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ABERRANT EXPRESSION PATTERN OF THE SS-B/La ANTIGEN IN THE LABIAL SALIVARY GLANDS OF PATIENTS WITH SJÖGREN’S SYNDROME

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Objective. Salivary glands of patients with Sjögren’s syndrome (SS) have been shown to be a site of anti-SS-B/La antibody production. The present study investigated differences in the localization of the SS-B/La antigen in labial salivary gland (LSG) tissue between SS and non-SS patients, which may explain the local antigen-driven anti-SS-B/La response.

Methods. Distribution of SS-B/La was studied immunohistologically in the LSG biopsy samples of 9 SS patients, 10 non-SS patients, and in normal tissues obtained at autopsy within 2 hours after death, using a mouse monoclonal antibody directed to SS-B/La. In 3 SS and 3 non-SS patients, LSGs were also studied with affinity-purified biotinylated human antibodies directed against SS-B/La.

Results. In the non-SS patients, SS-B/La was primarily observed in the nucleoli of acinic cells of the LSGs. Patients with either primary SS or secondary SS showed an accumulation of SS-B/La in the nucleoplasm of acinic cells. In the SS patients, SS-B/La was also detected in the cytoplasm as a diffuse or perinuclear staining. Sometimes, SS-B/La was found along the membrane of acinic cells as well. This aberrant nuclear and cytoplasmic distribution of SS-B/La in SS patients correlated well with abnormalities in the composition of the plasma cell population in the LSGs, but not with a lymphocytic focus score >1.

Conclusion. The accumulation and redistribution of SS-B/La in the LSGs may play an important role in the local antigen-driven anti-SS-B/La response in SS, and can also be used to improve the diagnostic possibilities of the LSG biopsy.

Antibodies against SS-B/La and SS-A/Ro RNPs are often found in the serum of patients with Sjögren’s syndrome (SS) (1–5). Although such autoantibodies are not disease-specific for SS (they can also be found in other autoimmune diseases), their presence has been included as a diagnostic parameter in the California criteria and the preliminary European criteria for the classification of SS (6,7).

SS-B/La is a 47-kd polypeptide associated with small RNA molecules in RNP complexes (1,2). The antigen is located in the nucleus and, to a lesser extent, in the cytoplasm of cells (2). The SS-A/Ro RNPs comprise SS-A/Ro proteins complexed with a subset of SS-B/La–associated RNAs, known as the RNA polymerase III–transcribed Y RNAs (2,3).

It is well known that exocrine tissues are a site of B cell activation and autoantibody production in SS (8–10). Patients with SS show increased numbers of IgG- and IgM-containing plasma cells in their labial salivary gland (LSG) tissue (11–14). Anti-SS-B/La antibodies have been demonstrated in the cytoplasm of plasma cells in the LSGs of SS patients (10). Saliva in these patients is enriched with anti-SS-B/La antibodies, as compared with the serum, and these antibodies have been found in the saliva of SS patients without detectable anti-SS-B/La activity in the serum (15). These findings point to the possibility that anti-SS-B/La antibodies are produced and secreted in the salivary glands of SS patients. However, it is unclear whether the SS-B/La antigen and anti-SS-B/La antibodies play a role in the pathogenesis of SS.
In the present study, the immunostaining pattern of SS-B/La in the LSGs of SS patients was compared with that in non-SS patients. Furthermore, the distribution of SS-B/La in various normal tissues was studied. The aim of this study was to search for differences in the expression pattern of SS-B/La between SS and non-SS patients, which might be related to the local production and secretion of antibodies to these antigens. In addition, the relationship between the SS-B/La immunostaining pattern and the composition of Ig-containing plasma cells in LSG tissue was studied.

**PATIENTS AND METHODS**

**Patients.** LSG biopsy samples were obtained from 9 SS patients, 6 with primary SS and 3 with secondary SS (2 associated with rheumatoid arthritis [RA] and 1 associated with systemic sclerosis). Informed consent was obtained in all cases. Six of the 9 SS patients fulfilled the criteria for the classification of SS as described by Daniels and Talal (16). In 3 patients, keratoconjunctivitis sicca (KCS) was absent; however, these patients had a lymphocytic focus score >1 and serologic abnormalities compatible with a diagnosis of 'possible SS' according to the criteria of Daniels and Talal (16). Two of these 3 patients had elevated levels of IgG and rheumatoid factor, antinuclear antibodies, and antibodies against SS-A/Ro and SS-B/La in their serum (patients 3 and 15 in Table 1). In addition, the 3 patients had an abnormal composition of the plasmacellular infiltrate consistent with a diagnosis of SS (Table 1).

As controls, LSG biopsy samples from 10 non-SS patients were used: 2 from RA patients without any clinical evidence of SS who voluntarily underwent lip biopsy, and 8 from patients without SS who underwent an LSG biopsy procedure for diagnostic purposes. Among the latter group, 3 patients who had KCS and xerostomia but did not show any other clinical, serologic, or histopathologic evidence of SS were included as controls (Table 1).

For diagnosis of SS via the LSG biopsy, a part of the biopsy specimen was fixed in a formalin sublimate solution (17,18). The lymphocytic focus scores (19) were determined from hematoxylin and eosin-stained sections. The percentages of IgA- and IgG-containing plasma cells were determined according to a procedure which has been described in detail previously (12,18). Briefly, a percentage of IgA-containing plasma cells <70%, and/or a discriminant function (DF) score (DF = [0.062 x % IgA] - [0.088 x % IgG] - 4.387) of lower than -2 was considered to be diagnostic for SS in the LSG biopsy (14). Because of the very high sensitivities (95%) and specificities (95%) of these immunohistologic criteria (14), we included the 3 patients with 'possible SS' in the group of patients with primary SS.

**Primary antisera.** In this study, the following primary antisera were used to assess the differences in immunoreactivity of SS-B/La in the LSGs of the SS and non-SS patients,
Human polyclonal antibodies directed against SS-B/La

Other mouse MAb used were directed against the

chains were used directly for immunohistochemical procedures.

continuing to standard procedures. Immunohistochemical

medicant antibodies against SS-B/La (2D-10) and 20B.2 (2D-10) were

and to compare the SS-B/La immunoreactivity with that of

Figure 1. Comparison of the immunohistochemical expression pattern of SS-B/La in the orbital salivary glands (LSOS) of patients with Sjögren's Syndrome.
La, obtained from an SS patient, were purified by affinity chromatography (28), and subsequently biotinylated according to a published protocol (29).

**Immunohistochemical analysis.** LSGs were snap frozen in liquid nitrogen, and 5-µm sections were cut, air-dried overnight, fixed in acetone for 10 minutes, and rinsed in phosphate buffered saline (PBS) (3 washes of 5 minutes each).

MAb SW5, 2.73, 4G3, and Y12 were used in an indirect immunoperoxidase (IP) technique. Incubations were performed with MAb SW5 (at 1:80 dilution for 60 minutes), with MAb 2.73 and 4G3 (at 1:100 dilution for 60 minutes), and with the MAb Y12 (at 1:10 dilution for 60 minutes), followed by incubation with peroxidase-labeled rabbit anti-mouse serum (at 1:80 dilution for 30 minutes).

To visualize SS-A/Ro with MAb 2G10, a 3-step indirect IP technique with undiluted MAb appeared to be necessary. This was subsequently incubated with peroxidase-labeled rabbit anti-mouse serum (at 1:80 dilution for 30 minutes) and peroxidase-labeled swine anti-rabbit serum (at 1:100 dilution for 30 minutes).

To visualize SS-B/La with the affinity-purified, biotinylated human anti-SS-B/La, the following protocol was used: endogenous biotin and avidin were blocked using an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA), followed by rinsing 3 times in PBS and incubations with biotinylated primary antibody (1:80 dilution, overnight at 4°C), mouse anti-biotin (1:50 dilution for 30 minutes), biotinylated horse anti-mouse (1:200 dilution for 30 minutes), and peroxidase-labeled avidin-biotin complex (1:100 dilution for 30 minutes).

All antisera were diluted in PBS (pH 7.4) containing 1% bovine serum albumin. The incubations were performed at room temperature and followed by 3 washes in PBS of 5 minutes each, unless otherwise noted. Peroxidase was developed with diaminobenzidine and hydrogen peroxide for 5 minutes, and intensified with 0.5% CuSO4 for 1 minute. In the control sections, incubation with the primary antibodies was replaced by incubation with only PBS. No nuclear or cytoplasmic counterstaining was used, in order to facilitate the microscopic assessment of the antigen expression pattern. Titration experiments with the primary antibodies were performed in order to determine the optimal concentrations and to obtain semiquantitative information about possible differences in the amount of antigens between SS and non-SS patients.

To study the SS-B/La distribution in normal tissues, specimens of liver, spleen, pancreas, adrenal gland, lymph nodes, striated muscle, and duodenum, obtained from 3 patients at autopsy within 2 hours postmortem, were used. The cause of death was cerebral hemorrhage, and none of these patients was known to have a systemic autoimmune disease. Frozen sections of these tissue specimens were stained with MAb SW5, according to the IP technique as described above.

**RESULTS**

The most striking difference between SS and non-SS patients was the very intense SS-B/La immunostaining of the nucleoplasm in the majority of acinic cells in all SS patients. In non-SS patients, the immunostaining was limited to the nucleoli of acinic cells, whereas the nucleoplasm was negative or very weakly stained. Figures 1A and B demonstrate the difference in the SS-B/La immunoreactivity between a patient with primary SS and a non-SS patient, using MAb SW5. The nuclear staining results with MAb SW5 and with the biotinylated human polyclonal anti-SS-B/La were essentially the same (Figures 1C and D). There was no difference between primary SS and secondary SS with regard to the SS-B/La nuclear immunoreactivity (Figures 1A and E). The expression patterns of SS-B/La in the LSG biopsy samples of both of the RA patients without any evidence of SS (Figure 1F) and the control subjects without any systemic connective tissue disease (Figure 1B) were essentially the same.

With regard to localization of U1 snRNP, U2 snRNP, and Sm, no differences between SS and non-SS patients were found. In all patients, there was high nucleoplasmic staining for these spliceosome-related antigens (Figures 2A–D). No obvious quantitative differences between SS and non-SS patients were observed in titration experiments (data not shown).

The 3-step indirect IP technique to visualize SS-A/Ro resulted in a very high background, which hampered the interpretation of staining results. In the acinic cells of SS and non-SS patients, both stained and nonstained nuclei were observed; however, in SS patients, the staining intensity and the fraction of positive nuclei seemed to be higher than in non-SS patients (Figures 2E and F). In some acinic cells of the non-SS patients, SS-A/Ro was found in the nucleoli, while in other acinic cells of the same patient, it was observed in the nucleoplasm (Figure 2F). The differences for expression of SS-A/Ro between SS and non-SS patients were obviously less convincing than those of SS-B/La.

At a higher magnification (×1,000), the nuclear staining pattern of SS-B/La in the acinic cells of SS patients appeared to be speckled (Figure 3A). In SS patients, SS-B/La was also observed in the cytoplasm of some acinic cells in a perinuclear or diffuse granular pattern, or along the membrane of these cells. In such acinic cells, a decrease or even absence of nuclear SS-B/La was noted (Figure 3B). In the non-SS patients, no cytoplasmic SS-B/La was observed in the LSG tissue. At higher magnification, U1 snRNP, U2 snRNP, and Sm showed a speckled nuclear staining pattern in acinic cells of both SS and non-SS patients, as expected.
Figure 2. Comparison of the immunohistochemical localization of U2 small nuclear RNP (snRNP), Sm, and SS-A/Ro in the LSG of SS and non-SS patients. LSG biopsy samples from an SS patient (A, C, and E) and a non-SS patient (B, D, and F) were stained with MAb-1E10 directed against U2 snRNP (A and B), with MAb-1/2 directed against Sm C and D, and with MAb-2020 directed against SS-A/Ro (E and F), arrows point to nuclear positively stained nuclei, and arrowheads point to 2 nuclei with positively stained nucleoplasm. (Original magnification × 400). See Figure 1 for other definitions. An aberrant SS-B/La pattern in the acinar cells. In contrast, the presence of SS-B/La in the serum of a non-SS patient (patient 10) was not accompanied by an aberrant expression pattern of SS-B/La in the acinar cells. An interesting finding was that 2 patients with R.A. (patients 7 and 8 in Table 1) without clinical evidence of SS had a grade IV lymphocytic adenitis, and immunoblotting, but all these patients exhibited an aberrant SS-B/La pattern in the acinar cells. In contrast, the presence of SS-B/La in the serum of a non-SS patient (patient 10) was not accompanied by an aberrant expression pattern of SS-B/La in the acinar cells. An interesting finding was that 2 patients with R.A. (patients 7 and 8 in Table 1) without clinical evidence of SS had a grade IV lymphocytic adenitis.
Figure 3. Staining pattern and localization of SS-B/La in the acinic cells of the LSG of SS patients. A, A speckled nucleoplasmic staining pattern of SS-B/La is apparent in the acinic cells of an SS patient, using MAb SW5. B, Arrow points to an acinic cell which exhibits that cytoplasm is stained while the nucleus is negative. (Original magnification × 1,000.) See Figure 1 for definitions.

which is suggestive of secondary SS. These patients had normal percentages of IgA-containing plasma cells (>70) and normal DF scores (>2), as well as a normal SS-B/La expression pattern in the acinic cells. In contrast, the 2 RA patients with SS (patients 4 and 5) combined abnormal immunohistologic findings (% IgA <70 and DF <2) with an aberrant expression pattern of SS-B/La in the acinic cells. All patients with an aberrant SS-B/La expression pattern had an abnormal composition of the plasmacellular infiltrate in the LSGs (% IgA <70 and/or DF <2) (Table 1).

In the normal tissues obtained at autopsy, there was inter-organ variation in SS-B/La expression (Table 2). SS-B/La was found exclusively in the nuclei. In almost all tissues, the nucleoli were stained and the intensity of the nucleolar staining appeared to be tissue dependent (Table 2). In most tissues, the nucleoplasm was also positive (Table 2). The nuclear staining patterns of SS-B/La in the acinic cells of both the exocrine pancreas and the LSGs of non-SS patients were the same, i.e., positive staining of the nucleoli and no or very weak staining of the nucleoplasm.

Using normal human liver tissue, comparison of the staining results with MAb SW5 and with biotinylated, affinity-purified human antibody against SS-B/La revealed an intense immunostaining of the nucleoplasm of the hepatocytes and a higher-intensity staining of the nucleoli by both antibodies. In the control sections, which were incubated with PBS instead of the biotinylated human anti-SS-B/La antibody, no nuclear staining was observed, while the cytoplasm of the hepatocytes remained positive due to the presence of endogenous biotin in the cytoplasm.

**DISCUSSION**

The present study demonstrates for the first time that in both primary and secondary SS, there is a nucleoplasmic accumulation of SS-B/La in the majority of acinic cells and a redistribution of SS-B/La from the nucleus to the cytoplasm in acinic cells of the LSGs. The presence of this aberrant distribution pattern of SS-B/La in 2 patients with primary SS without xerostomia and KCS, and the absence of such a pattern in non-SS patients with xerostomia and KCS, suggest that the differences between SS and non-SS patients are not related to functional activity of the acinic cells.

In vitro studies have revealed that in virally or

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* + = positively stained; ++ = more intensely stained; – = not or very weakly stained.
otherwise transformed cells, an up to 13-fold incremental increase of the nuclear SS-B/La concentration and a redistribution of SS-B/La, leading to cytoplasmic and cell membrane localizations, could be observed (30,31). In an immunofluorescence study using MAb SW5, it was shown that infection with cytomegalovirus, Epstein-Barr virus, and adenovirus was associated with a redistribution of SS-B/La from the nucleus to the cytoplasm of the HEp-2 cells, whereas cell membrane staining was observed only in the adenovirus-infected cells (30). These results could be partly reproduced in another study using adenovirus-infected HeLa cells and the same antibody against SS-B/La, but neither the membrane localization of SS-B/La nor an increase in the amount of total SS-B/La could be confirmed (32). Redistribution of SS-B/La was also observed in poliovirus-infected HeLa cells, and it was shown that it did not reflect a leakage of nuclear proteins to the cytoplasm (33).

Recently, translocation of SS-B/La to the cytoplasm was found in cultured epithelial cells of the LSGs of SS and non-SS patients, and it was shown that the fraction of cells with cytoplasmic SS-B/La increased after treatment with interferon-γ (34). Redistribution of SS-B/La was more frequently observed in SS than in non-SS patients, and the proportion of cells with cytoplasmic SS-B/La was greater in SS than in non-SS patients (34). An increase of cytoplasmic SS-B/La was also observed in proliferating peripheral blood lymphocytes after stimulation with phytohemagglutinin (20). In synchronized cells, it was shown that in the G₀ phase of the cell cycle, SS-B/La was nucleoplasmic in location, but during the G₁ and early S phases, strong immunostaining was found in the nucleoli. This pattern changed into a nucleoplasmic staining during the late S and G₂ phases of the cell cycle (4). In SS and non-SS patients, most acinic cells are in the G₀ phase, as we have shown by use of monoclonal antibody MIB1 (35), which recognizes the cell proliferation–associated Ki-67 antigen (which is expressed during the G₁, S, and G₂/M phases, but not in the G₀ phase) (data not shown). Therefore, the aberrant immunostaining pattern of SS-B/La in the majority of acinic cells in SS cannot be explained by differences in cell proliferation.

Cytoplasmic and membrane localization of SS-B/La has been reported in the epithelial cells of the conjunctiva of SS, KCS, and viral conjunctivitis patients, while in control subjects, a weak nuclear expression of SS-B/La was observed (36). Our observations suggest that redistribution also seems to occur in the LSG acinic cells of SS patients. The latter phenomenon cannot be explained by a diffusion artefact, since cytoplasmic localization of SS-B/La was not observed in normal liver, spleen, and adrenal cells, which also showed high nuclear SS-B/La immunoreactivity. U1 snRNP, U2 snRNP, and Sm did not exhibit such enhanced nuclear immunoreactivity or cytoplasmic localization in the acinic cells of the LSGs of SS patients, and differences between SS and non-SS patients were not observed. The absence of redistribution of Sm from the nucleus to the cytoplasm was also found in the experiments of Baboonian et al with virally infected cells (30). Cytoplasmic and cell membrane localization of snRNPs, Sm, SS-B/La, and SS-A/Ro has been described in cell lines after ultraviolet (UV) irradiation (32). This extranuclear localization may be caused by UV irradiation–induced apoptosis, because it has been shown that apoptotic bodies contain nucleosomal DNA, SS-A/Ro, SS-B/La, and snRNPs (37).

Although MAb 2G10, directed against SS-A/Ro, is suboptimal for immunohistochemical purposes, it is obvious that the differences in the expression patterns of SS-A/Ro between SS and non-SS patients are not as convincing as for SS-B/La. Studies with more suitable MAb directed against SS-A/Ro are necessary to confirm our observations. A possible explanation for the differences in the expression patterns of SS-B/La and SS-A/Ro is that the latter forms complexes only with hY RNAs, whereas SS-B/La can be complexed with all cellular RNA polymerase III transcripts, including the hY RNAs, as well as with several viral transcripts (1,2). Viruses, especially the Epstein-Barr virus and retroviruses, have been suspected to be major contributing factors in the etiology of SS (38-40).

Recently, we found a 45% prevalence of grade IV lymphocytic adenitis in the LSGs of RA patients who had no clinical evidence of SS and no immunohistologic abnormalities, as defined by a DF score <−2 and/or % IgA-containing plasma cells <70 (14). Although RA patients with a grade IV adenitis should be diagnosed as having secondary SS according to the criteria of Daniels and Talal (16), it seemed unlikely that our RA patients had SS, because of the absence of clinical and disease-specific immunohistologic features of SS in the LSG biopsy samples. The absence of the aforementioned abnormalities in the plasma cell composition prompted us to conclude that the focal lymphocytic adenitis in RA patients without SS is based on a different pathophysiologic mechanism, compared with the same type of LSG adenitis in SS patients (14).
The normal SS-B/La expression in the LSGs of these RA patients and the aberrant SS-B/La expression in RA patients with SS gives further support to this hypothesis.

The aberrant nuclear and cytoplasmic distribution of SS-B/La found in the LSG tissue of SS patients, together with the local production and secretion of anti-SS-B/La (10), support the hypothesis that membrane localization of SS-B/La might provoke the local SS-B/La antigen-driven immune response. Although the cause of the aberrant SS-B/La expression pattern in acinic cells in SS patients remains to be elucidated, and the cell membrane localization should be confirmed by more informative techniques, such as ultrastructural immunohistochemical procedures, it seems likely that the nuclear accumulation and redistribution of SS-B/La in the acinic cells plays a role in the pathogenesis of the B cell hyperreactivity and chronic lymphocytic adenitis of the salivary glands of SS patients. In addition, the consistently enhanced nuclear immunostaining of SS-B/La in acinic cells seems to be a promising tool to improve the diagnosis of SS in the LSG biopsy.

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REFERENCES

22. WILDE ET AL
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