10	1	—
19	1200: 20	1	—
20	1200: 80	ND	—
21	1000:300	1	NI

NI=not identified. ND=not done.

Raynaud's phenomenon and swollen hands. Although half the patients had no renal abnormality, 7 had increased urinary excretion of white cells and in 5 a renal biopsy was abnormal (one minimal change on light microscopy, 2 mesangioproliferative glomerulonephritis, 1 focal proliferative glomerulonephritis, 1 chronic glomerulonephritis). No patient had proteinuria, and only 1 had mild renal failure. The neurological abnormalities in our patients were a trigeminal neuropathy in 4 patients and a peripheral neuropathy in 1. Three patients had keratoconjunctivitis sicca. A high prevalence of hypergammaglobulinaemia and of positive rheumatoid factor was noted. Low serum complement and raised anti-DNA antibodies were uncommon. Those patients (Nos. 1, 8, 12, and 21) whose sera had residual titres of speckled ANA greater than 1:100 after RNase treatment were not distinguishable on clinical grounds from the group as a whole. As suggested in Table 1, these residual titres may represent antibodies to the Sm or Sjögren's-associated antigens.

Fig. 1 shows the association of the main clinical features as found in our 21 patients. Arthritis, Raynaud's phenomenon,[†] and swollen hands are strongly associated, as are myositis and trigeminal neuropathy.

Table 2 *Clinical and laboratory features of 21 patients with high-titre speckled antinuclear antibodies sensitive to RNase compared with other series*

Author	Present study	Sharp et al. (1976)	Farber and Bole (1976)	Leibfarth and Persellin (1976)	Parker (1973)
Number of patients	21	100	17	16	10
Basis of RNase sensitivity	Abolition of fluorescent speckled ANA	1000-fold drop in titre by HA	50-fold drop in titre by HA	400-fold drop in titre by HA	Abolition of agarose precipitation
	%	%	%	%	%
<i>Clinical</i>					
Arthritis	86	95	88	75	90
Swollen hands	55	66	53	38	30
Raynaud's phenomenon	90	85	76	50	100
Myositis	33	63	53	31	40
Sclerodermatous changes	29	33	44	38	—
Telangiectases	5	—	—		—
Vasculitis	38	30	29		—
Abnormal oesophageal mobility	3/7	50/75	35	31	—
Diminished pulmonary diffusion	4/9	29/43	5/5	44	—
Lymphadenopathy	24	39	18	31	—
Hepatosplenomegaly	10	19	12	—	—
Fever	19	33	41	94	—
Serositis	19	27	53	50	—
Renal abnormality	48	10	—	—	—
Neurological abnormality	23	10	—	—	—
<i>Laboratory</i>					
Speckled ANA	(100)	97	11/13	100	100
Raised anti-DNA binding	14	12	35	0	—
Positive rheumatoid factor	48	34/62	100	62	—
Hypergammaglobulinaemia	71	73	13/15	81	—
Low C3	14	3/70	5/12	44	0
Cryoglobulinaemia	2/19	—	—	—	—
Anaemia	10	41	71	25	—
Leucopenia	10	35	71	12	90

Fig. 1 Clinical features in 21 patients with anti-RNP antibodies.

GROUP B SERA

Of 35 patients with polymyositis 2 had sera containing speckled ANA but only 1 had a high titre of anti-RNP. This patient was clinically different, with Raynaud's phenomenon, swollen hands, and oesophageal involvement as well as myositis. Of 14 patients with systemic sclerosis 5 had speckled ANA, of whom 3 had high-titre anti-RNP. One has frank myositis as well as features of PSS; a second had myositis, has developed synovitis, and is showing improvement of grossly involved skin; the third is at present clinically indistinguishable from classical PSS. By the double diffusion technique 2 of 26 SLE sera showed a precipitin line believed to be anti-RNP. Both patients have marked Raynaud's phenomenon, and one has renal changes atypical for lupus with minimal light microscopy changes and deposits of IgA on the glomerular basement membrane. The other has continuing cutaneous vasculitis, membranous glomerulonephritis with vascular as well as glomerular immune complex deposits, and an antiribosomal antibody on routine serum immunofluorescence.

Discussion

Akizuki *et al.* (1977b) have compared immunofluorescence, haemagglutination, complement fixation, and immunodiffusion as methods for detecting antibodies to soluble nuclear antigens. These authors imply that elution of antigen renders the IF technique unreliable. The method described above does suffer from some disadvantages, but it does appear to provide clinically useful information with advantages in simplicity, time, and cost. It is not yet clear whether the greatest practical value is provided by the titre after treatment with buffer alone or the differential titre obtained by comparing buffer and

RNase. It should be emphasised that the present approach depends on the use of the titre of speckled rather than other types of ANA. The method was reproducible both on repeat examinations of the same serum and on examination of serial sera. Furthermore, the results of the double diffusion and immunofluorescence techniques correlate well, suggesting that they may detect the same anti-RNP antibodies. Other methods will help in classifying ANAs more completely.

We have shown that antibodies to RNP identify a group of patients with a clinical and laboratory profile similar to that associated with 'MCTD' (Sharp *et al.*, 1972, 1976; Parker, 1973; Farber and Bole, 1976; Leibfarth and Persellin, 1976). More specifically, all reports show that these antibodies appear to correlate with certain clinical features, in particular arthritis, Raynaud's phenomenon, myositis, and swollen hands. While renal disease appears to be uncommon, minor abnormalities in renal function tests may be associated with antibodies to RNP.

Reichlin and Mattioli (1972) noted that anti-RNP antibodies in a group of clinically defined SLE patients identified a subgroup with a paucity of serious renal disease. This subgroup also had an increased prevalence of Raynaud's phenomenon, myositis, and serositis compared with the subgroup of SLE patients lacking these antibodies. Arthritis and skin rash were equally prevalent in both groups. Parker (1973) found that the presence of anti-RNP in SLE patients was a marker for arthralgia, myositis, and Raynaud's phenomenon. Anti-RNP in our group of SLE sera identified 2 patients with marked Raynaud's symptoms and atypical renal features.

Anti-RNP antibodies in 2 of our systemic sclerosis patients were markers for myositis in both and synovitis in one. Parker (1973) noted that 2 out of 16

patients with scleroderma had antibodies to RNP: one had raised serum muscle enzymes, the other had had myositis, Coombs-positive haemolytic anaemia, and a softening of skin changes on corticosteroids. Chihara *et al.* (1977) found that the 8 of 33 patients with systemic sclerosis who had these antibodies had a higher prevalence of myositis, trigeminal neuropathy, and sicca symptoms. In our polymyositis patient these antibodies appeared as a marker for Raynaud's phenomenon and swollen hands.

Immunofluorescence screening can provide a simple method for detecting antibodies to RNP. The presence of these antibodies appears to correlate with a consistent set of clinical features and may be useful in identifying groups of patients whose clinical condition is distinct from that of others lacking such markers.

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