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Latest update on the Ro/SS-A autoantibody system

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ABSTRACT

Anti-SS-A (Ro52/Ro60) autoantibodies have been described as serological marker for Sjögren's syndrome but are also found in patients with other systemic autoimmune diseases. Historically, these autoantibodies were considered as a uniform autoantibody-system. However, recent studies provided evidence that Ro60 and Ro52 are not part of a stable macromolecular complex and that anti-Ro52 and anti-Ro60 (SS-A) antibodies have different clinical associations. The prevalence of anti-Ro52 in systemic sclerosis and myositis is significantly higher than anti-Ro60 (SS-A) and isolated anti-Ro52 can be found in up to 37% of myositis patients, often correlated with anti-Jo-1 reactivity ($p = 0.0002$). Furthermore, recent developments have made significant improvements in the quality of recombinant Ro60 showing excellent performance in Ro60 (SS-A) ELISA (Dr. Fooke Laboratorien). Of note, single reactivity to either Ro52 or Ro60 (SS-A) can be missed when measured with a classical SS-A ELISA based on a mixture of both antigens. Approximately 20% of anti-Ro52 or Ro60 (SS-A) positive samples may remain undetected using a mixture of both antigens. Moreover, the international reference sera from the Centers for Disease Control and Prevention (CDC 2, 3, 7, 10) were further characterized. It was concluded that Ro60 (SS-A) and Ro52 represent two distinct autoantibody systems and that separate detection is desirable in a clinical diagnostic setting.

Contents

1. Introduction

SS-A/Ro autoantibodies (aab) are among the most frequently detected autoantibodies (aab) and have traditionally been associated with systemic lupus erythematosus (SLE), Sjögren's

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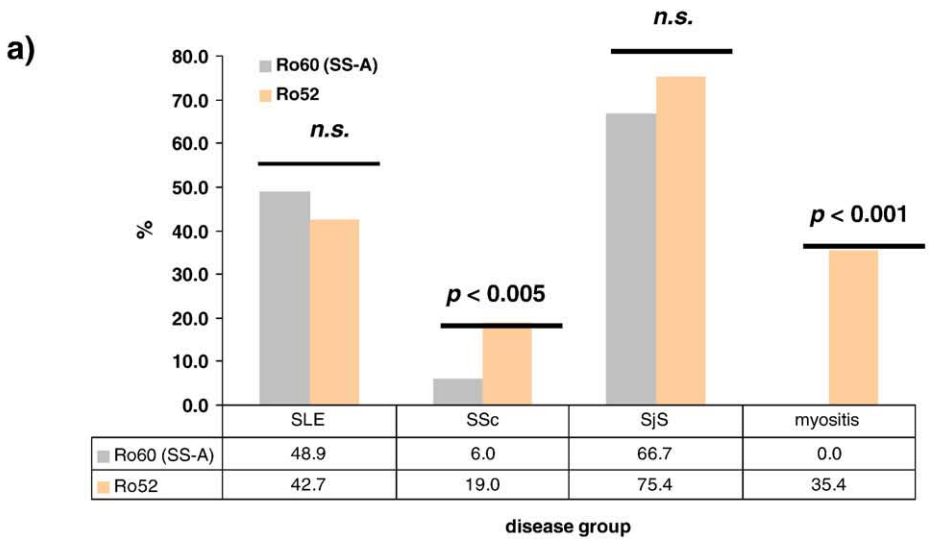

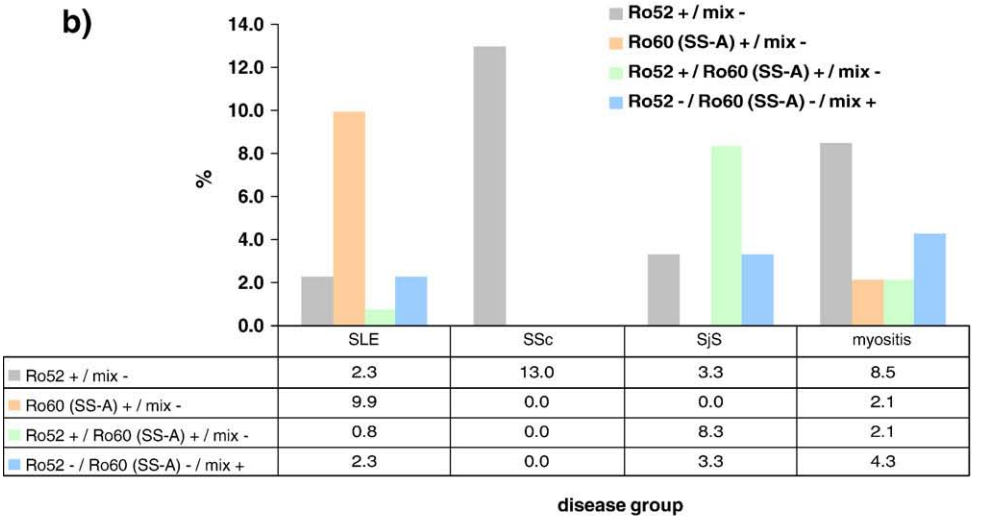

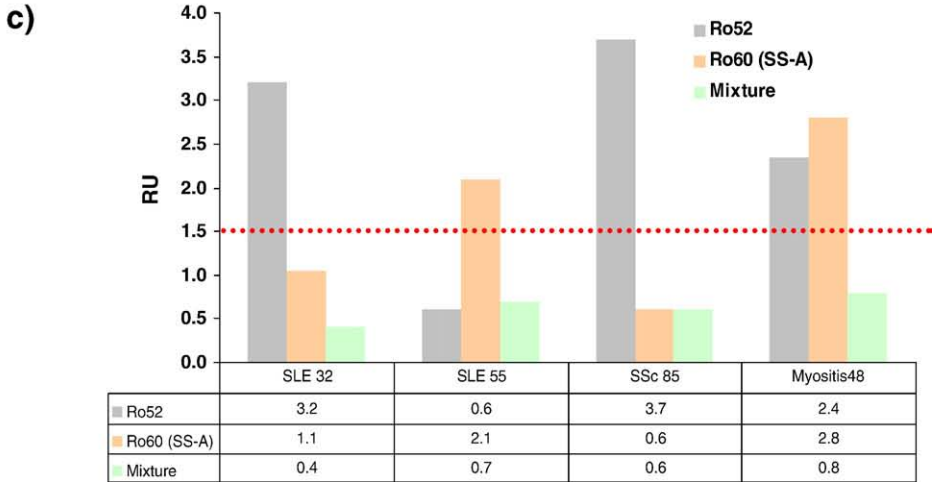

Fig. 1. Anti-Ro52 and anti-Ro60 (SS-A) reactivities in systemic autoimmune rheumatic diseases (SARD). a.) Prevalence of anti-Ro52 vs. anti-SS-A/Ro-60 in a cohort of 325 sera from patients with different SARD. The values reflect the consensus of three different detection methods: ELISA, line immunoassay (LIA) and addressable laser bead assay (ALBIA). Note the prevalence of anti-Ro52 in the SSc and especially in the myositis cohort. b.) Masking effect of anti-Ro reactivity in a mixture of Ro52 and Ro60 (SS-A) in different diseases and c.) as shown by selected examples (by ELISA). Values for c.) are expressed as relative units (RU) and the cut-off value of 1.5 RU is indicated by a red dotted line.

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Table 1
The masking effect of mixed Ro antigens in 338 sera of different systemic autoimmune rheumatic diseases

Sera	Single antigens		Mix	Isolated Ro reactivities				Maskings			Opposite
	rRo52	rRo60	rRo52 rRo60	rRo52+ / rRo60+	rRo52- / rRo60-	rRo52+ / rRo60-	rRo52- / rRo60+	Single rRo52+ mix neg	Single rRo60+ mix neg	Double single pos mix neg	Singles neg mix pos
SLE (n = 131)	65	87	78	61	40	4	26	3	13	1	3
SSc (n = 100)	23	5	10	5	77	18	0	13	0	0	0
SjS (n = 60)	45	39	45	37	13	8	2	2	0	5	2
Myositis (n = 47)	19	3	16	2	27	17	1	4	1	1	2
Σ (338)	152	134	149	105	157	47	29	22	14	7	7

SLE = Systemic lupus erythematosus; SSc = systemic sclerosis; SjS = Sjögren's syndrome.
Numbers of sera with an apparent masking effect when tested with the blended antigens are shown in the right shaded boxes.

syndrome (SjS), subacute cutaneous lupus and neonatal lupus syndrome [1–4]. Anti-SS-A/Ro aab have also been reported in systemic sclerosis (SSc) and myositis [5]. Anti-SS-A/Ro aab temporally precede other SLE associated aab such as anti-dsDNA, anti-ribonucleoprotein (RNP) and anti-Sm and are present on average 3.4 years before the diagnosis of SLE [6]. The target antigen, originally called "SjD", was first described in 1962 by Anderson et al. [7]. The subsequent double name Ro and SS-A derives from the description of this aab system by two research groups: one part of the nomenclature relating to the name of a SLE patient ("Ro") [8] and the other nomenclature related to its association with SjS ("SS"), the latter designation first published by Alspaugh and Tan in 1975 [9]. Eventually, after some of the molecular characteristics of target antigens were identified, the nomenclature became SS-A/Ro60 and SS-A/Ro52 to include the molecular masses of the respective antigens. In this manuscript we will refer to the antigens as Ro60 (SS-A) and Ro52. The primary target antigen for anti-Ro-aab was identified as a 60 kDa protein component of small cytoplasmic ribonucleoprotein complexes (hY-RNA complexes) in 1988 by Deutscher et al. [10] and shortly after by Ben Chetrit et al. [11], but only in 1991, it was confirmed by Chan et al. [12] that the Ro52 and the Ro60 (SS-A) antigens indeed consisted of two different proteins coded by different cDNAs. This review summarizes the historical milestones of the so-called Ro (SS-A) aab system and provides new insights into the association between anti-Ro52 and anti-Ro60 (SS-A) aab. Moreover, recent data on the detection of anti-Ro60 (SS-A) aab using recombinant Ro60 (rRo60) is presented and discussed. Finally, novel data of the anti-nuclear antibody (ANA) reference sera provided by the Centers for Disease Control and Prevention (CDC) is presented.

2. Ro52 is a biochemically and immunologically independent aab system

Although Ro52 and Ro60 (SS-A), which are encoded by different genes [12], were initially suggested to be closely related, a direct interaction of the proteins could never be conclusively proven. Their biological functions remained elusive for some time, but more recent studies indicate that they are localized to different cell compartments and they perform rather different functions. It was recently reported that the Ro60 (SS-A) protein, having a shape that resembles a doughnut, binds to misfolded, noncoding RNAs in vertebrate cells and acts as a quality checkpoint for RNA misfolding with molecular chaperones for defective RNAs. The misfolded RNAs are recognized and then tagged by Ro60 (SS-A) for degradation [13–15]. Epitopes on this protein were recognized by aab in sera of individuals who, within an average of 3.4 years later, developed SLE. The epitope spreading as described by McClain et al. in 2005 [16], included a peptide represented by amino acid (aa) 169 to 180 and a cross-reactive epitope encompassing aa 58–72 of the Epstein Barr virus nuclear antigen 1 (EBNA-1). The authors concluded that the Epstein Barr virus had a putative triggering effect enhancing the development of aab as a product of molecular mimicry, also reviewed by Doria et al. [17] and Poole et al. [18]. A similar observation, namely cross-reactivity between epitopes on human autoantigens and EBNA-1, was reported by Mahler et al. in 2001 [19].

The 52 kDa Ro antigen was eventually identified as a family member of the RING/Bbox/coiled-coil (RBCC) tripartite motif proteins (TRIM) and as an ubiquitin-ligase that is over-expressed in peripheral blood mononuclear cells in SjS and SLE patients [20,21]. Ro52 is reported to interact with several different molecules, among them calreticulin and a 78 kDa glucose-regulated protein (GRP78), also known as immunoglobulin heavy chain-binding protein (BIP) and formerly proposed as an early marker for rheumatoid arthritis [22]. Taking its function into consideration, Ro52 is thought to modify the role or stability of its substrates through ubiquitination, and this modification might result in the Ro52-mediated biological events [21,23].

Although the sequence of Ro60 (SS-A) was described more than 20 years ago, the 60 kDa protein seemed to be an extremely difficult recombinant protein to produce while maintaining immunological properties that were comparable to the native antigen. However, assays based on the newly available rRo60 (SS-A) antigen (Diarect AG Freiburg, Germany) show quite good agreement with its native counterpart as revealed by experiments using the setup of the Ro60 (SS-A) ELISA (Dr. Fooke Laboratorien GmbH) that studied a panel of 53 anti-Ro60 (SS-A) positive sera and a control group of 60 anti-Ro60 (SS-A) negative samples. The results showed a high degree of quantitative correlation between native and recombinant antigen with a regression correlation coefficient of 0.90 when only the positive sera were considered ($p<0.0001$) and 0.98 when positive and control sera were evaluated ($p<0.0001$). This data confirmed the excellent utility of rRo60 (SS-A) protein expressed in prokaryotes. The agreement between Ro60 (SS-A) ELISA (Dr. Fooke

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Table 2
Anti-Ro52 and anti-Ro60 (SS-A) reactivities in the CDC ANA reference serum panel, measured with three independent immunoassay systems

No.	Ro	CDC designation	LIA		ALBIA*		ELISA#		Proposed new definition
			Ro52	Ro60	Ro52	Ro60	Ro52	Ro60	
1		Homogeneous	-	-	73	63	0.4	0.6	–
2	±	Anti-La	+	-	**582$^{\pm}$**	**2098$^{\pm}$**	**2.1^{+}**	**2.4^{+}**	Anti-Ro52 (weak), Anti-Ro60, anti-La
3	+	Speckled	+	±	**512$^{\pm}$**	**3760^{+}**	**1.3$^{\pm}$**	**3.6^{+}**	Anti-Ro52 (weak), Anti-Ro60, anti-La
4		Anti-RNP	-	-	100	16	0.3	0.5	–
5		Anti-Sm	-	-	38	73	0.3	0.4	–
6		Nucleolar	-	-	149	51	0.2	0.3	–
7	+	Anti-Ro	+	+	**1313$^{\pm}$**	**5453^{+}**	**2.2^{+}**	**4.4^{+}**	Anti-Ro52 (weak), Anti-Ro60
8		Anti-CENP	-	-	184	97	0.1	0.2	–
9		Anti-Scl70	-	-	60	85	0.2	0.4	–
10		Anti-Jo-1	+	-	**17080^{+}**	130	**7.1^{+}**	0.2	Anti-Ro52, Anti-Jo-1
11		Anti-PM/Scl	-	-	55	86	0.3	0.4	–
12		Anti-Rib-P	-	-	47	70	0.2	0.4	–

*Cut-off values ALBIA are antigen dependent.
#ELISA: <1.0 RU negative; >1.0 RU = borderline; >1.5 RU positive. Borderline results are marked with ±, positives with +.

Laboratorien) and the Ro60 test contained in the Quanta Plex SLE Profile™ 8 (INOVA Diagnostics, San Diego, CA, USA; referred to as addressable laser bead assay = ALBIA) ranged from 88% to 90% dependent on the cut-off of ALBIA (data not shown). Since the new Ro60 (SS-A) is a recombinant antigen, contamination with other autoantigens can be ruled out and thus rRo60 enables true discrimination between anti-Ro52 and anti-Ro60 (SS-A) reactivities.

3. Association between anti-Ro52 and anti-Ro60 (SS-A) antibodies in different connective tissue diseases

Testing for anti-Ro60 (SS-A) and anti-Ro52 in different disease entities using three independent methods, namely line immunoassay (LIA), ALBIA and ELISA, revealed differing prevalence distribution (Fig. 1 a). The frequency of anti-Ro52 aab was similar to the frequency of anti-Ro60 (SS-A) in all groups except the myositis (35.4% vs. 0.0%, $p<0.001$) and SSc (19.0% vs. 6.0%, $p<0.005$) cohorts using the consensus of three methods. The percentages of anti-Ro52 aab that occur without anti-Ro60 (SS-A) aab also varied from 5.4% in childhood SLE to 35.4% in the myositis group. In the SjS group, 63.2% of anti-Ro52 sera had also aab to Ro60 (SS-A). If measured as the single or the stronger reactivity compared to anti-Ro60 (SS-A), anti-Ro52 aab seem, as observed by Rutjes et al. [24], to be merely associated with myositis and to a lesser extent with SSc, whereas reactivity against both antigens and to a lesser extent against Ro60 (SS-A) alone seemed to associate with SjS or SLE in the context of connective tissue diseases.

3.1. Coincidence of anti-Ro52 and anti-Jo-1 in patients with polymyositis

A strong correlation was observed in myositis sera for aab against Jo-1 and Ro52: in a panel of 43 sera of myositis patients coincidence of reactivities against Ro52 and Jo-1 was 70% in an average ($p=0.0002$, Odds ratio = 14.17, kappa = 0.54) in Jo-1 positive sera when tested with ELISA (Dr. Fooke Laboratorien) and ALBIA. Of the sera tested positive for anti-Jo-1 16/22 (72.7%) also tested positive by ELISA and 17/24 (70.8%) by ALBIA. These observations underline previous conclusions [25] that anti-Ro52 is indeed an independent aab in myositis. Rutjes et al. in 1997 [24] found anti-Ro52 reactivity in 58% of Jo-1 positive myositis sera, an observation confirmed in subsequent studies by Rozman et al., Brouwer et al. and Koenig et al. [26–28]. In contrast, Langguth et al. [29] indicated that isolated anti-Ro52 reactivity has limited clinical value in a non-obstetric population, a conclusion that could not be confirmed by our study. Our study demonstrated the importance of detecting anti-Ro52 and anti-Ro60 aab separately when considering the diagnosis of patients and in particular myositis patients. This perspective was not included in the study performed by Langguth and colleagues.

3.2. Detection of aab to Ro60 (SS-A) and Ro52

The different associations of anti-Ro aab have remained a matter of debate for over two decades, perhaps due to the lack of highly purified and diagnostically reliable recombinant antigens, which in the case of Ro60 (SS-A) has only lately become available. Traditionally, anti-Ro aab were detected by indirect immunofluorescence (IIF) on HEp-2 cells and confirmed by immunodiffusion (ID), immunoblot or ELISA, mostly using a mixture of both Ro52 and Ro60 (SS-A) as the antigens. In the mid nineties it was found that Ro60 (SS-A) is underrepresented in HEp-2 cells on certain IIF slides and, therefore, a significant portion of sera with anti-Ro reactivity had a negative IIF result [30]. As a consequence, HEp-2 cells transfected with Ro60 (SS-A) cDNA were used as an IIF substrate, an advancement that significantly increased the sensitivity of the ANA screening test [31–35]. More recently, with advances in the expression and purification of recombinant protein, immunoassays such as ELISA, LIA, ALBIA or autoantigen arrays became available that allow the separate detection of anti-Ro52 and anti-Ro60 (SS-A) aab. The importance of the separate detection of those two aab was analyzed in several studies and became a matter of debate especially since commercial ELISA kits are currently often marketed as SS-A ELISA that employ mixtures of both antigens together in a single assay.

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3.3. Hidden reactivities when using blended Ro52 and Ro60 (SS-A) antigens in one assay

A previously unreported effect of masked reactivity was observed when sera of different systemic autoimmune diseases were tested with a 1:1 mixture of rRo60 (SS-A) and rRo52 antigen (rRo60/rRo52) as well as with the respective single antigen (Table 1). We observed that 43/181 (23.8%) sera tested positive for reactivity against Ro52, Ro60 (SS-A) or both antigens in the single parameter tests were negative for anti-rRo60/rRo52. In detail, 22/152 (14.5%) anti-Ro52 positive sera were negative when tested on the antigen blend, 14/134 (10.5%) anti-Ro60 (SS-A) positive sera were negative and 7/181 (3.9%) additional sera positive in both single parameter Ro ELISAs showed no reactivity in the rRo60/rRo52 assay. The opposite effect rarely occurred: only 7/157 (4.5%) sera negative in the single parameter ELISA tests showed positive results when tested with rRo60/rRo52. Whether this effect arose due to protein–protein interaction or steric hindering of antibody binding remains a matter for future research. From what is currently known, a stable association between the two Ro proteins is unlikely, but a transient interaction is possible. Furthermore, the effect cannot be explained by lower concentration of the individual Ro antigen due to the co-coating because not only sera with a relative low titer turned negative when tested on the rRo60/rRo52 blend, but also sera with quite high titers of anti-Ro reactivity (Fig. 1 c). Additionally, no special anti-Ro reactivity seemed to be required for this effect to occur: instead the effect was found with anti-Ro52 positive sera, anti-Ro60 (SS-A) positive sera and sera with reactivity against both antigens. Although further research is mandatory to shed more light on this observation, these findings provide additional evidence and rationale for the recommendation that anti-Ro52 and anti-Ro60 (SS-A) should be detected separately with recombinant antigens.

3.4. Anti-Ro reactivities in the CDC ANA reference serum panel

With an increasing number of techniques to test aab, the availability of international standard sera represents an important tool for a better standardization of aab assays. The reference sera provided by the Serology Committee of the International Union of Immunological societies (IUIS)/World Health Organization (WHO)/Centers for Disease Control (CDC) [36–38] through the auspices of CDC are an important step forward. Although the Arthritis Foundation/CDC reference sera were originally described as reference reagents for IIF and double ID techniques, they are also useful for Western blotting and ELISA [37]. After further characterization of the ANA reference panel and publication of collected data, the reference preparations will also be useful for the standardization of multiplex tests such as LIA, and ALBIA. The anti-Ro52 reactivity of CDC 2 and CDC 7 are in good agreement with the findings of the CDC reference laboratories. In addition to CDC 2, CDC 3 and CDC 7 being described as anti-Ro positive samples, strong anti-Ro52 reactivity was detected in CDC 10 (designated an anti-Jo-1 positive specimen) with three different immunological detection methods (LIA, ALBIA and ELISA). Anti-Ro reactivity was previously detected in this serum with only a few assays, most likely because (as discussed above) anti-Ro52 was not tested as a unique and separate aab [37,39,40]. The coexistence of anti-Jo-1 and anti-Ro52 aab is in agreement with previous findings reported by Rutjes et al. [24]. Based on this data, a recharacterization of the CDC serum samples was proposed (Table 2).

4. Conclusion

In summary, differing anti-Ro52 and anti-Ro60 (SS-A) reactivities were found in a panel of various systemic autoimmune rheumatic diseases. We conclude that anti-Ro52 clearly differs in reactivity from anti-Ro60 (SS-A): Anti-Ro52 is seen in more frequently in myositis and SSc and anti-Ro60 (SS-A) in SjS and SLE compared to the respective other aab. Anti-Ro52 has a prevalence of up to 35% in myositis and in this disease group co-occurs in up to 72% of anti-Jo-1 positive sera. Based on the results of three independent assay systems, we present novel data for the CDC ANA reference sera (CDC 2, 3, 7 and 10). We further observed a previously unreported effect of masked reactivity when Ro52 and Ro60 (SS-A) antigens were blended in one assay. Further verification will be needed, but based on the observation that more than 20% of Ro positive samples remain undetected in assays that utilize blended antigens, we suggest that anti-Ro52 and anti-Ro60 (SS-A) can mask each other's reactivity. Therefore, we strongly recommend that diagnostic assays and kits should allow for the separate detection of anti-Ro52 and anti-Ro60 (SS-A) aab.

Take-home messages

- Ro52 is biochemically and immunologically distinct from Ro60 (SS-A).
- Anti-Ro52 co-occurs in up to 72% of anti-Jo-1 positive sera from myositis patients.
- Anti-Ro52 and anti-Ro60 (SS-A) reactivities can mask each other, thus more than 20% Ro positive samples can remain undetected in assays that utilize blended antigens.
- Further characterization of CDC ANA reference sera (for CDC serum 2, 3, 7 and 10) showed anti-Ro52 reactivity in sera previously defined as anti-Ro (SS-A) negative.
- Anti-Ro52 and anti-Ro60 (SS-A) should be tested separately.

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