Antibodies to nuclear antigens in polymyositis: relationship to autoimmune 'overlap syndromes' and carcinoma

P. J. W. VENABLES, P. A. MUMFORD, AND R. N. MAINI

From the Clinical Immunology Division, Mathilda and Terence Kennedy Institute of Rheumatology, 6 Bute Gardens, London W6 7DW

SUMMARY Thirty-two patients with polymyositis were categorised into 4 groups: (1) 'pure' polymyositis, (2) dermatomyositis, (3) myositis associated with autoimmune 'overlap syndrome', and (4) those with associated malignancy. Serum from each patient was examined for a range of antinuclear antibodies. Seventeen patients had ANA detected by immunofluorescence, 18 patients had raised DNA binding (>25 U/ml), of whom eight had levels greater than 50 U/ml (SI conversion: $U/l = U/ml \times 10^3$.) Antibodies to soluble nuclear antigens were detected in 23 (72%) by 1 or more of 3 methods, and in all of these anti-RNP was the main antibody detected. Antibodies to other soluble antigens were also present in 6 sera. In 2 cases, both patients with SLE/myositis overlap, these were shown to be anti-Sm. The remaining 4 had antibodies to various protein components of the extracts, but it was not possible to demonstrate an antibody of diagnostic specificity for polymyositis. Furthermore, quantitation of anti-RNP and anti-DNA antibodies failed to define a distinct clinical entity or exclude malignant disease. High levels of anti-RNP antibodies showed an association with Raynaud's phenomenon, sclerodactyly, and pulmonary fibrosis and an inverse correlation with the rash of dermatomyositis, suggesting that this antibody may be of pathogenetic rather than diagnostic significance.

The aetiology of polymyositis is unknown, but immunological mechanisms are thought to play a part in its pathogenesis. Tissue specific antibodies such as antimyosin antibodies are found in polymyositis¹ but are not thought to be diagnostic or pathogenic. Some studies have suggested that muscle damage is cell mediated because lymphocytes from polymyositis patients are sensitised to muscle antigens^{2,3} and are cytotoxic to muscle cultures *in vitro*.² Interest in antinuclear antibodies (ANA) has been stimulated by observations that antibodies to specific nuclear antigens may help to define syndromes in which myositis occurs. Such syndromes are said to include mixed connective tissue disease (MCTD)⁴, defined by antibodies to nuclear ribonucleoprotein (RNP), and systemic lupus erythematosus (SLE) characterised by antibodies to double-stranded DNA (ds-DNA)⁵ and anti-Sm.⁶ More recently Wolfe *et al.* have claimed

that antibodies to the soluble nuclear antigen PM-1 are highly specific for polymyositis.⁷ The relationship of these antibodies to myositis associated with malignancy has not been established. The purpose of this study was to ascertain whether reactions to PM-1 occurred in our patients and if quantitation of antibodies to RNP, DNA, and Sm in patients with myositis identified distinct clinical syndromes such as MCTD or SLE and excluded those with associated malignant disease.

Patients and methods

The 32 patients selected for this study fulfilled at least 3 of the 4 criteria for myositis based on those previously described by Bohan *et al.*⁸ The criteria were as follows: (1) symmetrical proximal muscle weakness; (2) raised creatine phosphokinase; (3) electromyographic changes characteristic of myositis (myopathic changes were not accepted); (4) muscle biopsy changes showing at least 2 of the

following: interstitial inflammatory infiltrate, fibre necrosis, and fibre regeneration.

Sera were taken at the time of the muscle biopsy. Only 8 patients were receiving treatment at this time. The patients were clinically classified into a scheme similar to that proposed by Walton and Adams⁹ as follows.

Group I: 'Pure' polymyositis. The five patients in this group had no evidence of major cutaneous or systemic involvement, though minor skin changes such as nail bed erythema or periorbital oedema were found.

Group II: Dermatomyositis. Dermatomyositis was diagnosed in the 9 patients who, in association with myositis, had the classical skin changes of heliotrope discolouration and oedema of the eyelids associated with erythema of the knuckles and extensor aspects of the fingers. Those patients with dermatomyositis associated with a major system involvement or malignant disease were allocated to groups III and IV. There was only 1 patient in our study who had childhood dermatomyositis with vasculitis, and she was therefore included in this group rather than classified as a separate subdivision.⁸

Group III: 'Overlap'. This group comprised 13 patients who had, in addition to myositis, evidence of major organ and system involvement, that is, arthritis, fibrosing alveolitis, scleroderma, nephritis, or keratoconjunctivitis sicca. The 13 patients in this group included 2 patients with SLE nephritis (fulfilling American Rheumatism Association (ARA) criteria),¹⁰ 3 with scleroderma, 2 with 'classical' rheumatoid arthritis,¹¹ 2 with pulmonary fibrosis, 1 with a transient nephritis, and 1 with keratoconjunctivitis sicca. The remaining 2 patients had a variety of associated features including arthritis, pulmonary fibrosis, and Raynaud's phenomenon.

Group IV: Malignancy associated. The 5 patients in this category had carcinomas. Of these, 2 had carcinoma of the ovary and the remaining 3 had carcinomas of the bronchus, breast, and rectum. Two of these patients had the rash of classical dermatomyositis, and 2 also had keratoconjunctivitis sicca.

Methods

ANA by immunofluorescence on a rat liver substrate was kindly performed by Professor B. W. Lacey (Westminster Hospital).

Antigens. Two antigens, an acetone extract of rabbit thymus (Pelfreeze Biol., Rogers, Arkansas) and a lyophilised extract of calf thymus were used as

previously described.¹² The rabbit thymus extract contained 10 mg/ml of protein (Lowry assay)¹³ and 2.6 mg/ml of ribonucleic acid (RNA) detected by the orcinol reaction. The calf thymus extract contained 42 mg of protein and 1.8 mg of RNA per 100 mg of lyophilised powder. This extract was reconstituted by dissolving it in phosphate buffered saline (PBS). (SI conversion: g/l = mg/ml.)

Double diffusion. 5 mm wells containing 30 μ l of serum or antigen were placed 3 mm apart in 0.4% agarose in 0.15 M PBS, pH 7.2. Both antigens were used: the rabbit thymus extract at a concentration of 10 mg of soluble protein per ml and the calf thymus extract at 3 different concentrations: 100 mg, 50 mg, and 10 mg of lyophilised extract per ml. The sera were grouped as a hexagon around the antigen and the plates read at 24, 48, and 72 hours. Each serum was tested with all 4 preparations. If a precipitin was observed, the identity of the reacting antigen was established by incubating the extract with RNase or trypsin and by seeking lines of identity with a reference serum which was placed in 2 of the wells adjacent to the test sera (Fig. 1).

Extractable nuclear antigen (ENA) haemagglutination test. The haemagglutination technique was performed as previously described.¹² A 5% solution of washed tanned sheep erythrocytes (SRBC) was coated with the calf thymus extract at a concentration of 5 mg of powder per ml of SRBC for 30 minutes at 37°C and then washed 3 times in phosphate buffered saline (PBS) containing 1% SRBC absorbed decomplexed horse serum and resuspended to a 1% cell solution. Half of the cells were then incubated with RNase at a concentration of 10 μ g per ml of cell suspension at 37°C for one hour. Sera which had been decomplexed and absorbed with SRBC were serially diluted with PBS/1% horse serum in U-well microtitre plates to a volume of 25 μ l per well. Each serum was tested against 3 cell populations: the ENA coated cells, the RNase digested ENA coated cells, and washed tanned cells which acted as a control.

Counter immunoelectrophoresis (CIE). Each serum was tested against the rabbit thymus extract alone and digested with RNase and trypsin as previously described.¹² Sera giving precipitins against RNase and trypsin sensitive antigens were, for the purposes of this study, regarded as containing anti-RNP. This antibody specificity was confirmed in all sera from polymyositis patients on double diffusion. The 2 sera which gave precipitins to trypsin resistant antigens were shown to contain antibodies to Sm on double diffusion. CIE was used because it was particularly sensitive for the detection of antibodies to RNase resistant, trypsin sensitive antigens

Fig. 1 Immunodiffusion showing RNP precipitins in 4 polymyositis sera: pure polymyositis (PM); polymyositis SLE/overlap (PM/SLE); polymyositis/scleroderma overlap (PM/Scler); and dermatomyositis associated with carcinoma (DM/Ca). Rabbit thymus extract (RTE) is in the centre well. On the left a line of identity is shown between the anti-RNP reference serum in the top and bottom wells, and all 4 sera (the serum from the SLE patient has a second precipitin line). After digestion of RTE with RNase (right-hand hexagon), the RNP precipitins have disappeared. The second precipitin in the SLE serum identified as anti-Sm, has remained.

(proteins) and because it separated multiple precipitin lines in sera containing antibodies to several antigens present in the extract.

Antibodies to double-stranded (ds) DNA. DNA binding was determined by the Farr assay using the standardised kit supplied by the Radiochemical Centre, Amersham.¹²

Results

PREVALENCE OF ANTINUCLEAR ANTIBODIES

ANA by immunofluorescence were positive at a screening dilution of 1:20 in 17 patients (Table 1), with 8 showing a speckled pattern, 2 nucleolar staining, and 7 a homogeneous pattern. Antibodies to ds-DNA were also frequent. 18 patients had values above the upper limit of normal (>25 U/ml) and eight had levels above 50 U/ml. Antibodies to ENA were detected in 23 (72%) patients by 1 or more of the 3 methods, 11 of these (34%) being demonstrated by all 3 techniques. The haemagglutination test was the most sensitive giving positive results (titre >32) in 20 patients. With double diffusion 19 patients gave precipitin lines with one or more of our antigen preparations; the calf thymus extract at 100 mg/ml giving precipitins with 15 of the sera and the rabbit thymus extract with 9. Counter-immunoelectrophoresis (CIE) yielded precipitins with 14 sera (Table 1).

Table 1 Antinuclear antibodies in diagnostic subgroups of polymyositis

	No patients	DNA U/ml		+ve ANF ($>1:20$)	ENA antibodies	
		Ab			Detected	HA titre
			>25 >50			$>100\ 000$
Polymyositis	5	3	1	1	3	0
Dermato-myositis	9	3	2	2	6	0
Overlap	13	8	5	11	11	5
Malignancy	5	4	0	3	3	0
Total	32	18	8	17	23	5

SI conversion: U/l = U/ml $\times 10^3$.

CHARACTERISATION OF ANTIBODIES TO SOLUBLE NUCLEAR ANTIGENS

Each polymyositis serum that reacted with the extracts showed evidence of antibodies to RNP. All 20 sera which gave a positive haemagglutination test ($>1:32$) showed a fall in titre of 2 or more dilutions following digestion with RNase (Fig. 2), and for this reason the ENA haemagglutination titre has been used as the method of quantitating antibodies to RNP. Similarly, the precipitin reactions observed on double diffusion and CIE disappeared after incubation of the antigen with RNase (Fig. 1). Furthermore, lines of identity with a reference anti-RNP serum (kindly donated by Professor E. M. Tan) were shown in 19 of the sera. Six sera, all with

Fig. 2 Effect of RNase digestion of ENA on the haemagglutination titre of 32 polymyositis sera.

relatively high titres of anti-RNP contained antibodies to RNase resistant antigens, which were most easily identified by CIE. Of these, 2 contained antibodies to Sm demonstrated by lines of identity with reference sera and by resistance of the antigen to trypsin digestion. The remaining 4 contained antibodies to RNase-resistant trypsin-sensitive (protein) antigens. Only 2 of these gave precipitins to RNase-resistant components of the extracts on double diffusion. These precipitins were non-identical, suggesting that we had failed to identify a specific reaction for polymyositis such as PM-1.

RELATIONSHIP OF ENA ANTIBODY TITRES TO ASSOCIATED CLINICAL FEATURES

The most frequently observed associated clinical features in our patients with polymyositis are shown in Fig. 3. The patients have been ranked according to their ENA haemagglutination titre, with the 12 haemagglutination negative patients on the left and the patients with the highest anti-ENA titres on the right. In general, patients with raised anti-ENA titres showed Raynaud's phenomenon, sclero-

dactyly, chronic synovitis, pulmonary fibrosis, and renal involvement. The typical rash of dermatomyositis (in 12 patients) was seen usually in those with absent or low titres of anti-PNA antibody. Other features not shown in Fig. 3 included Sjogren's syndrome (1 patient), decreased oesophageal mobility (seen in 2 out of 13 patients who had a barium examination), and subcutaneous calcinosis (2 patients).

The most striking clinical association was with Raynaud's phenomenon. Nine out of 10 patients with this feature had high-titre (1:512) anti-ENA by haemagglutination. All 7 patients with sclerodactyly had anti-ENA antibodies. In 5 patients changes consistent with scleroderma were found involving skin proximal to the fingers, and classical scleroderma was diagnosed in 3 of these (patients 21, 28, 30). When ANA antibodies occurred in association with arthritis (6 patients), the arthritis was symmetrical, nonerosive, and tended to be intermittent. The two ENA-negative patients had erosive joint disease with nodules and had 'classical' RA. Fibrosing alveolitis was also associated with

Fig. 3 The relationship of ENA antibodies to associated clinical features in polymyositis. The ENA haemagglutination titre of patients (nos. 1–32) is shown in the graph with those with the highest titres on the right. The clinical features are represented by hatched squares, and the presence of anti-RNP by CIE or double diffusion and antibodies to other antigens; Sm and P (protein) are shown vertically above each patient number.

high ENA antibody titres but was asymptomatic in 6 out of 7 patients. The exception (patient 21) died from pulmonary hypertension. Renal involvement was seen in 4 patients and restricted to the high-titre group. In only 1 patient, who had membranous nephritis with nephrotic syndrome, was it clinically significant. In the remainder serum creatinine levels were normal, and proteinuria reversed after treatment with steroids.

In this study the titre of ENA antibodies correlated with the number of overlapping features. Thus, in all patients who in addition to their myositis had 2 or more of the above systemic features, ENA haemagglutination titres of 1:512 or greater were found.

DIAGNOSTIC SIGNIFICANCE OF ANTI-RNP AND OTHER ANTINUCLEAR ANTIBODIES

In our laboratory antibodies to RNP were found in patients with SLE and RA (Table 2), and are by no means specific for myositis. However, they did not appear to occur in patients with polymyalgia, muscular dystrophy, or neurogenic atrophy.

Table 2 Prevalence of anti-RNP antibodies detected by CIE in various diseases

	No. positive	No. tested	%
Polymyositis	14	32	44
SLE	29	51	57
RA	12	89	13.5
Scleroderma (without myositis)	1	6	17
Polymyalgia rheumatica	0	14	0
Dystrophies neurogenic atrophy	0	9	0
Normal controls	1	75	1.3

In patients with polymyositis, antibodies to RNP were found in all 4 subgroups of the disease up to a titre of 1:4096. Titres higher than this were found only in the 'overlap' group. Antibodies to RNase resistant antigens were also restricted to the latter group, the 2 patients with anti-Sm both fulfilling ARA criteria for SLE. Antibodies to ds-DNA were above the upper limit of normal in all 4 groups of patients, and higher levels (>50 U/ml) occurred in 5 of the patients with overlap features, although in only 2 could a diagnosis of SLE be clinically supported. The presence of antinuclear antibodies in 4 out of 5 patients with an underlying malignant disease is particularly noteworthy. Of the 5 patients with carcinoma 3 had ANA demonstrated by immunofluorescence, 4 had raised DNA binding, and 3 had antibodies to RNP.

Discussion

In this study ANA of varying specificities, particularly anti-ds-DNA and anti-RNP, occurred frequently in polymyositis. The association of polymyositis with ANA shown by immunofluorescence has been well established in the past with prevalences varying from 16%⁸ to 37%.¹ The much higher prevalence (60%) in our study probably reflects a bias in patient referral to a unit with a particular interest in connective tissue diseases. Previous authors have suggested that the presence of ANA in polymyositis reflects the presence of an associated 'collagen vascular disease'.¹⁴ Our data support this assumption to the extent that the highest levels were found in those patients who had a multi-system disease with overlap features of 2 or more autoimmune diseases, but we have shown that ANA can occur in polymyositis and dermatomyositis without underlying

disease and in patients with an associated malignancy.

Recent reports have raised the possibility that antibodies to certain nuclear antigens may form the basis of a serological test of diagnostic specificity. Reichlin and Mattioli¹⁵ noted that sera from patients with polymyositis gave a specific reaction against a nuclear protein which could be demonstrated by an indirect complement fixation technique in 10 out of 17 (59%). Wolfe *et al.*⁷ claimed to be able to demonstrate by double diffusion, a precipitin reaction with a nuclear protein antigen, termed PM-1, which was highly specific for polymyositis. In our study the sera of only 4 patients contained precipitins to protein antigens, although a number of thymus and lymphoid cell extracts were examined. These precipitins were clearly visible on CIE, but because only 2 yielded precipitins on double diffusion it was not possible to determine whether these reactions were directed to a specific protein such as PM-1. More recently by using a concentrated rabbit thymus extract we have shown that one of these precipitins (in a patient with myositis/scleroderma 'overlap syndrome') was anti-Scl-1.¹⁶ This finding emphasises the heterogeneity of the precipitins that may be found in patients with myositis, and the need to characterise the antigen specificity of each reaction.

The very high prevalence (72%) of anti-RNP antibodies in polymyositis sera in this study may be in part due to the fact that 24 patients were not receiving treatment at the time of the study and in part due to the large number of detection systems that were used. The use of 2 antigen preparations at different concentrations on double diffusion made this system very sensitive for the detection of anti-RNP. Thus the rabbit thymus extract gave precipitins with the 9 sera with relatively high anti-RNP haemagglutination titres. However, anti-RNP precipitins were detected in a further 10 sera (all with lower haemagglutination titres) using the calf thymus extract. This apparent discrepancy could be related to the source of the antigen, different methods of extraction, and higher concentration of RNP in the rabbit thymus extract. In spite of the sensitivity incurred by the use of a range of assay systems, anti-RNP antibodies could be demonstrated by all detection systems in 11 out of the 32 sera, giving a prevalence of 34% in this study. This is still a surprisingly high prevalence of anti-RNP antibodies for polymyositis and would initially seem to be in direct contrast to a number of previous reports.^{3 17}

Antibodies to RNP are said to be characteristic of a distinct disease termed 'mixed connective tissue disease' (MCTD) the main clinical manifesta-

tations of which include Raynaud's phenomenon, sclerodactyly, arthritis, myositis, and pulmonary fibrosis.⁴ Our experience has questioned the existence of MCTD as a distinct clinical entity.^{18 19} Since myositis occurs in up to two-thirds of the patients with MCTD, it could be argued that the patients in our study with anti-RNP antibodies suffer from MCTD. The question then arises, Which patients have MCTD? Clearly the mere presence of anti-RNP cannot constitute a criterion, because such antibodies have been found in patients with uncomplicated myositis as well as patients with other connective tissue diseases such as RA and SLE and even an occasional healthy subject. Because of this lack of specificity, most authors now stipulate that a 'high titre' is necessary for the diagnosis of MCTD.^{20 21} If we use a titre of 1:1000 as the cut-off point,²⁰ 11 of our patients fulfil the criteria. Of these, 7 had Raynaud's phenomenon, 5 sclerodactyly, 5 arthritis, and 3 pulmonary fibrosis—all features which have been associated with MCTD. On the other hand these 11 also included 4 patients without any of these features, of whom 2 had uncomplicated dermatomyositis or polymyositis, 1 had myositis associated with glomerulonephritis, and 1 had myositis associated with malignant disease. Closer inspection of the 7 who apparently resembled MCTD revealed that 2 had typical SLE with renal involvement and 2 had classical changes of scleroderma. Three patients in this group have died, 1 with carcinoma of the bronchus and 2 with a fulminating myositis which in 1 was associated with a glomerulonephritis. It is clear that these patients are too heterogeneous to form a 'distinct clinical entity'. Stricter criteria for MCTD, suggested by Bennett and O'Connell,²¹ include ENA haemagglutination titre of greater than 1:6400 and an absence of antibodies to Sm and DNA. If these criteria are applied to our patients, none of them could be called MCTD, as all of the patients with high levels of anti-RNP had raised DNA binding and 2 also had anti-Sm. This suggests that defining a disease (MCTD) by a serological test (anti-RNP) is a fallacy. The high prevalence of anti-ds-DNA antibodies in polymyositis was surprising, particularly as those antibodies are often regarded as having a high degree of specificity for SLE.⁵ This could be due to the presence of single-stranded DNA contaminating the antigen used. However, in a previously published report the assay for ds-DNA used in this study showed good concordance with other assays using well characterised ds-DNA antigens.²² The association of DNA antibodies with myositis has been observed by Norman *et al.*,¹⁷ who found raised levels in 25% of their patients with 'dermatomyositis' and 50% with 'MCTD' (of

whom a large proportion presumably had myositis). In our study there was a tendency for the 'overlap group' to have the highest levels of DNA binding, though abnormal values were also present among the patients with 'pure' polymyositis, dermatomyositis, and myositis associated with malignancy.

Although the measurement of anti-DNA and anti-RNP antibodies has proved to be of limited use in clarifying the diagnosis of diseases associated with polymyositis, there is an interesting relationship between these antibodies and certain clinical features. The correlation between RNP haemagglutination titres and the presence of Raynaud's phenomenon and systemic features and their inverse correlation with the rash of dermatomyositis indicates that RNP antibodies may be related to the pathogenesis of some of the cutaneous and systemic features associated with myositis. Unfortunately, the occurrence of ANA in myositis does not allow their use as serological evidence against underlying malignancy, but raises intriguing questions about the relationship between autoimmunity and malignant disease. We believe that further research into the functional significance of autoantibodies is of far greater interest than the somewhat disappointing search for serological 'diagnostic markers'.

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