Clinical associations of IgG antibodies to the ribonucleoprotein p67 polypeptide in patients with systemic lupus erythematosus

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Abstract

The ribonucleoprotein (RNP) p67 antigen was purified from rabbit thymus and used in an enzyme linked immunosorbent assay (ELISA) with low interassay variability to detect IgG antibodies to p67 in patients with autoimmune connective tissue diseases. These antibodies were found in eight (80%) patients with a clinical diagnosis of mixed connective tissue disease (MCTD) but also in 27 (40%) patients with systemic lupus erythematosus (SLE). Sixty six per cent of the 12 patients with SLE with high levels of antibodies to p67 (>50 U) had three or more features of MCTD, including myositis, fibrosing alveolitis, Raynaud's phenomenon, and sclerodactyly. Antibodies to the p67 RNP were not associated with the presence or absence of renal disease in the patients with SLE. This study suggests that antibodies against the p67 RNP are markers for clinical features of MCTD even in the context of SLE.

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High titres of antibodies to nuclear ribonucleoprotein (nRNP) in the absence of antibodies to Sm are reported to occur in 95–100% of patients with mixed connective tissue disease (MCTD) but rarely in patients with systemic lupus erythematosus (SLE) and progressive systemic sclerosis.[1] Serum samples that have antibodies to the RNP antigen immunoprecipitate U1 snRNA-protein complexes, which contain a set of at least nine proteins denoted p67 and A–G.[2 3] Antibodies to nRNP bind to three polypeptides in this complex: p67, A, and C (molecular weight 67, 33, and 20 kilodaltons respectively).[4 5] Antibodies against the A and C polypeptides are found in patients with MCTD and SLE, but it has been suggested that antibodies to p67 are restricted to patients with MCTD.[3 5 6]

Current techniques of autoantibody detection such as immunodiffusion, passive haemagglutination,[7] or counterimmunoelectrophoresis[8] measure the total amount of antibodies to RNP against all three peptides p67, A, and C. Direct detection of antibodies to individual specific RNP polypeptides has been achieved by immunoblotting[4 6 9 10] and enzyme linked immunosorbent assay (ELISA) using natural antigen[11] or recombinant antigen.[12 13] The aim of this study was to examine the possibility of using naturally occurring RNP p67 antigenic polypeptide in an ELISA for the routine detection and measurement of antibodies to p67, and to investigate the clinical correlations of this antibody in patients with SLE.

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Patients and methods

PATIENTS, SERUM SAMPLES, AND ANTIBODIES

We used serum samples from five disease groups. (*a*) Twenty patients with dermatomyositis/polymyositis complex diagnosed according to the criteria of Bohan and Peter.[14] (*b*) Ten patients with at least three of the following features of MCTD: Raynaud's phenomenon, fibrosing alveolitis, sausage shaped fingers and toes, sclerodactyly, arthritis, or myositis. They were diagnosed without reference to the presence of the antibody to RNP and none of them fulfilled four or more American Rheumatism Association (ARA) criteria for SLE or had antibodies to Jo-1 present in their serum samples. (*c*) Sixty seven patients with SLE diagnosed using the ARA criteria.[15] (*d*) Forty eight patients with Raynaud's phenomenon. (*e*) Thirteen patients with progressive systemic sclerosis.[16]

Forty six normal serum samples were obtained from volunteer office staff (British Oxygen Company Ltd). The serum samples were used at a dilution of 1/100.

The murine monoclonal antibodies used were derived from fusions of MRL/lpr spleen cells. The Sm antibodies KSm2 (anti-D) and KSm5 (anti-B,B') were used as ascitic fluid,[6] and were used at a dilution that produces an absorbance greater than 2·0 in assays using the BB' and D antigens respectively. The RNP antibody K8–43, directed against p67, was used at a dilution of 1/10 of the cell culture supernatant.

ANTIGEN ISOLATION

Rabbit nRNP/Sm polypeptides were isolated from thymus acetone powder (PelFreeze Biological, Rogers, AR, USA) as described previously.[11] Five batches of p67 antigen were prepared and stored in glass tubes for one to seven weeks at 4°C. The protein content of the fractions was estimated with the BCA protein assay reagent (Pierce, Rockford, IL, USA) using an albumin calibration graph. The eluate was assessed by gel electrophoresis and immunoblotting.[9]

PURITY OF p67 ANTIGENS

Antigenic purity was checked by immunoblotting and ELISA. The proteins from each of the batches were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis, transferred to nitrocellulose paper,[9] and probed with either human serum containing antibody to RNP or monoclonal antibody K8–43. To confirm that the antigen preparation did not

contain any contaminating Sm polypeptides the separated antigen preparation was probed with the KSm2 and KSm5 monoclonal antibodies against the D and B,B' Sm antigens respectively.[11]

The p67 polypeptides from individual batches were coated onto microtitre plates for an ELISA.[11] The antigens were assessed with reference human serum containing antibodies to RNP/Sm and with normal human serum, or the monoclonal antibodies K8–43 (antibody to p67), KSm2 (antibody to D), and KSm5 (antibody to BB'), detected with an antihuman or antimouse IgG antibody linked to alkaline phosphatase (Sigma) followed by Sigma 104 substrate.

ENZYME LINKED IMMUNOSORBENT ASSAY

To establish the optimum concentration of antigen for coating the ELISA plates for use in the assay of patients' serum samples an initial 'chequerboard titration' of the p67 antigen was carried out. Antigen was coated onto ELISA plates at 5·0, 2·5, 1·2, 0·6, 0·3, and 0·15 μg/ml and probed with reference serum samples containing antibodies to p67 and normal serum samples at dilutions of 1/50, 1/100, and 1/200. For a working antigen dilution we used the concentrations which gave an absorbance (405 nm) of 1·5 with the reference serum after one hour's incubation with the ELISA substrate: 2·5 μg/ml (preparation 1) and 1·2 μg/ml (preparations 2–5).

Plates were stored sealed at 4°C. The ELISA on the serum samples was performed using a method modified from that of Williams *et al* [11] using a standard graph constructed by serial dilution of a human reference serum sample in duplicate.

STATISTICAL METHODS

Data were analysed using the χ^2 test. The analysis of variance (*F* test) was used for comparison of means.

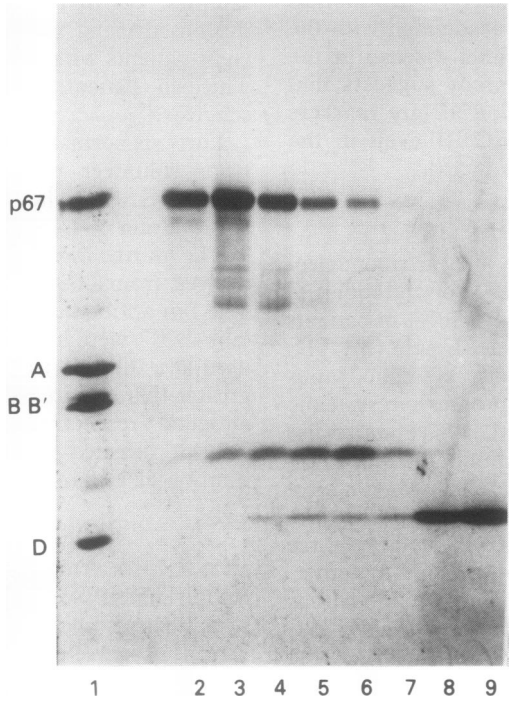

Figure 1 Immunoblotting analysis of consecutive fractions from reversed phase column chromatography of rabbit RNP/Sm (tracks 2–9). Track 1 is a whole RNP/Sm antigen with p67 (67 kilodaltons), A (33 kilodaltons), B,B' (29, 28 kilodaltons), and D (16 kilodaltons) peptides. The fraction shown in track 2 is uncontaminated p67 which was used for the immunoassay.

Results

PROTEIN PURITY OF p67

Fractions of reversed phase column eluate were electrophoresed, blotted, and probed with a mixture of antibodies to nRNP and Sm. Densitometry of stained gel electrophoretograms showed the protein purity of isolated p67 antigens to be 75, 82, 84, and 87%. Immunoblotting showed that the column fractions indicated the presence of p67 antigen in early eluting fractions from the reversed phase column (tracks 2–6; fig 1). Some fractions were contaminated with several weak immunoreactive bands with molecular weights of 64, 54, 45, and 21 kilodaltons (tracks 3 and 4), which may be degradation products of p67. Later fractions (tracks 5 and 6) containing p67 were also contaminated with D peptide and were discarded. The protein content of the fractions from five different preparations ranged from 25 to 41 μg/ml, giving a yield of pure peptides of between 100 and 164 μg from 5 g of rabbit thymus powder.

ANTIGENIC PURITY OF p67 ANTIGEN

Although the monoclonal antibody to p67 (K8–43) and a human serum sample containing antibodies to RNP bound in the ELISA to the purified p67 antigen from each preparation, the monoclonal antibodies KSm5 and KSm2 did

Table 1 Reproducible antibody specificity of p67 antigen. Values are duplicate results obtained in the enzyme linked immunosorbent assay (ELISA) using the human antibody against p67 (RNPs), normal human serum (NHS), and the three monoclonal antibodies against B,B' (KSm5), D (KSm2), and p67 (K8–43) on the purified p67 antigen

Batch of p67	Absorbance at 405 nm of ELISA well incubated with				
	KSm2	KSm5	K8–43	RNPs	NHS
1	0·025	0·022	1·53	1·85	0·097
2	0·028	0·039	2·47	1·86	0·097
3	0·032	0·019	2·58	1·90	0·120
4	0·033	0·040	2·70	1·92	0·093
5	0·034	0·055	2·76	1·85	0·116

Table 2 Interbatch variation of p67 antigen in six human serum samples containing antibodies to ribonucleoprotein determined by enzyme linked immunosorbent assay. Values are levels of antibody to p67 (U)

Batch of p67	Serum sample No					
	I	II	III	IV	V	VI
1	8·1	14	16	26	58	79
2	7·0	14	20	28	65	101
3	7·3	17	21	27	58	110
4	7·7	20	22	29	60	99
5	9·6	12	16	27	45	112
Mean	7·9	15	19	27	57	100
Standard deviation	1·0	3·3	2·7	1·7	7·4	13
Coefficient of variation (%)	13	22	14	6	13	14

not (table 1), confirming that this technique of immunoaffinity chromatography and high performance liquid chromatography can produce pure p67 antigen in a reproducible manner without contamination with the B,B', and D polypeptides from the Sm antigen.

COMPARISON OF INDIVIDUAL PREPARATIONS OF p67

The p67 antigens were diluted with phosphate buffered saline and coated onto ELISA plates as described. To confirm the reproducibility of the assay the binding of six reference serum samples with low to high binding activity to RNP was measured on each preparation (table 2). The coefficients of variation for individual serum samples were 13, 22, 14, 6, 13, and 13%.

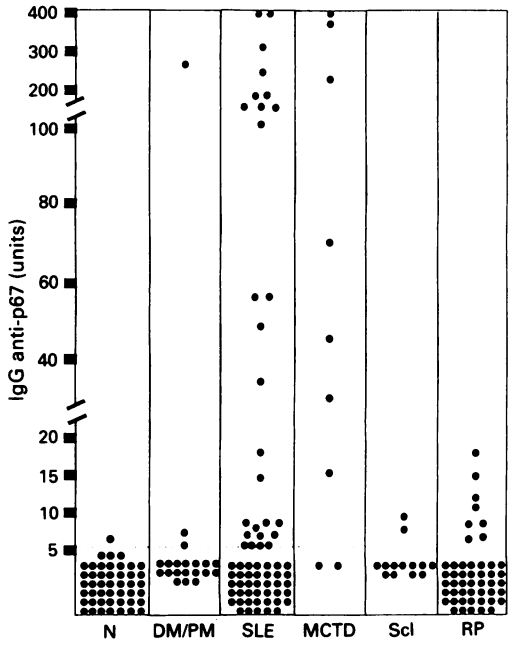

Figure 2 Distribution of IgG antibodies to p67 in the disease groups studied. N=normal controls (n=46); DM/PM=dermatomyositis/polymyositis (n=20); SLE=systemic lupus erythematosus (n=67); MCTD=mixed connective tissue disease (n=10); Scl=scleroderma (n=13); RP=Raynaud's phenomenon (n=48).

ANTIBODIES TO p67 IN AUTOIMMUNE CONNECTIVE TISSUE DISEASES

The upper limit of the normal range of antibodies to p67 (mean+three standard deviations) was 5·1 units, determined using serum samples from 46 healthy subjects (fig 2). Using this limit one normal subject had increased levels of antibodies against p67 (6 U). Increased levels of these antibodies were also seen in 3/20 (15%) patients with myositis, 8/10 (80%) with MCTD, 27/67 (40%) patients with SLE, 2/13 (15%) with progressive systemic sclerosis, and 8/48 (17%) with Raynaud's phenomenon. Our previous studies have suggested that levels of antibody to p67 greater than 50 U may be restricted to patients with MCTD.[11] High titres (>50 U) were found in 5/10 (50%) of the patients with MCTD, 12/67 (18%) of those with SLE, and 1/20 (5%) patients with dermatomyositis/polymyositis complex (fig 2).

CLINICAL FEATURES OF PATIENTS WITH SLE WITH ANTIBODIES TO p67

Patients with SLE were assessed for the presence of renal disease and the major clinical features of MCTD,[7] and then separated according to the presence or absence of antibodies against the p67 antigen (table 3). This showed that the incidence of renal disease, lung fibrosis, arthritis, and Raynaud's phenomenon was similar in the two groups, but that the antibodies to p67 were detected more often when myositis was a feature of the SLE (p=0·019). The mean antibody titre to p67 was also significantly higher in patients with myositis (122 U) than those without (31 U) (p=0·003 using the *F* test). In addition, in the patients with SLE with features of MCTD (alveolitis, Raynaud's phenomenon, myositis, and arthritis) the mean antibody titre to p67 was higher (167 U) than in those without these clinical features (33 U) (p<0·001).

Table 3 Associations of increased antibodies to p67 with some clinical features of mixed connective tissue disease in patients with systemic lupus erythematosus

Clinical feature	*Positive for antibodies to p67 (n=27)*	*Negative for antibodies to p67 (n=40)*	*p Value*
Renal disease (n=38)	18	20	NS
Alveolitis (n=15)	8	7	NS
Raynaud's phenomenon (n=39)	17	22	NS
Myositis (n=10)	8	2	0·019
Arthritis (n=57)	23	34	NS

Table 4 Association of high levels (>50 U) of antibodies to p67 with patients with systemic lupus erythematosus (SLE) and features of mixed connective tissue disease

Patient No	*No of SLE criteria*	*p67 titre (U)*	*Synovitis*	*Raynaud's phenomenon*	*Sclerodactyly*	*Myositis**	*Lung disease†*	*Oesophageal disease‡*	*Renal disease*
1	8	145	+	−	+	+	+	−	−
2	9	400	+	+	+	+	+	NR	+
3	4	232	+	+	+	−	+	+	−
4	7	90	+	+	+	+	+	NR	−
5	9	55	+	+	+	+	+	+	−
6	9	55	+	+	+	+	+	+	−
7	9	130	+	+	−	+	−	+	+
8	10	160	+	+	+¶	+	+	+	+
9	11	160	+	−	NR	−	+	+	+
10	7	409	+	+	+	−	+	NR	+
11	10	300	+	+§	+	+	+	−	+
12	7	129	+	+	−	−	+	−	+

Abbreviations: (+) Present; (−) not present; (NR) no record.
*Two or more criteria as defined by Bohan and Peter.[14]
†Either low K_{CO} or crepitations.
‡Detected either clinically or radiographically.
§Developed during illness.
¶Temporary.

In the group of 12 patients with SLE with more than 50 U of antibody to p67 (table 4) there was a high incidence of synovitis (100%), Raynaud's phenomenon (85%), sclerodactyly (75%), and myositis (75%), and 75% had decreased lung function. Five of 12 patients (42%) had four features of MCTD, three of 12 (25%) had three features, and four of 12 (33%) had two features of MCTD. Antibodies against p67 were present in seven of 12 (58%) patients with renal disease and five of 12 (42%) without (table 4), implying that these antibodies neither protect nor predispose patients with SLE to develop renal changes.

Thus 66% (8/12) of this subset of patients with SLE fulfilled three or more clinical criteria for MCTD, suggesting that high levels of antibodies to p67 delineate a group of patients with SLE who also have criteria for MCTD and thus may represent an overlap group between these two diseases.

Discussion

These results confirm that it is possible to prepare reproducibly pure p67 RNP antigen from rabbit thymus extract by a process of immunoaffinity and high performance liquid chromatography.[11] This technique produces antigen free from contamination with Sm polypeptides or other proteins as detected by immunoblotting and ELISA. The resultant antigen preparations can thus be used in a standard immunoassay provided each preparation is standardised using a chequerboard titration. Furthermore it is possible to use such low concentrations of antigen (1·2–2·5 μg/ml) that large numbers of microtitre plates can be prepared from one antigen purification, thus the assay can readily be used in routine laboratory experiments.

In the initial description of MCTD it was thought that the presence of high titres of antibodies to the nRNP antigen could be used as a serological marker for this distinct connective tissue disease.[7] Low levels of antibodies to RNP can be detected in many patients with other connective tissue diseases, however, as well as in a small percentage of normal subjects.[14] Early studies using immunoblotting suggested that the antibodies against the p67 antigen were found only in patients with MCTD and not in patients with SLE.[3 5 6] Our study aimed to investigate the occurrence of antibodies against the p67 antigen in patients with connective tissue diseases using the ELISA technique.

We have shown that antibodies to p67 above 5 U (mean+three SD of 46 normal subjects) were found in 8/10 (80%) patients with a clinical diagnosis of MCTD (based on only the clinical criteria of Sharp *et al*[7]). We also found increased levels in 27/67 (40%) patients with SLE, however, as well as 15% of patients with polymyositis/dermatomyositis, 8/48 (17%) with Raynaud's phenomenon and 1/46 (2%) healthy subjects. This confirms that antibodies against the p67 antigen are found in low titres in a wide spectrum of patients.[17]

High levels of antibodies to p67 (defined as >50 U/ml) were found only in the patients with connective tissue diseases (5/10 (50%) patients with MCTD, 12/67 (18%) patients with SLE, and 1/20 (5%) patients with dermatomyositis/polymyositis). During the course of the latter patient's disease systemic features developed, including Raynaud's phenomenon, decreased pulmonary diffusion capacity, dysphagia, oesophageal abnormalities on contrast radiographs, and finally digital vasculitis. Thus according to proposed clinical criteria,[7] she can now be considered as having MCTD. Hence high titres of antibodies to p67 occur only in patients with MCTD and SLE. This is in agreement with other studies that have shown the presence of antibodies to p67 in patients with SLE by ELISA using a recombinant p70 antigen[12] and using immunoblotting.[9 10]

To investigate whether antibodies to p67 were associated with features of MCTD we selected 67 patients fulfilling four or more ARA criteria for SLE and examined them for features of MCTD and the presence of antibodies to p67. Our findings did not confirm the protective influence of antibodies to p67 against renal changes in the SLE group.[7 18] Not only was the number of patients with SLE and renal disease almost identical to that in the group without renal disease, but also the mean values of antibodies to p67 in the group with and without renal disease were not statistically different.

The patients with SLE with antibodies to p67 at any titre often have clinical features of MCTD, however, particularly myositis (80%). This implies that antibodies to p67 are not restricted to MCTD but are particularly associated with myositis, which may be found in patients with MCTD and SLE. This confirms a previous study which often found the presence of MCTD features in patients with SLE,[19] and suggests that these antibodies may be a useful marker for these features in either group.

This is further corroborated by analysis of those patients with high titres of antibodies to p67 (table 4), as in this group of 12 patients with SLE 75% had myositis. The occurrence of other features of MCTD including sclerodactyly and reduced lung function was 75%, and all these patients had synovitis. In addition, the incidence of renal disease in these patients was similar in those with and without antibodies to RNP, which is in agreement with the data for the SLE group as a whole.

These results show that high levels of antibodies to p67 determined by ELISA are common in patients with MCTD but are also present in some patients with SLE. In this group of patients with SLE clinical features of MCTD are also seen, suggesting that the patients with SLE with antibodies to p67 form a subgroup of SLE/MCTD.

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