Is Identification of Anti-SSA and/or -SSB Antibodies Necessary in Serum Samples Referred for Antinuclear Antibodies Testing?

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Background: Anti-SSA and -SSB antibodies are clinically important antinuclear antibodies in patients with systemic rheumatic diseases. **Methods:** We evaluated fluorescence characteristics and clinical significance of anti-SSA and -SSB and explored whether identification of these antibodies was necessary in clinical application. **Results:** Of 4,978 consecutive samples, 259 showed anti-SSA or -SSB reactivity, clinical information of which were analyzed. Compared with SSA+SSB– and SSA–SSB+ group, SSA+SSB+ group showed a lower proportion (0.5%) of negative specimens detected with antinuclear antibody assay and a higher proportion (53.5%) of specimens with high titer. Anti-SSA– and/or -SSB-positive samples presented various patterns. However, 64.6% of SSA+SSB+ samples presented speckled pattern, significantly higher than SSA+SSB– and SSA–SSB+ samples. A total of 475 specimens containing anti-SSA or -SSB were ob-

tained from 302 individuals. Clinical information was obtained for 259 of them, which were further analyzed. The prevalence of Sjögren syndrome (SS) was significantly higher, and that of other diseases was lower in SSA+SSB+ than in SSA+SSB– group. Of the 259 individuals, 71 anti-SSA– and/or -SSB-positive patients had blood drawn on 2 or more occasions over the 2-year study period. The number of tests per patient was 2.7 ± 1.1 . In 7 of the 71 patients, anti-SSA was observed in some but not all samples, so was anti-SSB in 6. **Conclusion:** In conclusion, the identification of anti-SSA and -SSB antibodies is necessary, when corresponding diseases are suspected. The specificity of anti-SSA for the diagnosis of SS could be improved, when combined with anti-SSB. SS patients need not be identified frequently for anti-SSA and -SSB because of their stability. *J. Clin. Lab. Anal.* 26:447–451, 2012.

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Key words: anti-SSA antibody; anti-SSB antibody; antinuclear antibody; systemic lupus erythematosus; Sjögren syndrome

INTRODUCTION

Anti-SSA and -SSB antibodies are clinically important antinuclear antibody (ANA) in patients with systemic rheumatic diseases. These antibodies are found in Sjögren's syndrome (SS), as well as other systemic autoimmune diseases (ADs), such as systemic lupus erythematosus (SLE), myositis, scleroderma, etc. Even, these antibodies may also be seen in HCV-infected patients or those with primary biliary cirrhosis (PBC) (1–4).

Anti-SSA/Ro60 antibody is one of the most frequently identified antinuclear antibodies (ANA), the target antigen of which is Ro60 protein associated with one of several hYRNAs (5). Anti-SSB/La is another frequently identified ANA, which also recognizes the Ro-ribonucleoprotein (6). Commonly, SSA and/or SSB present speckled fluorescence pattern by indirect im-

munofluorescence screening for ANA, and false-negative ANA results of SSA were infrequent, although screening with conventional HEp-2 cells may miss SSA antigen (7–9). Especially, SSA-transfected cells, over-expressing SSA (60 kDa), were considered highly sensitive for detection of anti-SSA antibodies (10). However, some other

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Grant sponsor: China National Natural Science Foundation Council; Grant numbers: 81001333; 81102262; Grant sponsor: Changzheng Hospital Funds for Young Scholar; Grant number: 2011CZQN06.

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Received 7 June 2011; Accepted 2 August 2012

DOI 10.1002/jcla.21545

Published online in Wiley Online Library (wileyonlinelibrary.com).

studies reported that some ANA-negative samples may present positive antibodies to SSA, even though SSA-transfected cells were used (11, 12).

By far, fluorescence characteristics on HEp-2 cells and clinical significance of anti-SSA and -SSB antibodies have remained to be investigated in detail in Chinese population. Therefore, in the present study, we evaluated fluorescence characteristics and clinical significance of these antibodies and explored whether identification of these antibodies was necessary in serum samples referred for ANA testing.

MATERIALS AND METHODS

During 5th August 2008 and 20th August 2010, 4,978 consecutive serum samples were submitted to our laboratory to screen for the presence of antinuclear antibodies and identify the positive anti-SSA (Ro60) and -SSB (La) antibodies. In order to analyze clinical significance of positive anti-SSA (Ro60) and -SSB (La) antibodies, available medical records of patients could be reviewed. Screening for and titration of ANAs and identification of anti-SSA and -SSB antibodies were performed, respectively, using indirect immunofluorescence with HEp-2 cells and immunoblot (EUROIMMUN, Schleswig-Holstein, Germany), according to the manufacturer's instructions. All the sera were diluted by 1:100, 1:1,000 and 1:10,000 for routine screening of ANAs and were diluted by 1:101 for identification of anti-SSA and/or -SSB antibodies. In samples with positive anti-SSA or -SSB antibodies, sera with <1:100 titer were successively diluted by 1:80 and 1:40 according to suggestions by published series (13–15). Immunofluorescence readings, i.e., fluorescence strength and pattern, were carried out by two experienced medical technologists. Since samples were obtained from patients as part of routine screening for autoantibodies in the clinical laboratory, there was no informed consent.

In the present study, ADs included SS, SLE, rheumatoid arthritis (RA), scleroderma, mixed connective tissue disease (MCTD), polymyositis/dermatitis (PM/DM), PBC, autoimmune hepatitis (AIH), behcet's disease, and multiple sclerosis. All of ADs were diagnosed according to the international criteria for classification (i.e., Diagnostic Criteria of the European Study Group for SS (16), 1997 American College of Rheumatology [ACR] criteria for SLE (17), American Rheumatism Association 1987 revised criteria for the classification of RA (18), etc.).

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 11.0 software. Nonparametric statistics including Fisher's and/or chi-square tests (according to the number of patients) were used to determine the significance of the differences be-

tween groups. Results were considered significant when the two-tailed *P*-value was <0.05.

RESULTS

Of 4,978 samples submitted to the laboratory, 446 (8.9%) had positive anti-SSA antibody, and 227 (4.6%) had positive anti-SSB antibodies. Samples containing anti-SSA and/or -SSB antibodies could be divided into three groups: SSA+SSB–, SSA+SSB+, and SSA–SSB+. The numbers of SSA+SSB–, SSA+SSB+, and SSA–SSB+ samples were 248, 198, and 29, respectively (Table 1). There were 23 (9.3%) negative specimens with the antinuclear antibody assay, 76 (30.6%) with low-to-intermediate titer (1:40–1:1,000), 77 (31.0%) with intermediate-to-high titer (1:1,000–1:10,000), and 72 (29.0%) with high titer (>1:10,000) in SSA+SSB– group, respectively, versus 5 (17.2%), 13 (44.8%), 8 (27.6%), and 3 (10.3%) in SSA–SSB+ group. Compared with SSA+SSB– group and SSA–SSB+ group, SSA+SSB+ group showed a lower proportion (0.5%) of negative specimens ($\chi^2 = 14.9504$, $P < 0.001$ vs. SSA+SSB– group; $\chi^2 = 27.5338$, $P < 0.001$ vs. SSA–SSB+ group), and a higher proportion (53.5%) of specimens with high titer ($\chi^2 = 26.5515$, $P < 0.001$ vs. SSA+SSB– group; $\chi^2 = 17.2134$, $P < 0.001$ vs. SSA–SSB+ group). Of 29 ANA-negative samples containing anti-SSA and/or -SSB antibodies, three samples were obtained from SLE patients, three from PBC patients, two from RA patients, two

TABLE 1. Immunofluorescence Patterns or Titers on Hep-2 Cells of SSA and/or SSB Antibody-Positive Specimens (*n* = 475)

Immunofluorescence patterns or titers on Hep-2 cells	SSA+SSB– samples <i>n</i> = 248	SSA+SSB+ samples <i>n</i> = 198	SSA–SSB+ samples <i>n</i> = 29
Negative (<1:40)	23 (9.3%)	1 (0.5%)*,**	5 (17.2%)
1:40–1:80	17 (6.9%)	5 (2.5%)	4 (13.8%)
1:80–1:100	12 (4.8%)	3 (1.5%)	2 (6.9%)
1:100–1:1000	47 (19.0%)	15 (7.6%)*	7 (24.1%)
1:1000–1:10000	77 (31.0%)	68 (34.3%)	8 (27.6%)
≥1:10,000	72 (29.0%)	106 (53.5%)*,**	3 (10.3%)
Speckled pattern	121 (48.8%)	128 (64.6%)	6 (20.7%)
Homogeneous pattern	25 (10.1%)	15 (7.6%)	3 (10.3%)
Combined speckled and homogeneous pattern	23 (9.3%)	32 (16.2%)	3 (10.3%)
Centromere pattern	16 (6.5%)	6 (3.0%)	3 (10.3%)
Nucleolar pattern	4 (1.6%)	4 (2.0%)	0
Nuclear dots	4 (1.6%)	3 (1.5%)	0
Peripheral nucleoplasmic pattern	3 (1.2%)	1 (0.5%)	3 (10.3%)

* $P < 0.05$, compared with SSA+SSB– samples; ** $P < 0.05$, compared with SSA–SSB+ samples.

from systemic sclerosis (SSc) patients, nine from patients with other diseases, and ten from patients with unknown clinical information. Besides speckled pattern, anti-SSA-and/or -SSB antibody-positive samples presented various patterns on HEp-2 cells, such as homogeneous pattern, combined speckled and homogeneous pattern, centromere pattern, nucleolar pattern, nuclear dots, and peripheral nucleoplasmic pattern. Of samples with homogeneous pattern, 12 were from SLE patients, 2 from SS patients, 1 from RA patients, 10 from patients with other diseases, 18 from patients with unknown clinical information. Of samples with combined speckled and homogeneous pattern, 16 were from SLE patients, 4 from SS patients, 2 from RA patients, 1 from MCTD, 13 from patients with other diseases, 22 from patients with unknown clinical information. Of samples with centromere pattern, 1 was from SLE patient, 1 from PBC patient, 1 from scleroderma patient, 3 from patients with other diseases, 19 from patients with unknown clinical information. The data for other patterns were not described in detail, because they did not show any significance. Furthermore, 64.6% of SSA+SSB+ samples presented speckled pattern, significantly higher than that of SSA+SSB- (48.8%) and SSA-SSB+ samples (20.7%) ($\chi^2 = 11.2249$, $P < 0.001$ vs. SSA+SSB- group; $\chi^2 = 18.4327$, $P < 0.001$ vs. SSA-SSB+ group). Of note, considerable rate of anti-SSA- and/or -SSB antibody-positive samples presented centromere pattern (6.5% in SSA+SSB- group and 10.3% in SSA-SSB+ group).

A total of 475 specimens containing anti-SSA or -SSB antibodies were obtained from 302 different individuals. Reliable clinical information from the attending physicians was obtained for 259 of these 302 individuals, so clinical characteristics of the 259 patients were further analyzed (see Table 2). Eighty-eight (34.0%) patients had SLE alone; 34 (13.1%) had SS alone; 12 (4.6%) had RA alone; 7 (2.7%) had SLE and SS; 4 (1.5%) had RA and SS; 5 (1.9%) had MCTD; 5 (1.9%) had SSc; 3 (1.2%) had PBC; 2 (0.8%) had AIH; 1 (0.4%) had DM; 1 (0.4%) had Behcet's disease; 68 (26.3%) had other diseases. Compared with SSA+SSB- group, SSA+SSB+ group showed significantly higher prevalence of SS (22.4% in SSA+SSB+ group vs. 6.3% in SSA+SSB- group), and lower prevalence of other diseases (21.5% in SSA+SSB+ group vs. 47.2% in SSA+SSB- group). For SSA-SSB+ group, each of eight patients had unique disease (SS, MCTD, liver cirrhosis by hepatitis B virus, coronary heart disease, tuberculosis, nephrotic syndrome, glomerular nephritis, or purpura nephritis).

By analyzing the laboratory databank, we found that, of the 302 individuals, 71 anti-SSA and/or -SSB antibody-positive patients had blood drawn on two or more occasions over the 2-year study period. The number of tests per patients was 2.7 ± 1.1 (range, 2–7). In seven of the 71

TABLE 2. Clinical Data of Patients with Anti-SSA and/or Anti-SSB Antibodies ($n = 259$)

	SSA+SSB- samples $n = 144$	SSA+SSB+ samples $n = 107$	SSA-SSB+ samples $n = 8$
SLE	50 (34.7%)	38 (35.5%)	0
SLE+SS	3 (2.1%)	4 (3.7%)	0
SS	9 (6.3%)	24 (22.4%)	1 (12.5%)
RA	7 (4.9%)	5 (4.7%)	0
RA+SS	0	4 (3.7)	0
MCTD	2 (1.4%)	2 (1.9%)	1 (12.5%)
Scleroderma	2 (1.4%)	3 (2.8)	0
DM	0	1 (0.9%)	0
PBC	2 (1.4%)	1 (0.9%)	0
AIH	0	2 (1.9%)	0
Behcet's disease	1 (0.6%)	0	0
Other diseases ^a	68 (47.2%)	23 (21.5%)	6 (75%)

^aIncluding liver diseases (except autoimmune liver diseases), kidney diseases, diabetes mellitus, cardiovascular disease, and respiratory infection.

patients (two with SLE, one with SSc, three with other diseases, and one with unknown clinical information), anti-SSA antibodies was observed in some but not all samples, so was anti-SSB antibodies in six of the 71 patients (four with SLE, one with SSc, one with unknown clinical information).

DISCUSSION

In the present study, we analyzed fluorescence characteristics on HEp-2 cells and clinical significance of anti-SSA and/or -SSB antibodies in Chinese population. The results displayed that anti-SSA antibody was present in 8.9% of consecutive sera submitted to our laboratory and SSB in 4.6%. This proportion was significantly higher than that reported in previous studies (10, 19). We speculated that the reason may lie in different serum collection. In previous studies, consecutive sera were submitted to the laboratory to screen for ANA, and only ANA-positive samples were selected to further identify for anti-SSA and -SSB antibodies. However, in the present study, consecutive sera submitted to our laboratory for both screening for ANA and identifying anti-SSA and -SSB antibodies were ordered by clinicians. Under this condition, anti-SSA and -SSB antibody-associated diseases have been highly suspected by clinicians. Of note, considerable samples with anti-SSA or -SSB antibodies alone showed very low fluorescence intensities (with $<1:40$ titer) or negative reactivity in the indirect immunofluorescence antinuclear antibody assay. Furthermore, several ANA-negative samples containing anti-SSA and/or -SSB antibodies were obtained from clinically well-defined patients. The reason might be explained, in part, by low abundance of

SSA and diffusion of the antigen from the nucleus during fixation and subsequent sample preparation. However, Bossuyt et al. found that HEp-2000 cells over-expressing SSA also failed to detect anti-SSA antibodies in one-fifth of patients, which can not be explained well (10). In contrast, the prevalence of very high intensities (with titer $> 1:10,000$) was significantly increased in samples with both anti-SSA and -SSB antibodies (53.5%), compared with that in samples with single anti-SSA (29.0%) or -SSB antibodies (10.3%), suggesting that anti-SSA and anti-SSB antibodies might offset and enhance reactivity on HEp-2 cells each other. These results, combined by a previous study (12), suggested that only screening for ANA and selecting ANA-positive samples for identification for anti-SSA and -SSB antibodies may be insufficient, since a part of samples with very low fluorescence intensities presented anti-SSA or -SSB antibodies.

Although the majority of samples containing anti-SSA antibodies presented speckled fluorescence pattern on HEp-2 cells as expected (20), a number of samples showed other patterns, especially homogeneous and centromere patterns, as well as ANA-negative pattern, comparable with the result reported in a previous study (19). Furthermore, samples with anti-SSB antibodies but without anti-SSA antibodies showed no specificity for fluorescence patterns as described above, which needs to be confirmed in a larger samples, since, frankly speaking, the frequency of single anti-SSB reactivity without anti-SSA was slightly small. Anyway, our current results demonstrated that the identification for anti-SSA and -SSB antibodies is necessary regardless of titer and pattern on HEp-2 cells in ANA screening, when SSA- and SSB-associated diseases were suspected.

In addition, we found, in the current study, that the most common disease is SLE in patients with positive anti-SSA and/or -SSB antibodies, followed by SS, RA, MCTD, SSc, PBC, AIH, and other diseases. The presence of anti-SSA antibodies simultaneously with anti-SSB reactivity seems to be more specific for SS, since the prevalence of SS was significantly increased in SSA+SSB+ group for SS, compared with that in SSA+SSB- and SSA-SSB+ groups. To our surprise, the presence of single anti-SSB antibodies without anti-SSA reactivity did not show any indicative significance for ADs, which may be associated with too low prevalence of single anti-SSB-positive antibody in this study.

Because of limited number of samples, we, with caution, consider anti-SSA antibodies (especially with anti-SSB reactivity) as stable autoantibodies with indicative significance for SS. Since anti-SSA or -SSB antibodies rarely became negative once detected, frequent identifications of the two antibodies seems not to be needed for diagnosis of SS.

When interpreting above results, we have to address a limitation in the present study. Because only immunoblot method was used to identify anti-SSA and -SSB antibodies in our laboratory, the results were not confirmed by other methods. Although it has been reported that the immunoblot assay is more sensitive than immunodiffusion for the detection of anti-ENA antibodies and may replace immunoprecipitation as a second line test after immunofluorescence on HEp-2 cells (19), the sensitivity of this method still needs to be further improved, when compared with that of ELISA. Accordingly, some conclusions should be drawn with caution.

In conclusion, the present study has three important implications. First, the identification of anti-SSA and -SSB antibodies is necessary, regardless of ANA-screening result on HEp-2 cells, when corresponding diseases were suspected. Second, the specificity of anti-SSA antibody for the diagnosis of SS, when combined with anti-SSB antibody, although the presence of anti-SSB antibody alone seems to be insignificant for auxiliary diagnosis of diseases. Third, anti-SSA and -SSB antibodies are comparatively stable especially in SS, so SS patients need not be identified frequently for these antibodies.

ACKNOWLEDGMENTS

This study was supported by three grants from the China National Natural Science Foundation Council (81001333 and 81102262) and Changzheng Hospital Funds for Young Scholar (2011CZQN06).

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