

Use of recombinant RNP peptides 70K and A in an ELISA for measurement of antibodies in mixed connective tissue disease: a longitudinal follow up of 18 patients

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Abstract

In a three year prospective study disease activity variables and levels of antibody against the RNP-peptides 70K and A were measured in 18 patients with mixed connective tissue disease. Antibody measurement entailed use of cloned autoantigens in an enzyme linked immunosorbent assay (ELISA). Fluctuations in antibody levels against 70K and A were most commonly noted in patients who also had changes in disease activity, but these changes in serology and disease activity were synchronous in only a minority of the episodes. Even major disease flares were associated with changes in anti-A levels in only a few, and with changes in anti-70K levels in none of the episodes. The data indicate that measurements of anti-70K and anti-A levels are not useful in monitoring disease activity or response to treatment in mixed connective tissue disease, and suggest that these antibody specificities do not play a direct part in the pathogenesis of disease manifestations.

Connective tissue diseases are characterised by a variety of autoantibodies directed against nuclear and cytoplasmic antigens. Some of these autoantibodies are of diagnostic value—for example, anti-dsDNA and anti-Sm for systemic lupus erythematosus, anti-Scl-70 for diffuse scleroderma, and anti-Jo-1 for polymyositis.¹ Considering a possible pathogenic role for these autoantibodies, investigators have tried to correlate autoantibody levels with disease activity and prognosis. As anti-dsDNA was the first autoantibody that could reliably be measured most earlier studies have been done on this autoantibody.^{2–5} Although these studies vary in design, methodology, and results, at least one group of investigators has shown in careful, well reported, and prospective studies that the kinetics of anti-dsDNA can predict a disease flare.^{5–7} Recently, quantitative assays have been developed for antibodies against non-DNA antigens, such as RNP,^{8–9} Sm,^{10–11} Ro(SSA),¹² and La(SSB).¹³ Measurement was by titration in immunodiffusion, immunoblotting, or by enzyme linked immunosorbent assay (ELISA). The antigens used in an ELISA are most often purified by affinity chromatography and, more recently, obtained by molecular cloning.^{14–17}

Mixed connective tissue disease is a disease entity characterised by overlap symptoms of systemic lupus erythematosus, scleroderma, and polymyositis, as well as by the presence of antibodies to RNP.¹⁸ Analysis of the RNP complex by immunoblotting has disclosed con-

stituents 70K, A, and C as antigenic peptides.^{19–20} Data suggest that antibodies against 70K are almost always present in patients with mixed connective tissue disease²¹ but less commonly in patients with systemic lupus erythematosus positive for antibodies to RNP^{17–22–23} (Reichlin and van Venrooij, in preparation).

In this paper we describe 18 patients with mixed connective tissue disease studied prospectively for fluctuations in disease activity and levels of antibody against 70K and A. Antibody measurement was performed using cloned autoantigens in an ELISA.¹⁷ In this study we were interested in the possibility that antibody levels might be associated with disease activity and that changes in antibody level might predict disease flares or measure possible reactions to treatment. Our results indicate that this is not the case.

Patients and methods

PATIENTS

Between 1983 and 1986 18 patients with mixed connective tissue disease, who were followed up at or referred to the University Hospital St Radboud, Nijmegen, or the St Maartenskliniek, Nijmegen, were included in a prospective study. Patients had overlapping features of systemic lupus erythematosus, progressive systemic sclerosis, and polymyositis and antibodies to RNP.¹⁸ The patients were followed up for three years by one observer and seen as outpatients every two months and more often when in hospital. Systematic data collection included a complete medical history, physical examination, and laboratory data, including Westergren erythrocyte sedimentation rate, complete blood count, chemistry profile, creatine phosphokinase, serum protein electrophoresis, complement C3 and C4, antibodies to dsDNA (Farr assay⁵) and urine analysis. Chest radiograph, pulmonary function tests (vital capacity, carbon monoxide diffusing capacity), oesophageal motility study (barium swallowing), and Schirmer test results were also obtained. Table 1 shows these clinical and laboratory features before and during the study. Two patients had central nervous system manifestations that were possibly unrelated: subarachnoidal haemorrhage and involutive depression.

Three patients developed anti-dsDNA during the study; none of the patients had renal disease. Drugs were given as indicated by the clinical status and not influenced by the serological results. At each visit a disease activity index was obtained by an adapted version of the protocol described by Becker *et al*²⁴ by addition

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Accepted for publication
18 July 1989

Table 1: Clinical and laboratory characteristics of 18 patients with mixed connective tissue disease

Characteristics	n	%
Arthritis/arthralgia	18	100
Raynaud's phenomenon	18	100
Swollen hands	12	67
Mucocutaneous	5	28
Sclerodactyly	10	56
Myalgia	17	94
Myositis	8	44
Sjögren's syndrome	3	17
Central nervous system involvement	2	11
Fever (unexplained)	6	33
Pleuropericarditis	4	22
Impaired pulmonary function test	9	50
Oesophageal hypomotility	7	39
Leucopenia	9	50
Thrombocytopenia	4	22
Rheumatoid factor	11	61
Anti-dsDNA	3	17
Hypocomplementaemia	5	28
Corticosteroid treatment	9	50

of points given for each symptom present (table 2). A significant change in disease activity index was defined as a difference from the previous value of two or more points. Major disease manifestations included serositis, fever, vasculitis, leucopenia/thrombocytopenia, myositis, and central nervous system involvement.

Fifteen patients were seen for two years before the study period and these serum samples were also studied for changes over a total of five years (1981–1986).

SERA AND ANTIBODY MEASUREMENTS

Serum samples were obtained at each clinical visit and stored in aliquots at -70°C .

Counterimmunoelectrophoresis and immunoblotting were performed as described previously.²⁰

Isolation and identification of cDNA clones encoding the A and 70K ribonucleoprotein autoantigens have been described elsewhere.^{17, 25} In brief: a λ gt11 cDNA library from a human teratocarcinoma cell line was screened with human anti-(U1) RNP sera. Reactive phages were selected and rescreened until clonal purity. Authenticity of the clones was confirmed by hybrid selection, in vitro translation of in vitro transcribed RNA, and DNA sequencing.^{17, 25} Isolation of recombinant antigens was performed as we previously described.¹⁷

A reliable ELISA with these recombinant antigens was recently developed in our laboratory.¹⁷ About 500 serum samples from patients with connective tissue diseases were tested in this ELISA and the results compared with

earlier immunoblotting data.²⁶ The ELISA was found to be at least as sensitive as the immunoblotting test.¹⁷ Plates were read on a BioRad EIA reader at 450 nm. Serum samples were tested in triplicate and the results were averaged. The amount of antibody present in each sample was expressed in optical density (OD) units—that is, $\text{OD}_{450} \times \text{dilution}$. The same reference serum was always used as a standard on each plate. In longitudinal studies a twofold variation in antibody level was considered significant.

Results

Our group of patients with mixed connective tissue disease in general had mild disease as is reflected by the fact that none of the patients used cytostatic drugs. Thus only six patients showed a total of 10 major disease flares during the study. Characterisation of autoantibodies by counterimmunoelectrophoresis showed anti-RNP in only 17 patients and anti-RNP+Sm in one patient. In RNA precipitation eight patients precipitated (U1) snRNA, two patients (U1, U2) snRNAs, and seven patients (U1–U6) snRNAs. (In one patient the precipitation was not done.) By immunoblotting 16 patients had antibodies to 70K, all patients had antibodies to A, and 10 patients had antibodies to C. Seventeen patients also had antibodies to B'B; the only patient with a strong anti-D response had both anti-RNP and anti-Sm in the counterimmunoelectrophoresis. As serial serum samples of most patients were available for up to two years before the study period we were able to interpret immunoblot profiles for a period of five years. During this period anti-A activity disappeared in one patient and anti-70K activity in two patients; in 1 of these last two patients anti-centromeric antibodies (referred to as anti-CR-19) appeared after disappearance of the anti-70K band, whereas antibodies against B'B and C were continuously present. In some patients antibodies against undefined peptides were detected as well.

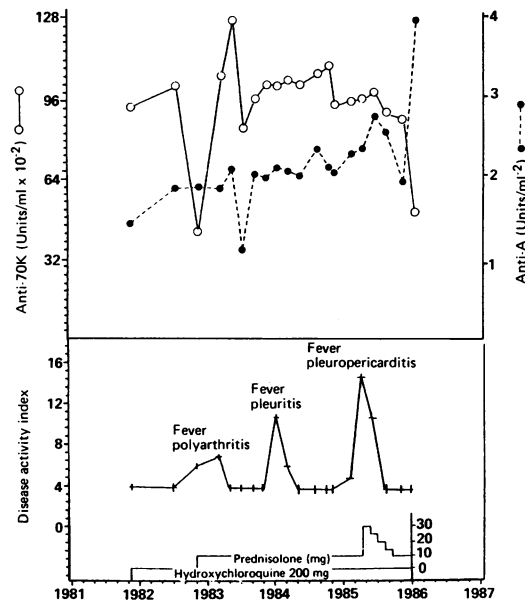
With the ELISA technique we determined the level of antibodies to 70K and A in serial serum samples and compared these values with the disease activity index. The figure shows a typical example of the profile obtained in one patient; most of the 18 patients showed similar fluctuations in antibody levels.

Table 3 classifies patients according to the presence or absence of significant increases in anti-70K, anti-A, and disease activity index

Table 2: Disease activity index obtained at each visit by addition of points given for each symptom present

Symptoms	Points	Symptoms	Points
Arthralgia	1	LE* rash	1
Arthritis 1–3 joints	1	Oral or nasal ulceration	2
Arthritis >3 joints	1	Sclerodactyly	1
Myalgia	1	Fever by history	1
Myositis CPK* <twofold	2	clinical	2
CPK <fourfold	2	Pleuropericardial pain	5
CPK >fourfold	2	Pleuropericardial rub	3
Raynaud's by history	1	CNS* cerebrovascular	4
present	1	depression	3
severe	2	Leucopenia $<4 \times 10^9$ cells/l	2
Swollen hands	1	$<3 \times 10^9$ cells/l	1
		Thrombocytopenia $<100 \times 10^9$ cells/l	2

*CNS=central nervous system; CPK=creatinine phosphokinase; LE=lupus erythematosus.



Longitudinal values for anti-70K and anti-A levels and disease activity index in one patient with mixed connective tissue disease. In the upper graph the values for anti-70K (○—○) and anti-A (●—●) are shown, in the lower, the disease activity index, showing three exacerbations, and drugs given.

Table 3: Number of patients with significant increases in anti-70K or anti-A levels and in the disease activity index during the study

Increase* in anti-70K or anti-A levels, or both	Increase in disease activity† index	
	+	-
	(n=12)	(n=6)
70K + A+	4	1
70K + A-	0	2
70K - A+	5	0
70K - A-	3	3

*Significant increase is defined as a minimally twofold difference from the preceding value.

†Significance increase is defined as a rise of two or more points in disease activity index as compared with the preceding value.

Table 4: Number of significant changes in anti-70K or anti-A levels concomitant with significant changes in disease activity

	Number of patients	Number of changes	In concert with disease activity change
Anti-70K Increase*	7	12	4
Anti-70K Decrease*	8	17	0
Anti-A Increase	10	11	4
Anti-A Decrease	11	14	4

*Twofold increase or decrease in antibody levels as compared with previous values.

Table 5: Major disease manifestations concomitant with significant changes in anti-70K or anti-A levels

Patient's code: disease manifestations	Change in anti-70K	Change in anti-A*	Change in treatment
D2 :serositis, arthritis, fever	0	+	Prednisolone 20 mg
D1 :fever, vasculitis, leuco/thrombopenia	0	0	0
D1 :fever, arthritis, myositis	0	0	0
D11 :fever, pleuritis	0	+	0
D11 :fever, pleuritis	0	0	Hydroxychloroquine
D11 :fever, pleuritis	0	-	0
S3 :fever, pleuritis	0	0	Prednisolone 5 mg
S3 :fever, pleuritis	0	0	Prednisolone 25 mg
A1 :depression	0	+	0
K1 :subarachnoidal haemorrhage	0	0	0

*+ = increase; - = decrease.

measured at some time during the study. Of 12 patients with significant increases in anti-70K or anti-A level, or both, nine had increases of disease activity, whereas out of six patients without serological changes, three had significant increases in disease activity index. Increases in anti-A occurred almost exclusively (nine out of 10) in patients who also showed significant increases in disease activity index at some time during the study, whereas an increase in anti-70K level occurred only in two patients without disease activity changes. These data show that increases in specific antibody level mostly occurred in patients who also had increases in disease activity index, irrespective of whether the changes were synchronous or not.

When looking for significant changes in antibody level occurring in concert with disease activity changes, we found only a small number of such episodes (table 4). Of 12 increases in anti-70K level, only four were concomitant with disease activity changes, whereas 17 decreases in anti-70K level had no relation at all with disease activity changes. Changes in anti-A level coincided with changes in disease activity in eight episodes out of 25. We conclude that only a minority of antibody level changes occurred in concert with disease activity fluctuations.

Even when clinical exacerbations characterised by major disease manifestations were studied, only a minority were accompanied by serological changes. None of the 10 major disease flares in our patients was associated with significant changes in anti-70K and only four with changes in anti-A level (table 5); one of these changes was in fact a decrease in antibody level.

When comparing the numerical values of anti-70K levels and disease activity index over a period of five years in patients from whom serum samples were available over this longer period, we found a statistical correlation (as determined by Pearson's correlation coefficient) in only three of the 15 patients. For anti-A levels we found significant correlation in only four out of 15 patients.

Discussion

Quantitative studies on autoantibody levels other than anti-RNP have indicated that correlations with disease activity may exist. The longitudinal studies of Swaak *et al* in a large number of patients with systemic lupus erythematosus showed that a rise in anti-dsDNA level with a doubling time of less than 10 weeks consistently preceded disease flares and there-

fore may have predictive value.⁵⁻⁷ At the onset of disease flares a sharp decrease in anti-dsDNA was usually found which could not simply be explained by institution of or increase in drug treatment. The methods used by this group differ from those of other groups as anti-dsDNA levels were obtained after appropriate dilution of serum samples, allowing for better measurement of antibody levels. For the moment these interesting observations await confirmation. It has been suggested that associations with disease activity exist for other autoantibody systems—for example, anti-La(SSB),⁹ anti-Ro(SSA),¹² and anti-Sm.^{10,11} The studies on antibodies against RNP antigens, on the other hand, have given inconsistent results. Houtman *et al*, using an ELISA with affinity purified RNP antigens, showed correlations of antibody levels with disease activity in a cross-sectional study.⁹ In a longitudinal study of the same group disease flares were mostly accompanied and often preceded by a rise in antibody level, though antibody kinetics were in most cases often slow and therefore of limited practical value.²⁷ Nishikai *et al* found fluctuations in anti-RNP titre were related to disease activity and steroid treatment in patients with systemic lupus erythematosus, whereas in patients with mixed connective tissue disease serological fluctuations present in two out of six patients were unrelated to disease activity.²⁸ Takeda *et al* found an association of disease activity with anti-68K (which is the same as the anti-70K in our study) and anti-C levels, but not with anti-A levels.²³ In a longitudinal study 68K reactivity consistently fell to a lower level during disease remission, while haemagglutination titres remained high. In our study no consistent correlation was found between disease activity and anti-70K and anti-A levels.

The reason for these varying results in studies on autoantibodies against RNP antigens is at present unclear. One should, however, realise that these studies differ considerably in experimental design and patient selection (disease activity, severity, duration, and treatment). Our patient group, for example, generally had only mild disease as is indicated by the limited number of major disease flares and by the fact that none of the patients used cytotoxic drugs. In the study of Pettersson *et al* a group of patients with severe mixed connective tissue disease (treated with high dose steroids and cytotoxic drugs) showed decreased RNP-peptide reactivity (particularly anti-A) in those patients who were in clinical remission.²² In patients with mild mixed connective tissue disease the RNP reactivity remained unchanged for many years,²² even in the patients in remission. Our patients, therefore, are more comparable with the latter group. Sullivan *et al* found a significant reduction in RNP antibody titre measured by haemagglutination in 42% of the patients in remission or with mild disease²⁹; persistently high titres were related to more continuing and severe disease activity. Grant *et al* also noted fluctuations in extractable nuclear antigen antibody titre in haemagglutination, mostly not associated with new clinical and serological abnormalities unless anti-Sm was also present.³⁰

There is also a considerable variation in the antibody tests used. They vary in antigen source, preparation, purification, and method of measurement. Antigens differ in composition, RNP being a more complex antigen than the single polypeptides 70K or A. As better correlations seem to be found for tests measuring antibodies against the more complex antigens²⁷ than for those against 70K, A, or C separately, it is possible that the tests for antibodies against complex antigens, which measure a more heterogeneous group of antibodies against a number of epitopes, show better association with disease activity than antibodies against more sophisticatedly defined antigens.

An important issue in autoantibody measurement is whether antibody changes are specific or merely a reflection of polyclonal B cell activation. The latter possibility is for example advocated by Houtman *et al*, who found concurrent rises in IgM rheumatoid factor, anti-tetanus antibodies, and total serum IgG,²⁷ and by Scopelitis *et al*, who showed concurrent rises in serum IgG in cases of increased anti-Ro(SSA) levels.¹² We used a quantitative immunoblotting assay to describe a case with concomitant fluctuations in levels of antibody against the 70K, A, and B'B peptides.⁸ On the other hand, in most other patients the levels of these antibodies seem to be regulated independently (as in this study).^{23,31} This indicates that B cell stimulation is not always of the same magnitude, at least not for these antigens during long term observations. Our results indicate that monitoring patients with mild mixed connective tissue disease by serial determinations of antibody levels against the RNP antigens 70K and A is probably not helpful in predicting disease flares or reaction to treatment. In addition, they do not support the hypothesis that antibodies to RNP are directly involved in the pathogenesis of mixed connective tissue disease manifestations.

We are grateful for expert computer programming by Michiel 't Hart and the secretarial assistance of Marloes Koldijk. This work was supported in part by the Netherlands League against Rheumatism and the 'Praeventiefonds' (The Hague).

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