Anti-Sa Sera from Patients with Rheumatoid Arthritis Contain at Least 2 Different Subpopulations of Anti-Sa Antibodies

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ABSTRACT. ***Objective.*** Anti-Sa antibodies have been described to be a highly specific marker for rheumatoid arthritis (RA). We demonstrate the existence of 2 different subsets of anti-Sa antibodies, only one of which is specific for RA. Our objective was to purify the Sa antigen, and to achieve partial characterization of these proteins.

Methods. Saline extract and mitochondrial extract from human placenta were used as antigenic sources. Antigens were purified by immunoaffinity chromatography and studied by ELISA and immunoblotting.

Results. Three antigenically active bands of 68, 50, and 46 kDa were purified from the saline extract by immunoaffinity chromatography. Two other bands of 29 and 10 kDa that do not react with anti-Sa antibodies were obtained as well. The 68 kDa band was purified from a mitochondrial extract. These bands are not the same as other known mitochondrial autoantigens such as M2, M4, or M9. The amino terminal sequence of the 68 kDa Sa band is DEPKXEVP. The sequence of the 68 kDa Sa band is not compiled in the databases we searched, as either aminoterminal or internal sequence. Antibodies to 50/46 kDa anti-Sa bands detected by immunoblotting were highly specific for RA, while the 68 kDa antigen reacted in ELISA with sera from patients with RA and systemic lupus erythematosus, the latter showing a marked increase in features of RA. Antibodies directed against the 68 and 50/46 kDa Sa bands fluctuated with time, the 50/46 kDa anti-Sa antibodies present during the active period of the disease, and the 68 kDa anti-Sa antibodies during the remission period.

Conclusion. At least 2 subsets of autoantibodies are present in anti-Sa sera, one directed against a 68 kDa Sa protein and another to the typical 50/46 bands of the Sa system. (J Rheumatol 2002;29:2053–60)

Key Indexing Terms:
RHEUMATOID ARTHRITIS ANTIGEN ELISA
PLACENTA MITOCHONDRIA IMMUNOPURIFICATION

Despres, *et al* identified a novel antigen-antibody system specific for rheumatoid arthritis (RA) called Sa[1], which differs from all other autoimmune systems associated with RA. Sa antigen can be obtained from human placenta or spleen. Anti-Sa antibodies are associated with extraarticular manifestations and more severe articular disease[2-5]. It is not known if anti-Sa antibodies are related to the pathogenesis of RA or if they are an epiphenomenon. However, the presence of Sa antigen in rheumatoid synovial tissue[6] suggests that these antigens could be involved in the development of the chronic articular destructive process in RA.

The very high specificity (92–98%) and positive predictive value (84–96%) of anti-Sa antibodies for RA suggest that detection of these antibodies may be a useful laboratory test for the diagnosis of RA[1-5,7,8]. However, immunoblotting is the only method available for detection of anti-Sa antibodies and its sensitivity is low (31–43%)[1-5,7,8], especially in early RA[5,9]. A more sensitive assay is needed. We obtained immunoaffinity purified Sa antigen and used it to develop an ELISA for the detection and measurement of serum anti-Sa antibody levels. Sera from patients with RA contain 2 different subsets of anti-Sa antibodies, one reactive to a 50/46 kDa Sa antigen and the other reactive to a 68 kDa Sa protein.

MATERIALS AND METHODS

Preparation of antigen sources

Saline extract. Human placenta extracts were prepared using the method described by Clark, *et al*[10], with modifications[1,11,12].

Differential centrifugation. A placental homogenate was fractionated by differential centrifugation following the method of Graham[13]. Human

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placental tissue, 25 g, was homogenized in 4 volumes of 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM ethylene diaminotetraacetic acid (EDTA), and 1 mM phenylmethylsulfonylfluoride (PMSF) in a blender for 5 min. It was centrifuged at 1000 g for 10 min. The pellet contained nuclei, plasma membrane fragments, intact cells, and debris. The supernatant was centrifuged at 3000 g for 10 min to obtain a pellet containing large mitochondria and plasma membrane fragments. The resulting supernatant was centrifuged at 10,000 g for 20 min to obtain a pellet containing mitochondria, lysosomes, and peroxisomes. The final supernatant was centrifuged at 100,000 g for 40 min to obtain a pellet containing microsomes of smooth and rough endoplasmic reticulum, Golgi apparatus membranes, and plasma membrane. The soluble cytoplasmic components remained in the supernatant. Pellets were resuspended in the buffer used for homogenization and studied by immunoblotting.

Mitochondrial extract. Human placental mitochondria were isolated as described by Kellis and Vickery[14] with modifications. The placental tissue was homogenized with 2 volumes of 25 mM phosphate buffer, pH 7.4, 0.25 M sucrose, and 1 mM EDTA. It was centrifuged at 800 g for 10 min. The pellet was rehomogenized and centrifuged in the same way. The supernatants were pooled and centrifuged 25 min at 15,000 g to pellet mitochondria. The pellet was resuspended in 10 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, and 0.01% butylated hydroxytoluene and recentrifuged at 15,000 g for 25 min. The washed mitochondria were resuspended in lysis buffer (50 mM sodium phosphate, pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mg/l butylated hydroxytoluene, 20% glycerol) and kept at –20ºC.

Mitochondria from 4 placentas were thawed and a solution of 10% deoxycholate was added to obtain a final concentration of 1.3%; it was stirred on ice for 30 min and then centrifuged at 15,000 g for 40 min. Aliquots of supernatant were obtained and frozen at –70ºC until use.

Microsomal extract. Human placental microsome isolation was as described[14]. The placenta was processed as described above. After sedimentation of the mitochondria, the supernatant was centrifuged at 140,000 g for 30 min to pellet microsomes. This pellet was resuspended in 100 mM sodium pyrophosphate, pH 7.4, 1 mM EDTA, and 5 mg/l butylated hydroxytoluene, and recentrifuged at 140,000 g for 30 min. The washed microsomes were resuspended in lysis buffer and frozen until use. To obtain the antigen extract, microsomes from 8 placentas were combined and proteins were extracted in the same way as for mitochondria.

Immunoblotting and specific anti-Sa antibody elution. Electrotransfer to nitrocellulose was performed as described[15,16]. Membranes were blocked with 3% bovine serum albumin (BSA) in Tris-saline and incubated 1 h at room temperature with 1:5 dilutions of sera from patients with RA or other rheumatic diseases and from healthy controls. After washing, membranes were incubated with an anti-IgG/peroxidase conjugate for 2 h. After 3 washing steps, reactions were visualized with H_2O_2/4-Cl,1-naphthol.

Purified anti-Sa antibodies were obtained from the immunoblotting. The antigenic extract was separated by electrophoresis and transferred to nitrocellulose as described. The membrane was then blocked and incubated with a positive anti-Sa serum diluted 1:5 for 1 h. Two strips were then cut from the left and right edges and incubated with an anti-IgG/peroxidase conjugate. The reaction was visualized with H_2O_2/4-Cl,1-naphthol. Once the reaction developed, the strips were used to locate the band of interest. This band was carefully cut and incubated with 200 μl of 100 mM Gly-HCl, pH 2.7, for 10 min while being shaken vigorously. The supernatant was collected and neutralized with 1 M Tris, pH 8; 3% BSA was added and antibodies were used for immunoblotting[17,18].

Antigen purification

IgG from a positive serum was purified by affinity chromatography with protein G-sepharose[19] and coupled to Affi-Gel Hydrazide (Bio-Rad) following the manufacturer's instructions.

A 100 ml volume of antigen extract, either saline extract or mitochondrial extract, was diluted in phosphate buffered saline (PBS) and filtered, then recirculated through the column overnight. Then the column was washed with phosphate buffer, pH 7.4, 500 mM NaCl until no protein was detected in the eluent. The bound antigen was eluted with 6 M urea and 0.5 ml fractions were collected. The fractions corresponding to the peak were pooled and concentrated by ultrafiltration in a Centricon-30 (Amicon).

The molecular weight of the Sa antigen was calculated by slab gel electrophoresis (SDS-PAGE), using phosphorylase B (94 kDa), BSA (67 kDa), ovoalbumin (43 kDa), carbonic anhydrase (30 kDa), soy trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa) as standards.

Aminoterminal sequencing and identification of the Sa antigens[20]. The 68, 54/46, and 30 kDa bands obtained from immunoaffinity chromatography using the saline extract were electrophoresed and blotted onto polyvinylidene difluoride (PVDF) membrane. The membrane was then stained with amido-black protein stain, and the bands were carefully excised. Each band was partially sequenced in a Procise 494 sequencer (Perkin-Elmer).

SwissProt and TrEMBL databases[22] were searched for the resulting sequences to identify the proteins.

ELISA[23,24]. A 96 well ELISA microplate was coated with purified 68 kDa antigen in carbonate buffer, pH 9, at concentration of 1.5 μg/ml. The remaining sites were blocked with 3% BSA in PBS for 1 h at 37ºC. After washing excess BSA, 100 μl of serum was diluted to 1:200 in PBS and 1% bovine gammaglobulin, then added to each well. Sera were tested in duplicate, either antigen coated or mock coated wells. The plate was incubated 1 h at room temperature and washed 3 times with PBS. The anti-human IgG/alkaline phosphatase conjugate was diluted to 1:3000 in the same buffer used for serum dilution and 100 μl were added to each well. The plate was incubated again for 1 h at room temperature, then washed 3 times with PBS. The substrate solution was then added (0.5 mg/ml p-nitrophenyl phosphate in diethanolamine) and the color reaction was allowed to develop for 2 h at 4ºC. The optical density (OD) was read at 405 nm using a reference wavelength of 655 nm.

ELISA units were calculated by subtracting the mean OD of the mock coated wells from the mean OD of the antigen coated wells and multiplying the result by 10^3 for each serum. Every plate contained a positive reference serum as a calibrator. The cutoff value was defined as the mean plus 2 standard deviations of the normal human sera tested. The specificity of the reaction was verified by inhibition assay under the same conditions as the ELISA, except that the serum was incubated with increasing antigen dilutions for 1 h at 37ºC prior to the assay.

Patients and controls. Sera from 54 patients who met American College of Rheumatology criteria for RA[21], 41 with systemic lupus erythematosus (SLE), 24 with chronic discoid lupus, 20 with scleroderma, 24 with arthrosis, 24 with fibromyalgia, and 21 healthy donors were studied by immunoblotting and ELISA. All patients were diagnosed and treated at the Rheumatology Service of the Hospital General Universitario Gregorio Marañón. Anti-Sa positive reference sera were selected on the basis of high reactivity in immunoblotting and absence of reactivity toward other known autoantigens. Reference antisera to Ro (SSA), La (SSB), RNP, Sm, Scl70, and Jo-1 were obtained from the Centers for Disease Control, Atlanta, GA, USA.

RESULTS

Antigen purification. When human placental and spleen saline extracts were tested with anti-Sa sera, a typical binding pattern was observed by immunoblotting, consisting of 3 bands between 48 and 36 kDa molecular weight (Figure 1). Unfortunately, the yield of this method was very low and the extraction was irreproducible because different pieces of the same specimen produced negative and positive extraction results. To optimize the extraction of Sa antigen from human placenta, immunoblotting was

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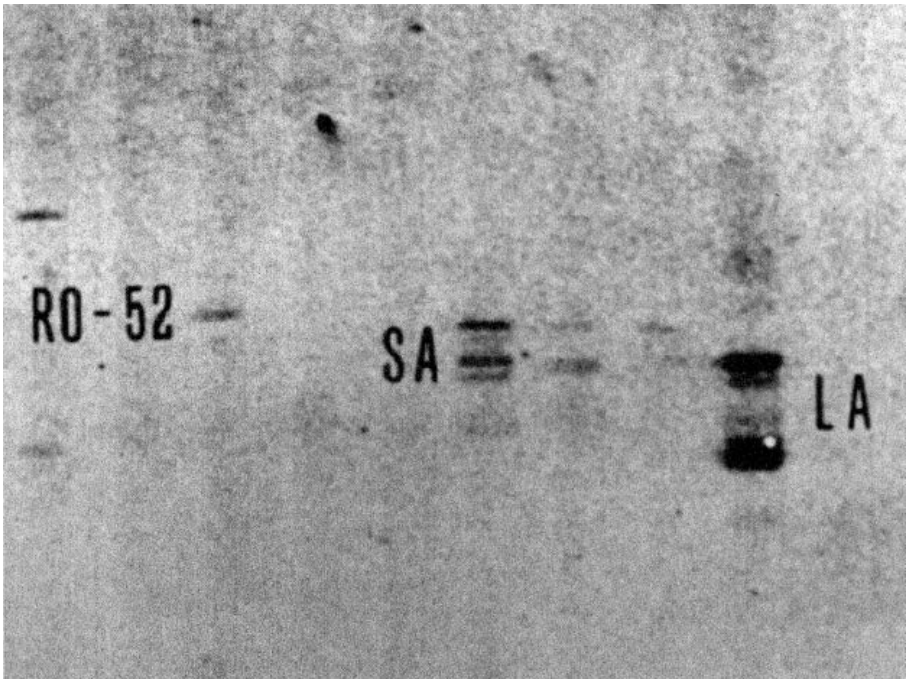

Figure 1. Detection of anti-Sa antibodies by immunoblotting, using a saline extract from human placenta. The difference between anti-Sa antibodies and anti-Ro/La antibodies is visible.

performed after each step of the process. It was found that the protein was always present in samples after saline extraction, demonstrating that Sa antigen was present in human placenta. The antigen was lost after ionic exchange and only appeared as an impurity in some preparations. To solve this problem, the ionic strength limits for the adsorption/elution of the protein to the resin were redetermined. It was found that Sa protein bonded poorly to the resin and was lost in the washing steps.

Affinity chromatography was performed using the crude saline extract to purify the Sa antigen. Using Gly-HCl, pH 2.7, as the eluent, bands of 68, 50, 46, 29, and 10 kDa were obtained (Figure 2). Only the 50 and 46 kDa bands were reactive by immunoblotting using anti-Sa sera (50/46 kDa

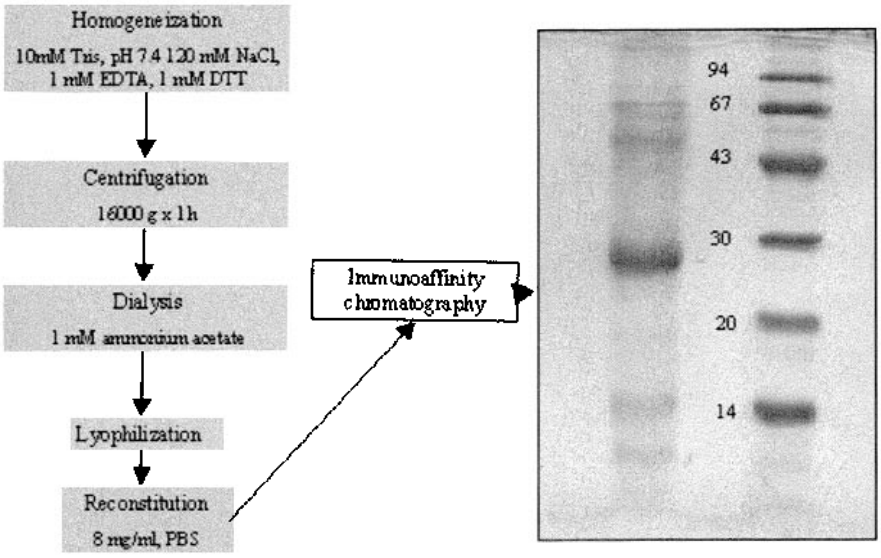

Figure 2. Electrophoresis of the Sa antigen, purified from a crude human placental saline extract. Electrophoresis was performed in 15% acrylamide gel. Coomassie blue stain. Lane 1: Sa antigen — 68, 50, 46, 29, and 10 kDa. Lane 2: MW standards.

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Sa antigens). If the elution step was carried out with 6 M urea, the 68 kDa band was reactive as well.

Fractions of the placental homogenate were obtained by differential centrifugation. Antigenic activity was detected by immunoblotting in a fraction that contained mostly mitochondria and in a fraction containing mitochondria, lysosomes, peroxisomes, endoplasmic reticulum, and Golgi apparatus microsomes.

Affinity chromatography was performed using the placental mitochondria extract and the column described above. If Gly-HCl buffer was used to elute the antigen from the column, a band was obtained at 68 kDa, but it was not reactive by immunoblotting. Using 6 M urea as the eluent, the same 68 kDa band was obtained and it retained antigenic activity by immunoblotting (68 kDa Sa antigen) (Figure 3).

48 kDa and 40/36 kDa Sa antigens from human placenta saline extract are antigenically related. The saline extract was electrophoresed and blotted under denaturing conditions. Purified antibodies (eluted from each band in a previous immunoblot) were used as probes. Both sets of antibodies reacted to all the bands (Figure 4). This cross reactivity between the 48 kDa and 40/36 kDa bands obtained from the placental saline extract suggests that the bands are derived from the same polypeptide, or they are very closely related proteins (e.g., Sm proteins B and D).

Aminoterminal sequence of the Sa antigen immunopurified from crude salt extracts from placenta. The 50 kDa band could not be directly sequenced, probably due to aminoterminal blockage, although this must be confirmed by other techniques.

The sequence of the 68 kDa band corresponds to DEPKXEVP, where X represents a residue that could not be determined. This sequence is not compiled in the databases we searched, either as an aminoterminal or an internal fragment.

ELISA for detection of anti-68 kDa Sa antibodies. The 68 kDa antigen was used to develop an ELISA for the detection of anti-Sa antibodies. Using a dilution to 1:20 of the sera, the sera from patients with RA was clearly discriminated from that of normal sera. However, when sera with marked hypergammaglobulinemia were tested, an intense background reaction was observed. This made it necessary to dilute the samples, at the risk of missing weak positives. Sera were tested at 1:200 using BSA coated wells with a blank included on each plate. Anti-Sa sera were incubated with progressively higher concentrations of 68 kDa antigen, obtaining up to 60% inhibition at an antigen concentration of 5 μg/ml (Figure 5).

Comparison between the detection of anti-50/46 kDa antigen and anti-68 kDa antigen antibodies. Sera from 208 subjects were studied by ELISA for anti-68 kDa Sa and by immunoblotting for anti-50/46 kDa Sa. Anti-68 kDa antigen antibodies were detected in the sera of 28 patients, of which 18 had RA, 9 SLE, and one arthrosis. Nine patients with SLE and anti-68 kDa Sa antibodies showed a marked increase in features of RA, although they did not meet criteria for this disease. However, 7 of these patients showed some criteria. Anti-50/46 kDa Sa antibodies were detected in 21 of the 208 patients, all of whom had RA. These results are summarized in Table 1.

Major discrepancies were observed in the results obtained with the 2 tests. In the group of 208 patients, the results of both tests coincided (both positive or both negative) in 81% of cases. Considering only the 54 patients with

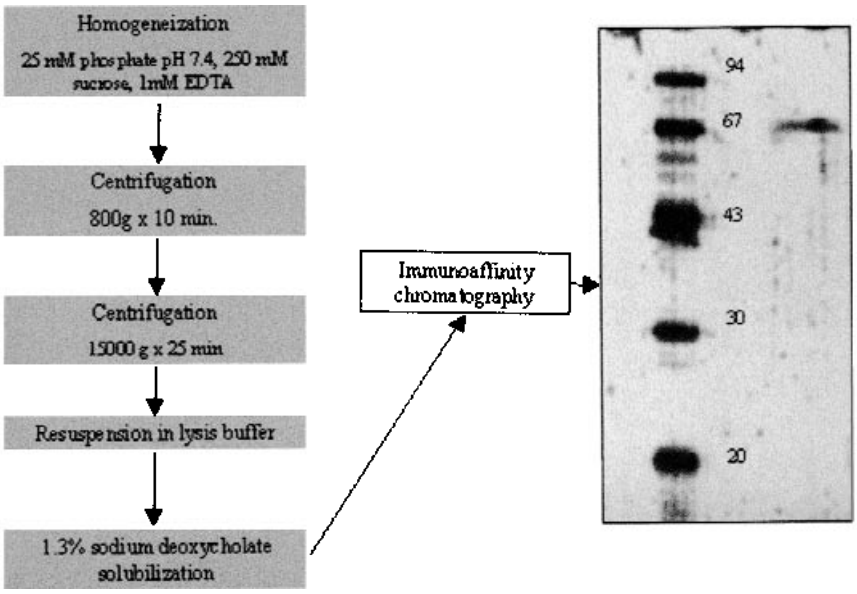

Figure 3. Electrophoresis of the Sa antigen, purified from a placental mitochondrial extract. Electrophoresis was in 15% acrylamide gel. Coomassie blue stain. Lane 1: MW standards. Lane 2: Sa antigen — 68 kDa band.

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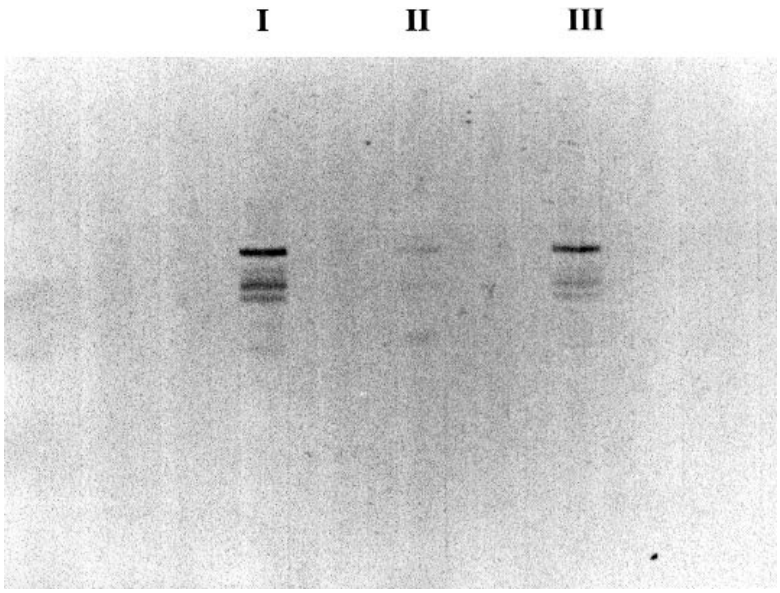

Figure 4. Reactivity of anti-Sa antibodies eluted from the immunoblot. Antibodies eluted from the upper (48 kDa) or lower (40/36 kDa) bands react with all of them. Lane 1: whole anti-Sa serum. Lane 2: antibodies eluted from the lower band. Lane 3: antibodies eluted from the upper band.

RA, the results coincided in only 54%, as summarized in Table 2. Immunoblotting of the purified 68 kDa Sa was performed using sera positive by ELISA. All sera positive by ELISA were also positive by immunoblot. No antiextractable nuclear antigen (ENA) control serum gave a positive reaction to the 68 kDa Sa antigen.

Fluctuation of the anti-Sa antibody concentration in serum. Anti-Sa antibody levels in serum were studied in 2 patients from 1989 to 1993. In both cases, the increase in anti-68 kDa Sa antibody levels quantified by ELISA was accompanied by negative immunoblot for anti-50/46 kDa Sa antibodies. This phenomenon coincided with remission of the disease, during which the clinical and biological indicators were normal. The immunoblot for anti-50/46 kDa Sa became positive again during a period of renewed disease activity. At this time, ELISA values for anti-68 kDa Sa were normal (Figure 6).

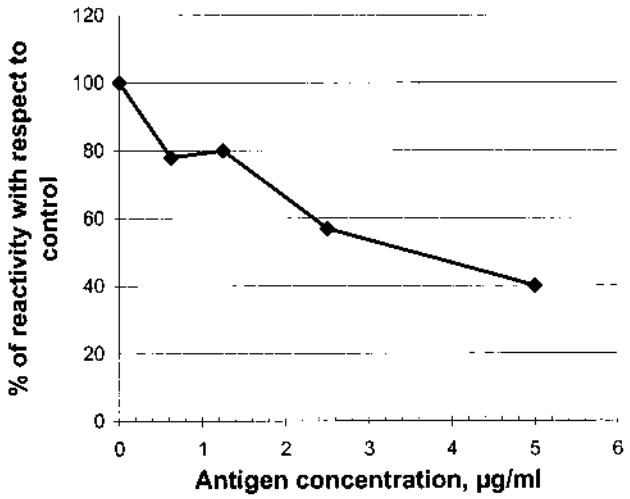

Figure 5. Inhibition of the reactivity of anti-68 kDa antibodies by ELISA. Sera were incubated with increasing concentrations of antigen prior to the assay.

DISCUSSION

Sa antigen was detected by immunoblotting for the first time

Table 1. Detection of anti-Sa antibodies in 208 patients. The antibodies directed against the 68 kDa band were measured by ELISA, and the anti-50/46 antibodies were detected by immunoblotting.

Patient	N	Anti-68 kDa Sa Antibodies	Anti-50/46 kDa Sa Antibodies
Rheumatoid arthritis	54	18	21
Systemic lupus erythematosus	41	9	0
Chronic discoid lupus	24	0	0
Scleroderma	20	0	0
Arthrosis	24	1	0
Fibromyalgia	24	0	0
Healthy controls	21	0	0

Table 2. Discordances between antibodies directed against the 68 kDa band and the 50/46 kDa bands. In each case, the left value corresponds to the study of 208 patients, and the right value is from the study of the 54 patients with RA.

	Anti-68 kDa Sa+	RA Patients Only	Anti-68 kDa Sa–	RA Patients Only
Anti-50/46 kDa Sa+	7	7	14	14
Anti-50/46 kDa Sa–	21	11	166	22

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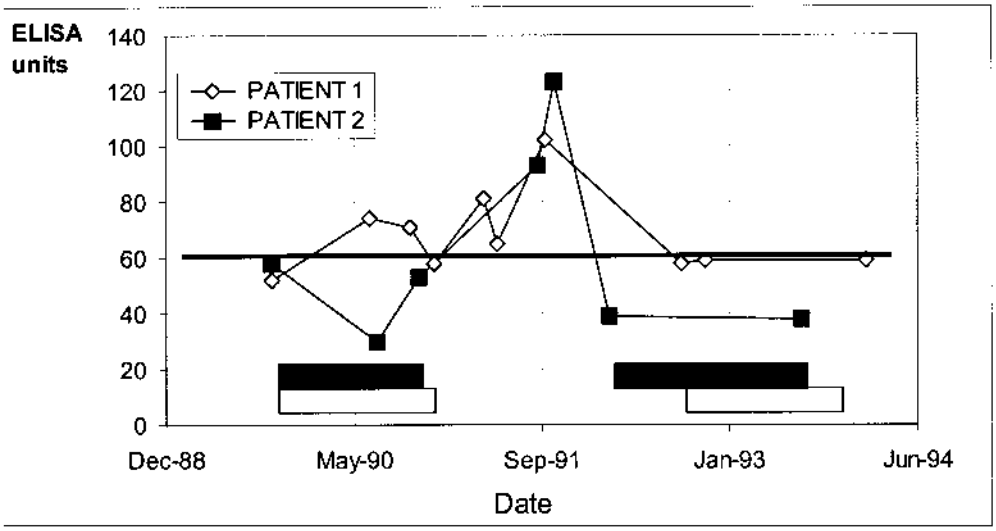

Figure 6. Fluctuation of anti-Sa antibodies in 2 patients with RA. The periods in which the anti-50/46 kDa antibodies are detected by immunoblot are coincident with the periods in which the values for anti-68 kDa antibodies are lower than the cutoff value (mean + 2 SD of values for controls), and correspond to the clinically active period of the disease. White bars indicate positive immunoblot for Patient 1, black bars positive immunoblot for Patient 2.

in placenta and spleen saline extracts. The saline extract in which Sa antigen was detected for the first time was initially prepared for study of the Ro particle[1,11,26,27]. This is an easy way to obtain an adequate extract for anti-Sa antibody determination by immunoblotting because semipurified extract is used and it does not require antigen purification. However, Sa antigen is a partially degraded impurity in this extract. As antibodies against this protein were detected only in patients with RA, they were thought to be a good serum marker of this disease. However, immunoblotting is not a useful detection method in clinical laboratories because it is a time-consuming technique that is unsuitable for processing batches of samples. Thus a screening assay for detection of anti-Sa antibodies, such as ELISA, is useful, with immunoblotting as a confirmation test.

To develop the ELISA, it was first necessary to purify the antigen. The Sa antigen was located by differential centrifugation in placental mitochondria and microsomes. When mitochondria and microsomes were purified, it was located only in mitochondria. Anti-Sa antibodies do not react to other mitochondrial autoantigens such as M2, M4, and M9 (data not shown).

Problems with purifying Sa antigen come from antigen degradation in low ionic strength buffers. We observed that the Sa protein was lost during ionic exchange, being washed out when the resin was washed. If the net charge of the protein, which determines binding to the DEAE cellulose resin, changes with degradation, the problem would be the same with any other ionic exchanger. Therefore, ionic exchange is not suitable for purifying Sa antigen.

The crude saline extract was used to perform affinity chromatography. This yielded 2 antigenically active bands of 50 and 46 kDa. Affinity chromatography was also performed using the mitochondrial protein preparation, which yielded a single band of 68 kDa. This band reacted with anti-Sa sera by immunoblotting, but the reaction was faint in most cases.

To study the structural relation between the 48 and 40/36 kDa Sa proteins, an immunoblot of the saline extract was performed using purified antibodies against each band, which had been eluted from a previous immunoblot. The aim of this experiment was to determine if both bands belonged to the same polypeptide. Both antibodies reacted to both bands — in other words, the epitope to which anti-Sa antibodies are reactive was present in all the Sa antigen bands detected in the saline extract. The differences in the electrophoretic mobility of the bands could be due to partial degradation of the protein during extraction.

The aminoterminal fragment of the 68 kDa band was sequenced, and the SwissProt and TrEMBL databases were searched for this sequence. No matches were found, neither for aminoterminal sequences nor internal fragments of the existing entries. New strategies must be found to identify this protein and its biological activity. The 50 kDa band could not be sequenced, probably due to aminoterminal blockage. This is very frequently observed in intracellular proteins. Another possibility for identification of this band could be a partial digestion and sequencing of the digestion fragments.

Significant discrepancies were observed in a large percentage of sera when the detection of anti-68 kDa antibodies by ELISA was compared with the detection of anti-50/46 antibodies by immunoblotting. Similar discrepancies between techniques have been reported for other Ag/Ab systems[26-28]. Boire, *et al*[11] identified sera reactive to the Ro

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particle by counterimmunoelectrophoresis, immunodiffusion, and ELISA, but not by immunoblotting. In these sera, antibodies to the native particle coexist with antibodies to denatured protein. The authors suggested that the antibody response is directed against native antigen because antibodies against the native antigen are immunodominant. It is possible that the anti-Sa response is similar to that of the Ro system. In both cases, there seemed to be 2 subsets of antibodies, one reactive to the 68 kDa antigen and the other to the 50/46 kDa protein. Both subsets coexist in the same serum, but often only one of them is detected by a single technique. As experimental autoimmunity models show, the autoantibodies induced by immunization are directed at first against nonconserved native sites present on the surface of native proteins[1]. The followup results of 2 patients with RA from 1989 to 1993 and from 1989 to 1994, respectively, are striking, although they are only preliminary data that need to be confirmed by study of a larger number of patients. The highest values for anti-68 kDa antibodies determined by ELISA were obtained in sera in which immunoblotting for anti-50/46 kDa antibodies was negative, coinciding with remission periods of the disease, including biological and clinical measures. That anti-68 kDa antibodies were detected by ELISA when anti-50/46 kDa antibodies were negative by immunoblotting suggests a heterogeneous anti-Sa response. Anti-50/46 kDa antibodies appeared when articular inflammation was active and tissue damage was taking place. The inverted fluctuation of the 2 subsets of antibodies suggests a response directed against 68 kDa protein that switched to an anti-50/46 kDa response during active periods of the disease, when the characteristic inflammatory phenomena were taking place. These data suggest 2 types of immunological response to Sa antigen, one involving the 68 kDa antigen and one the 50/46 kDa protein. Both types of antibodies coexist in the sera of patients with RA and only anti-50/46 kDa is disease-specific.

There may be an initial antibody response against a native epitope in the 68 kDa protein. If, as a consequence of denaturation, the protein reveals a hidden epitope, this could cause the switch in response, which would be initially directed against the native form. To check these possibilities, an immunoblot study of sera from patients positive by ELISA was performed using 68 kDa antigen for both assays. All sera positive for anti-Sa antibodies by ELISA were positive by immunoblot, while all control sera and sera positive for anti-Ro/La, anti-RNP, and other anti-ENA were negative. These results rule out the possibility of a single epitope with different conformations.

A second possibility would be an early antibody response to 68 kDa protein that switched to anti-50/46 kDa as a consequence of inflammation and tissue damage. Under these circumstances, these polypeptides are released as degradation products of the 68 kDa band and originate a second subset of antibodies.

Finally, another possibility is the existence of 2 subsets of antibodies against epitopes located on different polypeptide chains. Again, the initial response would be directed against the 68 kDa band and the other antigens would be exposed during periods of disease activity, inducing a switch in response.

Anti-50/46 kDa antibodies could be involved in the pathogenesis of RA, while anti-68 kDa antibodies could be related to remission, even if the initial response is driven by the anti-68 kDa antibodies. Determination of anti-68 kDa antibodies by ELISA and anti-50/46 kDa by immunoblotting may be useful for diagnosis and followup of patients with RA.

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