Evaluation of the different procedures in the detection of anti RO/SS-A 52 kDa and anti-RO/SS-A 60 kDa antibodies in primary Sjögren’s syndrome

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EVALUACIÓN DE DIFERENTES PROCEDIMIENTOS DE DETECCIÓN DE ANTICUERPOS ANTI RO/SS-A 52 kDa Y ANTI-RO/SS-A 60 kDa EN SÍNDROME DE SJÖGREN PRIMARIO

ABSTRACT

A group of patients with Sjögren’s Syndrome has been analysed in the present study in order to evaluate the different techniques used for the detection of anti Ro/SS-A of 52 and 60 kDa autoantibodies. The evaluated techniques were: double immunodiffusion performed in agarose gels using as antigen human spleen extract; Western Blot (WB) and enzyme-linked immune assays (ELISA) performed with recombinant protein antigens (Ro/SS-A 52 y 60 kDa in individual assays). Even though the ELISA and WB techniques are more sensitive than the gel precipitation method, the latter cannot be completely replaced by the former. It is necessary to take into consideration the nature of the antigen used in order to draw conclusions.

The recombinant antigens maintain the linear and conformational epitopes of the native molecules, but they are not able to bind to the other molecules of the RoRNP complex. On the other hand, in the antigenic extract used in the immunodiffusion technique, such interactions occur, and thus, the epitopes formed by the interaction of the different molecules might be present.

Our results indicate that, in spite of their higher sensitivity, the ELISA and WB techniques are not capable of entirely replacing the immunodiffusion method.

KEY WORDS: Sjögren’s Syndrome / Ribonucleoproteins / Autoantibodies/ Ro / SS-A 52 kDa / Ro / SS-A 60 kDa / La / SS-B.
INTRODUCTION

Sjögren’s Syndrome (SS) is a chronic autoimmune disease associated with the production of autoantibodies and characterised by a progressive lymphocytic and plasma cell infiltration of the salivary and lachrymal glands leading to xerostomia and xerophthalmia. SS may be primary or associated with other autoimmune diseases, most frequently Rheumatoid Arthritis (RA), Lupus Erythematosus (LE) and Progressive Systemic Sclerosis.

Although SS primarily affects the salivary and lachrymal glands, the skin, lungs, gastrointestinal tract and central nervous system also may be involved at variable rates. The mean age at presentation is 50 years, with more than 90% of the patients being women.

A possible retroviral aetiology has been proposed, based on the findings of viral particle expression in the salivary glands of the patients with SS and also anti-retrovirus antibodies in their serum. Mice which were infected with retrovirus also developed a pathology similar to SS. There are some hypothesis that mention the herpesvirus as the probable retrovirus related to SS, but they haven’t been conclusive at all yet.

In 1982, Anderson described for the first time the presence of precipitating antibodies in the serum of the patients with SS. The name Ro was established by Clark et al. in 1969 for a cytoplasmic antigen obtained from the spleen, liver, kidneys and human lymphatic ganglions. Mattiolo and Reichlin reported the finding of antibodies in the serum of patients with SS, which precipitated an antigen present in the cytoplasmic fraction of the bovine thymus. This antigen was called La protein. Alspaugh and Tan described, in the same type of patients, the presence of antibodies that precipitated Wil-2 from cell extracts, which were denominated SS-A and SS-B. Thus, different precipitating antibodies had been reported in different diseases by different groups, until a cooperative work between Alspaugh and Madison established the identity between Ro and SS-A and La and SS-B.

Although anti Ro/SS-A and anti La/SS-B antibodies were detected initially in sera from patients with SS, their presence was subsequently observed in patients with different kinds of LE (cutaneous subacute-CSLE, systemic-SLE, and neonatal-NLE), RA and other systemic autoimmune diseases. In SS these antibodies are related to glomerular injuries and extraglandular manifestations. These antibodies are also responsible for the congenital heart block, when they are transferred from mothers with SLE, SS or even totally asymptomatic, to their children. The cardiac blockade can be detected at birth or previously, and it shows no signs of structural anomaly.

The Ro ribonucleoproteins (RoRNPs) are complexes of RNA with highly conserved proteins, are expressed in every vertebrate cell and have also been recently found in nematodes. In spite of these characteristics, which predict an important role for this RNPs in the cell, their function is not totally clear at present. The complex Ro-RNP includes a hY-RNA molecule and at least two proteins: Ro/SS-A of 60 kDa and La/SS-B of 48 kDa. There is a third protein related to this complex, the Ro/SS-A of 52 kDa, which coprecipitates with the complex Ro-RNP, although the nature of its interaction in the complex is still controversial.

Nowadays there are a large number of commercial kits for the detection of autoantibodies against Ro/SS-A which include double immunodiffusion (DID, Ouchterlony), Western Blot (WB) and enzyme-linked immune assays (ELISA). The antigens Ro/SS-A used to make such kits can come from different sources, such as tissue extracts, cell culture extracts, and the most recent improvement is using recombinant proteins made in prokaryote systems. The assays that include gel precipitation methods, as the ones used to define these antibodies, involve the formation of immunocomplexes in the precipitation zone, which requires the existence of antibodies that are at least bivalent, as well as multiple antigenic determinants in the recognised molecule. On the other hand, WB and ELISA assays are based on different principles, where the existence of antibodies is detected without the necessity of complex formation. Taking this into account, even though it is known that these techniques show a higher sensitivity than the DID, their application to the detection of anti Ro/SS-A antibodies must be analysed carefully, besides bearing in mind the different nature of the antigens used in each assay.

In the present study we have tested different detection methods in a group of patients with primary SS. SS was the disease chosen because these patients show an autoantibody’s response quite limited compared to other autoimmune diseases like LE. The aim was to evaluate essentially diverse autoantibody detection methods: DID, WB and ELISA, using two sorts of antigens: Human Spleen Extract (HSE) and recombinant proteins: Ro/SS-A of 52 and 60 kDa.

MATERIALS AND METHODS

Production of Ro/SS-A of 52 and 60 kDa recombinant proteins

The plasmids, pET 3b and pET 3d, which contained the cDNA encoding for Ro/SS-A of 52 and 60 kDa, respectively, were kindly provided by Dr. Ger Pruijin from the University...
of Nijmegen. These were used to transform E-coli bacteria, strain Bl 21 pl.ys S-DE3®.

The transformed bacteria were cultured in LB medium at 37°C until they reached an optic density (OD) of 0.8 at 600 nm. Then IPTG (isopropyl-β-D-thiogalactopyranoside), inductor of the recombinant protein synthesis, was added in a final concentration of 1 mM in the culture medium. In this way the recombinant proteins were obtained into inclusion bodies.

The bacteria were lysated by sonication in three cycles of one minute each. This lysate was centrifuged at 12000 rpm for 15 minutes in order to collect the inclusion bodies. These ones were dissolved in a buffer containing Tris-HCl 50 mM, pH 8 and urea 8 M, subsequently the soluble proteins were dialysed exhaustively against Tris-HCl 50 mM, pH 8. The soluble fraction was later purified using an anionic interchange chromatography (Q-Sepharose, Amersham) and eluted with a NaCl gradient from 0 to 1 M.

Patients

Serum from the patients with a confirmed diagnosis of primary SS were provided by the Servicio de Reumatología, Hospital Rivadavia, Buenos Aires, Argentina. The diagnosis was performed according to the criteria indicated by the American Academy of Rheumatology. This criteria considers that a patient with a positive finding in a biopsy is included in the group of people with primary SS.

This group was comprised by thirty five female patients with an average age of fifty nine, which fluctuated between a minimum of forty six and a maximum of seventy one years old. The disease evolution was of six years, in average, among the people.

Ten ml of serum was obtained from each patient, which were stored at –20°C until the process began. Sera from 35 healthy patients were used as control of the assays, and the sex and age of these people were equivalent to the ones of the patients included in the tests.

Previously to these assays performed with recombinant proteins, 1ml from each serum of the SS patients as well as the ones used as control, were adsorbed with 50 ml of an extract from E-coli Bl 21 lacking of plasmid, during 16 hs. shaking constantly at 4°C.

Double Immunodiffusion

Commercial SS-A/SS-B antigens (INOVA Diagnostics, Inc. San Diego, USA) and Human Spleen Extract (HSE) were used as antigenic sources. The purification was performed according to Clark et al. Briefly, the tissue was homogenised in a buffer containing 50 mM Tris HCl pH 7.5, 150 mM NaCl, with 0.1 mM Phenylmethane sulfonyl fluoride (PMSF). Then, it was clarified by centrifugation and purified by anionic interchange chromatography, using DEAE cellulose (Bio-Rad, USA) as matrix and making the elution with 50 mM Tris-HCl pH 7.5, 300 mM NaCl. The presence of Ro/SS-A and La/SS-B in the samples was determined by DID with sera with a well-known specificity (anti Ro/SS-A and anti La/SS-B) provided by the Center for Disease Control (CDC, Atlanta, USA).

The gels used to carry out these assays were made of agarose 0.6% and 1% of polyethylene glycol-6000 (PEG-6000) in a phosphate-saline buffer (PBS, 0.01 M sodium phosphate, pH 7.4, 0.14 M NaCl). The antigen quantity to be used in the assays was determined by DID, which was performed trying different quantities of antigen and reactivity-known sera. The optimum volume was of 50 ml. The diffusion of the samples (50 µl) and of the antigen, were made during 24 hs at 4°C.

Western Immune Blot

The purified recombinant Ro/SS-A proteins (2.5 µg per lane) were separated in a SDS-10% PAGE gel and transferred into nitrocellulose filter papers. The filters, previously blocked with 3% non-fat dry milk in PBS over night at 4°C, were incubated one hour at room temperature with a 1:100 dilution of sera from patients and normal controls, which were previously adsorbed with E. coli extracts, as it was explained formerly. Next, the filters were washed four times with PBS and then incubated one hour at room temperature with peroxidase conjugated rabbit anti human Ig, in the dilution indicated by the manufacturer (Dako, USA). Afterwards four washes were performed with Tris-HCl 50 mM pH 7.5, NaCl 150 mM (TBS). Lastly the colour development was made with 4-Cl-naftol/H2O2 dissolved in 6 ml of methanol and 30 ml of TBS, and the reaction was stopped with water.

ELISA

Microtitre plates (Nunc MaxiSorp, USA) were coated at 37°C with 100 µl coming from a solution of 0.1 µg/ml of recombinant proteins Ro/SS-A of 52 and 60 kDa in TBS. After one hour the coated wells were blocked with 3% non-fat dry milk in PBS and incubated over night. Then, the wells were incubated one hour at 37°C with sera from patients and control sera in a 1:100 dilution with 1 non-fat dry milk in PBS. To detect the bound Ig, after four washes with PBS Tween-20 0.05, the plates were incubated with peroxidase conjugated rabbit anti human Ig (Dako, USA) in the indicated dilution. Later, the plates were washed with PBS Tween four times. The colour development was performed with O-phenidiamine/H2O2 in citrate buffer,
and the reaction was stopped with 4 N H2SO4. The criteria used to consider a reaction as positive or negative was the cut off. This was calculated as the mean of the figures of the control sera plus 3 standard deviations (SD). Any sera whose optic density (OD) figure exceeded the cut off was considered positive.

RESULTS

Production of Ro/SS-A of 52 and 60 kDa recombinant protein

The profiles of the recombinant proteins made in E-coli are shown in figure 1. The bacterial lysate is shown in the first gel (left). As it can be seen in lanes 3 and 5 the recombinant proteins are the ones mainly expressed. In lanes 4 and 6 the washed and solubilised inclusion bodies, corresponding to Ro/SS-A of 52 and 60 kDa, can be observed.

Lanes 1 and 2 correspond to bovine serum albumin and ovoalbumin, respectively, and they were used as molecular weight markers (66 and 45 kDa).

The second gel (right) shows the purified recombinant proteins. Ro/SS-A 52 kDa (0.73 mg/ml) was run in lane 1’, whereas Ro/SS-A 60 kDa (0.18 mg/ml) was run in lane 2’. Lane 3’ contains ovoalbumin (45 kDa) and bovine serum albumin (66 kDa).

Immune diffusion

A scheme of a DID in agarose gel using HSE as antigen is shown in figure 2, left. The precipitation bands can be differently observed in wells 1, 2 and 3.

The immunoreactivity of the HSE and the commercial antigen against the anti-Ro/SS-A and anti-La/SS-B were similar.

Western Immune Blot

The reactivity of series of patients’ sera were determined by western blot as shown in figure 2. The majority of the patients included in the series recognised the Ro/SS-A 52 kDa mainly. False positive results were not detected in the control series (data not shown).

ELISA

A number of 35 sera were analysed as healthy controls. These sera came from healthy patients, and their age and sex were similar to the patients whose sera were analysed as pathological ones. Using these negative controls the OD cut off was determined as the sera mean plus three SD. The sera from the patients with SS which were above that figure were considered positive. The relation between the evaluation by means of DID, WB and ELISA are shown in figure 3 and in charts 1 and 2.

Figure 4 shows the relation between the clinic parameters and the presence of autoantibodies anti Ro/SS-A 52 and 60 kDa in 20 patients of our group. The presence of anti Ro 52 antibodies showed a high correlation with most of the clinical parameters. For example the 83% of the patients with Raynaud’s Syndrome and the 85% of the ones with Arthritis have anti Ro 52 antibodies. In the same way, the absence of anti Ro 60 antibodies is related to the clinical parameters. For instance, 70% of the patients with parotid tumefaction and 75% of those with Peripheral Nervous System (PNS) manifestations have negative anti Ro 60 antibodies.
DISCUSSION

The methodologies used to determine the presence of anti Ro antibodies analysed in the present study are diverse in reference to the principles involved.

Even though it is true that ELISA and WB techniques are highly sensitive, more than the gel precipitation method, this difference does not necessarily imply that the first ones can replace this last technique.

Sera from the group of patients which were analysed in this work were divided into two groups, according to their capacity to precipitate the Ro/SS-A native antigen. Among the group which was not considered reactive by DID, 50 (8/16) turned out to be positive by any primary interaction technique that uses a recombinant antigen. 75% (6/8) of these sera were positive for Ro/SS-A 52 kDa by WB (data shown in table I). Considering this result it could be concluded that there are antibodies in the serum of these patients which recognise the Ro/SS-A 52 kDa antigen molecule in its denatured state. That is to say that these antibodies may be directed to the denatured epitopes of the Ro/SS-A 52 kDa antigen.
possibly be recognising linear epitopes that are hidden in the native antigen (used for DID) but that become exposed during the performance of SDS-PAGE. These results remark the importance of the analysis by WB of these sera.

It is worth to notice that some sera have antibodies which are able to recognise the proteins by WB but not by ELISA technique, or vice versa. This fact allows us to observe the diversity of the recognised epitopes, for example, hidden epitopes of the folded recombinant protein could be recognised when the protein is partially denatured, as it happens in the WB assay (this is the case of serum 34 that is negative by ELISA and positive by WB). It is also possible to find antibodies which are able to recognise conformational epitopes in the folded protein, those which are lost when the protein is denatured (an example is serum 35 that is positive by ELISA and negative by WB). As a result, of all the sera which are positive by WB, only 63 (12/19) are also positive by ELISA, and 86% (12/14) of the positive ones by ELISA is also positive by WB (17, 18).

Although antibodies against hidden epitopes in the native molecule could be recognised when performing an analysis by WB, and the ELISA technique is more sensitive than the DID, these two methodologies are not capable to replace completely the gel precipitation. Our study shows that 26% (5/19) of the sera which were positive by DID do not recognise the recombinant antigen by any technique, ELISA or WB (Table II). This shows the importance of the analysis with the native antigen due to, as formerly commented, the loss of epitopes present in the native ribonucleoproteic complex during the performance of the techniques using recombinant antigens.

An important point to have in mind is that the anti Ro autoantibodies, when present, are found in quite high concentrations in the sera of the patients with different autoimmune diseases. This allows the technique of DID to be useful, because a high sensitivity is not needed to detect them.

As a conclusion of all the facts exposed in this work, it could be said that no technique can be put aside or replaced but all of them have to be utilised together, in order to obtain valuable results.

**ACKNOWLEDGMENTS**

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**REFERENCES**


**TABLE II. Analysis of serum from patients Ro positive by DID**

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p: positive; n: negative.
17. Inub Y, Reichlin M. Autoantibodies to the Ro/SS-A antigen are conformation dependent. Anti-60-kD antibodies are mainly directed to the native protein; anti-52-kD antibodies are mainly directed to the denatured protein. Autoimmunity 1992;14:57-65.