Routine immunofluorescence detection of Ro/SS-A autoantibody using HEp-2 cells transfected with human 60 kDa Ro/SS-A

Wendy Pollock, Ban-Hock Toh

Abstract

***Background*—Ro/SS-A autoantibodies associated with systemic lupus erythematosus (SLE) and Sjögren syndrome may be missed during routine screening for antinuclear autoantibodies (ANA) by immunofluorescence using HEp-2 cells.**

***Aims*—To investigate the use of HEp-2 cells transfected with human 60 kDa Ro/SS-A for routine detection of these antibodies.**

***Methods*—10 500 sera were screened at a dilution of 1:200 for Ro/SS-A antibodies, identified by intense immunofluorescence staining in 10–15% of hyperexpressing cells of either the nucleus and nucleolus combined or the nucleus alone.**

***Results*—Ro/SS-A antibodies were identified in 160/2100 ANA positive sera (8%), of which seven were ANA negative (titre < 200) and 33 had weak ANA titres (200) in 85–90% of non-hyperexpressing "background" cells. Enzyme linked immunosorbent assay (ELISA) confirmed the presence of Ro/SS-A antibodies in 110 newly diagnosed Ro/SS-A positive sera. Of these, 50 reacted with Ro/SS-A, 51 with Ro/SS-A and La/SS-B, and nine with Ro/SS-A and other extractable nuclear antigen (ENA) specificities. Fifteen sera which did not show Ro/SS-A antibodies by immunofluorescence tested positive for Ro/SS-A by immunodiffusion, counterimmunoelectrophesis, or ELISA; of these, 14 had ANA titres > 200. Clinical data from 95 Ro/SS-A positive patients showed that 52% had SLE, 24% Sjögren syndrome, 8% rheumatoid arthritis, and 16% other diseases.**

***Conclusions*—(1) HEp-2 cells transfected with human 60 kDa Ro/SSA are useful for routine immunofluorescence detection for Ro/SS-A antibodies with a positive predictive value of 100%; (2) sera positive for Ro/SS-A antibodies by immunofluorescence should be tested for ENA by other methods because > 50% of these sera will have another ENA reactivity in addition to Ro/SS-A; (3) detection of Ro/SS-A by immunofluorescence may be missed in the presence of high titre ANAs; (4) with a detection sensitivity of 91%, a negative immunofluorescence result for Ro/SS-A does not exclude the presence of this autoantibody.**

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Gribble's Pathology, South Yarra, Melbourne and Department of Pathology and Immunology, Monash University Medical School, Prahran, Victoria 3181, Australia
W Pollock
B-H Toh

Correspondence to:
Dr Toh.
email:
bht@cobra.path.monash.edu.au

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Circulating antinuclear autoantibodies (ANA) are useful as diagnostic markers for a variety of autoimmune diseases including systemic lupus erythematosus (SLE) and Sjögren syndrome (reviewed by Tan, 1989).[1] The presence of ANA is usually tested by immunofluorescence using human HEp-2 cells. However, the use of this cell line does not detect the presence of all ANA, an observation which has given rise to the concept of "ANA negative lupus." The autoantigen that is most commonly not detected in these cases of "ANA negative lupus" is the Ro/SS-A antigen, which belongs to the group of extractable nuclear antigens (ENA).

Antibodies to Ro/SS-A were first identified by gel diffusion in patients with Sjögren syndrome in 1962[2] and in patients with SLE in 1969.[3] These autoantibodies are almost invariably associated with subacute cutaneous lupus erythematosus and neonatal lupus erythematosus and may be the only antibody present in patients with SLE or Sjögren syndrome (reviewed by Reichlin and Scofield, 1996[4]). Ro/SS-A together with La/SS-B is associated with hY (human cytoplasmic) uridine-rich RNA. These antibodies recognise two proteins of 60 kDa and 52 kDa, of which the 60 kDa protein is the major polypeptide.[5] Most if not all 60 kDa Ro/SS-A antibodies react with conformational epitopes on the native protein, demonstrable by immunodiffusion or by counterimmunoelectrophesis (CIEP), and are not detectable by immunoblotting.[6] In contrast, antibodies to 52 kDa Ro/SS-A protein preferentially react with denatured epitopes by immunoblotting.[6] The most sensitive methods for the detection of Ro/SS-A are by a sandwich ELISA and by immunoprecipitation.[7] However, the enhanced sensitivity of the latter two methods detects the presence of small amounts of antibody in asymptomatic patients,[8] therefore decreasing the diagnostic significance of the assays. Because of these considerations, the current preferred screening assay for Ro/SS-A is the less sensitive gel diffusion assay. This is less than ideal, given that the screening assay for ANA is the immunofluorescence test.

To overcome the problem of the detection of Ro/SS-A by immunofluorescence, HEp-2 cells have been transfected with human 60 kDa Ro/SS-A protein.[9–11] As only about 10–15% of transfected HEp2 cells overexpress the 60 kDa Ro/SS-A protein, the cell line can also be used for screening for other ANA specificities. Initial reports using this 60 kDa Ro-transfected HEp-2 cell line have been promising. Here we report our experience using this cell line for screening 10 500 serum samples for Ro/SS-A

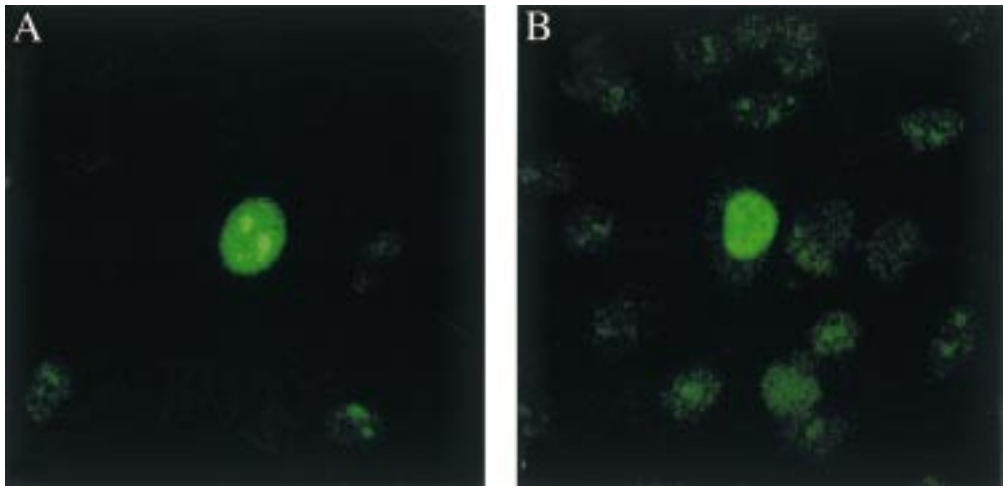

Figure 1 Immunofluorescence staining of HEp-2 cells transfected with human 60 kDa/Ro-SS-A by Ro/SS-A positive sera. Note the intense immunofluorescence staining of (A) nuclei and nucleoli and (B) nucleoplasm of hyperexpressing cells, compared with the much weaker staining of the adjacent non-hyperexpressing cells.

antibodies by immunofluorescence in a routine diagnostic laboratory. Our report is the largest survey for anti Ro/SS-A antibodies by immunofluorescence to date.

Methods

SERA

Sera from 10 500 patients were referred for routine tests for antinuclear antibody, predominantly from general practitioners but about 20% from specialists. These included sera from 946 patients referred for testing for antibodies to extractable nuclear antigens.

IMMUNOFLUORESCENCE

Sera were screened at a dilution of 1:200 in phosphate buffered saline containing a serum diluent provided by the manufacturers to reduce the background immunofluorescence. The slides were screened using a Leitz immunofluorescence microscope and a ×50 objective to enhance the sensitivity of the detection of positive tests. We selected a screening dilution of 1:200 because preliminary studies carried out to determine the normal reference range in our laboratory had established that this screening dilution gave optimal discrimination between patients with systemic autoimmune diseases and age and sex matched healthy controls. While this screening dilution of 1:200 may seem higher than the 1:40 and 1:80 screening dilutions used by other laboratories, in-house and external quality control programs have validated the use of this dilution for the routine detection of antinuclear autoantibodies in our hands. The slides were screened by either of two experienced scientists (WP or M Lewis), and reviewed by the consultant immunologist (B-HT).

Sera were reacted with the HEp-2000® cell line (Immuno Concepts) in which the cells have been transfected with the 60 kDa Ro/SS-A protein and grown on glass slides. After 30 minutes of incubation at room temperature, the slides were washed and incubated with FITC conjugated goat antihuman IgG (heavy and light chains; catalogue No 2009, Immuno Concepts). The slides were read under ultraviolet light using ×500 magnification. ANA patterns and titres were determined on the "background" non-hyperexpressing cells, while the Ro/SS-A pattern was identified on the hyperexpressing cells. For photomicrography, the stained cells were examined by confocal microscopy (Bio-Rad MRC 1024).

IMMUNODIFFUSION

Sera for routine testing for ENA antibodies were screened by immunodiffusion using a commercial kit (Auto ID®, Immuno Concepts) according to the manufacturer's instructions. Sera were tested neat and wells were routinely double filled. Results were read at 48 hours.

ELISA

Any sera giving a precipitin line on immunodiffusion or any sera showing the Ro/SS-A pattern by immunofluorescence were further characterised by ELISA using a commercially available ELISA kit (RELISA® ENA, Immuno Concepts). These kits comprise an eight well strip coated with affinity purified Sm, Sm/RNP, Ro/SS-A, La/SS-B, Scl70, and Jo-1. Sera positive by immunodiffusion but negative by ELISA were recorded as having unidentified precipitin lines (UPL). A result of > 40 units was considered positive. The manufacturers state that the ELISA kit has been compared with other similar ELISA assays as well as with other methods of detection including immunodiffusion, counterimmunoelectrophoresis, and immunoblotting. These comparisons showed that the ELISA kit gave a relative sensitivity, specificity, and overall agreement of 94–100%.

COUNTERIMMUNOELECTROPHORESIS

This test was carried out as described previously.[1]

Results

ROUTINE IMMUNOFLUORESCENCE SCREENING FOR Ro/SS-A ANTIBODIES

Antinuclear antibodies were detected in 2100 serum samples from 10 500 sera sent to Gribble's Pathology (Melbourne branch) for tests for ANA, giving a positive detection rate of

Table 1 Antinuclear autoantibody (ANA) titres of Ro/SS-A positive and Ro/SS-A negative sera detected by indirect immunofluorescence (IIF)

	HEp-2000 IIF	
ANA titre	*Ro/SS-A positive*	*Ro/SS-A negative*
<1:200	7	1
1:200	33	–
>1:200	71	14
Total	110	15

20%. Cells hyperexpressing Ro/SS-A (Ro/SS-A pattern) were detected by immunofluorescence in 160 serum samples, comprising 8% (160/2100) of all the positive ANA identified. The Ro/SS-A pattern seen in 10–15% of transfected cells typically comprised prominent staining of nucleoli combined with diffuse staining of the nucleoplasm as previously described[9 10] (fig 1A). Occasionally, hyperexpressing cells showed strong staining only of the nucleoplasm without staining of nucleoli (fig 1B), a pattern which has not previously been reported.

Fifty of the 160 sera which tested positive for Ro/SS-A by immunofluorescence using the Hep-2 transfected cells were previously shown to be positive for Ro/SS-A by immunodiffusion or by ELISA. Therefore only the remaining 110 newly diagnosed Ro/SS-A positive sera were subjected to further tests. Antibody titres were determined for these 110 sera. These titres were obtained by doubling serum dilutions screened on the 85–90% "background" non-hyperexpressing, transfected HEp-2 cells which did not show the Ro/SS-A pattern. These background non-hyperexpressing cells typically showed a speckled nucleus staining pattern with the exception of one serum showing a centromere pattern and three sera showing a homogeneous staining pattern. The results showed that of the 110 newly diagnosed Ro/SS-A positive sera, seven tested negative for ANA (titre < 200), 33 had ANA titres at 200, and 70 had titres > 200 (table 1). All 110 samples were also positive for Ro/SS-A antibodies by ELISA (table 2).

Seven of 110 Ro/SS-A positive sera (6%) had ANA titres below our reference range (< 1:200) in the non-hyperexpressing, transfected HEp-2 cells. Subsequent testing at lower serum dilutions failed to show the presence of ANA in the "background" cells by immunofluorescence. Therefore these sera would have been reported as "ANA negative" using conventional HEp-2 cells.

Table 2 Specificity of Ro/SS-A positive and Ro/SS-A negative sera detected by indirect immunofluorescence (IIF)

	HEp-2000 IIF	
RELISA specificity	*Ro/SS-A positive*	*Ro/SS-A negative*
Ro/SS-A	50 (46%)	3 (20%)
Ro/SS-A and La/SS-B	51 (49%)	4 (27%)
Ro/SS-A and other ENA	9 (6%)	8 (53%)
Total	110	15

RELISA, commercial enzyme linked immunosorbent assay kit (Immuno Concepts).

TESTS FOR EXTRACTABLE NUCLEAR ANTIGENS

Over the same period, we also carried out tests for ENA on 946 sera in which these tests were requested, irrespective of whether or not Ro/SS-A antibodies had been demonstrated by immunofluorescence. We tested for ENA antibodies by immunodiffusion followed by ELISA (on sera giving precipitin lines). In all, 192 sera tested positive for ENA, including the 110 Ro/SS-A positive sera detected by immunofluorescence. An additional 15 Ro/SS-A positive sera were identified, giving a total of 125 Ro/SS-A positive sera identified by ELISA. Therefore anti-Ro/SS-A antibodies comprised 65% (125/192) of all positive ENA antibodies. Thirteen of the 15 sera also tested positive for Ro/SS-A by CIEP, but the other two sera tested negative. Of the 15 sera which did not show Ro/SS-A antibodies by immunofluorescence, 14 had high ANA titres of the background cells (> 200) and one was ANA negative with a titre < 200 (table 1). The high ANA titres may have masked the detection of Ro/SS-A. The ELISA results from these 15 sera are shown in table 2.

SENSITIVITY AND SPECIFICITY OF Ro/SS-A IMMUNOFLUORESCENCE TEST

The positive predictive value of the immunofluorescence test for Ro/SS-A using the transfected cell line was 100% (160/160) and the sensitivity was 91% (160/175). However, as not all sera sent for ANA testing were screened for Ro/SS-A antibodies by immunofluorescence, it is possible that the sensitivity of the test may be less than 91%.

ELISA RESULTS

The ELISA values for the 125 Ro/SS-A positive sera ranged from a mean of 124 units for those with a background ANA titre of < 200, to 156 units for those with a titre of 200, and 170 units for those with a titre of > 200.

CLINICAL CORRELATION

Clinical information was available on 95 patients who tested positive for Ro/SS-A antibodies by immunofluorescence or by combined immunodiffusion and ELISA (tables 3 and 4).

Discussion

HEp-2 cells transfected with the human 60 kDa Ro/SS-A protein were developed to overcome the insensitivity of the detection of Ro/SS-A antibodies by immunofluorescence using conventional HEp-2 cells.[9] The increased sensitivity for the detection of these antibodies is based on

Table 3 Clinical diagnosis of 95 patients with or without Ro/SS-A antibodies detected by indirect immunofluorescence (IIF)

	HEp-2000 IIF		
Diagnosis	*Ro/SS-A positive*	*Ro/SS-A negative*	*Total*
SLE	41 (49%)	8 (67%)	49 (52%)
Sjögren syndrome	23 (28%)	0	23 (24%)
Rheumatoid arthritis	6 (7%)	2 (16%)	8 (8%)
Other	13 (16%)	2 (16%)	15 (16%)
Total	83	12	95

Table 4 Extractable nuclear antigen (ENA) specificity of patients with SLE and Sjögren syndrome

Diagnosis	*Ro/SS-A positive*	*Ro/SS-A La/SS-B positive*	*Ro/SS-A LA/SS-B and other ENA positive*	*Ro/SS-A and other ENA positive*	*Total*
SLE	20 (41%)	21 (47%)	4 (9%)	4 (8%)	49
Sjögren syndrome	5 (22%)	17 (74%)	1 (4%)	–	23

the overexpression of the 60 kDa Ro/SS-A protein in about 10–15% of cells. In a comparison of end point titres of 20 Ro/SS-A positive sera on transfected versus non-transfected HEp-2 cells, the overexpressing cells gave an increased mean titre which was 41-fold higher than that of the non-transfected cells. The concordance rate for the detection of Ro/SS-A antibodies in 39 of 53 patients with primary Sjögren syndrome using the 60 kDa Ro/SS-A transfected cell line and CIEP was 100%.[10] In another study, 69 of 73 sera (95%) which were positive for Ro/SS-A by immunodiffusion were also positive by immunofluorescence using the 60 kDa Ro/SS-A transfected cell line.[11]

In a previous study, 14 Ro/SS-A positive sera were detected from a total of 240 sera tested using the 60 kDa Ro/SS-A transfected cell line for routine ANA screening.[9] Here, we report the detection of 160 Ro/SS-A positive sera from 10 500 sera screened for ANA, representing therefore the largest experience with the use of this cell line for routine ANA screening to date. We used a screening serum dilution of 1:200, which is higher than the screening dilution of 1:40 used by other laboratories. It is possible that we may have missed the detection of some Ro/SSA positive sera by the use of this screening dilution. However, this is unlikely because cells transfected with human 60 kDa Ro/SSA dramatically overexpress this antigen, resulting in a 41-fold increase in titre compared with non-transfected cells.[9] The Ro/SS-A pattern was typically identified as hyperexpressing cells giving a combined immunofluorescence staining of both the nucleoplasm as well as the nucleolus, a pattern consistent with that of previous reports.[9–11] In the present study, we also describe one other immunofluorescence staining pattern which has not previously been reported—that is, strong immunofluorescence staining of the nucleoplasm only, without any staining of the nucleolus, in the hyperexpressing HEp-2 transfected cells. We have designated these two patterns of immunofluorescence staining in the hyperexpressing transfected HEp-2 cells as the "Ro/SS-A pattern."

All sera which gave the Ro/SS-A pattern by immunofluorescence were confirmed positive for this specificity by immunodiffusion or ELISA. The ANA status of the "background" non-hyperexpressing cells was negative in seven sera and weakly positive in 33. These observations highlight the value of using the transfected cell line. Importantly sera with negative or weakly positive ANA on the "background" cells had markedly increased ELISA units (> 100).

In a concurrent study of 192 sera which were tested for ENA by immunodiffusion followed by ELISA, 15 tested positive for Ro/SS-A in this way. These 15 sera did not give any immunofluorescence staining for the Ro/SS-A pattern in the HEp-2 cells transfected with the human 60 kDa Ro/SS-A protein. Given that the Ro/SS-A autoantigens comprise a 52 kDa as well as a 60 kDa protein, it is possible that antibody reactivity with the 52 kDa protein may have been missed in these 15 Ro/SS-A negative sera. However, in the present study, 14 of the 15 Ro/SS-A positive sera which tested negative for Ro/SS-A using the transfected cell line had ANA titres > 200, suggesting that the presence of these high titre antibodies may have masked the detection of Ro/SS-A antibodies.

CONCLUSIONS

Ro/SS-A antibodies comprise the majority of all ENA antibodies (65%) and are associated most commonly with SLE and Sjögren syndrome. With a positive predictive value of 100%, the HEp-2 cells transfected with human 60 kDa Ro/SS-A protein are a useful substrate for the routine detection of Ro/SS-A antibodies by immunofluorescence. Sera which test positive for Ro/SS-A antibodies by immunofluorescence should be subjected to further testing for other ENA antibodies by ELISA or by CIEP, because > 50% of sera may have other specificities in addition to Ro/SS-A, including La/SS-B antibodies. Given a sensitivity detection rate in our hands of 91%, Ro/SS-A antibodies should be tested by other methods if screening for these antibodies using the transfected cell line is negative but clinical suspicion that they should be present is high.

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