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EXTENDED REPORT

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

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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 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.

Autoantibodies to the ribonucleoprotein particles Ro/SSA and La/SSB are usually found in sera of patients with primary Sjögren's syndrome (pSS).[1] Their presence is associated with a longer disease duration, increased frequency of non-exocrine manifestation, and a higher intensity of lymphocytic infiltrates invading the minor salivary glands.[2] Previous studies suggest that this autoimmune response is antigen driven because the autoantibodies are produced in the immunopathological lesion,[3–5] class switched involving species-specific epitopes,[6] subject to intra- and intermolecular epitope spreading.[7]

Several laboratories have attempted to define the precise targets of the autoantibodies to Ro/SSA and La/SSB,[8 9] because the knowledge of B cell epitopes can offer new insights into the mechanisms responsible for autoantibody production and better antigenic substrates for their detection. Several methods showed that serum samples of patients with anti-Ro/SSA and anti-La/SSB reactivity recognise multiple different conformational and linear epitopes. Among them, the sequences 145–164aa, 289–308aa, 301–320aa, and 349–364aa of La/SSB as well as 169–190aa and 211–232aa of Ro 60 kDa, have been extensively studied, exhibiting high sensitivity and specificity for autoantibody detection.[10–13]

Early serological studies have shown positive associations of pSS with HLA-DR3, DR2, DR5, and DRw53.[14 15] Increased frequency of the haplotype HLA-DRB1*0301-DRB3*0101-DQA1*0501-DQB1*0201 has been found in Caucasoid patients with pSS, DRB1*0405-DRB4*0101-DQA1*0301-DQB1*0401 in Japanese patients, and DRB1*0803-DQA1*0103-DQB1*0601 in Chinese patients.[16 17] More recently, it has been suggested that HLA-DRB1*0301-*1501 heterozygosity may have a role in susceptibility to pSS.[18]

Abbreviations: BSA, bovine serum albumin; MHC, major histocompatibility complex; OR, odds ratio; PBS, phosphate buffered saline; pSS, primary Sjögren' syndrome; SLE, systemic lupus erythematosus

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.[19] In fact, HLA-DR2 and HLA-DR3 have frequently been reported to be associated with autoantibody production against Ro/SSA and La/SSB autoantigens.[19–21]

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.[22] Others have emphasised the role of the HLA-DQ locus, particularly DQ1-DQ2 heterozygotes, in anti-Ro/SSA and anti-La/SSB production.[21 23 24] 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).[19 21 25] A correlation of anti-Ro/SSA and anti-La/SSB with DR3, DQA4, and DQA4/DQA1 heterozygotes in white subjects has also been reported.[26] 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.[23 26] 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).[16]

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 HLA class II markers with autoantibody formation against particular Ro/SSA and La/SSB epitopes in these patients.

PATIENTS AND METHODS

Patients

Ninety patients with primary Sjögren's syndrome from six different European centres (Vienna, Austria; Erlangen, Germany; Bergen, Norway; Pisa, Italy; Athens, Greece; and London, UK) were enrolled in this study. Fifteen patients were studied from each centre. All patients fulfilled the European classification criteria for the diagnosis of primary Sjögren's syndrome.[27] The patients were selected on the basis of anti-Ro/SSA and anti-La/SSB positivity and each centre agreed to submit five cases considered to have both anti-Ro/SSA and anti-La/SSB antibodies, five cases with anti-Ro/SSA alone, and five cases with neither anti-Ro/SSA nor anti-La/SSB autoantibodies.

For each patient, biographical data, clinical and laboratory information were extracted from a Sjögren's syndrome data file common to all centres participating in the study.[28] Two millilitres of serum from each patient was submitted for antipeptide antibody detection and evaluation of anti-Ro/SSA and anti-La/SSB by RNA precipitation assay. Simultaneously, extracted DNA or purified peripheral mononuclear blood cells were provided for HLA genotyping to determine HLA-DRB1, DQA1, and DQB1 genotypes.

Biographical data and clinical presentation of the patients

Eighty eight patients were white, one was Asian, and one was black. Eighty nine patients were female and one was male, mean (SD) age 55.1 (14.3) years, and with mean (SD) disease duration 7.2 (4.8) years. Twenty five patients had disease limited to the exocrine glands while 65 patients had one or more of the following non-exocrine manifestations: arthritis (n=30), Raynaud's phenomenon as defined by tricolour change in the hands, after exposure to cold (n=27), palpable purpura (n=14), lung disease (n=10) as defined by abnormal chest *x* ray examination and/or impaired pulmonary function tests, peripheral nerve involvement (n=9) defined by nerve conduction studies, clinical or subclinical thyroid disease (n=9) defined by the measurement of thyroid hormones 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 urine 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 $\leqslant 100\times10^9$ cells/l), leucopenia (n=2) (leucocytes $\leqslant 3.5\times10^9$ 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.[10 11] The Ro 60 kDa epitopes were T^{169}KYKQRNGWSHKDLLRSHLKP^{190} (169–190aa) and E^{211}LYKEKALSVETEKLLKYLEAV^{232} (211–232aa). The four La/SSB related epitopes were H^{147}KAFKGSI^{154} (147–154aa), N^{291}GNLQLRNKEVT^{302} (291–302aa), V^{301}TWEVLEGE VEKEALKKI^{318} (301–318aa), and G^{349}SGKGKVQFQGKKTKF^{364} (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.[12 29]

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-Twin 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%/Tween 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 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.[30] RNAs 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-Y5 and Ro-Y3 RNA probes transcribed from constructs kindly provided by Dr WJ van Venrooij, in order to identify Ro-RNPs, and then by an oligonucleotide probe complementary to 5S RNA for discrimination between anti-Ro and anti-La RNP autoantibody specificity in the same serum.

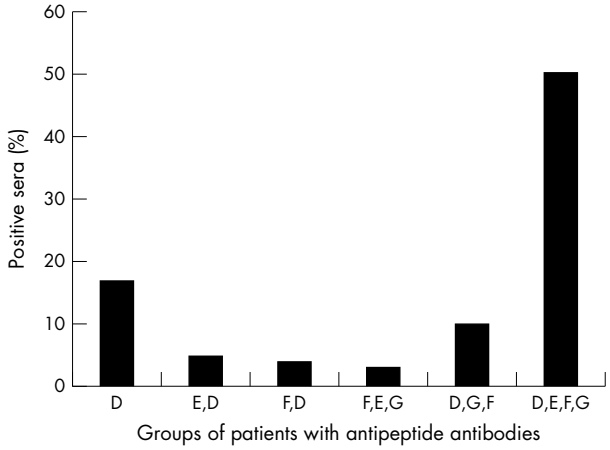

Figure 1 Distribution of the various groups of antibodies to La/SSB epitopes in patients positive for anti-Ro/SSA and anti-La/SSB antibodies (n=42). Group D: antibodies to p349–364aa; group E: antibodies to p301–318aa; group F: antibodies to p291–302aa; group G: antibodies to p147–154aa.

HLA genotyping

HLA genotyping was performed successfully in 88 patients by oligonucleotide hybridisation of enzymatically amplified DNA. Low resolution HLA-DRB1 typing comprised the DRB1*01 to DRB1*17 specificities by sequence-specific hybridisation of a panel of oligonucleotide probes to polymerase chain reaction products as published previously.[31] Similarly, DQB1 and DQA1 alleles were determined following the XI.IHWC protocol.[32] When this approach is used, most of the DQA1 (DQA1*0101/04, *0102, *0103, *0201, *03, *0401, *0501,*0503, *0601) and DQB1 (DQB1*0501,-*0504, *0601-*0604, *0605/06, *02, 0301-*0304) alleles can be differentiated. To assess the importance of individual amino acid positions, DQA1 position 34 (DQα-34Q (DQA1*0102, *0103, *0401, *0501-*0502, *0601) *v* DQα-34E (DQA1*0101, *0104, *0201, *0301, *0302)), and DQB1 position 26 (DQβ-26L (DQB1*0602-*0606, *02, 0302, *0303) *v* DQβ-26G/26Y (DQB1*05, *0601, 0301, *0304)), and the DQβ motif aa59–69 (EYWNSQKDILE), termed DQβ-DI, seen in DQB1*0601, *02, *04, were determined based on the sequence information and the genotyping results.

Statistical analysis

Statistical analysis was performed for the 88 patients who were genotyped. The patients were grouped into three major groups:

Group A: Patients (n=42) positive for anti-Ro/SSA and anti-La/SSB, as demonstrated by RNA precipitation assays.

Group B: Patients (n=19) positive for anti-Ro/SSA but negative for anti-La/SSB.

Group C′: Patients (n=27) negative for anti-Ro/SSA and anti-La/SSB.

Based on the specific antipeptide antibody assay, patients from the above groups were included in one or more of the following groups:

Group D: Patients (n=38) positive for La/SSB epitope 349–364aa.

Group E: Patients (n=33) positive for La/SSB epitope 301–318aa.

Group F: Patients (n=28) positive for La/SSB epitope 291–302aa.

Group G: Patients (n=29) positive for La/SSB epitope 147–154aa.

Group C: Patients (n=24) negative for anti-Ro/SSA and anti-La/SSB and B cell epitopes. Three of the 27 patients in control group C′ presented one or more antipeptide antibodies.

The biographical data, including age and sex, did not differ significantly between the groups of patients.

The association between particular HLA genotypes and autoantibody defined subsets of patients as well as clinical correlations were investigated by Fisher's exact test, Mantel-Haenszel χ^2, or Wilcoxon rank sum test where appropriate. Two sided p values and Bonferroni α levels are reported. Odds ratios (ORs) were calculated according to Woolf's method as cross product ratios of a 2×2 contingency table (OR=(a×d)/(c×b)).[33 34] Haldane's correction for the OR was used when either all patients were positive or all controls were negative for a particular specificity or allele.[35] The SAS statistical package was used for analysis.[36]

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

Patients	La p147–154 group G, (n=29)	La p291–302 group F, (n=28)	La p301–318 group E, (n=33)	La p349–364 group D, (n=38)	Ro p211–232 (n=5)	Ro p169–190 (n=24)
aRo/SSA, La/SSB positive (group A, n=42)	67	63	58	86	10	28
aRo/SSA positive (group B, n=19)	0	0	26	5	16	37
Without aRo/SSA, La/SSB (group C, n=27)	0	0	3	4	0	7
Normal controls (n=10)	0	0	0	0	0	0

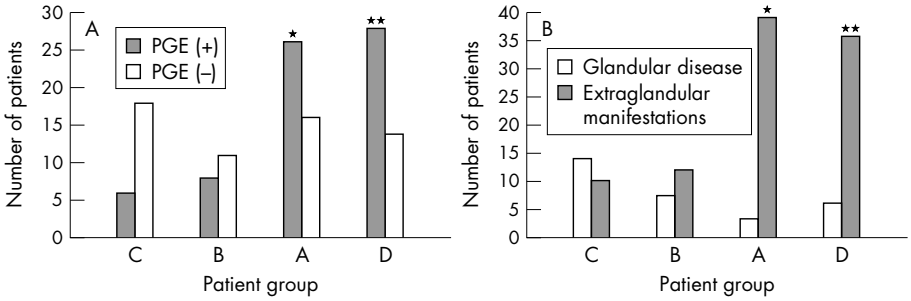

Figure 2 (A) Prevalence of parotid gland enlargement (PGE) in patients with pSS without autoantibodies (group C), with antibodies to Ro/SSA (group B), with antibodies to Ro/SSA and La/SSB (group A) and antibodies to the B cell epitope of La/SSB 349–364aa (group D) *p=0.005; **p=0.002. (B) Prevalence of non-exocrine manifestations in the same groups of patients *p<0.0001; **p=0.003.

Table 2 Comparison of the presence of several HLA alleles in five groups of patients with pSS and autoantibodies to La/SSB B cell epitopes and in patients with pSS without autoantibodies (group C)

HLA marker	Anti-Ro/SSA Anti-La/SSB (group A)	p349–364 (group D)	p301–318 (group E)	p291–302 (group F)	p147–154 (group G)
DRB1*03					
p Value	0.038*	0.043*	0.0009**	0.011*	0.014*
Odds ratio	3.33	3.00	7.50	5.00	4.4
95% CI	1.17 to 9.49	1.06 to 8.49	2.24 to 25.17	1.52 to 16.43	1.37 to 13.96
DQA1*0501					
p Value	0.099	0.099	0.0021*	0.016*	0.015*
Odds ratio	2.71	2.71	8.46	5.08	5.3
95% CI	0.93 to 7.91	0.93 to 7.91	2.02 to 35.46	1.35 to 19.17	1.40 to 19.92
DQB1*0201					
p Value	0.034*	0.064	0.0033*	0.0088*	0.045*
Odds ratio	3.33	2.96	6.62	5.44	3.71
95% CI	1.16 to 9.59	1.04 to 8.40	1.91 to 22.99	1.55 to 19.11	1.15 to 11.96

Note: Fisher's exact p value, odds ratio, and corresponding 95% confidence intervals (CI) are reported. Bonferroni α level=0.0017.
*Significant at p=0.05; **significant after Bonferroni adjustment.

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 *v* 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.

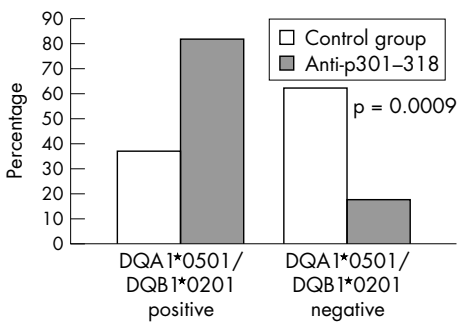

Figure 3. Prevalence of antibodies to the B cell epitope of La/SSB 301–318aa in patients with pSS and positive heterodimer HLA-DQA1*0501/DQB1*0201 compared with patients with pSS and negative heterodimer.

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*01/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

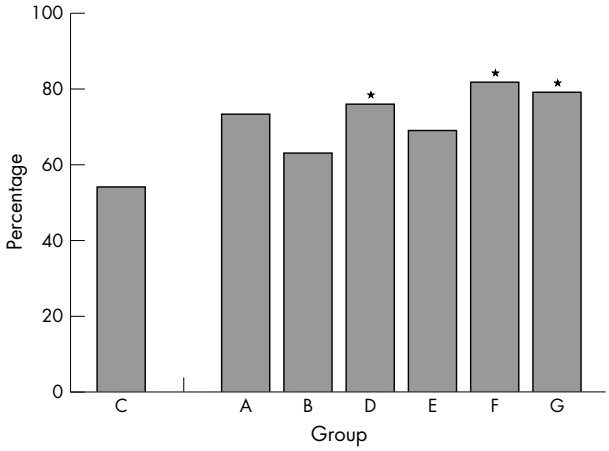

Figure 4 Prevalence of three or four alleles of DQβ-26L and DQA-34Q in the different groups of patients with pSS with autoantibodies to B cell epitopes of La/SSB (groups A, B, D-G) and without autoantibodies (group C). *Significantly different from the control group C at α=0.05.

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 DQβ-DI 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 DQβ-DI 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 DQβ-26L was found in 78/88 (89%) patients, and DQα-34Q was seen in 81/88 (92%) patients. Patients with antibodies to 291–302aa (group F) and 349–364aa (group D) presented in higher frequency a DQβ-26L positive DQB1 allele together with a DQα-34Q positive DQA1 allele (p=0.018 and p=0.038, respectively).

Two copies of DQα-34Q-positive DQA1 alleles were observed at a higher frequency in patients with anti-Ro/SSA antibodies (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 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 DQβ-26L or DQA-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 SS obtained from six different European countries confirms a previous study in Greek patients with pSS.[12] In contrast, antibodies to Ro 60 kDa linear epitopes were only infrequently detected, suggesting that the main body of anti-Ro/SSA antibodies 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-exocrine disease. These results are in accordance with previously reported findings.[2] 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*02) were present in higher frequency in anti-Ro/SSA and anti-La/SSB autoantibody positive patients than in autoantibody negative patients.[17] 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*02 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 autoantibody response, containing non-precipitating anti-La/SSB antibodies.[37] Scofield 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.[38] 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 NH_2-terminal epitope of Ro52 kDa were associated with the combination HLA-DRB1*0201, DQA1*0501, DQB1*0201, as compared with 30% of sera that recognised the central epitope.[39]

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^{291}GNLQLRNKEVT^{302} (291–302aa), V^{301}TWELVEGEVEKEAL KKI^{318} (301–318aa), and H^{147}KAFKGSI^{154} (147–154aa) were considered. Moreover, the presence of the DQβ-DI motif aa59–69 EYWNSQKDILE, located on the α 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 DQβ-26L and DQα-34Q, located on the floor of the antigen binding cleft of the HLA-DQαβ 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[40] and rabbits,[41] in conjunction with MHC class II binding prediction algorithms,[42–45] 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 (HLA-DRB1*03) on the antibody formation 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.[46]

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 permissive 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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REFERENCES

1 **Tzioufas AG**, Moutsopoulos HM. Clinical significance of autoantibodies to Ro/SSA and La/SSB. In: Venrooij WJ, Maini RN, eds. *Autoantigens and clinical significance of autoantibodies*. Vol II. The Netherlands: Kluwer Academic 1996:1.1–14.
2 **Manoussakis MN**, Tzioufas AG, Pange PJE, Moutsopoulos HM. Serologic profiles in subgroups of patients with Sjogren's syndrome. Scand J Rheumatol 1986;61:89–92.
3 **Horsfall AC**, Rose LM, Maini RN. Autoantibody synthesis in salivary glands of Sjogren's syndrome patients. J Autoimmun 1989;2:554–68.
4 **Tenger P**, Halse AK, Haga HJ, Jonsson R, Wahren-Herlemius M. Detection of anti Ro/SSA and anti La/SSB autoantibody producing cells in salivary glands from patients with Sjögren's syndrome. Arthritis Rheum 1998;41:2238–48.
5 **Tzioufas AG**, Hantoumi I, Polihronis M, Xanthou G, Moutsopoulos HM. Autoantibodies to La/SSB in patients with primary Sjögren's syndrome (pSS) are associated with upregulation of La/SSB mRNA in minor salivary gland biopsies (MSGs). J Autoimmun 1999;13:429–34.
6 **Manoussakis MN**, Kistis CG, Aidinis V, Guialis A, Piha L, Sekeris CE, *et al*. Detection of human-specific anti-La/SSB antibodies in patients with rheumatoid arthritis. J Autoimmun 1995;8:959–69.
7 **Topfer F**, Gordon T, McCluskey J. Intra and intermolecular spreading of autoimmunity involving the molecular self antigen La(SSB) and Ro(SSA). Proc Natl Acad Sci USA 1995;92:875–9.
8 **Wahren-Herlenius M**, Muller S, Isenberg D. Analysis of B cell epitopes of the Ro/SSA autoantigen. Immunol Today 1999;20:234–40.
9 **Scofield RH**, Farris AD, Horsfall AC, Harley JB. Fine specificity of the autoimmune response to the Ro/SSA and La/SSB ribunocleoprotein. Arthritis Rheum 1999;42:199–204.
10 **Routsias JG**, Tzioufas AG, Sakarellos-Daitsiotis M, Sakarellos C, Moutsopoulos HM. Epitope mapping of the Ro/SSA 60kd autoantigen reveals disease specific antibody-binding profiles. Eur J Clin Invest 1996;26:514–21.
11 **Tzioufas AG**, Yiannaki E, Sakarellos-Daitsiotis M, Routsias JG, Sakarellos C, Moutsopoulos HM. Fine specificity of autoantibodies to La/SSB epitope mapping and characterization. Clin Exp Immunol 1997;108:191–8.
12 **Yiannaki E**, Tzioufas AG, Bachmann M, Hantoumi J, Tsikaris V, Sakarellos-Daitsiotis M, *et al*. The value of synthetic linear epitope analogues of La/SSB; specificity, sensitivity and comparison of methods. Clin Exp Immunol 1998;112:152–8.
13 **Haaheim LR**, Halse A-K, Kvakestad R, Stern B, Normann O, Jonsson R. Serum antibodies from patients with primary Sjögren's syndrome and systemic lupus erythematosus recognize multiple epitopes on the La/SSB autoantigen resembling viral protein sequences. Scand J Immunol 1996;43:115–21.
14 **Chused TM**, Kassan SS, Opelz G, Moutsopoulos HM, Terasaki PI. Sjögren's syndrome, associated with HLA-DW3. N Engl J Med 1997;296:895–7.
15 **Mann DL**, Moutsopoulos HM. HLA DR alloantigens in different subsets of patients with Sjögren's syndrome and in family members. Ann Rheum Dis 1983;42:533–6.
16 **Kang HI**, Fei HM, Saito I, Sawada S, Chen SL, Yi D, *et al*. Comparison of HLA class II genes in Caucasoid, Chinese, and Japanese patients with primary Sjögren's syndrome. J Immunol 1993;153:3615–23.
17 **Tambur A**, Friedmann A, Safirman C, Markitziou A, Ben-Chetrit E, Rubinow A, *et al*. Molecular analysis of HLA class II genes in primary Sjögren's syndrome. A study of Israeli Jewish and Greek non-Jewish patients. Hum Immunol 1993;36:235–42.
18 **Guggenbuhl P**, Jean S, Jego P, Grosbois B, Chales G, Semana G, *et al*. Primary Sjögren's syndrome: role of the HLA-DRB1*0301*1501 heterozygotes. J Rheumatol 1998;25:900–5.
19 **Hamilton RH**, Harley JB, Bias WB, Roebber M, Reichlin M, Hochbera MC, *et al*. Two Ro (SSA) autoantibody responses in systemic lupus erythematosus. Correlation of HLA-DR/DQ specificities with quantitative expression of Ro (SSA) autoantibody. Arthritis Rheum 1988;31:496–505 .
20 **Harley JB**, Alexander EL, Bias WB, Fox OF, Provost TT, Reichlin M, *et al*. Anti-Ro (SS-A) and anti-La (SS-B) in patients with Sjögren's syndrome. Arthritis Rheum 1986;29:196–205.
21 **Harley JB**, Reichlin M, Arnett FC, Alexander EL, Bias WB, Provost TT. Gene interaction at HLA-DQ enhances autoantibody production in primary Sjögren's syndrome. Science 1986;232:1145–8.
22 **Wilson RW**, Provost TT, Bias WB, Alexander EL, Edlow DW, Hochberg MC, *et al*. Sjögren's syndrome. Influence of multiple HLA-D region alloantigens on clinical and serologic expression. Arthritis Rheum 1984;27:1245–53.
23 **Reveille JD**, Macleod MJ, Whittington K, Arnett FC. Specific amino acid residues in the second hypervariable region of HLA-DQA1 and DQB1 chain genes promote the Ro (SS-A)/La(SS-B) autoantibody responses. J Immunol 1991;146:3871–6.
24 **Reveille JD**, Anderson KL, Schrohenloher RE, Acton RT, Barger BO. Restriction fragment length polymorphism analysis of HLA-DR, DQ, DP and C4 alleles in Caucasians with systemic lupus erythematosus. J Rheumatol 1991;18:14–18.
25 **Fei HM**, Kang H, Scharf S, Erlich H, Peebles C, Fox R. Specific HLA-DQA and HLA-DRB1 alleles confer susceptibility to Sjögren's syndrome and autoantibody production. J Clin Lab Anal 1991;5:382–91.
26 **Scofield RH**, Harley JB. Association of anti-Ro/SS-A autoantibodies with glutamine in position 34 of DQA1 and leucine in position 26 of DQB1. Arthritis Rheum 1994;37:961–2.
27 **Vitali C**, Bombardieri S, Moutsopoulos HM, Balestrieri G, Bencivelli W, Bernstein RM, *et al*. Preliminary criteria for the classification of Sjögren's syndrome. Arthritis Rheum 1993;36:340–7 .
28 **Voulgarelis M**, Dafni U, Isenberg D, Moutsopoulos HM and the Members of the European Concerted Action on Sjögren's Syndrome. Malignant lymphoma in primary Sjögren's Syndrome. Arthritis Rheum 1999;42:1765–71.
29 **Routsias JG**, Sakarellos-Daitsiotis M, Tsikaris V, Sakarellos C, Moutsopoulos HM, Tzioufas AG. Structural, molecular and

immunological properties of linear B cell epitopes of Ro 60kd autoantigen. Scand J Immunol 1998;47:280–7.
30 **Manoussakis MN**, Kistis KG, Liu X, Aidinis V, Guialis A, Moutsopoulos HM. Detection of anti Ro/SSA in antibodies in autoimmune disease. Comparison of five methods. Br J Rheumatol 1993;32:449–55.
31 **Wagner U**, Kaltenhauser S, Sauer H, Arnold S, Seidel W, Hantzschel I, *et al*. HLA markers and prediction of clinical course and outcome in rheumatoid arthritis. Arthritis Rheum 1997;40:341–51.
32 **Bignon JD**, Fernandez-Vina MA, Cheneau ML, Fauchet R, Schreuder GMTh, Clayton J, *et al*. HLA DNA class II typing by PCR-SSOP: 12th International Histocompatibility Workshop experience. In: Charron D, ed. *HLA: genetic diversity of HLA, functional and medical implication*. Paris: EDK, 1997:21.
33 **Miettinen O**. Estimability and estimation in case-referent studies. Am J Epidemiol 1976;103:226–35.
34 **Woolf B**. On estimating the relation between blood groups and disease. Ann Hum Genet 1955;19:251–3.
35 **Haldane S**. The estimated significance of algorithm of a ratio of frequencies. Ann Hum Genet 1956;20:309–11.
36 **SAS Institute Inc**. *SAS procedures guide*. Release 6.03 edition. Cary NC: SAS Institute Inc, 1998.
37 **Rischmueller M**, Lester S, Chen Z, Champion G, Van Den Berg R, Beer R, *et al* . HLA class II phenotype, controls diversification of the autoantibody response in primary Sjögren's syndrome (Pss). Clin Exp Immunol 1998;111:365–71.
38 **Scofield RH**, Dickey WB, Hardgrave KL. Immunogenetic of epitopes of the carboxyl rerminus of the human 60kd Ro autoantigen (published erratum appears in Clin Exp Immunol 1995;100:377.). Clin Exp Immunol 1995;99:256–61.
39 **Buyon JP**, Slade SG, Reveille JD, Hamel JC, Chan EK. Autoantibody responses to the "native" 52Kda SSA/Ro protein in neonatal lupus syndromes, systemic lupus erythematosus and Sjögren's syndrome. J Immunol 1994;152:3675–84.
40 **Reynolds P**, Gordon TP, Purcell AW, Jackson DC, McCluskey J. Hierarchial self-tolerance to T cell determinants within the ubiquitous nuclear self-antigen La(SSB). Penuits induction of systemic autoimmunity in normal mice. J Exp Med 1996;184:1857–70.
41 **Yiannaki E**, Vlachoyiannopoulos PG, Manoussakis MN, Sakarellos C, Sakarellos-Daitsiotis M, Moutsopoulos HM, *et al*. Study of antibody and T cell responses in rabbits immunized with synthetic human B cell epitope analogues of La (SSB) autoantigen. Clin Exp Immunol 2000;121:551–6.
42 **Stille CJ**, Thomas LJ, Reyes VE, Humphreys RE. Hydrophobic strip of helix algorithm for selection of T cell presented peptides. Mol Immunol 1987;24:1021–7.
43 **Margalit H**, Sponge JL, Cornette JL, Cease KB, Delisi C, Berzofsky JA. Prediction of immunodominant helper T cell antigenic sites from the primary sequence. J Immunol 1987;138:2213–29.
44 **Rothbard JB**, Taylor WR. A sequence pattern common to T cell epitopes. EMBO J 1998;7:93–100.
45 **Sette A**, Buus S, Apella E, Chesnut R, Miles C, Colon SM, *et al*. Prediction of major histocompatibility complex binding regions of protein antigens by sequence pattern analysis. Proc Natl Acad Sci USA 1989;86:3296–300.
46 **Miyagawa S**, Shinohara K, Nakajima M, Kidoguchi K, Fujita T, Fukumoto T, *et al*. Polymorphisms of HLA class II genes and autoimmune responses to Ro/SSA, La/SSB among Japanese subjects. Arthritis Rheum 1998;41:927–34.