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ORIGINAL ARTICLE

The relationships between titers of anti-Ro or anti-La as measured by ELISA and salivary production rate with age correction

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Abstract The objective of this work was to clarify the clinical significance of titers of anti-Ro and anti-La, the relationships between titers of either anti-Ro or anti-La, and salivary production rate (SPR). These autoantibodies were titrated using enzyme-linked immunosorbent assay. The Saxon test was performed to measure SPR. Fifty-one females who had anti-Ro but not anticentromere antibodies or anti-U1RNP were enrolled. SPR decreased significantly with age. In order to exclude the effect of aging on SPR, we calculated the "SPR with age correction." According to the results of a multiple regression analysis, only the anti-La titer was significantly associated with SPR with age correction. The distribution pattern of the anti-La titers consisted of two subgroups (with a titer index cutoff of 100.0): a negative anti-La titer (anti-La < 25.0) and low anti-La titer ($25.0 \leq$ anti-La < 100.0) group, and a high anti-La titer group (anti-La ≥ 100.0). The concentration of serum IgG and the frequency of Sjögren's syndrome in the high anti-La titer group were significantly higher than those in the negative anti-La and low anti-La titer group. Several new aspects of the clinical significance of titrating anti-Ro and anti-La in comparison with SPR have been revealed.

Keywords Anti-Ro/SSA antibodies · Anti-La/SSB antibodies · Titer · Saliva · ELISA

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Introduction

Anti-Ro/SSA (anti-Ro) and anti-La/SSB antibodies (anti-La) are closely associated with Sjögren's syndrome (SS) [1]. Anticentromere antibodies (ACA) and anti-U1RNP antibodies (anti-U1RNP) are also associated with sicca syndrome [2–8]. We previously demonstrated that the salivary production rate (SPR) decreases with age in subjects with anti-Ro and/or anti-La. The degree of SPR with age among the groups was as follows: control group > group with anti-Ro alone > group with anti-Ro and anti-La. If a subject without sicca symptoms has these autoantibodies, they should not be diagnosed with SS. Nevertheless, sicca symptoms may become overt after several years in such a subject [2].

Most of the studies concerning anti-Ro and anti-La have focused on the use of their seropositivities to diagnose SS [9, 10]. Recently, the titers of anti-Ro and anti-La have been shown to be quantitatively measured by enzyme-linked immunosorbent assay (ELISA) [11–13]. Nevertheless, the clinical significance of the titers of anti-Ro and anti-La in the serum has not been fully elucidated.

The purpose of this study was to clarify the relationships between the titers of anti-Ro or anti-La and SPR in subjects with these autoantibodies, independent of clinical diagnoses. In order to accomplish this, we titrated these antibodies using ELISA.

Subjects and methods

Subjects

Female subjects who had anti-Ro but did not have ACA or anti-U1RNP were enrolled in this study. Specifically, when

subjects suffering from dry mouth and/or dry eyes visited our practice, their sicca-associated antibodies (ACA, anti-Ro, and anti-La) were examined. When subjects suffering from systemic sclerosis (SSc) and/or Raynaud's phenomenon visited our practice, their SSc-associated antibodies (ACA, anti-U1RNP, and anti-Scl-70) were examined. For subjects visiting our clinic for other reasons, their seropositivities for rheumatoid factor (RF) and antinuclear antibodies (ANA) were examined. If a subject showed positivity for at least one of these factors, their seropositivity for other autoantibodies (sicca-associated antibodies, SSc-associated antibodies, anti-Jo-1, and anti-Sm) was also examined. Through these systematic examinations, the autoantibodies in each subject were specified. The exclusion criteria in this study were subjects with diabetes mellitus, psychiatric disorders, fever (>37.3°C), dehydration, smoking habit, past head and neck radiation treatment, hepatitis C infection, acquired immunodeficiency disease, pre-existing lymphoma, sarcoidosis, graft versus host disease, and (1) taking drugs that affect SPR, such as anticholinergics, antidepressants, antihistamines and diuretics, or (2) consuming alcohol at levels of ≥40 g/day. SPR was measured at least 48 h after the last intake of alcohol.

Fifty healthy females were used as the control group.

Methods

The seropositivity for ANA was examined by an indirect immunofluorescence kit using HEp-2 cells (Fluoro-HEPANA® test, MBL, Nagoya, Japan). Anti-U1RNP, anticentromere protein B (CENP-B), anti-La, anti-Scl-70, anti-Jo-1, and anti-Sm antibodies were titrated by ELISA kits using a recombinant protein (MESACUP®-2 test, MBL); anti-Ro was titrated using an affinity-purified native antigen (MESACUP®-2 test, MBL). RF and IgG were titrated by immunonephelometry (Dade Behring, Eschborn, Germany). Index readings of ≥22.0 for anti-U1RNP, ≥16.0 for anti-CENP-B, ≥25.0 for anti-La, ≥24.0 for anti-Scl-70, ≥18.0 for anti-Jo-1, ≥30.0 for anti-Sm, ≥30.0 for anti-Ro, or >20 IU/ml for RF were considered to be positive. The titers of anti-Ro or anti-La were measured at least once. They were repeatedly measured in 13 subjects.

An index reading of <1.0 for anti-La as measured by ELISA was negative: the cutoff value was determined using 162 normal sera by the manufacturer. Index readings of between 1.0 and 25.0 were determined as being in "a gray zone" when the results obtained using double immunodiffusion (DID) were compared with those obtained using ELISA (MESACUP SS-B/La test, instruction booklet, MBL). Yoshida et al. [14] also compared the results from 243 sera samples as measured by DID and ELISA. They described that if the data in the gray zone were taken to be negative, the sensitivity, specificity, and accuracy were the same as or higher than those obtained if the data were taken to be positive.

The Saxon test was performed in order to measure SPR (g/2 min), as previously described by Kohler and Winter [15]. The Schirmer I test was performed in order to measure the tear production rate (mm/5 min) according to published guidelines [16]. The data for the eye that showed the lowest tear production rate in the subject were adopted for the analysis.

Statistical analysis

Comparisons between two groups were performed using a Mann–Whitney U test. Comparisons between the means of multiple groups were made using ANOVA. Post hoc significance was assessed using a Games–Howell multiple comparison test. Correlations between the two parameters were analyzed using Pearson's correlation coefficient. A multiple regression analysis using a stepwise procedure was performed in order to examine the association between a specific parameter and several factors. P values of <0.05 were considered to be significant. For statistical analysis, each titer of ANA was converted to "log of ANA titer" (formula used to calculate this: base 2 logarithm of ANA value/20).

Results

The number of anti-Ro-positive subjects, who were all female, was 51; the age, 54 ± 14 years (mean ± SD, range 22–83); SPR, 2.69 ± 1.45 g/2 min; tear production rate, 2.8 ± 3.5 mm/5 min; anti-Ro titer index [for those attaining the cutoff level or more (index ≥30.0)], 109.9 ± 32.8; and anti-La titer index [including the 19 subjects who were lower than the cutoff level (index <25.0)], 69.9 ± 75.2. Forty subjects (78.4%) were diagnosed as having SS; 8 (15.7%) had systemic lupus erythematosus (SLE) 6 (11.8%) had rheumatoid arthritis (RA); 3 (5.9%) had SSc. The control group comprised 50 females (and no males); age, 52 ± 14 years (mean ± SD); SPR, 4.98 ± 2.20 g/2 min. The SPR values for the positive anti-Ro subjects were significantly lower than those for the control group ($P < 0.001$).

SPR in all of the positive anti-Ro subjects significantly decreased with age [Fig. 1b; simple linear regression: $y = -0.0253x + 2.69$, where y = SPR (g/2 min), x = years of age, $r = -0.292$, $P < 0.05$]. In contrast, the SPR of the control group did not decrease with age (Fig. 1a; $y = -0.0088x + 5.465$, $r = -0.036$, $P = 0.806$). Schirmer I tests were performed in 27 positive-anti-Ro subjects. The results showed that tear production rate did not

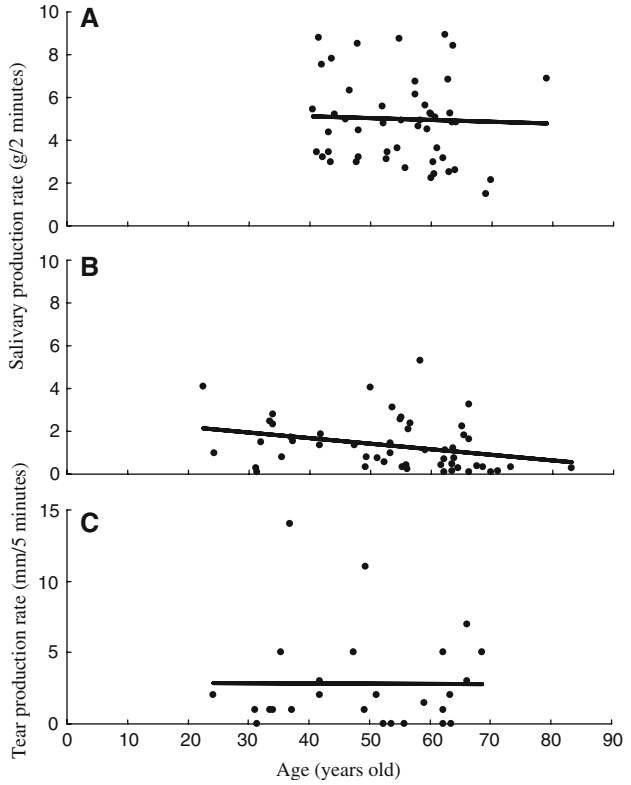

Fig. 1 The relationship between the salivary production rate (SPR) (*vertical axis*) and age (*horizontal axis*) in the control group [**a**, $n = 50$, $y = -0.0088x + 5.465$, where $y =$ SPR (g/2 min), $x =$ years of age, $r = -0.036$, $P = 0.806$], and in all of the positive-anti-Ro subjects (**b**, $n = 51$, $y = -0.0253x + 2.69$, $r = -0.292$, $P < 0.05$). The relationship between the tear production rate and age in 27 positive-anti-Ro subjects [**c**, $n = 27$, $y = -0.0016x + 2.90$, where $y =$ tear production rate (mm/5 min), $x =$ years of age, $r = -0.006$, $P = 0.977$] is also shown. The SPR was measured using the Saxon test. The tear production rate was measured using the Schirmer I test

decrease with age [Fig. 1c; $y = -0.0016x + 2.90$, where $y =$ tear production rate (mm/5 min), $x =$ years of age, $r = -0.006$, $P = 0.977$].

There was a relationship between SPR and anti-Ro titer that was not statistically significant [simple linear regression: $y = -0.0101x + 2.44$, where $y =$ SPR (2 g/2 min), $x =$ titer of anti-Ro (index), $r = -0.276$, $P = 0.050$, Fig. 2a], and a relationship between SPR and anti-La titer that was statistically significant [simple linear regression: $y = -0.0062x + 1.76$, where $y =$ SPR (2 g/2 min), $x =$ titer of anti-La (index), $r = -0.453$, $P < 0.01$, Fig. 2c]. In this study, the mean age of the positive-anti-Ro subjects was 54 years. Therefore, in order to exclude the effect of aging on SPR, we used a parameter termed "SPR with age correction," which was calculated using the following formula that assumes that all of the positive-anti-Ro subjects were 54 years of age: SPR + 0.0253 × (age − 54). There were significant relationships between "SPR with age correction" and anti-Ro or anti-La titer [simple linear regression: $y = -0.011x + 1.37$, where $y =$ SPR with age correction, $x =$ titer of anti-Ro (index), $r = -0.314$, $P < 0.05$, Fig. 2b; $y = -0.0061x + 1.74$, where $y =$ SPR with age correction, $x =$ titer of anti-La (index), $r = -0.398$, $P < 0.01$, Fig. 2d; respectively].

The constant "SPR with age correction" was compared simultaneously with seven variables: anti-Ro, anti-La, log of ANA titer, RF, IgG, WBC, and C-reactive protein in all of the positive-anti-Ro subjects. Only anti-La titer was significantly associated with "SPR with age correction" (Table 1).

The distribution pattern of the anti-Ro titers showed just one peak (upper panel, Fig. 3). In contrast, that of the anti-La titers exhibited two subgroups (cutoff anti-La titer index was 100.0; see lower panel in Fig. 3). Based on the titers of anti-La, all of the positive-anti-Ro subjects were subdivided into two groups: a combined group comprising a negative-anti-La group and a low anti-La titer group (anti-La <100.0 index, $n = 36$) and the group of high anti-La titer group (anti-La ≥ 100.0, $n = 15$). Among the various demographic features, the concentration of serum IgG and the frequency of SS in the high anti-La titer group were significantly higher than those in for the group with negative anti-La titers ($P < 0.01$ and $P < 0.01$, respectively), and the group with low anti-La titers ($P < 0.01$ and $P < 0.05$, respectively). The frequency of disease(s) other than SS in the negative-anti-La titer group was significantly higher than that in the high-anti-La titer group ($P < 0.05$, Table 2).

Figure 4 shows the titers of anti-Ro or anti-La measured repeatedly during 2001–2006 in 13 subjects (panels A and B). These titers were almost unchanged over this period, and none of the subjects in the low-anti-La titer group or the high-anti-La titer group crossed the anti-La titer index cutoff of 100.0. There was no significant relationship between the titers of anti-Ro and age [panel C; simple linear regression: $y = -0.195x + 120$, where $y =$ titer of anti-Ro (index), $x =$ age (years old), $r = -0.082$, $P = 0.565$].

Discussion

We previously demonstrated that SPR decreased with age in groups with any of ACA, anti-Ro and/or anti-La, but which did not fulfill the classification criteria for SS [2]. In contrast, in healthy control subjects, SPR did not decrease with age (Fig. 1a). It wasecently found that it is not rare for subjects to have these sicca-associated autoantibodies long before the sicca symptoms become overt [2, 17]. Therefore, in the present study, we analyzed subjects with these autoantibodies irrespective of whether they were diagnosed with SS or not.

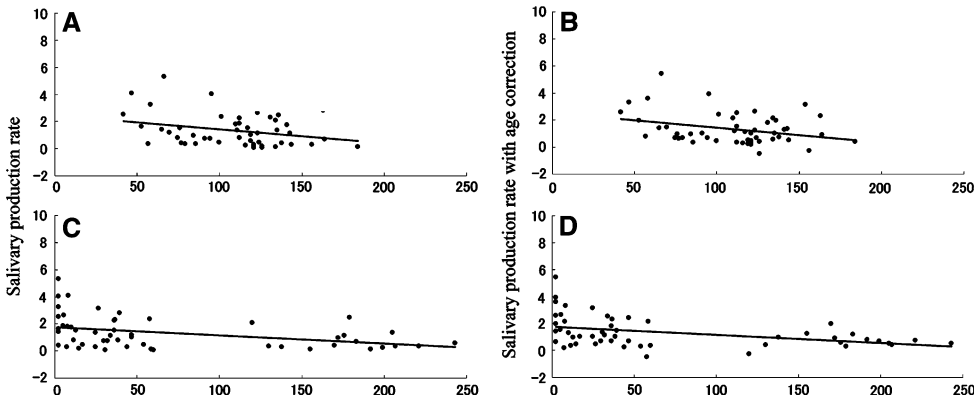

Fig. 2 The relationships between SPR and anti-Ro titer [**a** simple linear regression: $y = -0.0101x + 2.44$, where y = SPR (2 g/2 min), x = titer of anti-Ro (index), $r = -0.276$, $P = 0.050$] and SPR and anti-La titer [**c** simple linear regression: $y = -0.0062x + 1.76$, where y = SPR (2 g/2 min, x = titer of anti-La (index), $r = -0.453$, $P < 0.01$]. In order to exclude the effect of age on SPR, we investigated the relationships of the parameter termed "SPR with age correction" to the titers of the autoantibodies. "SPR with age correction" was calculated assuming that all of the subjects were 54 years of age using the following formula: SPR + 0.0253 × (age − 54). The relationships between "SPR with age correction" and the anti-Ro titer [**b** simple linear regression: $y = -0.011x + 1.37$, where y = SPR with age correction, x = titer of anti-Ro (index), $r = -0.314$, $P < 0.05$] and "SPR with age correction" and anti-La titer [**d** simple linear regression: $y = -0.0061x + 1.74$, where y = SPR with age correction, x = titer of anti-La (index), $r = -0.398$, $P < 0.01$] are therefore also shown

Table 1 Results from a multiple regression analysis using a stepwise procedure that was performed to examine the associations between factors and "SPR with age correction" in all of the positive-anti-Ro subjects

Independent variable	Unbalanced recurrence coefficient		Standard unbalanced recurrence coefficient β	t	P
	B	SD			
Fixed number	2.392	0.613		3.900	0.000
Titer of anti-Ro (SSA) antibodies	−0.007098	0.005	−0.203	−1.452	0.153
Titer of anti-La (SSB) antibodies	−0.005365	0.002	−0.352	−2.342	0.024
Log of ANA titer[a]	0.02555	0.078	0.047	0.327	0.745
C-reactive protein (CRP)	−0.06161	0.158	−0.052	−0.389	0.699

SPR salivary production rate, *ANA* antinuclear antibodies

Induced variable: SPR with age correction, $R^2 = 0.202$, $F = 2.906$, $P < 0.05$

Rheumatoid factor, IgG and white blood cells were excluded by stepwise selection

Calculating formula: SPR (g/2 min) + 0.0253 × [age (years old) − 54]

[a] For statistical analysis, each titer of ANA was converted to "log of ANA titer" (calculated using: base 2 logarithm of ANA value/20)

Some researchers have pointed out that the sensitivity and specificity of the Schirmer I test for evaluating sicca symptoms of the eyes are not very high [18, 19]. Hay et al. [20] stated that the association between oral symptoms and whole salivary flow was stronger than that between ocular symptoms and tear production rate as measured by the Schirmer I test. Among all of the positive-anti-Ro subjects, SPR decreased with age (Fig. 1b). In contrast, tear production rate did not decrease with age (Fig. 1c). Moreover, SPRs were measurable (results were expressed as >0 g/2 min) in all of the positive-anti-Ro subjects (Fig. 1b), whereas, tear production rate was not measurable (results were expressed as 0 mm/5 min) in 6 of the 27 positive-anti-Ro subjects (Fig. 1c). These differences between the Saxon test and the Schirmer I test may stem from the difference between the sizes of the lachrymal glands and the salivary glands. Therefore, we adopted the data obtained by the Saxon test, namely on SPR alone, for comparison with anti-Ro or anti-La titers.

SPR decreased with age in subjects with anti-Ro and/or anti-La (Fig. 1b). In other words, age is a factor that affects SPR in subjects with these antibodies. When the effect of subject age was excluded, the titers of these antibodies were negatively correlated with SPR (Fig. 2a, c). The ages of the subjects of this study ranged from 22 to 83 years old. To exclude the effect of age on SPR, we introduced a parameter termed the "SPR with age correction," which was calculated by assuming that all of the subjects were 54 years of age, and then used this parameter in the following analysis. SPR with age correction was significantly negatively

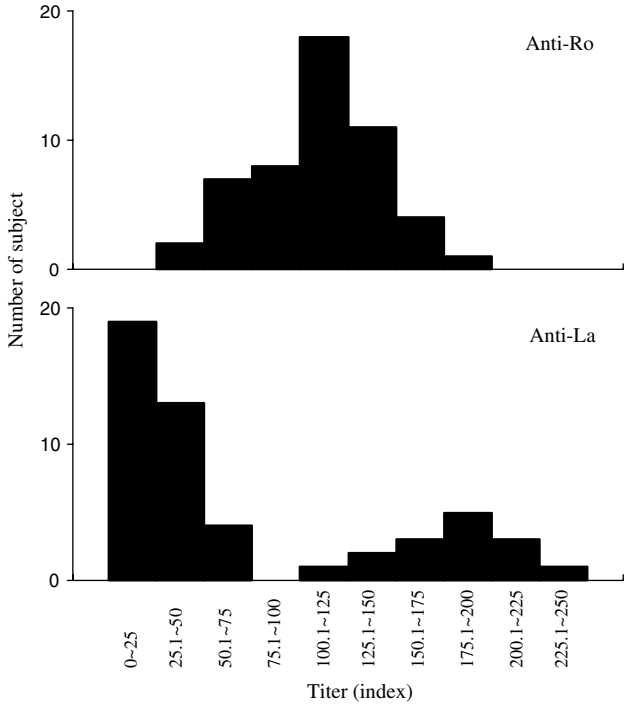

Fig. 3 Histograms of the anti-Ro titers (*upper panel*) and the anti-La titers (*lower panel*), as measured by ELISA for all of the subjects. *Vertical axis* number of patients, *horizontal axis* titers of autoantibodies

correlated with the titers of anti-Ro (Fig. 2b) and anti-La (Fig. 2d). Therefore, "age" and "the titers of these antibodies" are both factors that affect SPR. More accurately, "disease duration" should be a factor that influences SPR. Nevertheless, we did not use "disease duration" in any analysis in this study, because patients with sicca symptoms often cannot accurately recall when their sicca symptoms started. Instead, we used "age," which should be a surrogate parameter for "disease duration."

In SLE patients, Hassan et al. [13] analyzed the sequential levels of these antibodies over 18–44 months. In most patients, little variation in the titers was observed. In the present study, when the titers of anti-Ro and anti-La were repeatedly measured in 13 patients, they were almost unchanged during the observation period (Fig. 4), indicating that the production levels of these antibodies were maintained at a constant level in each subject. The fact that the titers of these autoantibodies remained constant over the years (Fig. 4) may be due, at least in part, to the fact that immunosuppressive therapy is not usually performed in patients with sicca syndrome alone. The titers of these autoantibodies may be closely associated with the intensity of the destructive process in the salivary glands. Focal mononuclear cell infiltration of exocrine glands and the presence of autoantibodies (especially anti-Ro and anti-La) are hallmarks of SS. Tengnér et al. [21] stated that anti-Ro and anti-La were produced and presented at sites of inflammation, and indicated their potential involvement in the autoimmune exocrinopathy of SS. Along with activated B cells, T cells, macrophages, and mast cells infiltrate into such exocrine tissues and take part in the tissue destruction [22]. Nguyen et al. [23] demonstrated that the Th17/IL-23 system was upregulated in the minor salivary glands of SS patients. We speculated that the intensity of cellular immunity in the salivary glands might be closely associated with the titers of these autoantibodies; therefore, the titers of these antibodies might be used as surrogate markers for the intensity of salivary gland destruction. In fact, Halse et al. [24] stated that there was a strong correlation between the focus score in lip biopsy specimens and titers of anti-Ro and anti-La in the serum or the saliva, as measured using ELISA.

Franceschini and Cavazzana [11] indicated that, upon comparing these two autoantibodies, the appearance of

Table 2 Demographic features

	Negative-anti-La group (group N), anti-La index < 25.0	Low-anti-La titer group (group L), $25.0 \leq$ anti-La index < 100.0	High-anti-La titer group (group H), anti-La index ≥ 100.0	*P* value		
				N versus L	N versus H	L versus H
n	19	17	15	NS	NS	NS
Age (years old ± SD)	51 ± 13	53 ± 16	58 ± 13	NS	NS	NS
IgG (mg/dl ± SD)	2,044 ± 587	2,246 ± 464	3,075 ± 857	NS	<0.01	<0.01
Rheumatoid factor (%)	12 (63.2)	10 (58.8)	11 (73.3)	NS	NS	NS
SS (%)	12 (63.2)	13 (76.5)	15 (100.0)	NS	<0.01	<0.05
Have disease(s) other than SS (%)	12 (63.2)	8 (47.1)	4 (26.7)	NS	<0.05	NS
Systemic lupus erythematosus (%)	3 (15.8)	4 (23.5)	1 (6.7)	NS	NS	NS
Rheumatoid arthritis (%)	2 (10.5)	3 (17.7)	1 (6.7)	NS	NS	NS
Systemic sclerosis (%)	3 (15.8)	0 (0.0)	0 (0.0)	NS	NS	NS
Interstitial pneumonia (%)	2 (10.5)	3 (17.7)	0 (0.0)	NS	NS	NS

SS Sjögren's syndrome

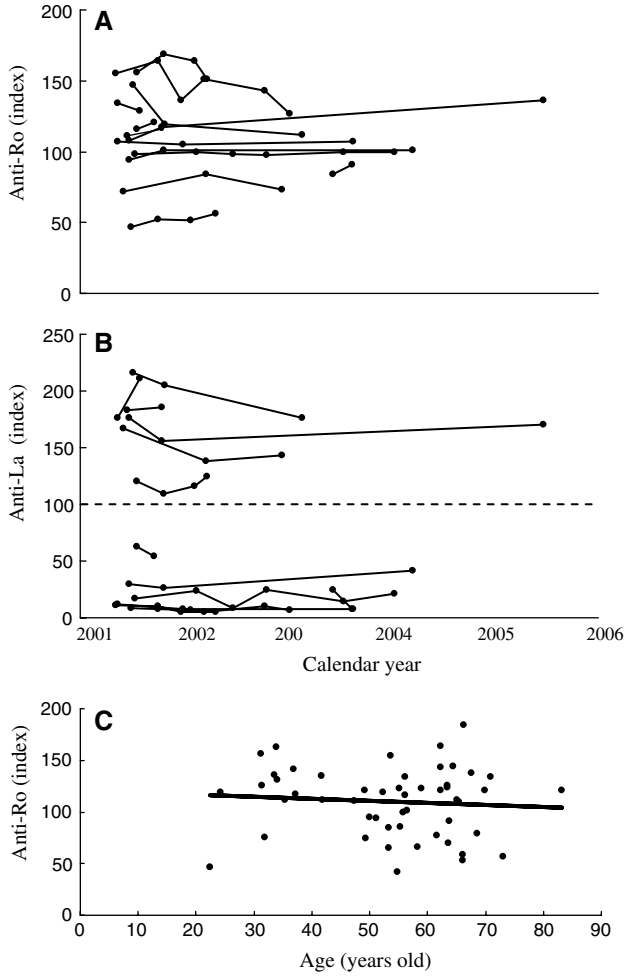

Fig. 4 The sequential titers of anti-Ro (**a**) and anti-La (**b**) in subjects who were measured twice or more ($n = 13$). Data were obtained from 2001 to 2006. The relationship between the anti-Ro titer (*vertical axis*) and age (*horizontal axis*) [**c** simple linear regression: $y = -0.195x + 120$, where $y =$ titer of anti-Ro (index), $x =$ age (years old), $r = -0.082$, $P = 0.565$] is also shown

anti-La was found to be related to the severity of SS. In the present study, we also demonstrated that the anti-La titer alone among several factors was significantly associated with SPR with age correction in all of the positive-anti-Ro subjects (Table 1).

ELISA is a sensitive and specific technique for the detection of autoantibodies. Using ELISA for the titration of anti-Ro and anti-La, we found that the distribution pattern of the anti-La titers consists of two subgroups (the anti-La titer index cutoff was 100.0; see lower panel, Fig. 3). To our knowledge, no studies demonstrating that subjects can be subdivided into subgroups according to the anti-La titer distribution have been reported previously. This distribution pattern indicates that the basic characteristics of the patients may be different between the subgroups. Tsuzaka et al. [25] described that the frequency of hypergammaglobulinemia was 67% in the group of anti-Ro alone and 96% in the group of anti-Ro and anti-La ($P < 0.025$), indicating that the appearance of anti-La is more closely associated with nonspecific immunoglobulin production than the appearance of anti-Ro alone. In the present study, the concentration of serum IgG in the high-anti-La titer group was significantly higher than that in the negative-anti-La group or the low-anti-La titer group ($P < 0.01$ or $P < 0.01$, respectively). The frequency of SS in the high-anti-La titer group (100.0%) was significantly higher than that in the negative-anti-La group or in the low-anti-La titer group (63.2%, $P < 0.01$; 76.5%, $P < 0.05$, respectively). The frequency of disease(s) other than SS, such as SLE and RA, in the negative-anti-La group (63.2%) was significantly higher than that in the high-anti-La titer group (26.7%, $P < 0.05$; Table 2). These results indicate that a high anti-La titer is closely associated with SS.

Conflict of interest statement We declare that there is no conflict of interest in this paper.

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