Anti-La positive, anti-Ro negative subset of primary Sjögren's syndrome: anti-La is a reality but is the disease?

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Abstract

Objective

To characterise the serological and clinical findings in primary Sjögren's syndrome in which anti-La was found without anti-Ro. We hypothesised that a significant portion of these are falsely negative for anti-Ro60.

Methods

Twenty-nine sera from primary Sjögren's syndrome patients were tested for antibodies directed against La and Ro. Anti-La was detected using bovine La treated with or without DNAase and RNAase to identify potential false positivity. Anti-Ro60 antibodies were detected using HEp-2000 substrate (in which cells are transfected with human Ro60) and HEp-2 substrate. Anti-Ro60 and Ro-52 were also tested by in vitro transcription/translation followed by immunoprecipitation assay.

Results

All 29 sera bound La, even after treatment with DNAase and RNAase. Of the 29 sera, 25 were unequivocally negative on HEp-2000 (1:40 dilution). Four samples were anti-Ro60 positive with a speckled pattern, three of the four at 1:320 dilution. Thus, false negative anti-Ro60 exists in a small fraction (14%) of the Ro-negative/La-positive primary Sjögren's patients. However, all the samples were negative for Ro60 and Ro52 by in vitro immunoprecipitation assay. Clinically these patients tended not to have salivary gland pathology characteristic of Sjögren's syndrome.

Conclusion

We found only a small fraction of Ro negative/La positive sera to show positive HEp-2000 pattern. These subjects did not have characteristic findings on pathological examination of minor salivary glands, suggesting these subjects have a process distinct from Sjögren's syndrome.

Key words

primary Sjögren's syndrome, autoimmunity, autoantibodies

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Introduction

Sjögren's syndrome is a systemic autoimmune disease characterised by dryness of the eyes and mouth along with an immune cell infiltrate of the salivary and lacrimal glands. There can also be manifestations involving other organs (1). Like many other autoimmune diseases, Sjögren's syndrome has an increased prevalence in women with onset commonly between the 4th and 6th decades. The disease may be second most common autoimmune rheumatic illness (2).

Sjögren's syndrome patients produce autoantibodies that target nuclear and cytoplasmic complexes. The most common autoantibodies found in the sera of Sjögren's syndrome patients are anti-Ro (or SSA) and anti-La (or SSB). The presence of these autoantibodies is part of the commonly employed classification criteria (3, 4), and are highly useful in a clinical setting (5). Historically, using the original method of detection, double immunodiffusion, anti-La was never detected in the absence of anti-Ro (6, 7).

However, double immunodiffusion has been supplanted in almost all commercial laboratories by high-throughput techniques that lend themselves to automation. Under these new methods of detection, a few sera are identified as anti-Ro negative, in spite of their anti-La positive status. We undertook this study to fully characterise such anti-Ro negative, anti-La positive patients attending our comprehensive Sjögren's syndrome research clinic.

Materials and methods

Subjects and sera

Subjects with dry eyes and dry mouth were evaluated in the Oklahoma Medical Research Foundation Sjögren's Syndrome Research Clinic, with complete medical/rheumatological, dental and ophthalmology examinations, as previously described (8). Blood, sera, plasma, DNA, RNA, and minor salivary gland tissue was acquired and stored on all subjects, each of which was classified as to the presence of primary Sjögren's syndrome (3, 4). Sera used in the present study were stored at -80°C until use. We assessed the presence of extraglandular manifestations of Sjögren's syndrome by the criteria of the EULAR Sjögren's syndrome disease activity index (ESSDIA) (9). However, pulmonary function testing was performed beginning in October of 2014 on only about 20% of the subjects; thus, this aspect of the ESSDIA was not considered. In addition, we also determined whether small fibre peripheral neuropathy was present by clinical neurological examination. Institutional Review Board approved all procedures and each subject provided written informed consent.

Materials

Bovine La and Ro were purchased from Immunovision, Springdale, AK. HEp2 ANA kits were procured from INOVA diagnostics, San Diego, CA. HEp-2000 ANA kits were purchased from Immuno Concepts, Sacramento, CA. The TNT Quick Coupled Transcription/Translation system was from Promega, Madison, WI. All other reagents were of reagent grade.

Serology

Anti-Ro and anti-La antibodies were determined by double immunodiffusion, line immunoassay (InnoLia), and multiplex bead assay (BioPlex). The last two assays included analyses for both 60 kD Ro and 52 kD Ro. We also determined anti-60 kD Ro by enzyme linked immunoassay, as previously described (10, 11).

Digestion of bovine La with DNAase, RNAase

Bovine La antigen (~12 μg) was treated with 6 units of DNAase (1 unit degrades 1 μg DNA in 10 min at 37ºC) and incubated for 3 h at 37ºC. The sample was also digested with 50 units of RNAase at 37ºC for 90 min. Bovine La was incubated for a similar period without DNAase or RNAase at 37ºC. These La samples as well as untreated La were used as the antigen source for ELISA.

La ELISA

A direct antigen ELISA to determine reactivity towards the La antigen was carried out as described for 60 kD Ro (9). Briefly, bovine La was coated on

96-well microtitre plates overnight at 4°C at 5 μg/ml in coating buffer. The plates were blocked with 3% non-fat dry milk in phosphate buffered saline (PBS) for 2 h at room temperature. Primary sera were added and incubated for 2 hours at room temperature. After washing 4x with PBS containing 0.05% Tween-20 (PBST), appropriate alkaline phosphatase conjugate was added and incubated for another 2h at room temperature. Following washing 4x with PBST, substrate was added and absorbance read at 405 nm.

Indirect immunofluorescence

HEp-2000 (12) and HEp-2 slides were used according to manufacturer's instructions for determination of antinuclear antibodies (ANA) by indirect immunofluorescence assay (IFA). Essentially, sera were diluted with PBS (1:40, 1:80, 1:160 or 1:320). The diluted samples were added to the slide, incubated for about 40 minutes and washed with PBS. The slides were then incubated with the provided FITC conjugate. The slides were washed and viewed using fluorescence microscopy.

Anti-Ro60 and anti-Ro52 quantitative immunoprecipitation assay

^{35}S-Ro60 and ^{35}S-Ro52 proteins were generated by *in vitro* transcription and translation using Promega, Quick TNT kit. IgG antibodies from patient sera were immobilised on Protein A beads. The Protein A beads coupled to patient IgG were added to radiolabelled proteins. The beads were washed extensively and the bound radioactivity was measured by scintillation counting.

Statistics

Categorical data were compared using Chi square or a two-tailed Fisher's exact test, as appropriate, with Bonferroni's correction for multiple comparisons. Continuous data were analysed by Student's *t*-test.

Results

We classified 503 subjects attending the OMRF Sjögren's Research Clinic as primary Sjögren's syndrome. No primary Sjögren's syndrome subject was found to have anti-La without anti-Ro by immunodiffusion. This means all anti-La antibody detected by immunodiffusion was found in subjects who also had anti-Ro antibody by the same method.

However, when anti-Ro and anti-La were determined by the other methods (line immunoassay, multiplex bead assay, and ELISA), we found 29 sera were anti-Ro negative and anti-La positive. We hypothesised that some of these sera contained autoantibodies specific to human Ro60, which would not be detected by these assays that use purified bovine Ro60. In order to detect such antibodies we employed the HEp-2000 cells for ANA determination. The cells used in this assay are transgenic for the human 60 kD gene; and, therefore, over-express the gene product. Positive and negative controls bound as expected (Fig. 1). However, only 4 of the 29 anti-Ro negative, anti-La positive subjects (13.7%) tested positive in the HEp-2000 ANA assay at titres greater than that found using HEp-2 cell ANA assay. All 4 subjects demonstrated strong speckled patterns with the HEp-2000 ANA assay at titres up to 1:320, as against much weaker patterns with the HEp-2 ANA assay or completely negative fluorescence with the non-transfected cells (Fig. 2). Thus, these 4 sera had negative anti-Ro results on immunodiffusion, ELISA, line immunoassay and the bead assay, but results on HEp-2000 ANA consistent with anti-Ro60. While HEp-2000 over-expresses recombinant human 60 kD Ro, these other assays use purified bovine 60 kD Ro.

We also assessed the presence of anti-Ro directed against the human antigens by an *in vitro* translation expression system followed by immunoprecipitation. This assay was used with expression of 60 kD Ro as well as 52 kD Ro. We found that none of the 29 anti-Ro negative, anti-La positive sera bound human 52 kD Ro. Likewise, none of these 29 sera bound 60 kD Ro in this assay. So, while we found 4 sera negative for anti-60 kD Ro on the basis of human-specific binding seen by use of the HEp2000 cells, no additional anti-52 kD Ro or anti-60 kD Ro positivity was uncovered by the *in vitro* transcription immunoprecipitation assay.

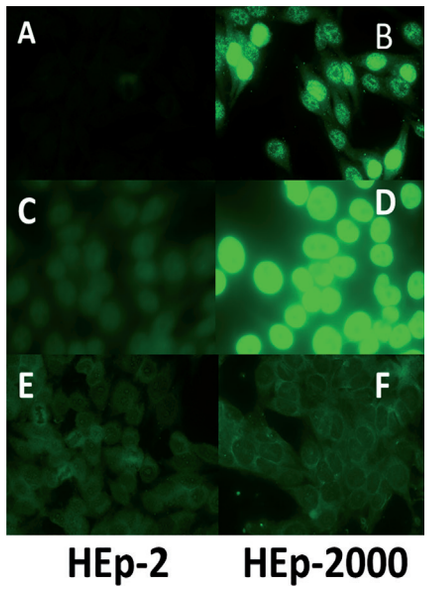

Fig. 1. Antinuclear antibody (ANA) test using permeabilised HEp-2000 and HEp-2 cells immobilised on slides with positive and negative controls (supplied in the HEp-2000 and HEp-2 ANA kits) and pSS sera. **A**: HEp2000 negative control; **B**: HEp2000 positive control; **C**: Hep-2 negative control; **D**: HEp-2 positive control; **E** and **F**: Representative anti-Ro negative, anti-La positive sera with no increased binding to HEp-2000. All sera were used at 1:80 dilutions.

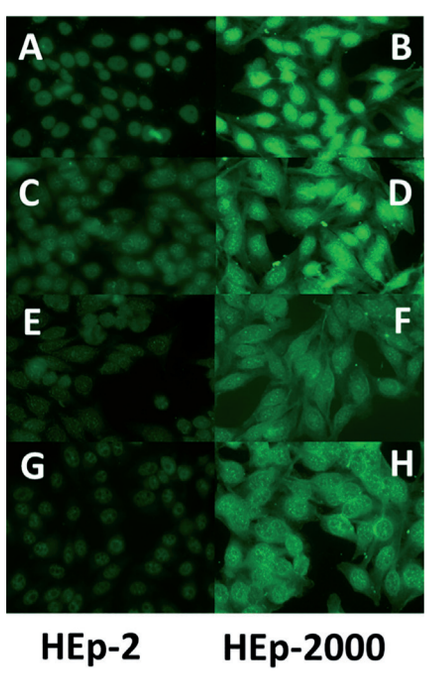

Fig. 2. ANA testing using four Ro60 negative and anti-La positive pSS sera on HEp-2 and HEp-2000 slides at a dilution of 1:80. Each individual serum is shown horizontally, tested on HEp-2 slides (**A**, **C**, **E**, **G**) and HEp-2000 (**B**, **D**, **F**, **H**). The HEp-2000 testing demonstrates increased fluorescence when compared to with use of HEp-2 as the substrate.

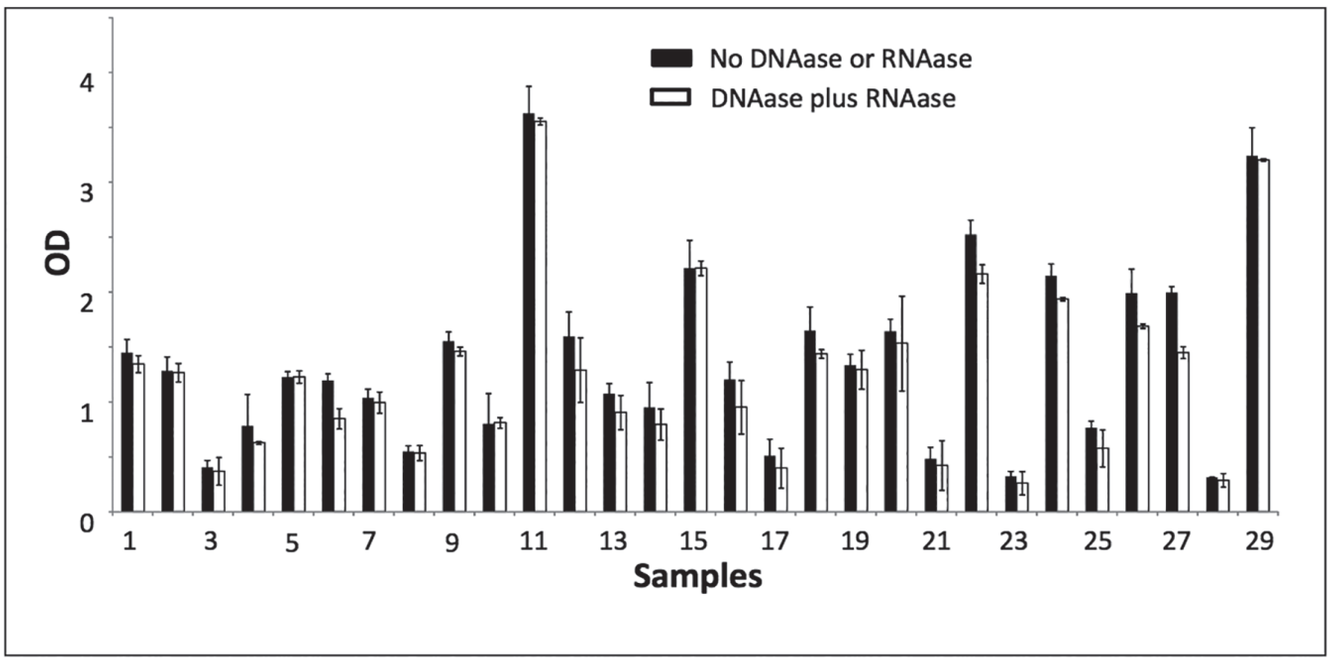

Fig. 3. Anti-Ro60/SSA negative, anti-La/SSB positive pSS sera binding to La/SSB antigen (treated with or without DNAase, RNAase) coated on ELISA plates. The solid bars correspond to anti-Ro60 negative, anti-La positive sera binding to La not treated with neither DNAase nor RNAase. The open bars correspond to anti-Ro60 negative, anti-La positive sera binding to La treated with DNAase, RNAase as mentioned in Methods.

We next asked whether any of the anti-La responses were false positives. In particular, we hypothesised that, since the La protein binds RNA (13), false positives might result from the presence of anti-RNA or anti-ssDNA autoantibodies, either of which is commonly found in autoimmune rheumatic diseases (14, 15). Such false positivity would occur if the purified bovine La preparation used in the various immunoassays was contaminated with RNA or DNA. So, we treated the commercially available La with RNAase and DNAase, with conditions to ensure complete digestion of any residual RNA and DNA. We found there was no significant change in anti-La activity after treatment of the La with RNAase and DNAase (Fig. 3). Thus, no false positivity was demonstrated, confirming that these subjects classified as primary Sjögren's syndrome had anti-La without the presence of anti-Ro-antibodies.

We then compared the clinical manifestations of Sjögren's syndrome in these anti-Ro-negative, anti-La-positive subjects to the clinical manifestations found in other groups of primary Sjögren's syndrome patients (Table I). Objective findings of lacrimal or salivary gland dysfunctions namely, Schirmer's test, ocular staining and whole unstimulated salivary flow, were not statistically different between the groups. We also looked for an association of rheumatoid factor with this subset having anti-Ro negativity, anti-La positivity (See Table I). About two-thirds of subjects with both anti-Ro and anti-La antibodies had rheumatoid factor, while only 3 of 29 (10.3%) of those with anti-Ro negative and anti-La positive had rheumatoid factor (χ^2=29.9, p<0.001, odds ratio=17.2, 95%CI=4.9-59.1). But, there was no statistical difference in rheumatoid factor positivity between the anti-Ro negative, anti-La positive group and either those with only anti-Ro positive or those with neither anti-Ro nor anti-La positive (χ^2=2.61, p=0.11, odds ratio=3.1, 95%CI=0.89-10.09 and Fisher's exact test p=0.46, odds ratio=1.33, 95%CI=0.37-4.8, respectively). One of the 3 anti-Ro negative, anti-La positive sera with rheumatoid factor was also 1 of the 4 that had human specific anti-Ro60 identified by binding to HEp-2000 cells.

We also examined the age at presentation of these Sjögren's syndrome subjects, most of whom were diagnosed for the first time during their evaluation at our clinic. In fact, the anti-Ro-negative, anti-La-positive Sjögren's subjects had an average age of 44.5 years (SD=12.1), which was significantly younger than the other 3 serological subgroups (see Table I).

There were differences between the groups in regards to a focus score of ≥1.0 on pathological evaluation of minor salivary gland biopsy specimens. Of course, as defined by the classification criteria, 100% of those with negative serology (negative anti-Ro and anti-La) had a focus score ≥1.0. In addition to this difference, we found that the anti-Ro-negative, anti-La-positive primary Sjögren's syndrome patients were several-fold less likely to have a positive biopsy compared to those subjects with either both anti-Ro and anti-La positive or anti-Ro alone positive (see Table I). Nineteen of the 29 anti-Ro-negative, anti-La-positive Sjögren's syndrome subjects had a focus score of zero.

We also examined the distribution of manifestations of Sjögren's syndrome captured by the ESSDIA in the 29 anti-La positive, anti-Ro negative Sjögren's syndrome subjects and compared the incidence of each manifestation to subjects with other serological combinations. (Table II). In general the anti-

Table I. Manifestations among the anti-Ro-negative, anti-La-positive subjects classified as primary Sjögren's syndrome compared to other serological groups of primary Sjögren's syndrome. More than 98% of all subjects had both dry eyes and dry mouth; thus, these near universal manifestations are not shown. Number (percentage) of subjects with an abnormal value are given for each test.

	Age (Ave±SD)	Schirmer's (<5 mm)	Ocular score (>3 vB)	WUSF (<1.5/10')	Biopsy (FS>1.0)	RF (+)
Anti-Ro-/La+ n = 29	44.5 (12.1)	11 (40%)	11 (40%)	17 (59%)	6 (21%)	3 (10%)
Anti-Ro+/La-n = 144	53.2 (13.7)$^{\lambda}$	65 (45%)	73 (51%)	91 (63%)	77 (54%)*	38 (26%)
Anti-Ro+/La+ n = 173	55.2 (14.6)†	114 (66%)	111 (64%)	129 (75%)	120 (69%)**	115 (66%)‡
Anti-Ro-/La-n = 157	57.6 (13.2)$^{\Psi}$	83 (53%)	85 (54%)	106 (58%)	157 (100%)	21 (13%)

Ocular score positivity ≥3 by van Bijsterveld system; WUSF: whole unstimulated salivary flow <1.5 mls in 10 minutes; Biopsy: minor salivary gland biopsy with a focus score ≥1; RF: rheumatoid factor.
$^{\lambda}p$=0.002 compared to Ro-/La+
$^{\dagger}p$=0.0004 compared to Ro-/La+
$^{\Psi}p$<0.0001 compared to Ro-/La+
*χ^2=9.12, p=0.0025, odds ratio=4.4 (95%CI 1.7-11.5) compared to anti-Ro-/La+
** χ^2=23.1, p<0.0001, odds ratio=8.6 (95%CI 3.3-22.6) compared to anti-Ro-/La+
‡This group is statistically different from the other three, but there is no statistical difference between anti-Ro-/anti-La+ and either anti-Ro+/anti-La- or anti-Ro-/anti-La-. See text.

Table II. Extraglandular manifestations of Sjögren's syndrome seen among the four serological groups evaluated herein. Values are given for number.

	Anti-Ro-/La+ n=29	Anti-Ro+/La- n=29	Anti-Ro+/La+ n=29	Anti-Ro-/La- n=29
Low grade fever/ night sweats	16	10	11	9
Lymphoadenopathy	3	3	0	2
Parotid enlargement	1	4	3	4
Submandibular gland	5	1	1	6
Arthralgia	7	23*	19	25†
Arthritis	2	5	6	7
Persistent cough	2	8	8	9
Raynaud's phenomena	6	8	8	9
Peripheral neuropathy	8	5	5	7
Leucopenia	2	2	5	1
Neutropenia	0	0	0	0
Lymphopenia	0	2	1	0
Haemolytic Anaemia	ND	ND	ND	ND
Thrombocytopenia	0	1	0	0
Low C3	1	0	0	0
Low C4	1	0	3	0
Low CH 50	3	8	11	6
High IgG	2	4	14‡	1
High IgM	3	3	4	1
High IgA	0	1	11$^{\text{¥}}$	1
Low IGs (total)	2	3	0	0

ND: not done. No patient had clinical evidence of haemolytic anaemia but Coomb's test was not performed.
*p=0.0011, odds ratio 0.83
$^{\dagger}p$=0.00006, odds ratio 0.05
$^{\ddagger}p$=0.016, odds ratio 0.07
$^{\text{¥}}p$=0.006, odds ratio 0

Ro negative, anti-La positive subjects had fewer systemic or extra-glandular manifestations, such as joint disease, persistent cough, cytopenias, decreased complement and elevated immunoglobulin. However, only a few of these differences were statistically significant (see Table II).

Discussion

Anti-Ro and anti-La were originally described by Ouchterlony immunodiffusion assay in the sera of patients with systemic lupus erythematosus (16), and then as anti-SSA and anti-SSB in the sera of Sjögren's syndrome patients (17). Subsequent data showed Ro60 and SSA were identical antigens. Further, La and SSB were one and the same (18). These papers, as well as all subsequent papers using immunodiffusion or counter immunodiffusion, always reported anti-La was found only in sera that also had anti-Ro. Likewise antibodies binding 52 kD Ro (also now known as TRIM21) were, similar to anti-La, found only in sera containing anti-Ro60 antibody, at least when patients with either systemic lupus erythematosus or Sjögren's syndrome were studied (19).

Over the few decades since their original description, many investigators have applied other assays for the determination of these autoantibodies (10). Western immunoblot, bead assays, and immunoprecipitation, among other techniques, have been deployed to determine anti-Ro and anti-La antibody. Commercial referral laboratories use high-throughput, easy-to-automate techniques, but in general purified bovine antigen is used. We found 4 sera that were negative on multiple assays, both commercial and in-house, in which bovine 60 kD Ro is the antigen, but had binding consistent with anti-Ro60 on HEp-2000, which uses cells over-expressing human 60 kD Ro. Thus, we conclude these sera have anti-Ro60 that is human antigen specific. However, these same sera did not bind 60 kD Ro using immunoprecipitation of *in vitro* translated 60 kD Ro. This discrepancy is not easily explained but perhaps the antigen is present in a more native conformation when over-expressed in the HEp-2000 cells. Antibodies binding 60 kD Ro typically bind the native molecule but poorly bind denatured antigen in an assay such as immunoblot (20-22).

We found no evidence of anti-Ro52 in these anti-Ro-negative, anti-La-positive subjects as well as markedly lower rates of rheumatoid factor. Thus, the anti-La only sera were distinct from anti-Ro and anti-La positive sera, where rheumatoid factor and anti-Ro52 are both common. Finally concerning serology, no false positive anti-La was found as we did not find antibodies binding either RNA or DNA.

In addition to the serological differences, the anti-La only patients had

clinical differences when compared to other subgroups of Sjögren's syndrome (Tables I and II). In particular, the anti-Ro negative, anti-La positive subjects were much less likely to have a lymphocytic infiltrate found on pathological evaluation of minor salivary glands. Such an infiltrate is one of the cardinal features of the disease, but 66% of the anti-Ro-negative, anti-La-positive subjects had a focus score of zero; that is, no focal infiltration of the salivary glands at all. In terms of systemic or extra-glandular manifestations, which contribute to the ESSDIA, many of these were numerically less frequent in the anti-Ro-negative, anti-La-positive group but only a few were statistically significant after correction for multiple comparison (Table II).

Another clinical difference we found was age. The anti-La only subjects were younger than all three of the other serological groups. A younger age might suggest an earlier stage of disease, and that these anti-La only individuals may go on to develop anti-Ro positivity in the future. However, our previous work in SLE (23) as well as the work of others in Sjögren's syndrome (24, 25) demonstrates that anti-Ro appears many years before clinical disease. Thus, the development of anti-Ro once clinical disease has developed is uncommon (26).

One other study has presented data that characterised a cohort of anti-La only subjects. Baer and colleagues reported on subjects from the Sjögren's International Collaborative Clinical Alliance and concluded that subjects with low titre anti-La but negative anti-Ro were phenotypically more like those subjects with both anti-Ro and anti-La negative (27). The key features of Sjögren's syndrome, including ocular staining score, salivary gland lymphocytic focal infiltration, unstimulated whole saliva flow, and Schirmer's testing were all statistically worse in subjects with anti-Ro (with or without anti-La) compared to those with anti-La only. Furthermore, many of the disease features were found less often in the anti-La only subjects compared to subjects negative for both autoantibodies (27). For instance, statistically significant differences were found for salivary flow, positive ANA, dry mouth symptoms, and abnormal Schirmer's test. Thus, in this cohort anti-Ro negative, anti-La positive subjects lacked the key features of Sjögren's syndrome. The investigators concluded that other support for the diagnosis or research classification should be present, such as lymphocytic sialadenitis with focus score >1.0 on minor salivary gland biopsy. Furthermore, the authors state that newer, highly sensitive detection methods may find low levels of anti-La that are not clinically important in the evaluation of patients with sicca or suspected Sjögren's syndrome on other bases (27). While in logistic regression analysis age did not explain the difference in the anti-La only subjects in this study, a comparison of average age is not given (27).

Comparison of our results and those of Baer *et al*. (27) is not straight forward as we studied subjects meeting classification criteria for Sjögren's syndrome, while the previous study was of the entire cohort regardless of Sjögren's classification. Thus, only about half of the subjects met either the AECG or ACR classification criteria. Nonetheless, similar to the Baer study, we found only a small percentage of our anti-Ro negative, anti-La positive subjects had a minor salivary gland focus score of ≥1 (see Table I). Some of the subjects with low levels of anti-La, no focal salivary lymphocytic infiltration and few of the key clinical features of Sjögren's syndrome probably do not have a disease process that should be considered together with those subjects with anti-Ro and/or a focus score of >1.0. On the other hand, some anti-La only patients may instead have a distinct subtype of Sjögren's syndrome. For clinical research, where homogenous populations with certainty that the disease is present is desirable, the differences in clinical presentation found in the present study as well as by Baer *et al*. (27) suggest that such patients are probably best not included in research cohorts.

On the other hand, when considering the practice of medicine, the gold standard for Sjögren's clinical diagnosis remains expert opinion as there is no single diagnostic test. Thus, the best clinical diagnosis for at least some of these patients with only anti-La along with features of sicca is likely Sjögren's syndrome.

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