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Autoantibodies against eukaryotic protein L7 in patients suffering from systemic lupus erythematosus and progressive systemic sclerosis: frequency and correlation with clinical, serological and genetic parameters

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SUMMARY

Recent studies have shown that sera of patients suffering from systemic autoimmune diseases contain autoantibodies directed against the eukaryotic ribosomal protein L7 [1]. In the present study we screened a large panel of sera from patients with systemic lupus erythematosus (SLE) for the presence of anti-L7 autoantibodies and their relationship to clinical, serological and genetic parameters of SLE. By means of an ELISA employing recombinant protein L7 as antigen we detected anti-L7 autoantibodies in 172 of 506 SLE sera (34%). Negative correlations were observed between the presence of anti-L7 autoantibodies, serum IgG levels and proteinuria; a potentially positive relationship existed with lung fibrosis. In order to analyse further this possibility we screened sera of 129 patients suffering from progressive systemic sclerosis (PSS) for anti-L7 reactivity; 45 of these patients had lung fibrosis. Of the PSS patients, 41% exhibited anti-L7 autoantibodies, but positive reactions were evenly distributed among patients with and without lung fibrosis. Protein L7 thus represents a major autoantigen of systemic autoimmune diseases, but does not so far define a distinct subpopulation of patients.

Keywords autoantibodies L7 frequencies correlations

INTRODUCTION

Autoimmune diseases are characterized by the presence of autoantibodies targeted against cellular proteins and nucleic acids. Many autoantibody specificities are characteristic of a given disease. They serve as useful diagnostic markers for the clinician. Anti-DNA or anti-Sm autoantibodies for example are used as markers for systemic lupus erythematosus (SLE). Anti-SS-A/Ro and anti-SS-B/La autoantibodies are indicative of Sjögren's syndrome (SS). However, this does not imply that these autoantibody specificities are exclusively associated with these particular diseases (for review [2,3]).

Although there is evidence supporting a pathogenic role of autoantibodies in some diseases, e.g. myasthenia gravis [4], their significance and clinical relationship in most systemic diseases including SLE is unclear. For instance, the pathophysiological significance of anti-ribosomal P protein autoantibodies remains controversial. Bonfa *et al.* suggested that anti-P autoantibodies correlate with lupus psychosis [5]. In contrast, Derksen *et al.* reported on two SLE patients who failed to show an obvious association between the level of anti-P autoantibodies and lupus psychosis [6]. Further studies on anti-P autoantibodies have confirmed these conflicting results [7]. Yet for a small number of autoantibody specificities in SLE a potential pathological role is described. Anti-DNA autoantibodies are thought to cause tissue injury by deposition of antigen–antibody complexes in the kidneys, skin and vessel walls [8,9]. Anti-Ro autoantibodies seem to play a role in cutaneous manifestations and congenital heart block [10].

We have shown in a previous study that sera of patients suffering from SLE, mixed connective tissue disease (MCTD), rheumatoid arthritis (RA), SS and progressive systemic sclerosis (PSS) frequently contain autoantibodies which specifically precipitate the eukaryotic ribosomal protein L7, and anti-L7

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autoantibody titres seemed to be augmented during active phases of the disease [1]. The autoantigen L7 is a 29-kD protein which associates with the large ribosomal subunit, but presumably is not an integral structural component of ribosomes [11]. We have shown that it binds specifically to sites on mRNA and 28S rRNA [12]. Protein L7 resides in both the nucleus and the cytoplasm, but is not detected in the nucleolus [1]. Anti-L7 autoantibodies target three major linear epitopes (von Mikecz *et al.*, this issue of *CEI*, page 205).

The aim of this study was to determine anti-L7 autoantibody titres in SLE patients, and to investigate a potential relationship between the occurrence of such autoantibodies and clinical, serological and genetic parameters of SLE. To this end we developed an ELISA and screened 506 sera collected from SLE patients enrolled in a German multicentre SLE study [13]. In order to elucidate a potential correlation of elevated anti-L7 autoantibody titres with lung fibrosis we included 129 sera collected from PSS patients into our study.

PATIENTS, MATERIALS AND METHODS

Sera from patients and healthy donors

Sera of SLE patients ($n = 506$) registered in a German multicentre SLE study were tested. The sera were collected between 1986 and 1990 in the out-patient clinics of the university hospitals at Düsseldorf (D), Erlangen (Er), Hannover (H) and Freiburg (Fr) from Caucasian SLE patients of European descent [13]. Overlap syndromes were carefully excluded, and all patients fulfilled the revised American Rheumatism Association (ARA) criteria for SLE [14]. Based on a slightly modified lupus activity index established by Morrow *et al.* [15], a great majority of the patients had inactive (40%) and weakly or moderately active disease (57%). Only 3% of the patients had severely active SLE. All SLE patients are HLA-typed [13]. Sera of 129 patients suffering from PSS were collected at the Rheumaklinik Aachen and at the Freiburg University hospital. For control purposes, sera of 50 healthy subjects comprising blood donors or members of the clinical and laboratory staff were collected.

Purification of GST-L7 and GST

The expression of protein L7 fused to *Schistosoma japonicum* glutathione-S-transferase (GST-L7) and the expression of glutathione-S-transferase (GST) in *Escherichia coli* and their purification by affinity chromatography were performed as described previously [12].

ELISA for anti-L7 autoantibodies

Ninety-six-well polystyrol plates (Greiner, Frickenhausen, Germany) were coated with GST-L7 (500 ng/well) in PBS pH 7·0. The plates were incubated overnight at 4°C. After washing the wells four times with PBS/0·1% Tween-20, non-specific binding was blocked by incubation with 1% non-fat dried milkpowder in PBS/0·1% Tween-20, for 1 h at 37°C. Sera were added at a dilution of 1 : 100 in blocking buffer into the first well and were then serially diluted 1 : 2. The plates were incubated for 2 h at room temperature. After four washes with PBS/0·1% Tween-20, 100 μl of peroxidase-conjugated goat anti-human IgG Fcγ antibody (0·8 mg/ml; Dianova, Hamburg, Germany) at a 1 : 20 000 dilution in blocking buffer were added to each well and the plates were incubated for another 2 h at room temperature. Similarly, peroxidase-conjugated goat anti-human IgM Fcμ (0·8 mg/ml; Dianova) and goat anti-human IgA Fcα (0·9 mg/ml; Dianova) were used at a 1 : 10 000 dilution in blocking buffer. Subsequently, the wells were washed four times with PBS/0·1% Tween-20 and 100 μl of the substrate solution were added. The substrate solution contained 0·4 g 1,2-phenylendiamin in 1 *l* 0·1 M citric acid and 0·2 M Na_2HPO_4 pH 6, and 5 μl of 30% H_2O_2 per 20 ml substrate solution. The enzyme reaction was allowed to proceed for 15 min at room temperature, and was stopped by addition of 25 μl 1 M H_2SO_4. The absorbance in each well was determined at 492 nm (OD_{492}) by means of a microplate reader (Anthos HT III). As a specificity control, plates were coated with 500 ng/well GST and ELISAs were performed exactly as described above. For large scale screening, sera were routinely tested in parallel on ELISA plates coated with GST-L7 and GST.

For the quantification of anti-L7 titres the same procedure was performed with serially diluted patient sera. As a measure for specific anti-L7 titres the difference of the optical densities at 492 nm wave length obtained in GST-L7, or respectively, GST-coated wells was used: $OD_{492}L7 = OD_{492}GST\text{-}L7 - OD_{492}GST$.

Statistical analysis

The χ^2 test, with Yates' correction when necessary, was used to analyse statistical significance. $P<0{\cdot}05$ indicates significance. By χ^2 analyses the presence or absence of a clinical, serological or genetic finding was compared with the presence or absence of anti-L7 reactivity detected by ELISA.

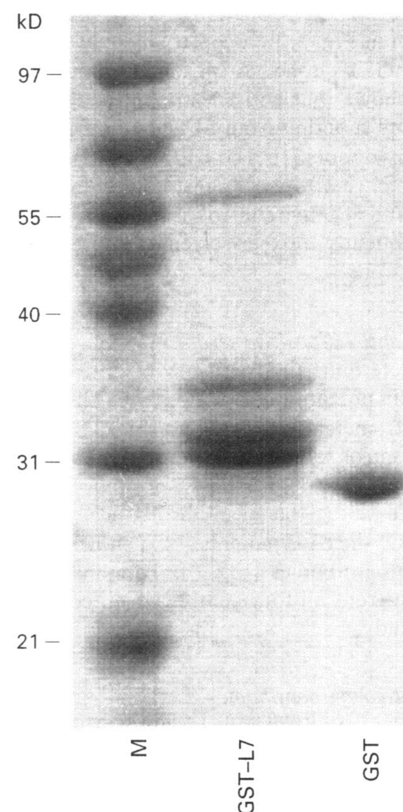

Fig. 1. Coomassie blue-stained SDS–PAGE of GST-L7 and GST Molecular weight markers (M) are shown.

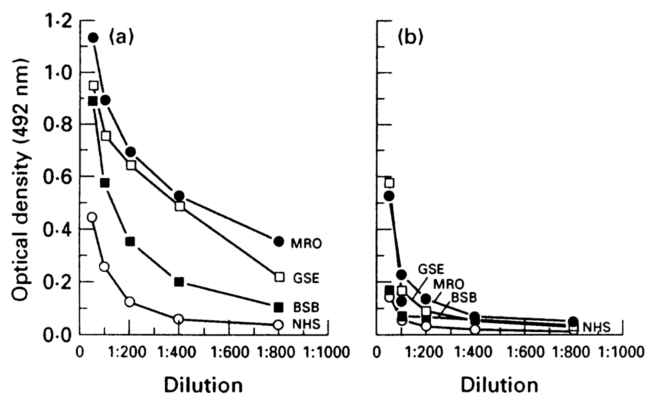

Fig. 2. Result of a typical ELISA performed with (a) GST-L7-coated microtitre wells, and (b) GST-coated microtitre wells. A three-letter code designates sera of patients. NHS, Normal serum of a healthy individual.

RESULTS

Detection of anti-L7 autoantibodies by ELISA

An ELISA was developed in order to screen a large number of sera from SLE patients for the presence of autoantibodies against the eukaryotic protein L7. To this end recombinant protein L7 was expressed as a fusion protein with GST in *E. coli* and purified by affinity chromatography on glutathione agarose beads. The purity of GST-L7 was verified by SDS–PAGE (Fig. 1). The 55-kD band represents the complete GST-L7 fusion protein, whereas the smaller bands contain GST-L7 molecules presumably truncated at the C-terminus [12].

A typical ELISA performed with GST-L7-coated microtitre wells is shown in Fig. 2. In a control assay we employed wells coated with GST alone. A previous immunoprecipitation analysis has shown that anti-L7 autoantibodies are present in sera of patients suffering from SLE, but are not detectable in sera of healthy donors [1]. The ELISA confirms this result. A maximal difference of optical densities obtained with anti-L7-positive sera and negative controls is seen at a serum dilution of 1 : 400. Subsequently, further screenings were performed with 1 : 400 diluted sera.

Frequency of anti-L7-positive sera

In order to determine the frequency of anti-L7 autoantibody titres and their potential correlation with clinical, serological and genetic parameters, 506 SLE sera (Fig. 3, lane D, Er, H and Fr) and 50 control sera (Fig. 3, lane NHS) were screened by ELISA. The cut-off to define anti-L7 positivity (OD_{492} = 0·200) was set at 2 s.d. above the mean absorbance obtained with the normal sera. Of the SLE patients, 34% showed elevated levels of anti-L7 autoantibodies (Fig. 3). This high frequency was similar in sera from all four centres which contributed to the multicentre study.

Isotype of anti-L7 autoantibodies

We checked the isotype of anti-L7 autoantibodies by ELISA using anti-human γ-chain, μ-chain and α-chain antibodies as detecting reagents. The anti-L7 autoantibodies detected by ELISA in this study are all of the IgG isotype (data not shown). We could not detect anti-L7 autoantibodies of the IgM and IgA isotype.

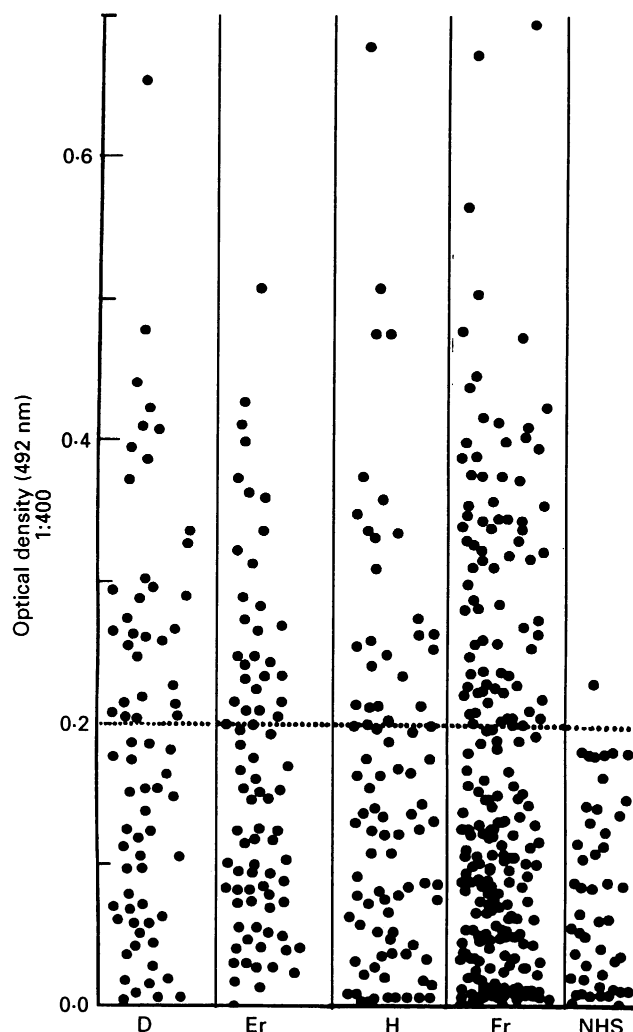

Fig. 3. Prevalence of autoantibodies against eukaryotic protein L7 detected by ELISA in systemic lupus erythematosus (SLE) sera from a German multicentre study and in normal control sera (NHS). The patient sera were collected from four centres: Düsseldorf (D), Erlangen (Er), Hannover (H) and Freiburg (Fr). The cut-off point for anti-L7 positivity is set at OD_{492} = 0·2.

Correlations of anti-L7 autoreactivity with clinical, serological and genetic parameters

Using the χ^2 test we surveyed the data bank of the German SLE multicentre study for statistical correlations between the presence of anti-L7 autoantibodies and serological, clinical and genetic parameters of SLE. Tables 1 and 2 show the parameters examined and a summary of the results. A given diagnosis is correlated with the frequency of elevated levels of anti-L7 antibodies in the patients concerned, and *P* values indicate the level of significance. We found negative correlations of proteinuria and elevated IgG levels with anti-L7 titres. That is, only a minority of patients suffering from proteinuria or showing elevated IgG levels were anti-L7-positive (Table 1). Thus, the autoimmune B cell response against the protein L7 is unlikely to be the consequence of a polyclonal stimulation. As far as clinical parameters are concerned, we observed a negative correlation of vasculitis with anti-L7 titres (Table 2), though

Table 1. Frequencies of elevated levels of anti-L7 antibodies in different patient groups with different serological findings and *P* values, giving the level of significance

SLE study group (*n*)	Positive for anti-L7 (*n*)	Laboratory finding (number of positive patient sera)	Positive for anti-L7 (*n*)		Positive for laboratory finding (*n*)		*P*
			Positive for laboratory finding	Negative for laboratory finding	Positive for anti-L7	Negative for anti-L7	
356	121	ANA-pattern:					
		Homogen (294)	102	19	102	192	>0·05
		Speckled (58)	19	102	19	39	>0·05
		Nucleolar (4)	0	121	0	4	>0·05
259	94	Autoantibodies:					
		Anti-dsDNA (218)	80	14	80	138	>0·05
		Anti-ssDNA (183)	65	29	65	118	>0·05
		Anti-Crlp IgG (163)	60	34	60	103	>0·05
		Anti-Crlp IgM (87)	29	65	29	58	>0·05
		Anti-68kD (22)	9	85	9	13	>0·05
		Anti-RNP/Sm (77)	29	65	29	48	>0·05
		Anti-Sm (36)	9	85	9	27	>0·05
		Anti-Ro (105)	40	54	40	65	>0·05
		Anti-La (55)	20	74	20	35	>0·05
		Anti-Scl70 (11)	3	91	3	8	>0·05
		Anti-Cenp (6)	1	93	1	5	>0·05
		Anti-Jo1 (7)	4	90	4	3	>0·05
237	86	Crithidia (82)	29	57	29	53	>0·05
216	72	C3d high (117) (> 10/ml)	39	33	39	78	>0·05
330	105	CH_{50} (56) (<20/ml)	19	86	19	37	>0·05
236	71	Proteinuria (116) (>0·1 g/day)	26	45	26	90	0·012
141	38	Clearance (82) (>80 ml/min)	25	13	25	57	>0·05
435	151	IgG high (143) (>18 g/*l*)	61	90	61	82	0·015
279	106	Number of lymphocytes (110) (<1000/ml)	37	69	37	73	>0·05
472	163	Number of leucocytes (92) (<4000/ml)	38	125	38	54	>0·05
296	109	Rheumatoid factor (57)	21	88	21	36	>0·05
165	64	Cryoglobulin (12)	4	60	4	8	>0·05
179	71	Coombs' test (5)	1	70	1	4	>0·05

Crlp = Cardiolipin, ANA = anti-nuclear antibodies; *n*, number of patient sera.

the level of significance was weak ($P = 0{\cdot}049$). A potentially positive correlation was observed between lung fibrosis and anti-L7 autoantibodies (Table 2). Eleven out of 20 lung fibrosis patients were positive for anti-L7 autoantibodies and nine were negative. In order to analyse further this correlation we screened sera of 129 patients suffering from PSS for the presence of anti-L7 autoantibodies. PSS patients show lung fibrosis as a frequent clinical symptom [16]. In our group of PSS patients 45 out of 129 (35%) showed clinical and spirometric signs of lung involvement. Figure 4 illustrates that 35% of PSS patients with lung fibrosis and 46% of those without were positive for anti-L7 autoantibodies. This distribution strongly suggests that there is no correlation of lung fibrosis with the presence of anti-L7 autoantibodies. However, as in SLE, the high frequency of 41% anti-L7-positive PSS sera should be noted.

SLE in Caucasians has been found to be associated with distinct MHC class II genes. Both HLA-DR2 and -DR3 [17,18] and the haplotypes HLA B7-DR2 and HLA B8-DR3 [13] are more frequent among SLE patients compared with the healthy population. The presence of anti-L7 autoantibodies was not associated with one of these SLE haplotypes.

DISCUSSION

In the present study we developed an ELISA to screen on a large scale sera of SLE patients for anti-L7 autoantibodies. Of 506 sera obtained from a German multicentre SLE study, 34%

Table 2. Frequencies of elevated levels of anti-L7 antibodies in different patient groups with different clinical features and *P* values, giving the level of significance

SLE study group (*n*)	Positive for anti-L7 (*n*)	Clinical feature (number of positive patient sera)	Positive for anti-L7 (*n*)		Positive for clinical feature (*n*)		*P*
			Positive for clinical feature	Negative for clinical feature	Positive for anti-L7	Negative for anti-L7	
475	162	Vasculitis (25)	4	158	4	21	0·049
480	163	Skin* involvement (250)	82	81	82	168	>0·05
479	163	CNS† involvement (65)	23	140	23	42	>0·05
473	162	Nephritis (62)	17	145	17	45	>0·05
435	151	Arthritis (49)	15	136	15	34	>0·05
435	151	Serositis‡ (10)	6	145	6	4	>0·05
463	157	Lung fibrosis (20)	11	146	11	9	0·042
475	162	Sicca (71)	29	133	29	42	>0·05
474	159	Myalgia (67)	23	136	23	44	>0·05

n, Number of patient sera; CNS, central nervous system.

* Skin involvement: presence of one or more of the following symptoms: butterfly rash, discoid rash, erythema, photosensitivity, alopecia areata or diffusa.

† CNS involvement: presence of convulsions and/or psychosis and/or neuropathy.

‡ Serositis: presence of pleuritis and/or carditis.

had anti-L7 autoantibody titres. We have previously shown in a small-scale immunoprecipitation study that 18 of 24 sera (75%) obtained from SLE patients attending one centre (Fr) contained anti-L7 antibodies [1]. This discrepancy in frequencies is possibly due to the different sensitivities of the assays, different detecting reagents and different L7 antigen preparations. In the immunoprecipitation study, radiolabelled L7 protein translated in a cell-free system was employed as target antigen. This approach yielded a low background and consequently allowed the detection of low anti-L7 autoantibody titres, but the procedure is unsuitable for large-scale screening. Recombinant L7-protein fused to glutathione-S-transferase (GST-L7) was used to coat the ELISA plates. The cut-off to define disease-specific reactivity was set at 2 s.d. above the mean reactivity of sera from 50 healthy individuals. Moreover, in order to measure pure anti-L7 reactivity we subtracted the reactivity against GST from the reactivity against GST-L7. Actually, less than 2% of the sera (10 of 506) contained anti-GST titres (between OD_{492} 0·1 and 0·3) (data not shown). Such sera were treated as anti-L7-negative in the statistical analysis. These precautions let the frequency of 34% anti-L7-positive SLE sera seem to be a minimal estimate. In comparison with the frequency of other autoantibody specificities, for instance anti-ribosomal P protein autoantibodies, which occur in SLE at a frequency of 5–10% [19] and 20% [20], the frequency of at least 34% for anti-L7-positive sera is notably high. Anti-ribosomal antibodies were not routinely determined in the German multicentre SLE study. However, of nine sera diagnosed as anti-ribosomal protein-positive, only one contained anti-L7 antibodies. This, and the difference of frequencies of anti-P and anti-L7 antibody titres among SLE patients, argues against a correlation of anti-L7 and anti-ribosomal autoreactivity.

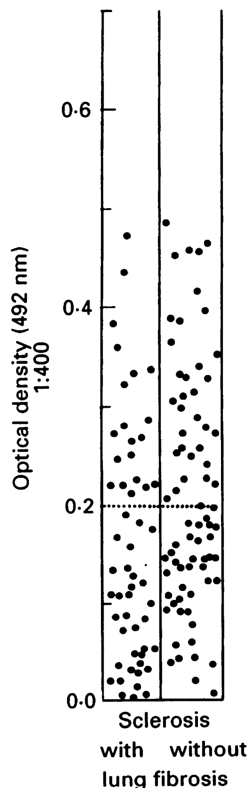

Fig. 4. Prevalence of autoantibodies to eukaryotic protein L7 detected by ELISA in sera of progressive systemic sclerosis (PSS) patients with and without lung fibrosis. The cut-off point is set as in Fig. 3.

We participated with the L7-ELISA in a German multicentre SLE study in order to correlate the presence of anti-L7 autoantibodies with more than 30 serological, clinical and genetic parameters of SLE. A negative correlation was found between elevated anti-L7 autoantibody titres and high IgG levels. This supports the notion that anti-L7 autoantibodies are not generated through antigen-independent, polyclonal B cell stimulation. They are rather the result of a specific immune response driven by antigen. In keeping with this is the finding that almost all anti-L7 autoantibodies belong to the IgG class.

A negative correlation was also found between the presence of anti-L7 autoantibodies and proteinuria. This argues against a kidney damaging potential of containing immune complexes.

We observed a weak negative correlation between vasculitis and the presence of anti-L7 autoantibodies, but as the level of significance was weak ($P = 0{\cdot}049$), we doubt whether this is meaningful.

A potentially positive correlation was found between the presence of anti-L7 autoantibodies and lung fibrosis ($P = 0{\cdot}042$). However, the multicentre SLE study contained only 20 patients suffering from lung fibrosis, and the statistical basis for the calculation of this correlation was therefore weak. In order to investigate further the potential correlation between the presence of anti-L7 autoantibodies and lung fibrosis with a larger number of sera, we screened anti-L7 titres in sera obtained from 129 patients suffering from PSS. Of them, 45 had lung fibrosis. The frequency of anti-L7-positive sera was high in both groups of PSS patients, 35% among the patients with lung fibrosis and 46% among the patients without lung involvement. This almost even distribution argues against a relationship of anti-L7 titres with lung fibrosis. A missing correlation of autoantibody titres with specific disease symptoms is not unusual. Anti-histone antibodies, for example, also do not correlate with any specific disease symptom in SLE [21]. Interestingly, we did not find a correlation of anti-L7 autoantibodies with the SLE-prone MHC haplotypes B7-DR2 and B8-DR3. Similar results were obtained for antibodies against the recombinant 60-kD Ro protein [22], suggesting that SLE patients carrying the MHC haplotypes B7-DR2 or B8-DR3 are not automatically predisposed to raise an autoimmune B cell response against 60-kD Ro and L7.

Most patients of the German multicentre SLE study were in an inactive or moderately active stage of the disease. Only 3% of them were in a severely active stage according to the slightly modified SLE disease activity index of Morrow *et al.* [15]. This and the limited resolution of the ELISA for high anti-L7 autoantibody titres makes it difficult to correlate anti-L7 autoantibody titres with active phases of the disease. In our previous study we have shown in 11 SLE patients that serum samples collected from the same patient over a period of 2–3 years contain high anti-L7 titres during the active phase of the disease and low titres during remission [1].

Epitope mapping should be useful for characterizing the anti-L7 autoantibody response. The implict understanding of doing so is, that this might be useful to discriminate between subsets of the disease with a particular prognosis [23]. We show in the accompanying study that autoantibodies to the protein L7 target three major epitopes (see this issue, p. 205). Future testing of larger numbers of patient sera will show whether the recognition of particular L7 epitopes by autoantibodies occurs irrespective of the clinical stage of the disease.

In conclusion, protein L7 is a major autoantigen in patients suffering from systemic autoimmune diseases such as SLE and PSS, but anti-L7 autoantibodies apparently do not define a distinct disease subset as yet. This impression may change when the response against distinct epitopes of protein L7 is analysed.

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