Centromere Protein C Is a Target of Autoantibodies in Sjögren's Syndrome and Is Uniformly Associated with Antibodies to Ro and La

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ABSTRACT. ***Objective.*** To determine which centromere proteins are recognized in Sjögren's syndrome (SS) and whether antibodies recognizing centromere proteins (CENP) B and CENP C identify a specific serologic subset.

Methods. Sera from 47 patients with SS, 12 xerostomic controls without SS, and 12 healthy controls were studied. All 47 patients met San Diego criteria for SS. Of these, 45 patients had primary SS and 2 had secondary SS with CREST. Sera were analyzed by immunoprecipitation of [^{35}S] methionine-labeled Ro 52, La, and CENP B and C generated by coupled *in vitro* transcription/translation. Human salivary gland cells were also lysed and immunoprecipitated to determine antibody status against Ro 60. Serological and clinical profiles of patients recognizing CENP were defined. Proportions of sera recognizing CENP B, CENP C, Ro, or La across the 3 groups were compared using Fisher's exact test.

Results. Twenty-eight of 45 primary SS patients (62%) recognized Ro 52, and 24 patients (53%) recognized La. Ten of these 45 (22%) sera recognized CENP B or C. Furthermore, 7 of these 10 recognized exclusively CENP C; these 7 (100%) all tested positive for antibodies to both Ro 52 and La. This was in contrast to the group of SS patients that did not recognize CENP C alone, in whom anti-Ro 52 antibodies were found in 21 of 38 (55%; p = 0.034), and antibodies to La in 17 (45%; p = 0.01). Five of 7 CENP C positive sera were also positive for Ro 60. One of 3 patients with antibodies to CENP B also had antibodies to Ro 52, while none of these 3 had antibodies to La. Only patients with antibodies to CENP B showed a centromere pattern on immunofluorescence staining.

Conclusion. Antibodies to both CENP B and CENP C occur in SS. In a subset representing 15% of SS patients studied, these anticentromere antibodies recognize exclusively CENP C, and were uniformly associated with antibodies to Ro 52 and La. (J Rheumatol 2004;31:1121–5)

Key Indexing Terms:
CENTROMERE ANTIBODIES SJÖGREN'S SYNDROME

Sjögren's syndrome (SS) is a systemic autoimmune disorder associated with mononuclear infiltration of epithelial tissues, most notably salivary and lacrimal glands[1]. Systemic autoimmune manifestations include synovitis, neuropathy, vasculitis, and autoantibodies. Autoantibodies occurring in primary SS include antinuclear antibodies, anti-Ro/anti-SSA, anti-La/anti-SSB, and rheumatoid factor. SS may be divided into primary and secondary forms[2]. In secondary SS, the disorder occurs concomitantly with other autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and scleroderma. Primary SS has been reported to affect from less than 1% up to 5% of the population, with a peak onset in middle age, with female predominance, and an incidence of 4 per 100,000 population per year[3].

Although antibodies recognizing the centromere are typically associated with limited scleroderma, they have also recently been recognized in patients with other systemic autoimmune diseases, including SS[4-9]. Limited information exists, however, as to whether distinct components of the centromere are recognized in the various autoimmune rheumatic disorders. To determine which centromere proteins (CENP) are recognized in SS, and whether such antibodies identify a particular subset of patients, we examined the frequency of anticentromere antibodies (ACA) directed against CENP B and CENP C in SS patients, as compared to xerostomic and healthy controls. We also investigated the relationship of such autoantibodies to clinical and objective features of exocrine gland function in SS.

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MATERIALS AND METHODS

Patients. Forty-seven patients met the San Diego classification criteria for SS[10]. All these patients had primary SS apart from 2 who also had CREST (calcinosis, Raynaud's, esophageal dysmotility, sclerodactyly, telangiectasias). In addition, 12 xerostomic control patients, who did not meet the San Diego criteria, and 12 healthy controls were also studied. The healthy volunteer subjects participated in a separate control protocol designed to allow for healthy controls to serve as a comparison for SS patients. The protocol included the collection of saliva and blood samples as well as the performance of a labial minor salivary gland biopsy for each participant. The San Diego classification criteria were uniformly ascertained in all participants; they include: objective evidence of keratoconjunctivitis sicca (KCS) as determined by ocular dye tests (van Bijsterveld score ≥ 4) and/or a low Schirmer I test (≤ 5 mm/5 minutes), a labial minor salivary gland biopsy demonstrating focal infiltrates of lymphocytes replacing normal salivary acini and ducts, and positive immunological laboratory findings in sera for antinuclear antibodies, anti-Ro/anti-SSA, anti-La/anti-SSB, or rheumatoid factor. Subjects were excluded if they had a past history of head and neck irradiation, hepatitis B or C infection, acquired immunodeficiency syndrome (AIDS), pre-existing lymphoma, sarcoidosis, or graft versus host disease. Patients and healthy volunteers were all participants in protocols approved by the Institutional Review Board of the National Institute of Dental and Craniofacial Research (NIDCR), National Institutes of Health, and were evaluated at the Sjögren's Syndrome Clinic, NIDCR.

Laboratory analysis. [^{35}S] methionine-labeled Ro 52, La, and CENP B and C were generated by coupled *in vitro* transcription/translation[11,12]. Patient sera were used to immunoprecipitate these labeled proteins to assess antibody status for these antigens. [^{35}S] methionine-labeled cultured human salivary gland cells were lysed and immunoprecipitated with patient sera to determine which sera contained antibodies to Ro 60 (reference sera containing antibodies to Ro 60 were included in this assay). All immunoprecipitates were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels, and the labeled proteins were visualized by fluorography. Control sera did not precipitate any of these antigens. The serological and clinical profiles of patients recognizing CENP were examined.

Statistical analyses. Disease measures for the 3 study groups were compared by analysis of variance. The proportion of sera recognizing Ro 52, Ro 60, La, CENP B, and CENP C according to group status was compared using Fisher's exact test. Spearman correlations were calculated for the presence of antibodies in relation to disease measures. The analyses were performed using SAS V8.2 statistical package.

In addition to the study criteria applied above, we also analyzed the data by applying the new American-European Consensus Group revised European criteria[13]. Briefly, these criteria include (1) symptoms of dry eyes; (2) symptoms of dry mouth; (3) keratoconjunctivitis sicca by the Schirmer test or van Bijsterveld score; (4) focus score ≥ 1 on minor salivary gland biopsy; (5) salivary dysfunction, including a salivary flow of less than 1.5 ml/min; and (6) a positive test for serum anti-Ro/anti-SSA antibodies, anti-La/anti-SSB antibodies, or both. The criteria can be met by fulfilling 4 of 6 of the criteria elements provided that the criteria elements 4 or 6 are met. Alternatively, cases may be classified as SS if 3 of 4 objective criteria elements, i.e., 3 through 6, are fulfilled. The American-European criteria allow for more subjective symptoms than the San Diego criteria. However, the American-European criteria only include the anti-Ro/anti-SSA and anti-La/anti-SSB antibodies, whereas the serological component of the San Diego criteria can be met by having a positive test for anti-Ro/anti-SSA, anti-La/anti-SSB, anti-nuclear antibodies, or rheumatoid factor.

RESULTS

Objective characteristics of salivary and lacrimal gland function as well as markers of inflammation differed among the 3 study groups (Table 1). On average, the patients with SS showed more evidence of tissue inflammation (higher focus scores), decreased unstimulated salivary flow ($p < 0.0001$) as well as stimulated salivary flow ($p = 0.047$), and decreased tear production. Tear production as measured by the Schirmer test was lower and ocular dryness was greater in SS patients than controls, as measured by higher van Bijsterveld scores. In addition, SS patients exhibited elevated IgG levels and erythrocyte sedimentation rate (ESR) as compared with controls.

Figure 1 shows representative immunoprecipitation profiles obtained using sera from SS patients, as well as xerostomic and normal controls. In the 47 SS patients studied, reactivity was seen for one, both, or neither of the CENP examined. For example, patient SS1, a case of secondary SS with CREST, showed the presence of all 4 antigens depicted (CENP B, CENP C, Ro 52, and La). The remaining 3 patients had primary SS: patient SS2 had autoantibodies to CENP B, while SS3 had antibodies to CENP C, Ro 52, and La. The immunoprecipitation profile represented by SS4 depicted seronegative sera with none of the 4 autoantibodies. None of the sera from the xerostomic and healthy controls were positive for any of the 4 autoantibodies tested by immunoprecipitation.

Of the sera from the 45 primary SS patients, 28 (62%) recognized Ro 52, and 24 (53%) recognized La. Ten (22%) of these patients recognized CENP B or CENP C. Of these 10 primary SS patients, 7 recognized exclusively CENP C, and 3 recognized exclusively CENP B. The 7 sera that exclusively recognized CENP C all tested positive for antibodies to both Ro 52, and La; 5 tested positive for antibodies against Ro 60. Among the remaining 38 SS patients whose sera did not recognize CENP C alone, anti-Ro 52 antibodies were found in 21 (55%) of these sera ($p = 0.034$) with antibodies to La in 17 (45%; $p = 0.01$). One of 3 (33%) patients with antibodies to CENP B also had antibodies to Ro 52, while none of the 3 had antibodies to La. The 2 sera, from SS patients who had been excluded from the definition of primary SS since they also had CREST, both recognized CENP B and CENP C. Finally, by antinuclear antibody testing, a centromere immunofluorescence staining pattern was seen in all 3 of the primary SS patients with CENP B positive sera and in the 2 patients with CREST who were positive for both CENP B and CENP C. In contrast, none of the 7 primary SS patients with CENP C positive sera manifested this immunofluorescent staining pattern. This suggests that positive immunofluorescence of the centromere is associated with the presence of CENP B autoantibodies.

We next examined the correlation between the occurrence of autoantibodies to Ro 52, La, CENP B and CENP C and objective disease measurements in the 45 primary SS patients (Table 2). The van Bijsterveld score correlated with Ro 52 ($r = 0.33$; $p = 0.03$), La ($r = 0.39$; $p = 0.01$), and CENP C ($r = 0.35$; $p = 0.02$), but not CENP B. The unstimulated

Table 1. Objective characteristics of salivary and lacrimal gland function and of inflammation among patients with SS, and xerostomic and healthy controls. All values for Sjögren's syndrome, xerostomic controls, and normal volunteers are given as mean (standard error). Parotid, submandibular and total salivary flow rates are reported in ml/min; the total salivary flow rate reported here is the sum of the parotid and submandibular flow rates. The van Bijsterveld score is obtained by summing the values of subscores for the ocular dye staining of the nasal (0–3) and temporal (0–3) conjunctiva and the cornea (0–3) on slit lamp examination, giving a range of scores from 0–9.

Variable	Sjögren's Syndrome n = 47	Xerostomic Controls n = 12	Healthy Volunteers n = 12	p
Age (yrs)	56.4 (1.9)	56.8 (3.9)	42.5 (3.4)	0.0052
Focus score	8.2 (0.6)	1.9 (0.6)	1.3 (0.6)	< 0.0001
Parotid flow unstimulated, ml/min	0.01 (0.004)	0 (0)	0.05 (0.02)	0.0046
Parotid flow stimulated, ml/min	0.59 (0.12)	0.75 (0.10)	0.84 (0.21)	0.4993
Submandibular unstimulated, ml/min	0.05 (0.02)	0.09 (0.03)	0.30 (0.06)	< 0.0001
Submandibular stimulated, ml/min	0.24 (0.04)	0.57 (0.16)	0.66 (0.11)	0.0002
Total unstimulated saliva, ml/min	0.05 (0.02)	0.09 (0.03)	0.35 (0.07)	< 0.0001
Total stimulated saliva, ml/min	0.82 (0.14)	1.32 (0.23)	1.43 (0.21)	0.0469
Schirmer, mm/5 min	4.7 (0.7)	7.9 (1.9)	21.5 (7.1)	< 0.0001
Van Bijsterveld score	6.1 (0.4)	2.7 (0.6)	1.8 (0.6)	0.0001
IgG, g/dl	1661 (102.6)	999 (48.8)	1124 (70.4)	0.0005
ESR, mm/h	43 (3.9)	25 (3.7)	19 (2.5)	0.0012

Table 2. Correlations between the presence of autoantibodies and objective clinical disease measures in patients with SS. For the correlations, r values are given followed by p values in parentheses. Correlations for Ro 60 with other measures given in the table were generally similar in magnitude and significance to those for Ro 52.

	Ro 52	La	CENP B	CENP C
Age, yrs	–0.13 (0.39)	–0.23 (0.14	–0.04 (0.78)	–0.13 (0.40)
Schirmer test, mm/5 min	–0.29 (0.053)	–0.14 (0.37)	–0.21 (0.17)	–0.15 (0.31)
Tear film break up time, s	–0.13 (0.41)	–0.28 (0.07)	–0.05 (0.74)	–0.25 (0.11)
Van Bijsterveld score	**0.33 (0.03)**	**0.39 (0.01)**	–0.05 (0.73)	**0.35 (0.02)**
Parotid flow, unstimulated, ml/min	0.03 (0.84)	0.10 (0.51)	–0.11 (0.49)	0.22 (0.15)
Parotid flow, stimulated, ml/min	–0.19 (0.21)	–0.02 (0.88)	–0.07 (0.66)	–0.01 (0.94)
Submandibular unstimulated, ml/min	**–0.37 (0.01)**	**–0.43 (0.003)**	–0.03 (0.87)	**–0.30 (0.049)**
Submandibular stimulated, ml/min	–0.29 (0.053)	–0.26 (0.08)	–0.15 (0.31)	–0.21 (0.17)
Focus score	**0.46 (0.001)**	**0.32 (0.03)**	–0.08 (0.61)	0.03 (0.84)
IgG, g/dl	**0.49 (0.0007)**	**0.48 (0.0008)**	–0.20 (0.18)	**0.31 (0.04)**
IgA, g/dl	**0.34 (0.02)**	**0.34 (0.02)**	–0.15 (0.32)	0.07 (0.67)
IgM, g/dl	–0.22 (0.15)	–0.18 (0.25)	0.14 (0.37)	–0.03 (0.87)
ESR, mm/hr	**0.37 (0.01)**	**0.37 (0.01)**	**–0.36 (0.02)**	**0.30 (0.045)**

ESR: erythrocyte sedimentation rate; the van Bijsterveld scores represent the extent of staining of the conjunctival and corneal surfaces of the eyes. Data in bold face: statistically significant at $p < 0.05$.

submandibular salivary flow correlated inversely, while IgG correlated positively with Ro 52, La, and CENP C, but not CENP B. Focus scores and IgA correlated positively with the presence of antibodies to Ro 52 and La. No relationship was found between age and the presence of antibodies against Ro 52, La, CENP B, and CENP C. No other correlations were noted between these antibodies and the disease measures depicted in Table 2. It is notable that the presence of CENP B was not associated with any of the objective disease measures of exocrine gland dysfunction.

We also investigated the relationship between autoantibodies to CENP B and CENP C and clinical manifestations

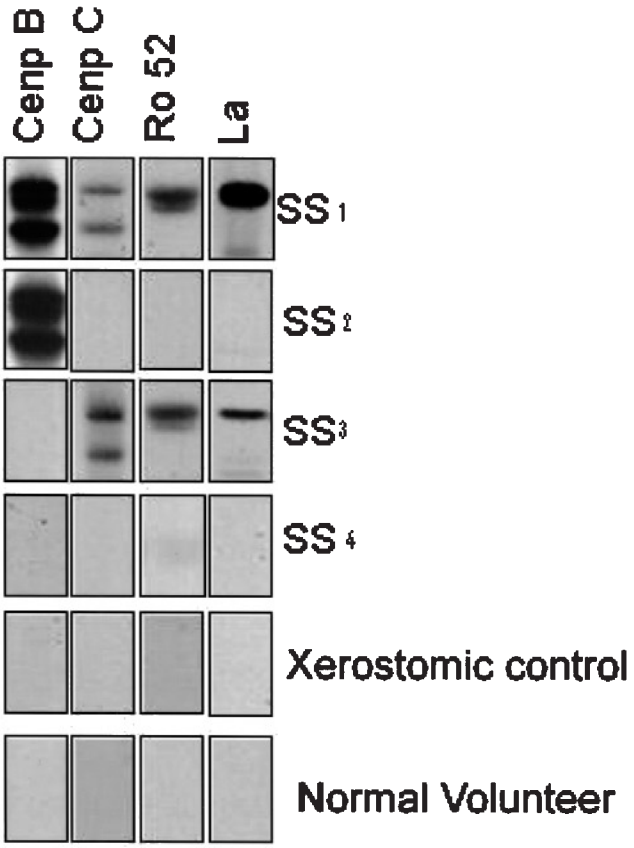

Figure 1. Representative immunoprecipitation profiles obtained using sera from SS patients and xerostomic and healthy controls. Sera from SS patients were used to immunoprecipitate [^{35}S] methionine-labeled CENP B, CENP C, Ro 52, and La. The sera were found to react with one, both, or neither of CENP B and C. For the 4 SS patients shown, SS1, a secondary SS patient with CREST, had all 4 autoantibodies. Of the remaining 3 patients who had primary SS, SS2 has antibodies only to CENP B, SS3 has antibodies to CENP C, Ro 52, and La, and patient SS4 has none of the 4 autoantibodies. The xerostomic and healthy controls had no antibodies to CENP B, CENP C, Ro 52, or La.

for patients with SS, including parotid swelling, cough, fatigue, fever, arthralgia, arthritis, Raynaud's phenomenon, vasculitis, and peripheral neuropathy. However, no significant relationships were found (data not shown). The 2 secondary SS patients who were positive for both CENP B and CENP C were the only 2 who had CREST. None of the patients with CENP B or CENP C alone had CREST.

To evaluate if the subset of CENP C positive patients was affected by the manner in which cases were classified, we repeated the analyses, which had initially used the San Diego criteria, according to the American-European Consensus Criteria. Both SS classification criteria sets were applied to the whole study population. Five cases, classified as xerostomic by the San Diego criteria, met the American-European criteria for SS. The CENP C positive patients all satisfied the SS classification using both criteria sets.

DISCUSSION

We hypothesized that primary SS patients with serum antibodies to certain centromere proteins would represent a subset of the SS population. We found antibodies to centromere proteins in 22% of patients with primary SS. In 16% of these 45 primary SS patients, the ACA recognized exclusively CENP C and were uniformly associated with serum antibodies to Ro 52 and La. Thus, antibodies to CENP C do identify a subset of Ro 52/La-seropositive patients with SS.

Recent studies have consistently noted the presence of ACA in SS patients. In 1994, Chan, *et al* found that sera from 14.8% (4 of 27) SS patients had ACA compared with sera from 4.2% (55 of 1323) of individuals with other autoimmune diseases (which included rheumatoid arthritis, systemic lupus erythematosus, Raynaud's disease, scleroderma, and Grave's disease), 0.5% (11 of 2215) of sera from patients with non-autoimmune disease, and 0.2% (1 of 500) of normal control individuals[4]. ACA appear to be more common in patients over the age of 50 years[8] and may occur in limited scleroderma, Raynaud's disease, lupus, and SS[5]. In one study, most ACA positive SS patients had scleroderma-like changes on capillaroscopy[7].

Katano, *et al* compared the clinical and laboratory features of 12 ACA positive primary SS patients who were SSA negative with 19 primary SS patients who were positive for anti-SSA[9]. The ACA positive group had lower IgG levels, more frequent Raynaud's phenomenon, higher natural killer cell activity, less frequent leukocytopenia, and lower Epstein Barr virus viral capsid antigen titers than the anti-SSA positive group. Labial salivary gland biopsies were similar in the 2 groups. They concluded that ACA positive primary SS differs clinically from the classic anti-SSA positive SS. However, Katano, *et al* designed their study to specifically exclude patients who were positive for both anti-SSA and ACA. In contrast, we investigated the clinical and objective measures of exocrine gland function among patients who had one, the other, or both antibody profiles.

We were interested in whether there were differences in the objective clinical measures between CENP B and CENP C positive subsets of patients. Apart from the correlation with the ESR, only CENP C showed correlations with the disease measures in Table 2, in particular the Van Bijsterveld score, submandibular gland salivary flow, and serum IgG levels. However, these correlations should be interpreted with caution until data are available in a larger number of patients. It is notable that CENP B did not correlate with any of the ocular, salivary, or serological measures in Table 2 other than the ESR, suggesting that CENP B, in contrast to CENP C, probably has little or no role as an important marker of disease activity in SS. Interestingly, the presence of antibodies to CENP B was associated with the appearance of a centromere pattern on immunofluorescent staining.

In our study, 28 (62%) of the 45 sera from primary SS

patients recognized Ro 52, and 24 (53%) recognized La. Ro and La have both been reported as occurring in 50 to 90% of primary SS patients[14]. As illustrated in Figure 1, sera from SS patients in our study showed the 4 possible patterns of reactivity for CENP B and C: reaction with both, CENP B only, CENP C only, or reaction with neither. We found that 10 of these 45 (26%) sera recognized CENP B or CENP C. The 2 SS sera that recognized both CENP B and CENP C were both cases of secondary SS with CREST. All of the sera that exclusively recognized CENP C tested positive for antibodies to both Ro 52 and La, whereas only about half of the remaining patients whose sera did not recognize CENP C alone had antibodies to Ro 52 and La. We also found that only 5 of 7 exclusively CENP C positive patients studied were Ro 60 positive. Neither CENP B nor CENP C were related to fatigue, Raynaud's phenomenon, vasculitis, or peripheral neuropathy.

Importantly, all patients with sera positive for CENP C fulfilled both of the SS classification criteria sets, showing that the presence of such antibodies is not dependent on the criteria used to classify individuals as having SS. However, only for the San Diego criteria did all individuals with antibodies against Ro 52, La, CENP B, and CENP C fall exclusively within the patients classified as SS. This suggests that the more objective the SS classification criteria applied, the more closely they parallel the autoimmune markers of the disease.

In conclusion, antibodies to CENP are found in about 22% of patients with primary SS. In most of these ACA positive SS patients, the ACA recognize exclusively CENP C (7 of 10) and are uniformly associated with antibodies to Ro 52 and La. Antibodies to CENP C therefore identify a subset of Ro 52/La-seropositive SS patients.

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