Clinical, immunological, and immunogenetic aspects of autoantibody production against Ro/SSA, La/SSB and their linear epitopes in primary Sjögren’s syndrome (pSS): a European multicentre study


Objectives: To investigate the clinical and immunogenetic aspects of antibody formation against Ro/SSA and La/SSB as well as their linear B cell epitopes in patients with primary Sjögren’s syndrome (pSS) from different European countries.

Patients and methods: Ninety patients with pSS from six European centres were studied. Serum samples from all patients were tested in a control laboratory for anti-Ro/SSA and anti-La/SSB autoantibodies by RNA precipitation assay and autoantibodies to the previously reported B cell linear epitopes of Ro 60 kDa (p169–190aa and p211–232aa) and La/SSB (p147–154aa, p291–302aa, p301–318aa, and p349–364aa). DNA from 88 patients was used for the determination of HLA-DRB1, -DQA1, and -DQB1 genotypes. Analysis of the results was performed in the 88 patients who were genotyped and tested also for antipeptide antibodies.

Results: Antibodies to B cell epitopes of Ro 60 kDa were detected at a low frequency (range 10–37%). In contrast, B cell epitopes of La/SSB were detected frequently (range 58–86%) among the anti-La/SSB positive sera. Autoantibodies to the La/SSB epitope p349–364aa, were significantly positively associated with longer disease duration (p<0.05), recurrent or permanent parotid gland enlargement (p<0.005), and a higher proportion of non-exocrine manifestations (p<0.005), compared with patients without autoantibodies. The presence of anti-Ro/SSA and anti-La/SSB autoantibodies was significantly associated with the presence of HLA-DRB1*03 and DQB1*02 (p=0.038 and p=0.034, respectively). This association was even more prominent and extended to HLA-DQA1*0501 when patients were stratified according to the presence of autoantibodies to discrete La/SSB B cell epitopes in comparison with autoantibody negative patients (p<0.01). They were found also to be highly associated with the alleles HLA-DQB1*02 and HLA-DQA1*0501 as well as the presence of a shared amino acid motif in the region 59–69aa of DQB1 first domain (p<0.01, respectively).

Conclusions: Autoantibodies against La/SSB, binding to four synthetic peptides, derived from the sequence of the La protein were identified with increased frequency in sera of patients with pSS. The formation of autoantibodies against B cell epitope analogues of La/SSB in European patients with pSS may be dependent on the presence of a permissive HLA-DQ heterodimer, most prominently represented by the HLA-DQA1*0501/DQB1*0201 heterodimer, suggesting that a model of HLA restricted presentation of La/SSB peptide determinants is crucial for the autoimmune response against La/SSB.
Several studies have reported the association between Ro/SSA and La/SSB responses with certain class II alleles, suggesting the major histocompatibility complex (MHC) dependent nature of the immune response to these autoantigens. In fact, HLA-DR2 and HLA-DR3 have frequently been reported to be associated with autoantibody production against Ro/SSA and La/SSB autoantigens. Most of these investigations were performed in patients who were followed up at a single centre.

Moreover, stronger associations have been found with HLA-DR2 and HLA-DR3 and the Ro/La antibody responses than with the disease itself. Others have emphasised the role of the HLA-DQ locus, particularly DQ1-DQ2 heterozygotes, in anti-Ro/SSA and anti-La/SSB production. Heterozygotes for HLA-DQw1/DQw2 have the highest level of anti-Ro/SSA and anti-La/SSB both in pSS and systemic lupus erythematosus (SLE). A correlation of anti-Ro/SSA and anti-La/SSB with DR3, DQA4, and DQA4/DQA1 heterozygotes in white subjects has also been reported. Specific amino acid residues in the second hypervariable region of HLA-DQA1 and DQB1 chain genes, glutamine in position 34 of DQA1, and leucine in position 26 of DQB1, promote the anti-Ro/SSA and anti-La/SSB responses. A shared amino acid motif in the DQB1 first domain has been found in a disease associated haplotype in different ethnic groups (Caucasoid, Chinese, and Japanese patients with pSS).

Our study aimed at investigating the prevalence of antibodies to B cell epitopes of Ro/SSA and La/SSB in a multicentre study of patients with pSS and elucidating the association of the specificity of thyroid and/or antibodies to thyroid peroxidase and thyroglobulin, lymphadenopathy (presence of lymph nodes of more than 1 cm in diameter) (n=6), liver disease (n=5) as manifested by the raised levels of aspartate aminotransferase, alanine aminotransferase, and confirmed by liver biopsy, kidney disease (glomerulonephritis (n=3) as defined by an active urinary sediment with red blood cell count and proteinuria, interstitial kidney disease (n=2) as defined by a low specific gravity in the urine, alkaline pH, and positive morning urine acidification tests. In all cases the diagnosis was confirmed by renal biopsy. Myositis (n=3) was defined by raised creatine kinase levels and electromyographic studies and/or muscle biopsy, thrombocytopenia (n=2) (platelet count <100x10^9 cells/l), leucopenia (n=2) (leucocytes <3.5x10^9 cells/l) and, finally, central nervous system involvement defined by the clinical picture and brain magnetic resonance imaging (n=2).

Detection of antibodies to B cell linear epitopes of Ro/SSA and La/SSB

The six different B cell epitope synthetic analogues were prepared in soluble forms as previously described. The Ro 60 kDa epitopes were T"KYKQRNGWSHKDLRLSHKP" (169–190aa) and E"12LYKEKALSETVEKLKLYE" (211–232aa). The four La/SSB related epitopes were H"KAFKG" (147–154aa), N"GNLQRNLKEV" (291–302aa), V"TWVLEGVEKEALKK" (301–318aa), and G"SGGKVQFGKKTFT" (349–364aa). Previous experiments showed that these peptides either alone or connected to sequential oligopeptide carriers can be effective as antibody substrates for the development of enzyme linked immunosorbent assays (ELISAs) for autoantibody detection.

Ninety six well polystyrene plates (Nunc, Denmark) were coated with 5 µg peptide/ml in phosphate buffered saline (PBS) and incubated overnight at 4°C. Wells were then washed three times with PBS-Tween 20 0.1%. Afterwards they were blocked with 100 µl bovine serum albumin (BSA) 10% in Tris (50 mM/0.9% NaCl, pH 7.4) per well and incubated overnight at 4°C. The plates were then thoroughly washed (four times) and 50 µl/well of serum samples were added in a dilution of 1/200. The serum samples were diluted in PBS/BSA 2%/Twene 20 0.01% and were shaken while incubated for 30 minutes at room temperature (24°C). Four more washes followed and then 50 µl of conjugated second antibody (goat antihuman alkaline phosphatase; Sigma Chemicals Co, Saint Louis) was used as a substrate for alkaline phosphatase; Sigma Chemicals Co, Saint Louis was added for a 30 minute incubation at room temperature. Finally, the plates were washed five times and the wells were incubated with 50 µl/well substrate for 30 minutes at 37°C. p-Nitrophenyl phosphate disodium (Sigma Chemicals Co) was used as a substrate for alkaline phosphatase and the optical density was then measured at 410 nm (Dynatech, London, UK). The cut off point for positivity was set at the mean of OD + 3 standard deviations of 10 normal sera, all taken from the same centre. The specificity of the antipeptide assays was evaluated by inhibition experiments (data not shown).

RNA precipitation assay

RNA precipitation assays were performed on HeLa whole cell extracts as previously described. RNA were extracted with phenol from immune complexes, then analysed on a denaturing RNA gel and transferred onto a nylon membrane (northern blotting). Sequential hybridisation was achieved by the use of antisense Ro-15 and Ro-13 RNA probes transcribed from constructs kindly provided by Dr WJ van Venrooj, in order to identify Ro-RNPs, and then by an oligonucleotide probe complementary to 55 RNA for discrimination between anti-Ro and anti-La RNP autoantibody specificity in the same serum.
**RESULTS**

Prevalence and clinical significance of antibodies to B cell epitopes of Ro/SSA and La/SSB

All sera positive for anti-Ro/SSA and anti-La/SSB (group A), were also found to be positive for at least one B cell epitope of La/SSB (fig 1). Table 1 shows the prevalence of antibodies to B cell epitopes in each group of patients. Within group A, the most frequently detected antibodies were against the epitope 349–364aa of La/SSB (86%) (group D). Antibodies against Ro/SSA B cell epitopes were detected in a small number of sera. More specifically, antibodies to epitope 169–190aa were detected in 37% of the patients with anti-Ro/SSA antibody alone and in 28% of sera with anti-Ro/SSA and anti-La/SSB antibodies.

Patients with anti-Ro/SSA and anti-La/SSB antibodies had longer disease duration than patients without autoantibodies. Furthermore, patients with antibodies to B cell epitope 349–364aa of La/SSB had significantly longer disease duration than patients without autoantibodies (8.6 (5.6) years v. 6.71 (4.9) years, p<0.05).

Patients positive for anti-Ro/SSA and anti-La/SSB antibodies (group A) presented with recurrent or permanent parotid gland enlargement more often than patients without autoantibodies (group C), (p=0.005). The same was also true for patients positive for antibodies to B cell epitope 349–364aa (group D) (p=0.002) (fig 2A). These patients had also a higher prevalence of non-exocrine manifestations than patients without autoantibodies (p<0.0001 and p=0.0003, for groups A and D respectively) (fig 2B). There was no association between non-exocrine manifestations and the titre of antibodies to p349–364aa.

**Table 1** Prevalence (%) of autoantibodies to Ro/SSA and La/SSB linear epitopes in different groups of patients with pSS and normal controls

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<tr>
<td>αRo/SSA, La/SSB positive (group A, n=42)</td>
<td>67</td>
<td>63</td>
<td>58</td>
<td>86</td>
<td>10</td>
<td>28</td>
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<tr>
<td>αRo/SSA positive (group B, n=19)</td>
<td>0</td>
<td>0</td>
<td>26</td>
<td>5</td>
<td>16</td>
<td>37</td>
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<td>Without αRo/SSA, La/SSB (group C, n=27)</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>7</td>
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<td>Normal controls (n=10)</td>
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No correlation was found between the presence of antibodies to B cell epitopes and the tests which assess the exocrine function, where they were available (Schirmer’s test, rose bengal staining of the cornea, and unstimulated parotid saliva).

**Association of HLA class II markers with antibody formation against B cell epitopes of La/SSB**

Forty nine (56%) of 88 patients with pSS were HLA-DRB1*03 positive. However, DRB1*03 was more frequently detected in patients with anti-Ro/SSA and anti-La/SSB reactivity than in patients without autoantibodies (groups A vs C, p=0.038). These patients also carried the DQB1 allele DQB1*02 (p=0.034) more frequently than patients lacking autoantibodies. No significant difference was observed for DQA1*0501 allele (p=0.01). There was no association between the Ro60 kDa linear epitopes and any HLA marker.

When the analysis included comparisons of groups D, E, F, G (autoantibodies directed against the La/SSB B cell epitopes 349–364aa, 301–318aa, 291–302aa, and 147–154aa, respectively) with group C (patients without autoantibodies), statistically significant associations emerged (table 2). In fact, group D was positively associated with HLA-DRB1*03 (p=0.043), group E was highly positively associated with HLA-DRB1*03 (p=0.0009), DQB1*0201 (p=0.0033), and DQA1*0501 (p=0.0021). Patients with antibodies to a B cell epitope 291–302 (group F) more often carried HLA-DRB1*03 (p=0.011) as well as the alleles DQB1*0201 and DQA1*0501 (p=0.0088 and p=0.016), respectively.

Finally, DRB1*03 (p=0.014) and DQA1*0501 (p=0.015) occurred more frequently in group G than in patients without autoantibodies. When HLA-DQA1*0501 and DQB1*0201, implicating the DRB1*03-linked DQ2 heterodimer, were taken together, a more pronounced difference between groups E and G compared with patients without autoantibodies was seen. In fact, patients with antibodies to the p301–318aa epitope of La/SSB showed a stronger association with this allele combination (p=0.0009) than with each individual allele (fig 3). The same was also true for the groups of patients with antibodies to the B cell epitope 147–154aa (p=0.014).

Among patients with anti-Ro/SSA and anti-La/SSB autoantibodies (group A), a higher proportion of DQB1*02/DQB1*02 (DQB1*06/DQB1*02) heterozygosity was seen than among patients without autoantibodies (p=0.046). This heterogeneity became more prominent when the individual groups of
patients with antibodies to synthetic B cell epitope analogues were analysed (group D: p = 0.043, group E: p = 0.064, group F: p = 0.028, group G: p = 0.0075).

Next, the different groups of patients were compared for the presence or absence of the DQB1-D1 motif aa59–69 EYWNSQK-DILE, which in a previous study was shown to be associated with anti-Ro/SSA and anti-La/SSB response. Patients with anti-Ro/SSA and anti-La/SSB autoantibodies (group A) or patients with anti-Ro/SSA autoantibodies alone (group B) did not show a significant increase of the DQB1-D1 motif over patients lacking autoantibodies (p = 0.064 and p = 0.35, respectively). Interestingly, however, this shared DQB1 sequence motif was found at significantly higher frequency in patients with antibodies to p301–318aa (group E) and p291–302aa (group F) (p = 0.0026 and p = 0.019, respectively).

The analysis of the contribution of individual DQA1 and DQB1 amino acid positions showed that DQB1-26L was found in 78/88 (89%) patients, and DQβ-34Q was seen in 81/88 (92%) patients. Patients with antibodies to 291–302aa (group E) and p291–302aa (group F) (p = 0.0026 and p = 0.019, respectively).

Two copies of DQα-26L-positive DQA1 alleles were observed at a higher frequency in patients with anti-Ro/SSA autoantibodies (group B, 14/19 (74%), p = 0.024), and antibodies to 349–364aa (group D, 24/38 (63%), p = 0.029), to 301–318aa (group E, 22/33 (67%), p = 0.035), to p291–302aa (group F, 20/28 (71%), p = 0.028), and to p147–154aa (group G, 22/29 (76%), p = 0.006) compared with patients without autoantibodies (group C, 11/24 (46%).)

The number of alleles containing either DQB1-26L, or DQβ-34Q, or both, was compared in each group of patients with the control group C. More patients presented higher number of alleles in groups D (three or four alleles in 29/38 (76%) patients, p = 0.038), F (23/28 (82%), p = 0.018), and G (23/29 (79%), p = 0.015), but not in groups A, B, and E (31/42 (74%), p = 0.052; 12/19 (63%), p = 0.31; and 23/33 (70%) p = 0.13, respectively), compared with the control group C 13/24 (54%) (fig 4), suggesting a gene dosage effect for the production of these autoantibodies.

**DISCUSSION**

In this multicentre European study we have evaluated clinical and immunogenetic aspects of antibody formation directed towards the B cell epitopes of Ro/SSA and La/SSB intracellular autoantigens. All sera were evaluated for anti-Ro/SSA and anti-La/SSB antibodies by RNA precipitation assay, because this method presents high sensitivity and specificity. The frequent detection of anti-La/SSB antibodies using synthetic analogues as substrates in serum samples from patients with pSS obtained from six different European countries confirms a previous study in Greek patients with pSS. In contrast, antibodies to Ro 60 kDa linear epitopes were only infrequently detected, suggesting that the main body of anti-Ro/SSA autoantibodies is directed towards conformational epitopes or other still unidentified linear determinants. Comparison of autoantibodies to Ro/SSA and La/SSB with the clinical picture of the patients showed that their presence is higher in patients with longer disease duration and systemic non-erythematous. These results are in accordance with previously reported findings. Interestingly, this correlation was also evident for autoantibodies directed against the B cell epitope 349–364aa, which in both this and in previous studies appears to be the major La/SSB epitope. Thus antibodies to the synthetic B cell epitope analogue of the 349–364aa fragment of La/SSB may serve as a diagnostic tool in patients with pSS.

In agreement with previous reports, the analysis of HLA class II markers indicated that alleles of the DR3-DQ2 haplotype (DRB1*03-DQA1*0501-DQB1*0201) were present in higher frequency in anti-Ro/SSA and anti-La/SSB autoantibody positive patients than in autoantibody negative patients. In addition, several investigators have analysed the associations of specific HLA class II alleles with the fine specificity of these autoantibodies. Thus Rischmueller et al studying Australian patients with pSS, reported that the HLA-DR3-DQA1*0501-DQB1*020 haplotype was primarily associated with a diversified anti-Ro/SSA and anti-La/SSB response containing precipitating antibodies to La/SSB, while the haplotype HLA-DR2-DQA1*0102-DQB1*0602 was associated with a more restricted antibody response, containing non-precipitating anti-La/SSB antibodies. Scefield and coworkers, reported that antibodies binding the 13 kDa fragment of Ro 60 kDa autoantigens are more likely to be found in sera of patients with particular DQA1 and DQB1 alleles. Finally, Buyon and associates showed that 81% of sera from patients with either SLE or pSS who had children with neonatal lupus erythematosus, reacting with an NH2-terminal epitope of Ro 52 kDa were associated with the combination HLA-DRB1*0201, DQA1*0501, DQB1*0201, as compared with 30% of sera that recognised the central epitope.

In our study, particular HLA class II alleles were positively associated with autoantibodies to short peptide B cell epitopes of La/SSB in patients with pSS. This was even more pronounced when the association of alleles, predisposing to anti-La/SSB autoimmune response (HLA-DQA1*0501 and DQB1*0201—that is, DQ2) with antibodies to the epitopes N\(^\text{301}\)GKLRLKNEV\(^\text{131}\) (291–302aa), V\(^\text{318}\)TWELVEGEVEK\(^\text{414}\) (301–318aa), and H\(^\text{414}\)KAFKGS\(^\text{528}\) (147–154aa) were considered. Moreover, the presence of the DQ-D1 motif aa59–69 EYWNSQKDILE, located on the \(\alpha\) helix of the antigen binding groove, was found to be highly positively associated with an antibody response to the synthetic epitope analogue 291–302aa and 301–318aa.

When DQB1-26L and DQβ-34Q, located on the floor of the antigen binding cleft of the HLA-DQβ heterodimer and which contributes to anti-Ro/SSA and/or anti-La/SSB autoantibody formation, were considered, the strongest associations in comparison with autoantibody negative patients were seen for the group of patients presenting antibodies to p291–302aa of La/SSB. Furthermore, associations with these specific amino acids of the DQ heterodimer were subject to gene dosage effects.

Taken all together, our results indicate that immunogenetic markers relevant for autoantibody formation against La/SSB are more strongly associated with the presence of antibodies directed against synthetic epitope analogues, corresponding to the region 291–302 and 301–318 of La/SSB, compared with
the associations seen with anti-La/SSB as such. Our findings support the hypothesis that HLA class II molecules may be directly involved in the presentation of discrete epitopes of La/SSB and thus contribute to antibody formation against the La/SSB autoantigen. Previous studies in mice and rabbits, in conjunction with MHC class II binding prediction algorithms, have suggested that these neighbouring sequences of La/SSB possess also features of a T cell epitope. In this regard, the heterodimer DQA1*0501/DQB1*0201 may exhibit preferential binding ability for peptides derived from the aforementioned sequences of La/SSB. However, it should be kept in mind that owing to the strong linkage disequilibrium, a contribution from the HLA-DR locus cannot be excluded. Because the association analyses of individual amino acids and a pSS associated sequence motif of the DQ heterodimer indicated that structural requirements for anti-La/SSB antibody formation may also be met by other heterodimers and gene dosage may have a role, a direct impact HLA-DQ could be envisioned.

In our study no association between anti-Ro/SSA antibodies alone or its epitopes with HLA-DQA1 or HLA-DQB1 alleles was noted. This finding is in agreement with a previous investigation demonstrating that the development of anti-Ro/SSA and anti-La/SSB responses is probably influenced by different HLA class II molecules.

In conclusion, autoantibodies to La/SSB were detected by the use of synthetic peptide analogues corresponding to B cell epitopes of La/SSB, using serum from 90 patients with pSS from different countries in Europe. Autoantibodies against the B cell epitope of La/SSB 349–364aa are the most prevalent and they possess clinical correlations similar to those seen with anti-La/SSB autoantibodies. Thus antibodies against this epitope may be used as a tool for clinical practice. In addition, the formation of autoantibodies against B cell epitope analogues of La/SSB in European patients with pSS may depend on the presence of a permissible HLA-DQ heterodimer, most prominently represented by the HLA-DQA1*0501/DQB1*0201 heterodimer, suggesting a model of HLA restricted presentation of La/SSB peptide determinants to autoreactive T helper cells.

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