Differential Expression of Ro/SSA 60 kDa and La/SSB, But Not Ro/SSA 52 kDa, mRNA and Protein in Minor Salivary Glands from Patients with Primary Sjögren's Syndrome

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ABSTRACT. ***Objective.*** To analyze the protein and messenger RNA (mRNA) expression of La/SSB, Ro/SSA 60, and Ro/SSA 52 antigens in minor salivary glands (MSG) from patients with primary Sjögren's syndrome (pSS).

Methods. La/SSB, Ro/SSA 60, and Ro/SSA 52 protein expression was studied by immunohistochemistry in MSG from 26 patients with pSS and 16 controls. mRNA expression was determined by real-time polymerase chain reaction in MSG of 10 patients with pSS and 7 controls.

Results. La/SSB and Ro/SSA 60, but not Ro/SSA 52, mRNA expression was higher in samples from patients with pSS compared to controls ($p < 0.05$). La/SSB protein had higher expression in the cytoplasm of ductal cells than in the cytoplasm of mucous acinar cells in patients with pSS ($p = 0.013$) but not in controls. Ro/SSA 60 had higher expression in the cytoplasm of ductal cells than in the cytoplasm of serous acinar cells in patients with pSS ($p = 0.006$) but not in controls. The Ro/SSA 52 expression pattern was similar in patients and controls. There was no association between circulating autoantibodies to Ro/SSA or La/SSB and the aberrant expression of the cognate autoantigens.

Conclusion. The increased Ro/SSA 60 and La/SSB mRNA expression in MSG of patients with pSS as well as the differential Ro/SSA 60 and La/SSB protein expression in ductal cells of MSG in patients with pSS suggest that these these 2 autoantigens, but not Ro/SSA 52, are probably involved in triggering and maintaining the tissue-specific autoimmune response in pSS MSG and may contribute to the antigen-driven immune response and local autoantibody production in pSS. (J Rheumatol 2007; 34:1283–92)

Key Indexing Terms:
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Sjögren's syndrome (SS) is associated with a variety of high-titer circulating autoantibodies. Anti-Ro/SSA and anti-La/SSB are the autoantibodies found most often in the serum of patients with SS[1]. Although not specific, anti-Ro/SSA and anti-La/SSB are considerably selective for SS. The Ro/SSA antigen is a ribonucleoprotein (RNP) complex that is composed of the 60 kDa and 52 kDa polypeptides in association with small cytoplasmic RNA known as hY1-5 RNA[2]. There are 2 alternative splicing mRNA forms for Ro/SSA 60, the predominant α isoform and the minor ß form that is restricted to a few cell types[3,4]. The Ro/SSA 52 antigen also exists in 2 forms (α and ß), the 52α predominant form and the 52ß form derived from the splicing of exon 3 to exon 5[5]. The cellular function of Ro/SSA RNP is not completely known. There is some evidence that the Ro/SSA 60 protein is involved in a discard pathway for defective 5S ribosomal RNA precursors[6] and recent data have shown that Ro/SSA 52 is an E3 ubiquitin ligase[7,8]. The nature and stability of the association of the 60 and the 52 Ro/SSA components are controversial and it has not been definitely established that the 52 polypeptide is a resident component of the Ro/SSA RNP[9]. La/SSB consists of a 48 kDa protein that is transiently associated with almost all RNA polymerase III transcripts, including hYRNA[1,10]. La/SSB has been shown to be required for efficient and correct termination of RNA polymerase III transcription[11], although other

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functions related to RNA metabolism have been reported[12].

The histopathology of salivary glands in patients with SS has been extensively studied and consists mostly of lymphocytic infiltration with subsequent destruction of the glandular tissue. Major and minor salivary glands (MSG) are similarly affected with good histopathologic correlation. The inflammatory infiltrate begins around ductal epithelial cells, extending and replacing the functional tissue[13]. It consists predominantly of CD4+ T cells, with few CD8+ T cells and B cells[14]. In addition there are a considerable number of IgG- and IgM-containing plasma cells[15]. It has been consistently shown that MSG of patients with SS are a site of anti-La/SSB and anti-Ro/SSA autoantibody production[16,17].

The mechanisms that stimulate the local autoantibody production and initiate the inflammatory process in MSG of patients with SS remain unknown. Further, it is not known why the La/SSB and Ro/SSA 60 and 52 proteins are preferential targets for the autoimmune response in SS and how these antigens participate in the pathogenesis of SS. Since autoantibody production is thought to be antigen-driven, it is conceivable that the Ro/SSA and La/SSB autoantigens are aberrantly expressed in the MSG of patients with SS.

To test this hypothesis, we analyzed mRNA and protein expression of Ro/SSA (60 and 52 kDa forms) and La/SSB antigens in the MSG of patients with pSS and control subjects. The mRNA expression was determined by real-time polymerase chain reaction (PCR) and the protein expression was analyzed by immunohistochemistry with specific monoclonal antibodies.

MATERIALS AND METHODS

Patients and samples. Specimens were selected from 120 patients subjected to lower lip MSG biopsy for diagnostic purposes. Criteria selection included an appropriate amount of MSG tissue and the establishment of definite pSS diagnosis or the exclusion of this diagnosis (control subjects). No patient with possible SS was included. Patients with pSS were classified according to the American-European Consensus Group criteria for SS[18]. Biopsy samples from MSG of 26 patients with pSS (25 women, mean age 53.2 ± 16.9 yrs) and 16 control subjects (15 women, mean age 45.1 ± 11.5 yrs) fulfilled the selection criteria and were used in the immunohistochemistry assays. For analysis of mRNA expression, MSG samples from 10 patients with pSS (all women, mean age 52.3 ± 10.8 yrs) and 7 controls (all women, mean age 44.4 ± 4.6 yrs) yielded appropriate material. Informed consent approved by the São Paulo Federal University (UNIFESP) Ethics Committee was obtained from all studied subjects. The clinical, serologic, and histopathologic features of patients with SS and controls are shown in Tables 1 and 2, respectively.

MSG dissected from the lower lip were fixed in formalin for diagnostic focus scoring and for the immunohistochemistry assays. Snap-frozen biopsy samples were kept in liquid nitrogen until the RNA extraction procedure. All patients with pSS had a MSG focus score ≥ 1 according to the classification of Chisholm and Mason[19]. The control subjects did not have inflammatory infiltrates or they had only low-grade inflammation in their MSG.

Serum autoantibodies to Ro/SSA and La/SSB were detected by standard double immunodiffusion against calf spleen extract[20]. The discrimination of reactivity to the 52 component of Ro/SSA was achieved by immunoblot with HeLa cell extracts as described[21,22]. Rheumatoid factor and antinuclear antibodies were determined by standard latex and indirect immunofluorescence on commercial HEp-2 cells, respectively.

RNA extraction. Total RNA was extracted from snap-frozen MSG tissue using the TRIzol reagent. Minced frozen MSG were mixed (vortex) in 1.5 ml TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) for 5 min. After adding 300 μl chloroform, the homogenate was vortexed for 15 s and centrifuged at 14,000 rpm for 15 min at 2°C. The aqueous phase was transferred to a new tube and homogenized with 750 μl isopropanol. The mixture was allowed to precipitate at –20°C for 24 h, and then centrifuged at 14,000 rpm for 15 min at 2°C. The pellet was washed twice with 70% ethanol, air-dried at 37°C, and solubilized in 30 μl diethylpyrocarbonate (DEPC)-treated H_2O.

Reverse transcription. Single-stranded cDNA was synthesized from 2 μg of the total RNA preparation using the ThermoScript reverse transcription (RT)-PCR System kit (Invitrogen). The RNA sample was mixed with 2 μl Oligo-dT (50 μM) and DEPC-treated H_2O (qsp 10 μl), and then incubated at 65°C for 5 min. To the reaction tube 4 μl 5× cDNA synthesis buffer, 1 μl dithiothreitol (DTT) (0.1 M), 1 μl RNaseOUT 40 U/μl, 1 μl DEPC-treated H_2O, 2 μl dNTP mix (10 mM), and 1 μl ThermoScript RT (15 units/μl) were added, mixed, and incubated at 60°C for 60 min. The reaction was terminated by incubation at 85°C for 5 min.

Real-time PCR. The mRNA expression was determined by real-time PCR using specific primers corresponding to the cDNA for Ro/SSA 60α (forward 5'-GTC CAT CCT GCT ATT GCT CTG and reverse 5'-AAG CCG CAC ATA TCC AAC AT), Ro/SSA 52α (forward 5'-GGC TGA GAA GTT GGA AGT GG and reverse 5'-GCA GCT GCC TCT GTT CTT CT), and La/SSB (forward 5'-GTT TCA GGG CAA GAA AAC GA and reverse 5'-TGT TGT TTG GAT GCA GGT TC). Only the α isoforms of Ro/SSA 60 and Ro/SSA 52 mRNA were analyzed, since our preliminary studies have shown absence of Ro/SSA 60ß and Ro/SSA 52ß mRNA in MSG of patients with pSS and controls.

The samples were run in triplicate using the Rotor-Gene 3000 (Corbett Research, Sidney, Australia). Real-time PCR was performed at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min using the following mixture: 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.75 μl each primer (18 μM), 10 μl DEPC-treated H_2O, and 1.0 μl cDNA for a total volume of 25 μl. Reactions were run for each gene (Ro/SSA 60α, Ro/SSA 52α, and La/SSB) and for each of the housekeeping genes (GAPDH and ß-actin) in the same batch of cDNA for each sample. Gene expression was calculated from the threshold cycle (Ct) of the target gene and normalized with respect to the Ct of the housekeeping genes ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$). Relative quantitation was performed by comparison with a healthy control sample used as a calibrator for all samples ($\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$). The relative level of expression of each gene was calculated as a function of $2^{-\Delta\Delta Ct}$ [23]. Relative mRNA expression ($2^{-\Delta\Delta Ct}$) was compared between patients with pSS and controls.

Immunohistochemistry. Mouse monoclonal antibodies 2E7, A8, and A2 (kindly provided by Prof. E.K.L. Chan, University of Florida, Gainesville, FL, USA) were used in the immunohistochemistry assays as probes for Ro/SSA 52, Ro/SSA 60, and La/SSB proteins, respectively. Monoclonal antibodies 2E7, A8, and A2 have been previously characterized[24,25] and shown to yield the expected nuclear fine speckled pattern in immunofluorescence with HEp-2 cells and to recognize bands compatible with the molecular weights of Ro/SSA 52, Ro/SSA 60, and La/SSB, respectively, in Western blots with HeLa cell total extracts. Mouse anti-human monoclonal antibodies to p53 (DO7 clone; ref M7001; Dako, Glostrup, Denmark) and to proliferating cell nuclear antigen (PCNA; PC10 clone; ref M0879; Dako) were used as irrelevant monoclonal controls. Negative controls were also performed by using a mouse negative control (code V1617; Dako, Carpinteria, CA, USA) and by omitting the primary antibodies.

Biopsy samples fixed in buffered formalin were paraffin-embedded, and then the paraffin blocks were cut into 2–3 μm slices, mounted on 3-aminopropyl-triethoxy-silane (Sigma, St. Louis, MO, USA) treated slides, and dried overnight at 60°C. Subsequently, sections were dewaxed in xylene and rehydrated through graded alcohol, then briefly immersed in water. Tissue sections were subjected to heat-induced retrieval of antigens in 10 mM citrate buffer, pH 6.0, in a pressure cooker for 4 min. After cooling, endogenous peroxidase activity was quenched with 6% H_2O_2 in 4 rounds of 5 min and sec-

Table 1. Clinical and laboratory features of 26 patients with primary Sjögren's syndrome.

Patient	Sicca Symptoms	Altered Eye Tests	Saliva, ml/15 min	Focus Score*	Immunodiffusion	Western Blotting	ANA Titer	RF Titer
1	KCS + XT	RB	1.4	> 1	∅	∅	∅	1:80
2	KCS + XT	RB	5.5	1	∅	∅	1:80	1:80
3	KCS + XT	Sch + RB	2	> 1	∅	∅	1:160	1:2560
4	KCS + XT	Sch + RB	3	> 1	Ro	∅	∅	1:1280
5	KCS + XT	RB	1	> 1	∅	∅	1:80	∅
6	KCS + XT	Sch + RB	NM	> 1	Ro	NM	1:160	∅
7	KCS + XT	RB	0	> 1	Ro	Ro60 + Ro52 + La	1:1280	∅
8	KCS + XT	Sch	0.5	> 1	∅	∅	∅	∅
9	KCS + XT	RB	1.5	> 1	∅	∅	1:160	∅
10	KCS + XT	RB	0	> 1	Ro	NM	1:80	∅
11	KCS + XT	None	1.2	1	Ro + La	NM	1:1280	∅
12	KCS + XT	None	1.3	> 1	Ro	Ro52 + La	1:80	1:640
13	KCS + XT	Sch + RB	5	> 1	Ro	NM	1:160	1:160
14	KCS + XT	Sch + RB	0	> 1	Ro	Ro52 + La	1:10,240	1:80
15	KCS + XT	None	1.3	1	Ro	NM	1:160	1:1280
16	KCS + XT	RB	5	> 1	∅	∅	1:160	∅
17	XT	Sch	1.2	> 1	∅	∅	∅	∅
18	KCS + XT	None	4	1	Ro + La	Ro60 + Ro52 + La	1:10,240	∅
19	KCS + XT	Sch + RB	0.5	> 1	∅	NM	∅	∅
20	XT	Sch + RB	1	> 1	∅	∅	1:2560	∅
21	KCS + XT	Sch + RB	1.2	> 1	Ro + La	Ro60 + Ro52 + La	1:10,240	1:320
22	KCS + XT	RB	1	> 1	∅	∅	1:80	∅
23	KCS + XT	Sch	NM	1	∅	NM	∅	1:80
24	KCS + XT	NM	0.5	> 1	Ro + La	Ro60 + Ro52 + La	1:1280	1:160
25	KCS	Sch + RB	7	1	∅	∅	1:1280	∅
26	KCS + XT	RB	0	1	∅	∅	1:80	1:320

* Number of inflammatory infiltrates containing > 50 mononuclear cells per 4 mm^2 area. KCS: keratoconjunctivitis sicca; XT: Xerostomia; Sch: altered Schirmer's I test (≤ 5 mm in 5 min); RB: altered Rose Bengal score (≥ 4, van Bijsterveld score); NM: not measured; ANA: antinuclear antibodies (normal < 1:80); RF: rheumatoid factor (normal < 1:80); ∅: negative result.

Table 2. Clinical, serologic, and histopathologic features of 16 control subjects.

Patient	Sicca Symptoms	Altered Eye Tests	Saliva, ml/15 min	Focus Score*	Immunodiffusion	ANA Titer	RF Titer	Diagnosis
1	None	None	10	0	∅	∅	∅	None
2	None	NM	11	0	∅	∅	∅	None
3	None	RB	8	0	∅	∅	1:160	RA
4	None	None	6	0	∅	1:160	∅	SLE
5	None	None	6.5	0	∅	1:640	∅	SLE
6	None	None	7	0	∅	∅	∅	RA
7	KCS + XT	None	9.5	0	∅	∅	∅	None
8	KCS	None	2.8	0	∅	1:80	∅	None
9	KCS + XT	None	8	0	∅	∅	∅	RA
10	None	Sch + RB	5	0	∅	∅	1:320	RA
11	KCS + XT	None	5	0	∅	∅	∅	SPA
12	KCS	None	3	0	∅	∅	1:80	None
13	KCS + XT	None	4	0	∅	∅	∅	None
14	KCS + XT	None	3.5	0	∅	∅	1:320	RA
15	KCS + XT	None	4	0	∅	1:640	∅	SLE
16	XT	None	1.5	0	∅	∅	1:160	RA

* Number of inflammatory infiltrates containing > 50 mononuclear cells per 4 mm^2 area. KCS: keratoconjunctivitis sicca; XT: Xerostomia; Sch: altered Schirmer's I test (≤ 5 mm in 5 min); RB: altered Rose Bengal score (≥ 4, van Bijsterveld score); ANA: antinuclear antibodies (normal < 1:80); RF: rheumatoid factor (normal < 1:80); NM: not measured; ∅: negative result. RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SPA: spondyloarthropathy.

tions were then washed with phosphate buffered saline (PBS), pH 7.4. The primary antibodies 2E7 (1:20), A8 (1:25), A2 (1:200), p53 (1:50), and PCNA (1:6000) diluted in PBS with 1% bovine serum albumin (BSA; Sigma) and 0.1% sodium azide were incubated for 30 min at 37°C, and then overnight (18 h) at 4ºC. This was followed by incubation with Dako EnVision+ system labeled polymer, HRP reagent (Dako) for 60 min at 37°C. All incubations were followed by three 5-min washes in PBS. Staining was revealed with diaminobenzidine substrate 60 mg/dl, H_2O_2 0.06%, and DMSO 1% in PBS

for 5 min at 37°C, followed by washing in distilled H_2O for 3 min. Sections were counterstained with hematoxylin, dehydrated through graded series of ethanol, and mounted in Entellan (Merck, Darmstadt, Germany) for evaluation under light microscopy at 400× magnification.

The staining intensity for the 3 different proteins in the cytoplasm and in the nucleus of mucous acinar, serous acinar, and ductal cells was scored semiquantitatively and graded as negative (Ø) (no positive cells); weakly positive (+), 1–25% positive cells; moderately positive (++), 26–50% positive cells; strongly positive (+++), 51–75% positive cells; and very strongly positive (++++), more than 75% positive cells. Sections were scored by 2 blinded observers, including one experienced pathologist.

Statistical analysis. Relative mRNA expression ($2^{-\Delta\Delta Ct}$) was compared between patients with pSS and controls using the Mann-Whitney test. The chi-square test was used to compare the intensity of the staining for Ro/SSA 60, Ro/SSA 52, and La/SSB proteins in MSG between patients with pSS and controls and between different cellular compartments of the same sample. P values < 0.05 were considered significant.

RESULTS

La/SSB, Ro/SSA 60, and Ro/SSA 52 mRNA expression. The following data were calculated taking GAPDH gene expression as reference, but similar results were obtained when using ß-actin gene expression as reference. La/SSB and Ro/SSA 60 mRNA expression was higher in MSG from patients with pSS (5.326 ± 5.107 and 6.866 ± 7.868, respectively) as compared to controls (0.856 ± 1.255 and 1.045 ± 1.329, respectively; Figure 1A, 1B). In contrast, Ro/SSA 52 mRNA expression in samples from patients with pSS (5.616 ± 4.885) did not differ statistically from controls (2.648 ± 4.223; Figure 1C). The presence of antibodies to La/SSB, Ro/SSA 60, and Ro/SSA 52 in the serum of patients with pSS was not associated with the expression of the respective mRNA in MSG (data not shown). An interesting finding was that La/SSB, Ro/SSA 60, and Ro/SSA 52 mRNA had lower expression rates in pSS patients with focus score above 1 (1.253 ± 1.167, 1.345 ± 0.760, and 2.863 ± 2.491, respectively) compared to those with focus score of 1 (10.420 ± 2.342, 12.383 ± 7.913, and 10.641 ± 2.956, respectively) ($p < 0.05$; Figure 2). No correlation was found between the intensity of La/SSB, Ro/SSA 60, and Ro/SSA 52 mRNA expression and the MSG degenerative changes such as acinar atrophy, ductal dilatation, fatty cell infiltration, and fibrosis (data not shown).

La/SSB, Ro/SSA 60, and Ro/SSA 52 protein expression. Representative immunohistochemistry images of MSG from patients with pSS are shown in Figure 3. No staining of samples from patients with pSS and controls was observed with the negative controls (monoclonal anti-p53 antibody, mouse negative control, and omitting primary antibody) and anti-PCNA antibody produced the expected nuclear staining pattern (data not shown). The protein expression of the 3 polypeptides varied widely among the several patients with pSS and controls (Table 3). In general, however, La/SSB, Ro/SSA 60, and Ro/SSA 52 protein immunostaining had higher intensity in the cytoplasm compared to the nucleus in ductal and acinar cells of patients with pSS and controls. Consistent nuclear expression of La/SSB and Ro/SSA 60 but not Ro/SSA 52 protein was observed only in ductal cells of patients and controls. The staining intensity for the 3 polypeptides in the nucleus and in the cytoplasm of all MSG cell types did not differ between patients with pSS and controls (data not

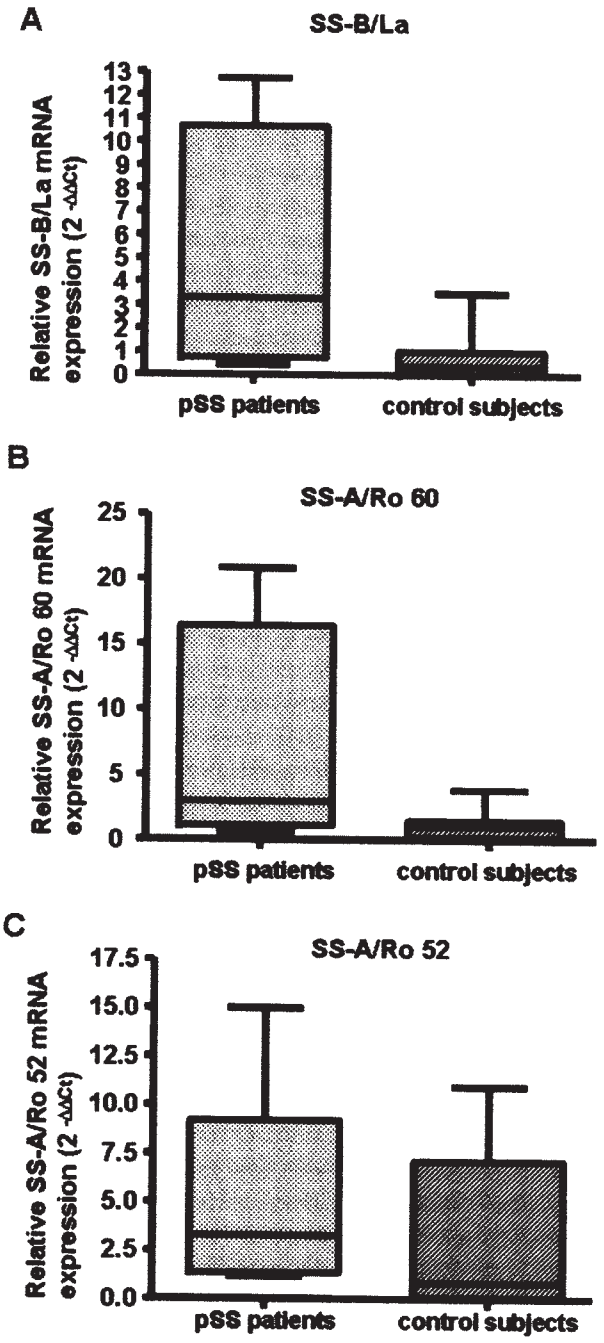

Figure 1. Distribution of patients with primary Sjögren's syndrome (pSS) and controls according to relative mRNA expression ($2^{-\Delta\Delta Ct}$): A. La/SSB; B. Ro/SSA 60; and C. Ro/SSA 52 genes normalized by GAPDH expression. La/SSB and Ro/SSA 60 mRNA expression was higher in minor salivary glands (MSG) samples from patients with pSS compared to controls ($p = 0.0311$, $p = 0.0330$, respectively). Ro/SSA 52 mRNA expression in MSG samples from patients with pSS did not differ statistically from controls ($p = 0.088$). These results were confirmed when normalized with ß-actin housekeeping gene.

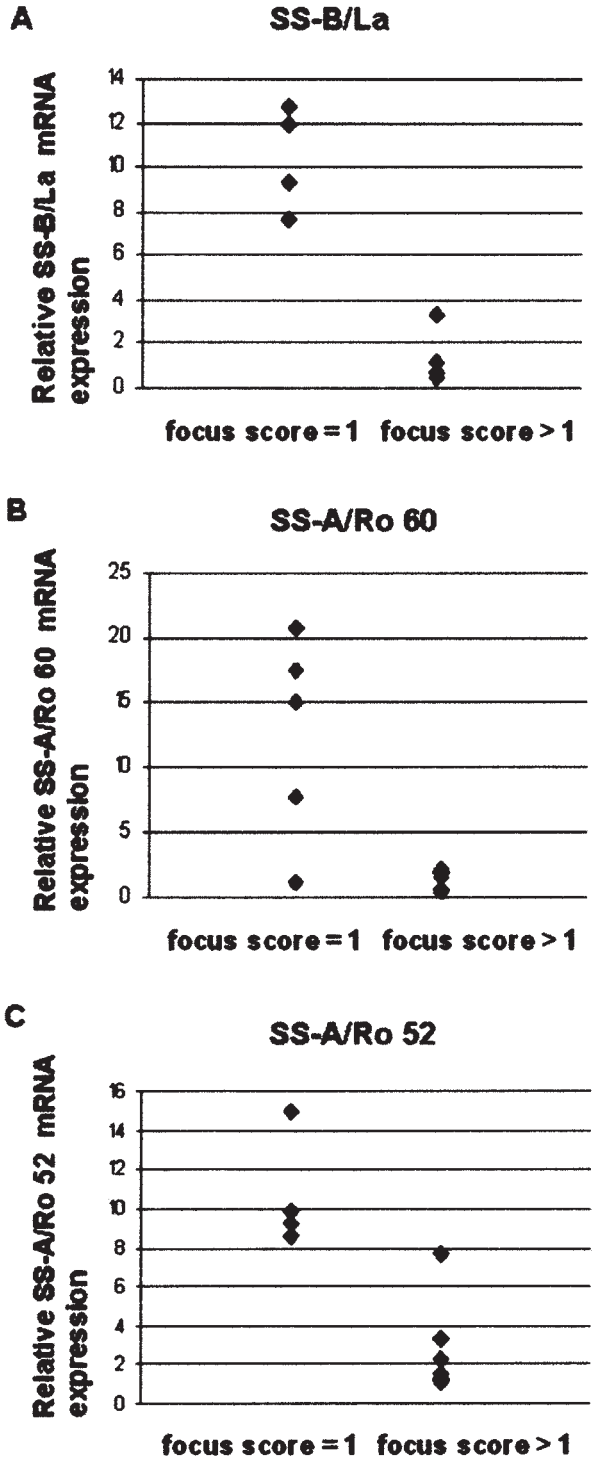

Figure 2. Distribution of patients with pSS according to the intensity of the inflammatory infiltrate and the relative mRNA expression: A. La/SSB; B. Ro/SSA 60; and C. Ro/SSA 52 in minor salivary glands. La/SSB, Ro/SSA 60, and Ro/SSA 52 mRNA showed lower expression rates in pSS patients with focus score above 1 compared to those with focus scores of 1 ($p < 0.05$).

shown). However, some subtle differences in the protein expression pattern between patients with pSS and controls were observed among the several cell types. For the La/SSB protein the intensity of immunostaining was higher in the cytoplasm of ductal cells compared to the cytoplasm of mucous acinar cells in MSG of patients ($p = 0.013$) but not in controls ($p = 0.722$; Figure 4A). Within the pSS group, this difference was not associated with the presence of circulating antibodies to La/SSB (data not shown). For the Ro/SSA 60 protein the immunostaining had higher intensity in the cytoplasm of ductal cells than in the cytoplasm of serous acinar cells of patients ($p = 0.006$), while no significant difference was detected in controls ($p = 0.289$; Figure 4B). Within the pSS group, this difference was not associated with the presence of circulating antibodies to Ro/SSA 60 (data not shown). The Ro/SSA 52 immunostaining pattern was similar in patients with pSS and controls, with a more intense immunostaining in the cytoplasm of ductal cells compared to the cytoplasm of mucous acinar cells (both $p < 0.001$) and to the cytoplasm of serous acinar cells (both $p < 0.05$).

No correlation was found between the intensity of La/SSB, Ro/SSA 60, and Ro/SSA 52 protein expression in each cellular compartment studied and the focus score in MSG of patients with pSS (data not shown). No association was observed between La/SSB, Ro/SSA 60, and Ro/SSA 52 relative mRNA expression and the intensity of the staining in different cellular compartments for La/SSB, Ro/SSA 60, and Ro/SSA 52 proteins in MSG of patients with pSS (data not shown).

DISCUSSION

We analyzed the mRNA and protein expression for the major Sjögren's syndrome autoantigens La/SSB, Ro/SSA 60, and Ro/SSA 52 in MSG of patients with pSS and controls. mRNA expression for La/SSB and for Ro/SSA 60 but not for Ro/SSA 52 was increased in pSS samples. In a broad sense the protein expression for the 3 autoantigens did not differ between patients with pSS and controls, and was more prominent in the cytoplasm compared to the nucleus in all cell types. Subtle differences in the topographic distribution of La/SSB and Ro/SSA 60 but not Ro/SSA 52 protein were observed in patients with pSS. The mRNA and protein expression pattern for the autoantigens was not associated with the respective circulating autoantibodies. Samples with the highest histological inflammation score from MSG showed decreased expression of Ro/SSA 60, Ro/SSA 52, and La/SSB mRNA.

Evidence that anti-Ro/SSA and anti-La/SSB responses are antigen-driven includes the fact that autoantibodies in patients with pSS recognize multiple epitopes of these autoantigens[26-28] and that these autoantibodies recognize species-specific epitopes[28-31]. In other autoimmune systems it has been previously indicated that aberrant expression of the autoantigen is involved in triggering the cognate autoimmune response[32,33]. MSG have been shown to be a site of B cell activation and autoantibody production in pSS[15,16]. Anti-La/SSB antibodies are found in the cytoplasm of MSG plasma cells and are

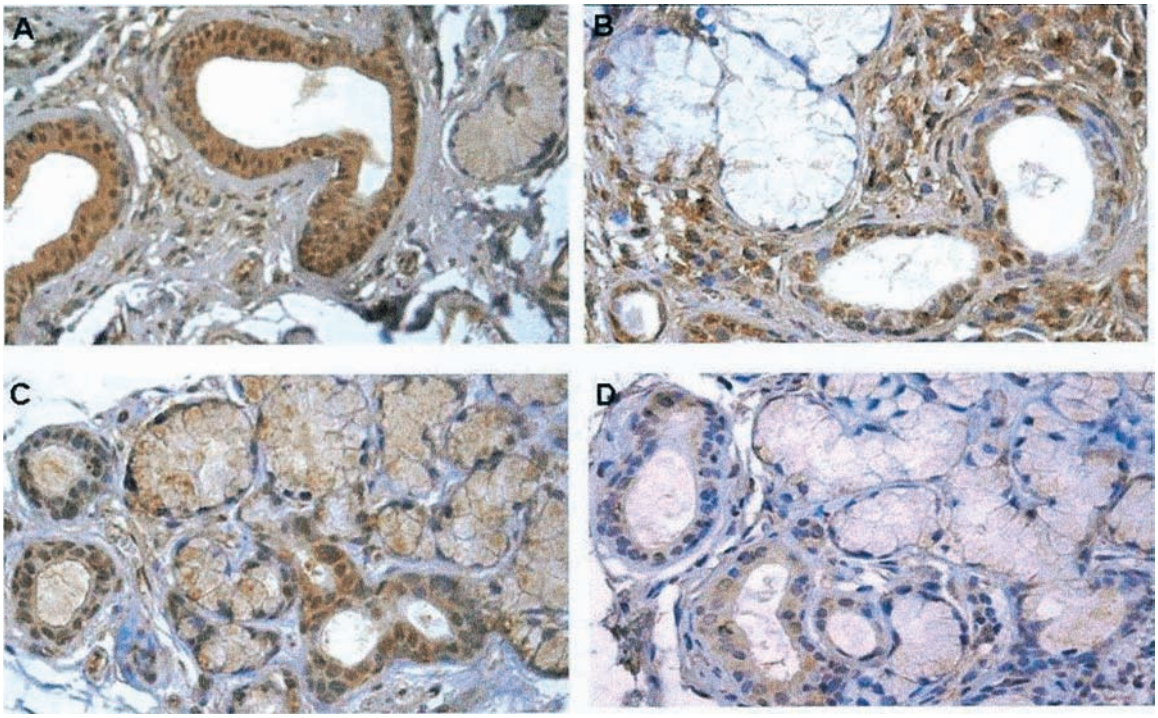

Figure 3. Immunohistochemistry with monoclonal antibodies 2E7, A8, and A2 in MSG specimens of patients with pSS. Magnification ×400. A. Representative sample of very strongly positive La/SSB expression in cytoplasm and nucleus of ductal cells and cytoplasm of acinar cells in a MSG sample with ductal dilatation and glandular destruction. B. Strongly positive La/SSB staining in cytoplasm and nucleus of ductal cells and minimal La/SSB expression in acinar cells in MSG specimen with ductal dilatation and inflammatory infiltrate. C. Very strongly positive Ro/SSA 60 expression in cytoplasm of ductal and acinar cells, strongly positive anti-Ro/SSA 60 staining in nucleus of ductal cells, and negative staining in the nucleus of acinar cells. D. Moderately positive Ro/SSA 52 expression in cytoplasm of ductal cells, weakly positive expression in the nucleus of ductal cells and cytoplasm of acinar cells, and no Ro/SSA 52 staining in the nucleus of acinar cells.

enriched in the saliva of patients with pSS. Indeed, anti-La/SSB antibodies can be found in the saliva of patients with pSS without circulating anti-La/SSB antibodies[34]. Cells producing anti-Ro/SSA and anti-La/SSB antibodies are localized to the salivary glands of patients with pSS, and there is a strongly positive correlation between serum levels of autoantibodies and the frequency of autoantibody-producing cells within the salivary glands[17]. This background appears to indicate that the MSG would be an appropriate site to look for aberrant expression of Ro/SSA and La/SSB autoantigens. Our observations of differential mRNA and protein expression of La/SSB and Ro/SSA 60 in pSS MSG appear to confirm for this disease the general principle previously demonstrated for other autoantibody systems. Such evidence supports the hypothesis that differences in the expression of La/SSB and Ro/SSA 60 autoantigens in the salivary glandular tissue of patients with pSS might initiate a local antigen-driven immune response to these autoantigens.

Analysis of the cells participating in the pathogenesis of SS has shown that antigen-presenting cells (monocytes and macrophages) compose less than 5% of the existing cells in the MSG[35]. However, acinar and ductal epithelial cells in pSS MSG express HLA-DR antigens and B7 costimulatory molecules and therefore may participate in the antigen presentation[35,36]. The expression of HLA-DR molecule is especially prominent in epithelial cells close to the lymphocyte infiltrates[35,37,38]. The consistently increased expression of HLA-DR by ductal and acinar epithelial cells in pSS MSG plus the increased expression of Ro/SSA 60 and La/SSB antigens in the salivary tissue argue for the participation of these 2 autoantigens in the local immunologic process in SS MSG.

A remarkable finding of our study was the higher degree of protein expression for La/SSB and Ro/SSA 60 in the cytoplasm of ductal epithelial cells as compared to the cytoplasm of acinar cells in patients with pSS. This observation is of interest because one of the major immunopathologic events observed in pSS MSG is the inflammatory infiltrate that initiates around ductal epithelial cells, extending and leading to the subsequent replacement of the functional tissue. In addition, the expression of B7 molecules in SS MSG is significantly stronger and more frequent in ductal epithelial cells as compared with acinar cells[36]. Moreover, ductal epithelial cells are the main cell type expressing chemokines in patients with SS[39]. Altogether these findings suggest that the inflammatory process in SS MSG is initiated at and around salivary ducts and that acinar epithelial cells play a minor or secondary role.

The fact that Ro/SSA 52 mRNA expression in pSS MSG was dissociated from that observed for Ro/SSA 60 and

Table 3. Intensity of immunohistochemistry staining for La/SSB, Ro/SSA 60, and Ro/SSA 52 antigens in different cellular compartments of MSG from patients with pSS and controls. NS: nucleus of serous acinar epithelial cells; CS: cytoplasm of serous acinar epithelial cells; NM: nucleus of mucous acinar epithelial cells; CM: cytoplasm of mucous acinar epithelial cells. –: no positive cells; +: weakly positive; ++: moderately positive; +++: strongly positive; ++++: very strongly positive.

	SS-B/La						SS-A/Ro 60						SS-A/Ro 52					
	NS	CS	NM	CM	ND	CD	NS	CS	NM	CM	ND	CD	NS	CS	NM	CM	ND	CD
Patients pSS																		
1	-	++	-	+	-	+++	-	+	-	+++	-	+++	-	+	-	-	-	+
2	-	+	-	+	+	++	-	++	-	++	-	+++	-	-	-	-	-	+
3	-	++	-	+	++	+++	-	++	-	+++	-	++++	-	+	-	+	-	++
4	-	+	-	+++	+	++	-	++	-	+	-	+++	-	-	-	+	-	+
5	-	+++	-	+	+++	++++	-	++	-	-	++	++++	-	+	-	-	+	++
6	-	++	-	++	-	++	-	++	-	+++	-	++	-	+	-	++	-	++
7	-	+++	-	+	-	+++	-	++	-	-	-	++	-	++	-	-	-	++
8	-	++	-	-	-	++	-	++	-	-	-	+++	-	-	-	-	-	++
9	-	+++	-	-	+++	+++	-	+++	-	-	+++	+++	+	++	-	-	-	++
10	-	+	-	+	+++	++++	-	+++	-	++	+++	+++	-	++	-	-	-	++
11	-	+++	-	++++	++++	++++	-	+++	-	++	++	++++	-	++	-	-	+	+++
12	-	+++	-	++	-	++	-	+++	-	+++	-	++	-	+	-	+	-	+
13	-	+	-	++++	-	+++	-	++	-	+	-	+++	-	-	-	-	-	+
14	-	+	+	++++	+++	+++	-	++	-	+	+	+++	-	+	-	-	-	++
15	-	-	-	++++	-	++	-	++	-	++	-	++	-	+	-	-	-	+
16	-	+	-	+++	-	+++	-	-	-	++	-	++	-	+	-	-	-	++
17	-	+	-	+++	-	++	-	++	-	+	-	+++	-	+	-	-	-	+
18	-	+++	-	++	-	++	-	+++	-	+	-	++	-	+	-	-	-	++
19	-	++	-	+	+	+++	-	++	-	+++	++	+++	-	++	-	+	-	++
20	-	+	-	++	+++	+++	-	++	-	++	-	+++	+	+	-	-	-	+
21	+	+++	+	+	+++	++++	-	+++	-	++	+++	++++	-	+++	-	+++	++	+++
22	-	+++	-	++	+++	+++	-	++	-	+	++	+++	-	++	-	-	+	++
23	-	++	-	+	+++	+++	-	++	-	-	-	++	-	+	-	-	-	+
24	-	+++	-	+	+++	+++	-	+++	-	++	+++	+++	-	++	-	+	++	+++
25	-	+++	-	+++	++++	++++	-	++	-	+++	++++	++++	-	++	-	++	-	++
26	++	+++	-	+	+++	+++	-	+++	-	+	+++	+++	-	++	-	-	+	++
Controls																		
1	-	++	-	+++	+++	++	-	++	-	+	++	+++	-	++	-	-	-	++
2	-	+++	-	+	+++	++++	-	+++	-	-	++	+++	-	+	-	-	-	++
3	-	-	-	++++	-	++++	-	+++	-	-	-	++++	-	+	-	-	-	++
4	-	-	-	+	+	++	-	+	-	-	-	++	-	+	-	-	-	+
5	-	++	-	+++	+	+++	-	+++	-	+	-	+++	-	+	-	-	-	++
6	-	+++	-	++	-	++	-	++	-	+++	-	++	-	+	-	+	-	++
7	-	+	-	++	+	++	-	+++	-	+	++	+++	-	++	-	-	-	++
8	-	++	-	+++	+++	+++	+	++	-	-	-	++++	-	+	-	-	-	++
9	-	++	+	++++	-	+++	-	-	-	-	-	+++	-	-	-	-	-	+
10	-	+	-	++	-	++	-	++	-	+++	-	++	-	++	-	-	-	++
11	-	++	+	+++	++	+++	-	++	-	+	++	++	-	+	-	-	-	++
12	-	++	-	++++	+++	+++	+	++	-	+	-	+++	-	++	-	+	-	++
13	-	+++	-	-	++++	++++	-	+++	-	++	+	+++	-	++	-	-	+	+++
14	-	++	-	+	+++	+++	-	++	-	-	++	+++	-	+	-	-	-	++
15	++	+++	-	-	+++	+++	-	+++	-	++	-	++	-	+	-	-	-	+
16	-	+++	-	++++	-	++	-	++	-	+	-	++	-	++	-	-	-	++

La/SSB is in agreement with recent evidence that the 52 kDa polypeptide may in fact not be a constitutive part of the Ro/SSA RNP, and that transient interactions occur only in special circumstances. In general it has not been possible to immunoprecipitate Y RNA with antibodies specific to the 52 kDa polypeptide in contrast to the 60 kDa polypeptide that is tightly complexed to the Y RNA series. Recent data indicate that Ro/SSA 52 is an ubiquitin ligase involved in the addition of ubiquitin to the final substrate[7,8]. It is possible that Ro/SSA 52 interaction with Ro/SSA 60 represents a transient step in the ubiquitination of Ro/SSA 60. From a different perspective, the observed dissociation adds credibility to the differential expression pattern for La/SSB and Ro/SSA 60 we observed. Due to the known molecular interactions of the 2 latter polypeptides with Y RNA, our data suggest an altered expression pattern for this series of cytoplasmic RNA in pSS MSG. Since Y RNA is thought to interact with foreign viral RNA, it is tempting to speculate that a putative aberrant expression of Y RNA in pSS MSG might be associated with some sort of local viral infection. This hypothesis is intriguing in view of several previous suggestions on the participation of viral agents in the pathogenesis of SS[40,41].

De Wilde, *et al*[42] described an aberrant expression pattern for the La/SSB antigen in SS MSG. They demonstrated redistribution of La/SSB from the nucleus to the cytoplasm in acinar cells of pSS and secondary SS MSG. This aberrant pattern was not accompanied by the presence of serum anti-La/SSB, which is in accord with our observations. They also observed La/SSB protein expression in the cytoplasm of acinar cells only in patients with SS, but not in the control subjects, which is also in accord with our observation of subtle differences in the topographic distribution of La/SSB protein immunoex-

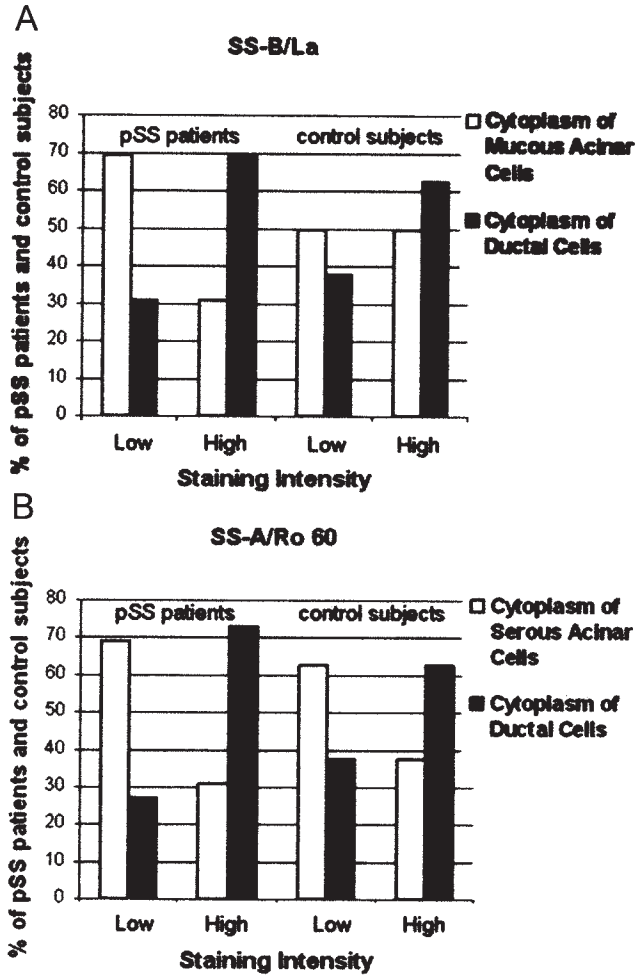

Figure 4. Distribution of patients with pSS and controls according to staining intensity of: A. La/SSB protein in the cytoplasm of mucous acinar cells and cytoplasm of ductal cells in MSG; B. Ro/SSA 60 protein in the cytoplasm of serous acinar cells and ductal cells in MSG. La/SSB protein had higher expression in the cytoplasm of ductal cells than in cytoplasm of mucous acinar cells in patients with pSS ($p = 0.013$) but not in controls ($p = 0.722$). Ro/SSA 60 had higher expression in the cytoplasm of ductal cells than in cytoplasm of serous acinar cells in patients with pSS ($p = 0.006$) but not in controls ($p = 0.289$). Low intensity = scores Ø, +, and ++; high intensity = scores +++ and ++++.

pression between patients with pSS and controls. Tzioufas, *et al*[43] showed by *in situ* hybridization that pSS and secondary SS patients with serum anti-La/SSB antibodies had upregulation of La/SSB mRNA in acinar and mononuclear cells of MSG. We were not able to confirm this finding in our patients and also could find no association between circulating anti-Ro/SSA 60 antibodies and increased expression of this autoantigen in MSG.

The cellular localization of Ro/SSA and La/SSB proteins has long been a matter of controversy. Although generally considered as nuclear proteins, their association with small cytoplasmic RNA (Y RNA) suggests that at least part of the pool should be in the cytoplasm. Indeed, O'Brien, *et al*[44] have determined that Ro/SSA RNP particles reside primarily in the cytoplasm of mammalian cells, and Peek, *et al*[45] have demonstrated that 60%–70% of both La/SSB and Ro/SSA proteins are present in the cytoplasm. A recent study, however, reports on the functional requirement of Y RNA for DNA replication, which may also justify the nuclear localization of Ro/SSA proteins[46]. These carefully conducted studies support our findings that Ro/SSA and La/SSB proteins were predominantly localized in the cytoplasm of acinar and ductal cells of MSG from patients and control subjects.

The lack of association between the presence of serum antibodies to Ro/SSA and La/SSB and the abnormalities in the expression of their respective autoantigens is not unexpected. The method used for autoantibody screening was double immunodiffusion, which has limited sensitivity but brings very specific results. In addition, sera were also tested by Western blot. Therefore, we believe that the methodology did not induce a significant bias in this observation. It is known that in general autoantibody production is strongly conditioned by MHC alleles. Several studies have reported on the association between anti-Ro/SSA or anti-La/SSB antibodies and the MHC class II alleles DR2, DR3, DR8, DQ1, and DQ2[47-49]. Further, MSG samples and sera were obtained at a specific moment in the disease pathophysiology and do not necessarily reflect possible past or future causal effects. This is especially relevant since anti-La/SSB and anti-Ro/SSA antibodies may fluctuate across the course of disease development, as we observed in a longitudinal analysis in patients with systemic lupus erythematosus[50]. In addition, anti-Ro/SSA and anti-La/SSB antibodies may be present locally in MSG of patients with SS without the presence of circulating autoantibodies[34].

An original finding was the demonstration of an inverse relationship between the degree of inflammatory infiltrate and the mRNA expression of La/SSB and Ro/SSA. A possible interpretation for this result is that La/SSB and Ro/SSA hyperexpression may occur in the early stages of the inflammatory process in pSS MSG, and that a complete local inflammatory response in this tissue is associated with a decline in the autoantigen mRNA expression.

The absence of association between the relative mRNA and protein expression for La/SSB, Ro/SSA 60, and Ro/SSA 52 may have been due to methodological constraints. The amount of tissue available from the biopsy of a single lip MSG is not enough to provide material for both immunohistochemistry and mRNA quantitation. Therefore the MSG used for mRNA extraction was not the same MSG used for immunohistochemistry assays, although both originated from the same lip sample. As described, there is considerable variability in the degree of lymphocytic infiltration and histopathologic changes among MSG situated close together in SS patients[51]. Therefore, it is not unexpected that the degree of mRNA and protein expression would vary among different MSG in the same lip sample.

Our study has demonstrated an increased mRNA expres-

sion for Ro/SSA 60 and confirmed previous reports of increased La/SSB mRNA expression in the preferential tissue target of pSS, i.e., MSG. We have also documented a dissociation of Ro/SSA 52 in relation to Ro/SSA 60 and La/SSB gene expression in pSS MSG. In addition to aberrant mRNA expression we could also detect qualitative differences in La/SSB and Ro/SSA 60 protein expression in pSS MSG. Although one cannot rule out the hypothesis that our findings were a consequence of the disease, it is quite possible that the observed differential expression of Ro/SSA 60 and La/SSB mRNA and protein may contribute to the antigen-driven immune response with local autoantibody production and to the tissue-specific autoimmune response in pSS salivary tissue. However, the exact mechanisms of La/SSB and Ro/SSA 60 autoantigen processing and presentation and their exact participation in SS pathogenesis remain to be elucidated.

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