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Specificity of Autoantibodies to SS-A/Ro on a Transfected and Overexpressed Human 60 kDa Ro Autoantigen Substrate

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The objective of this study was to analyze apparently discrepant results that arose during the use of an indirect immunofluorescence (IIF) assay using transfected HEp-2 cells to detect anti-SS-A/Ro autoantibodies in human sera. Fourteen sera that had SS-A/Ro antibodies as detected on this commercial substrate, but did not have antibodies to SS-A/Ro as determined by double immunodiffusion (ID) or enzyme-linked immunosorbent assay (ELISA), were studied by immunoprecipitation (IP) of radiolabeled cell extracts and full-length recombinant SS-A/Ro. A multiantigen strip immunoblotting (IB) assay containing both the 52- and 60-kDa antigens was included in the analysis. We confirmed that 12 of 14 of the sera under study had antibodies to SS-A/Ro protein antigens as determined by at least one other immunoassay. One serum had antibodies to hyRNA but no detectable reactivity with the 52- or 60-kDa antigens. One serum remained negative in all assays for SS-A/Ro autoantibodies. J. Clin. Lab. Anal. 16:103–108, 2002.

Key words: autoantibodies; Sjögren's syndrome; systemic lupus erythematosus; indirect immunofluorescence; transfected cells

INTRODUCTION

Autoantibodies directed against a variety of intracellular macromolecules are a hallmark of sera from patients with systemic rheumatic diseases. One of the more common autoantibodies seen in these diseases is directed against SS-A/Ro antigens. The first description of the SS-A/Ro system is attributed to Anderson et al. (1), who described two precipitating autoantibody specificities referred to as SjD and SjT from patients with Sjögren's disease. Clark et al. (2) subsequently defined the Ro system as a protease- and nuclease-resistant cytoplasmic antigen that was extracted from various tissues and reacted with antibodies in 40% of systemic lupus erythematosus (SLE) sera. In 1975 Alspaugh and Tan (3) described SS-A and SS-B as two distinct precipitating autoantibody specificities that occurred predominantly in patients with Sjögren's syndrome (SS). It was subsequently showed that SS-A and Ro were identical (4), and since then this antigen system has been referred to as SS-A/Ro, Ro/SS-A, or simply Ro. Based on observations that the Ro antigen associates with small cytoplasmic RNAs referred to as hyRNA (5), this system is known as the Ro ribonucleoprotein (Ro RNP). Human sera containing anti-SS-A/Ro antibodies recognize at least two proteins: one of 52 kDa and one of 60 kDa (6–8).

A number of techniques, including indirect immunofluorescence (IIF) (9–11), immunodiffusion (ID) (2,12), immunoprecipitation (IP) (13,14), immunoblotting (IB) (15–18), enzyme-linked immunosorbent assay (ELISA) (19,20), and counter immunoelectrophoresis (CIE) (19–21), have been used to identify SS-A/Ro antibodies. It is recognized that some of these techniques have limitations in a clinical laboratory. For example, ID lacks sensitivity and can take up to 48 hr before precipitin lines are visible or interpretable. IB is expensive and time-consuming, and not all SS-A/Ro autoantibodies are detected by this technique (22,23). CIE is useful for detecting high titer SS-A/Ro antibodies, but does not discriminate between reactivity of sera with the 52- or 60-kDa antigens (19,20). IP protocols that use extracts from radiolabeled cells are not suitable for the clinical detection of autoantibody to 52-kDa SS-A/Ro protein because many sera are negative in this assay (24). ELISA techniques are the protocol of choice for clinical laboratories that rely on high volume and rapid reporting of results. Unfortunately, it appears that standardization of these assays has not been achieved, since in a previous study different laboratories using a num-

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ber of commercial kits produced discrepant results (25). ELISA using recombinant SS-A/Ro can be a highly specific and sensitive assay for SS-A/Ro protein, but it is expensive and has not been validated in multicenter trials. Recently, natural and recombinant antigens, including 60- and 52-kDa SS-/Ro antigens, have been placed in discrete lines on strips of a solid phase backing that allow convenient detection of autoantibodies using modified IB techniques (21).

Although IP, CIE, and ELISA have certain advantages, IIF using HEp-2 or other tissue culture cells as substrate remains an inexpensive and rapid technique to perform. However, the use of IIF on tissue culture cells to detect SS-A/Ro antibodies is limited by a lack of specificity and sensitivity due to the low abundance of the SS-A/Ro antigen (26) and diffusion of the antigen from the nucleus during fixation and subsequent sample preparation (10). This has resulted in commercial HEp-2 cell substrates that do not reliably detect SS-A/Ro antibodies. In addition to variation between kits produced by various manufacturers, significant lot-to-lot variation from single manufacturers has also been noted (24). These limitations can be compounded by sera that contain multiple autoantibodies, which necessitate the use of more expensive and time-consuming techniques to definitively identify SS-A/Ro antibodies. With these limitations in mind, some commercial vendors use fixatives and procedures to ensure that the SS-A/Ro antibodies produce a characteristic fine-speckled pattern of staining by IIF (10).

A more recent approach to improve the sensitivity and specificity of assays that detect SS-A/Ro antibodies is to employ the technique of transfection, in which the appropriate gene(s) are inserted into cells. This technique introduces multiple copies of the gene that, under the control of specific promoter-regulator genes, leads to stable "overexpression" of the gene of interest. This approach has been utilized in the development of an IIF substrate that utilizes HEp-2 cells transfected with a full-length human 60 kDa SS-A/Ro cDNA (27,28). Since its introduction, a number of clinical laboratories have evaluated this commercially available substrate and reported relatively high sensitivity and specificity for the detection of SS-A/Ro antibodies (21,28–32). We identified a number of sera that reacted with the overexpressed 60 kDa antigen by IIF, but on routine testing by ELISA and ID the sera had no apparent reactivity with this antigen. We report the results of our analysis of these sera in this work.

MATERIALS AND METHODS

Sera

The sera used in this study were referred to the Advanced Diagnostics Laboratory at the University of Calgary for specialized testing because of discrepant results between the IIF assay on the commercially prepared SS-A/Ro HEp-2 substrate (HEp-2000, Immuno Concepts Inc., Sacramento, CA). Control sera with anti-SS-A/Ro autoantibodies from the Center for Disease Control/Arthritis Foundation (CDC/AF) serum bank (33,34), as well as previously characterized sera from the Advanced Diagnostics Laboratory were used in this study (35). All sera were retested to confirm the discrepant results, aliquoted to avoid repetitive freeze–thaw cycles, and then stored undiluted at –20 or –70°C until required for assays.

IIF

Autoantibodies directed against SS-A/Ro antigens were first evaluated on the basis of IIF microscopy on commercial HEp-2 cell substrates (HEp-2000, ImmunoConcepts Inc.) using a fluorescein-conjugated polyvalent goat anti-human immunoglobulin (light and heavy chain), as previously described (28). This substrate employs a full-length cDNA encoding a human 60-kDa SS-A/Ro protein that was stably transfected and overexpressed in the HEp-2 cell line, as described by Keech et al. (27,29). Slides were viewed on a Zeiss Universal microscope fitted with a TEC-470 CCD videocamera system (Optronics Engineering, Goleta, CA) and images were processed with a Sony color video printer and recorded on Sony Type 1010 photography paper.

ELISA

Sera were tested for reactivity with SS-A/Ro with a commercial kit (RELISA ENA; ImmunoConcepts Inc.), following the protocol suggested by the manufacturer.

ID, SDS-PAGE, and IB

All sera were tested for antibodies to SS-A/Ro by ID using a commercial kit (AutoID; ImmunoConcepts Inc.). A novel IB "line" assay was used to confirm reactivity with the 52- and 60-kDa SS-A/Ro antigens. Relevant antigens were placed on mylar-backed nitrocellulose strips which were then used in a conventional IB assay to measure a broad spectrum of autoantibodies (InnoLIA; Innogenetics, Norcross, GA). Sera were diluted 1/500 and antibodies were detected using the reagents and protocol provided by the manufacturer.

IP

IP of [^{35}S]-labeled in vitro translation products was performed using Protein A-Sepharose beads (36). Briefly, 10 µl of human serum and 2–4 µl of in vitro translation product were incubated with 100 µl of Protein A-Sepharose beads in 66.7 µl of 15 mg/ml BSA, and 400 µl of NET2 + F buffer for 2 hr at 4°C. After incubation, the Sepharose beads were washed five times with NET buffer and resuspended in SDS sample buffer. Samples were then analyzed by SDS-PAGE and autoradiography as described above.

In Vitro RNA Transcription and Translation

The full-length 60-kDa SS-A/Ro cDNA (a gift from Dr. E.K.L. Chan, La Jolla, CA) was used to produce recombinant

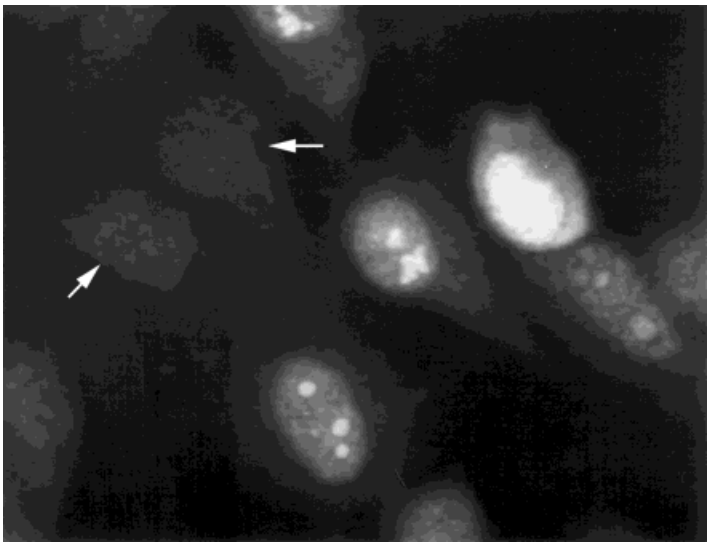

Fig. 1. IIF of human sera containing antibodies to the 60-kDa SS-A/Ro antigen on transfected HEp-2 cells produces intense nuclear staining of ~1/4 of the cells in a high-power field. The staining pattern is typically strongest in the nucleus (speckled) and nucleolus with weaker cytoplasmic staining. Untransfected cells (arrows) show much weaker nuclear staining. Original magnification ×400.

protein in a rabbit reticulocyte-based transcription and translation kit (TnT; Promega, Madison, WI) with previously described protocols (36). Next, 1 μg of the purified plasmid DNA used as template for in vitro transcription with T3 RNA polymerase was added in a 50-μl translation reaction containing rabbit reticulocyte lysate, [^{35}S]-methionine (trans-^{35}S-label, ICN Biochemicals, Irvine, CA), and RNAse block II (Stratagene, Inc., La Jolla, CA) as suggested by the manufacturer. The reaction was carried out at 30°C for 1 hr, followed by SDS-PAGE of a 2–5-μl aliquot to confirm the presence of translation products. The recombinant proteins were stored at –80°C until the analysis was performed.

RESULTS

The IIF staining pattern produced by a prototype anti-SS-A/Ro serum on the transfected HEp-2 cells is illustrated in Fig. 1. Typically, 15–25% of the cells in a high-power field (40× objective) demonstrated a markedly increased intensity of nuclear and nucleolar staining. Cells showing this staining pattern have been previously shown to be overexpressing the SS-A/Ro antigen (27–29). In the calendar year 2000, we received 2,576 sera for autoantibody testing, and 114 (4.4%) were positive for SS-A/Ro antibodies by any technique. Out of this group, 101/114 (89%) had the typical SS-A/Ro staining pattern on HEp-2 cells (Table 1). Of the 101 sera that produce the typical staining pattern, one (1%) did not have SS-A/Ro antibodies as tested by conventional ID or ELISA. Fourteen sera (collected over several years) that demonstrate this "discrepant" reactivity are the subject of this study.

Results from the study of these 14 sera are summarized in Table 2. Six of the 14 sera immunoprecipitated proteins that comigrated with either the 52- or the 60-kDa SS-A/Ro antigen (Fig. 2). Two of these sera precipitated a ~85 kDa protein which on further analysis was not reactive with known antigens that have a similar molecular mass (e.g., Ku, PM/Scl, golgins-95 and -97, and Scl-70). Four of the sera that IP radiolabeled SS-A/Ro, and another six sera that did not, immunoprecipitated the 60-kDa radiolabeled full-length recombinant protein (Fig. 3). Three sera had antibodies to the 52-kDa SS-A/Ro protein by IB in the InnoLIA assay (Fig. 4). At this stage of sample analysis, 12/14 demonstrated reactivity to an SS-A/Ro antigen by at least one assay. When the remaining two samples were tested for reactivity by IP of radiolabeled RNA, one precipitated hy5RNA (data not shown). The other serum did not react with any known autoantigens.

TABLE 1. Anti-SS-A/Ro antibodies in clinical laboratories

Reference	Total ANA	IIF ANA + ve (%)	SS-A/Ro + ve/Total ANA (%) /ANA + ve (%)	HEp2000 + ve/ Routine SS-A/Ro + ve (%)	HEp2000 + ve/ Extra SS-A/Ro + ve (%)[a]
Fritzler et al., this study	2576	969 (37.6)	114/2576 (4.4) 114/969 (11.7)	101/114 (89)	101/101 (100)
Bossuyt et al. [2000] (21)	2427	1394 (57)	106/2427 (4.4) 106/1394 (7.6)	107/122 (88)	108/108 (100)
Pollock and Toh [1999] (30)	10500	2100 (20)	160/10500 (1.5) 160/2100 (7.6)	145/160 (91)	159/160 (99)
Peene, et al. [2000] (32)	4303	1061 (24.5)	70/4303 (1.6) 70/1061 (6.6)	70/91 (77)	16/16 (100)

[a]Extra refers to additional, more sophisticated testing such as immunoprecipitation and immunoblotting.

TABLE 2. Fourteen SS-A/Ro sera positive on transfected HEp-2 cells

Serum ID	IIF	ID/ ELISA	IP ^{35}S-labeled MOLT-4 extract	IP TnT	IB InnoLIA	Other
8E	+	–/–	+	+	nd	
8B	+	–/–	+	+	nd	
8C	+	–/–	+	+	nd	
8D	+	–/–	+	+	nd	
2D	+	–/–	+	–	+	85kDa
2B	+	–/–	+	–	+	85kDa
2C	+	–/–	–	+	–	
7A	+	–/–	–	+	–	
7B	+	–/–	–	+	+	
8A	+	–/–	–	+	nd	
8F	+	–/–	–	+	–	
2A	+	–/–	–	+	–	
2E	+	–/–	–	–	–	hyRNA
7C	+	–/–	–	–	–	
Prototype	+	+/+	+	+	+	hyRNA
NHS	–	–/–	–	–	–	–

ELISA, enzyme linked immunoassay; IB, immunoblotting; hyRNA, small cytoplasmic RNA (hy5 RNA); ID, immunodiffusion; IP, immunoprecipitation; nd, not done; NHS, normal human serum; TnT, transcription and translation reaction of cDNA.

DISCUSSION

Transfection techniques have been widely used to study a number of autoantibody systems. However, the transfected HEp-2 substrate has significant potential as a routine test because SS-A/Ro antibodies are frequently detected in a clinical autoantibody laboratory. For example, SS-A/Ro antibodies are found in 30–50% of SLE sera, 70% of Sjögren's syndrome sera, 10–20% of systemic sclerosis sera, 5–15% of rheumatoid arthritis sera, and 80–95% of sera from mothers of babies with neonatal lupus erythematosus (reviewed in Refs. 24 and 37). As shown in this study and others, anti-SS-A/Ro antibodies are detected in 1.5–4.4% of all sera tested and in up to 11.7% of ANA positive sera (see Table 1).

Many commercially available IIF substrates are not reliable and lack specificity for the detection of SS-A/Ro antibodies, and ID, IB, CIE, ELISA, and IP techniques add markedly to the cost and turnaround time of the test. The availability of a serological test, such as the HEp2000® kit, that will allow rapid, accurate, and relatively inexpensive detection of SS-A/Ro antibodies in the clinical laboratory setting has obvious advantages. However, it was anticipated that the IIF test for SS-A/Ro antibodies would produce results that did not agree with other assays. Despite the fact that ID and ELISA were unable to confirm the presence of anti-SS-A/Ro antibodies in the 14 sera described here, we showed that 13/14 of these sera contained antibodies directed against the 52- or 60-kDa, or the hyRNA SS-A/Ro antigens when other techniques were used. These observations are consistent with other reports (21,30,32) that showed that virtually all sera with an apparent spurious result by IIF on transfected HEp-2000 substrate had anti-SS-A/Ro antibodies that could be demonstrated by other techniques (see Table 1). These results also support earlier studies, using unselected sera, that showed that the HEp-2000 substrate had higher sensitivity and specificity than either IB or ID in detecting SS-A/Ro antibodies (27,28).

SS-A/Ro sera can be subdivided by IB into four groups: 1) those that contain antibodies to the 60-kDa antigen; 2) those that contain antibodies to the 52-kDa antigen; 3) those that contain antibodies to the 60- and 52-kDa antigens; and 4) those that contain antibodies to the "native Ro particle" (22,23). In this context, a few additional points regarding the HEp-2000 substrate are worth mentioning. First, as illustrated in this study and others (32), it appears that some sera that contain only antibodies to the 52-kDa SS-A/Ro antigen can demonstrate typical reactivity with the stably-transfected HEp-2 substrate. The reasons for this are not clear since only the 60-kDa SS-A/Ro cDNA has been transfected into the cells. It is probably not due to cross-reactivity of anti-52-kDa with anti-60-kDa antibodies because they share virtually no sequence homology, and numerous studies have shown that they are distinct autoantigen systems. Second, as shown in this study and a previous one (28), some rare sera that appear to target only the hyRNA component of the small cytoplasmic

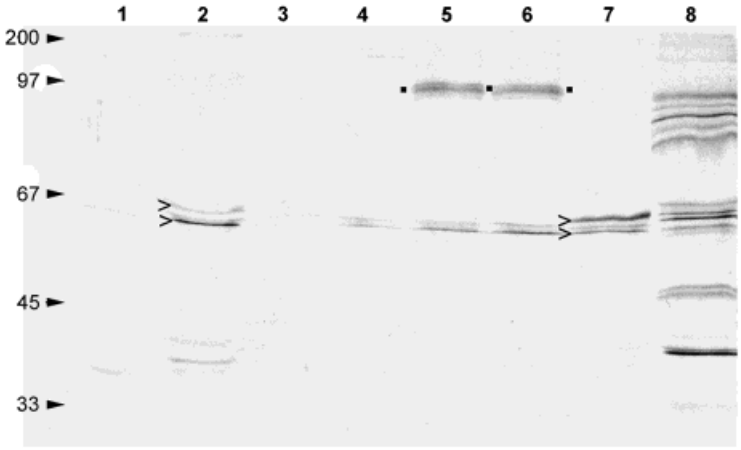

Fig. 2. IP of an extract from [^{35}S]-methionine-labeled HeLa cells using (lane 1) normal human serum and SS-A/Ro prototype sera with primarily (lane 2) 52-kDa and (lane 7) 60-kDa antibodies resolved by SDS-PAGE on a 12.5% gel. Lanes 3–6: Test sera. Lane 8: A control autoimmune serum containing anti-Sm and anti-SS-A/Ro. The specific signals at ~85 kDa are indicated by dots, and the 60- and 52-kDa SS-A/Ro proteins by open arrows. Molecular mass markers are shown on the left.

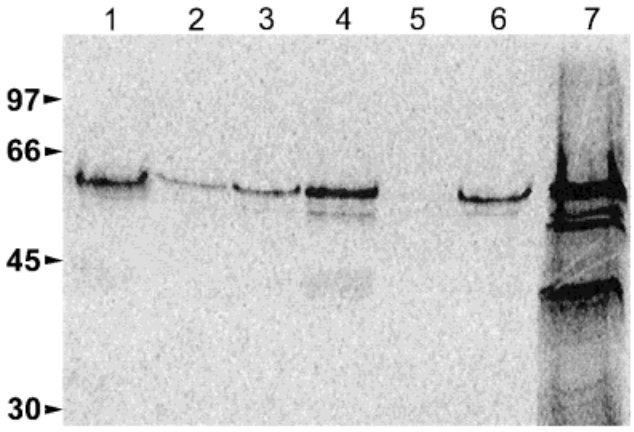

Fig. 3. The full-length 60-kDa SS-A/Ro cDNA was produced in the rabbit reticulocyte system (TnT) to produce a radiolabeled 60-kDa protein. The radiolabeled protein was incubated with human sera, the immune complexes were bound to Protein A, and the eluted protein was separated by SDS-PAGE. Lanes 1–4: Representative test sera. Lane 5: A control normal serum. Lane 6: A prototype serum with anti-60-kDa SS-A. Lane 7: An aliquot of the total TnT product.

RNP SS-A/Ro complex can also show reactivity with the 60-kDa SS-A/Ro transfected substrate. Again, the reason for this reactivity with transfected substrates is not clear, but perhaps stable overexpression of the 60-kDa protein results in binding larger amounts of the hyRNA ligand and presentation of a reactive hyRNA epitope. The reactivity with RNA epitope(s) may be reflected by the dramatic nuclear and nucleolar staining observed in these transfected cells (see Fig. 1). On balance, these observations suggest that the overexpressed 60-kDa antigen alters the metabolism of the hyRNA, making larger amounts or epitopes available for antibody binding in transfected cells.

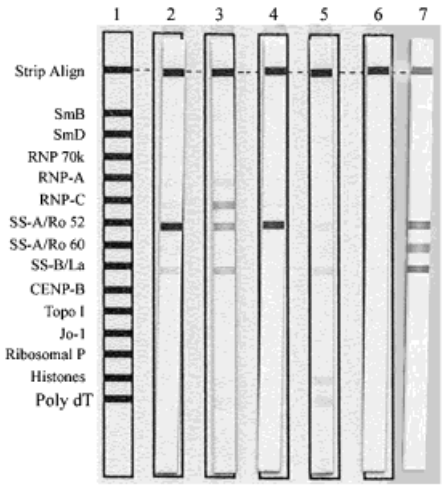

Fig. 4. Analysis of selected sera by IB using a line assay (InnoLIA). Lane 1: The array of antigens included in the assay. Representative test sera show reactivity with the (lanes 2–5) 52-kDa SS-A/Ro, (lanes 2, 3, and 5) SS-B/La, and (lane 3) RNP-C. (Lane 6) The control normal serum shows no reactivity, and (lane 7) a control prototype serum shows reactivity with the 60- and 52-kDa SS-A/Ro and SS-B/La.

In our study and others, we observed that some sera that contain SS-A/Ro antibodies by conventional techniques may not show the typical IIF pattern on the HEp-2000 substrate. Other studies have emphasized that when other high-titer autoantibodies are present they may mask the reactivity of SS-A/Ro antibodies (21,30). This is particularly a problem in laboratories that screen sera with a single serum dilution. In our previous study (28), we experienced little difficulty in identifying reactive sera if two serum dilutions were performed.

It can be concluded from these studies that the sensitivity and specificity of the stably-transfected HEp-2000 substrate to detect anti-SS-A/Ro is remarkable, and if sera are diluted to avoid masking by other high-titer antibodies, it is at least equivalent to other conventional assays in common use today. The HEp-2000 and similar stably-transfected cell substrates have several advantages. First, a substrate that can be used to test sera for a wide variety of autoantibodies, and at the same time identify a specific autoantibody, is beneficial to the clinical laboratory. If the product is competitively priced, the ability to detect SS-A/Ro antibodies with a single test should result in savings for the clinical laboratory and the health-care system. Second, the turnaround time for reporting the results from this IIF assay should be decreased. Third, two or three assays are typically required to reliably detect SS-A/Ro antibodies in human sera. The use of this substrate could replace expensive (ELISA and IB) and hazardous (radioisotopes) techniques to detect SS-A/Ro antibodies in the clinical laboratory.

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