New Coupled-Particle Light-Scattering Assay for Detection of Ro/SSA (52 and 60 Kilodaltons) and La/SSB Autoantibodies in Connective Tissue Diseases

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The diagnostic and analytical performance of the coupled-particle light-scattering assay in detecting anti-Ro/SSA autoantibodies (the 60-kDa [Ro60] and the 52-kDa [Ro52] antibodies) and anti-La/SSB autoantibodies was evaluated. The antigens were obtained by recombinant DNA procedures to include the most immunogenic epitopes for each protein by using a prokaryotic expression system. Serum samples from 151 patients with connective tissue diseases and 52 control subjects (including patients with viral infections, patients with Lyme disease, and healthy subjects) were studied. Sensitivities for detection of anti-Ro/SSA and anti-La/SSB were 88.2 and 95.2%, respectively; specificities were 97.6 and 98.1%, respectively. The intra-assay coefficient of variation (CV) ranged from 4.3 to 10.9% for anti-Ro/SSA and from 2.8 to 12.5% for anti-La/SSB; interassay CVs ranged from 6.5 to 13.2% and from 8.2 to 14.5%, respectively. Among the anti-Ro/SSA-positive samples, Ro60 was recognized by 66% of the test sera and Ro52 was recognized by 95% of the test sera. Thirty-four percent of the Ro/SSA-positive sera were reactive only with the Ro52 antigen, indicating that anti-Ro52 is the most common antibody specificity recognized by anti-Ro/SSA autoantibodies. No differences were found between the prevalences of anti-Ro60 and anti-Ro52 in relation to systemic lupus erythematosus or Sjögren’s syndrome. The results of the present study indicate that this new immunoassay is an efficient diagnostic tool for the detection of anti-Ro/SSA and anti-La/SSB autoantibodies in patients with autoimmune disorders.

Anti-Ro/SSA and anti-La/SSB autoantibodies are two of the specific antibodies associated with connective tissue diseases (CTDs). Depending on the assay performed and how the patients are selected, the evidence shows that from 40 to over 90% of patients with Sjögren’s syndrome (SS) have anti-Ro/SSA autoantibodies and that 20 to 50% have anti-La/SSB autoantibodies (22, 28, 37). Both autoantibodies can also be detected in 10 to 50% of patients with systemic lupus erythematosus (SLE); they are less frequent in patients with other CTDs, such as mixed CTD, dermatopolymyositis, and systemic sclerosis (16), and are only occasionally detected in patients with rheumatoid arthritis (RA) (35) or primary biliary cirrhosis (27). The presence of anti-Ro/SSA and/or anti-La/SSB is one of the criteria for the diagnosis and classification of SS (36).

Their presence also has a prognostic value: anti-Ro/SSA autoantibodies are more frequently detected in sera from SS patients with early disease onset, long disease duration, and intensive lymphocytic infiltration of salivary glands (35). Furthermore, their presence correlates with the presence of extraglandular manifestations, such as nephropathy, hypergammaglobulinemic purpura, photosensitive rash, lymphadenopathy, splenomegaly, and vasculitis (7, 31, 35). Patients with anti-La/SSB autoantibodies tend to have an increased incidence of cutaneous manifestations, vasculitis, leukopenia, and lymphopenia compared to the incidences among patients without these antibodies (18). Furthermore, the presence of anti-Ro/SSA antibodies (particularly the anti-Ro/SSA antibody of 52 kDa [Ro52]) in pregnant women may cause neonatal lupus in the newborn, whose most serious clinical feature is congenital heart block (9, 10).

Several methods are commonly used in clinical laboratories to detect these autoantibodies, but none was shown to be better than the others concerning diagnostic accuracy (2, 4, 5, 8, 14, 15, 20, 21, 23, 25, 33). Enzyme-linked immunosorbent assay (ELISA) methods exhibit high degrees of sensitivity but low degrees of specificity; counterimmunoelectrophoresis and immunodiffusion are generally acknowledged to be very specific, but they lack sensitivity; immunoblot techniques show good specificity but are less sensitive than ELISA for the detection of anti-Ro/SSA antibodies due to conformational changes in Ro proteins during assay procedures, leading to alteration of epitopes (15, 25). Moreover, at present, only ELISA is suitable for the extensive routine workups that are performed in clinical immunology laboratories.

We evaluated the sensitivity, specificity, and precision of a new immunoassay based on recombinant antigens, carried out on a novel instrument, the Copalis system (Diasorin, Stillwater, Minn.), for the determination of autoantibodies directed against Ro/SSA and La/SSB. In addition, in view of reports that autoantibodies to the 52-kDa component are more frequently found in the sera of SS

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patients, whereas autoantibodies to the 60-kDa component are more often observed in SLE patients (3, 29, 32), and that this different behavior may be helpful in the differential diagnosis of these autoimmune disorders, we also evaluated the prevalence and distribution of the two anti-Ro antibodies in patients with an established diagnosis of SS or SLE.

MATERIALS AND METHODS

Recombinant DNA procedures and protein purification. The cDNAs corresponding to Ro52 and Ro60 and Lg genes were isolated from HEP-2 and HeLa cells, respectively. Total RNA was purified by standard methods, and poly(A)+ mRNA was purified by oligo(dt) cellulosic chromatography. For cDNA synthesis and gene isolation, we designed specific primer pairs according to published sequences (11, 12, 17, 34) to include the most immunodominant epitopes for each protein. Primer synthesis was performed in-house on a Beckman Oligo 1000 instrument.

Ro52 cDNA included the sequence coding for amino acids 1 to 316 of the original protein, comprehensive of two zinc fingers and one leucine zipper motif; the Ro60 cDNA sequence coded for amino acids 60 to 484, comprehensive of an RNA-binding domain and a zinc finger (13). Lg cDNA was amplified and included the sequence coding from amino acids 9 to 389, comprehensive of a ribonuclease domain and a PEST region.

The cDNA fragments thus obtained were then inserted into bacterial (E. coli) expression vectors (pET); those for both Ro/SSA autoantibodies were included in a 1000 instrument.

Recombinant clone expression was induced by adding 1 mM isopropyl-

β-D-thiogalactopyranoside to the growth medium, and the specific proteins were then visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting (30).

Recombinant Ro52 and Ro60 were generated as His-tag fusion proteins of 45 and 57 kDa, respectively, and were purified on a chelating affinity column, taking advantage of the N-terminal His tail (19).

Recombinant La expression led to a 50-kDa protein which was subsequently purified by cationic exchange and hydrophobic interaction chromatography (Pharmacia Biotech, Uppsala, Sweden).

The Copalis system. The Copalis (coupled-particle light-scattering) system is an innovative particle-based homogeneous immunoassay technology that allows the rapid and simultaneous detection of multiple analytes in a single fluid sample (6). Microparticle beads ranging in diameter from 1.6 to 1.7 μm are separately coated with the Ro52, Ro60, and La/SSB antigens and are differentiated on the basis of light-scatter measurements as they flow across a finely focused elliptical beam produced by a semiconductor laser. To ensure that the microparticles are centered in the flow stream, a concentric stream of sheath fluid is introduced around the sample stream. As the microparticles flow, a pulse of scattered light is produced according to their aggregation status (monomers, doublets, triplets, or larger multimers), and this scattering is converted into a decrease in the monomer number if the reaction is positive.

The Copalis I system also uses an internal quality control to verify analyzer and assay performance. The contents of each test include an inert microparticle (not coated with any antigen), in addition to the microparticles used for the individual assays. Critical parameters for the internal reference inert particle are monitored during data acquisition to verify proper operation of the analyzer and the reaction condition of the test; this provides real-time quality control with every sample tested.

Patients and sera. A total of 203 serum samples were studied. A total of 119 anti-Ro/SSA-positive samples, of which 42 were also anti-La/SSB positive, came from 113 patients with CTDs (36 with SS, 34 with SLE, 1 with mixed CTD, 2 with overlapping syndromes, and 40 with undifferentiated CTDs, diagnosed according to the recommendations of the American College of Rheumatology or other accepted international criteria) and from 6 patients with other autoimmune diseases (2 with Hashimoto’s thyroiditis, 3 with RA, and 1 with autoimmune hepatitis type 1). For this group of 119 samples we determined assay sensitivity.

To provide data on assay specificity, 84 control samples were included, of which 32 were from patients with CTDs (8 with SS, 2 with SLE, 11 with systemic sclerosis, and 11 with undifferentiated CTDs), 13 were from patients with viral infections (3 with Epstein-Barr virus infection and 10 with cytomegalovirus infection with immunoglobulin G [IgG] and IgM antibodies), 10 were from rheumatoid factor-positive RA patients (rheumatoid factor concentration range, 72 to 1,700 mg/ml), 10 were from patients with IgG and IgM antibodies to Borrelia burgdorferi (the Lyme disease agent), and 19 were from age- and sex-matched healthy subjects.

All serum samples were selected on the basis of preliminary findings by one ELISA and one immunoblot method (24) for anti-Ro/SSA and anti-La/SSB antibodies. Sera were considered positive or negative if both assays gave the same result. Discrepancies between assigned seroreactivity and results obtained with the Copalis system were resolved by restesting of the sera by the Western blot method.

The samples were all tested by two different microparticle formats. In the first cup, the Ro60 and the Ro52 antigens were coated together on the same particle (A format); in the second cup (B format), they were coated on two different particles so that it was possible to separate the fine specificities of the two anti-Ro antibodies in order to assess their prevalence in patients with CTDs, particularly SS and SLE.

Test procedure. Fifty microliters of the sample was placed into the side well of the test cup before the cup was put into the instrument carousel. The antigen-coated microparticles were supplied as dried reagents and were solubilized with buffer, which is automatically added by the instrument to the test cups. The serum sample was then added by the instrument and incubated at room temperature for 10 min, followed by reading of the test result.

The InterCal (internal calibration) represents a zero level of analyte and is used in the calculation of sample reactivity. The Copalis test result (CTR) unit is calculated as a ratio of the number of monomeric particles counted in the InterCal sample divided by the number of the monomers counted in the patient sample; the ratio is then multiplied by 100. The relative frequencies of the unreacted monomeric microparticles and their reacted aggregates are quantified by means of a histogram. The results for each analyte are based on the values associated with their peaks. These data are analyzed, and the results are printed as a report.

Precision study. To determine intra-assay and interassay precisions, three anti-Ro/SSA-positive samples and three anti-La/SSB-positive samples, arbitrarily chosen among the test sera, with low (350 and 310 CTR units, respectively), intermediate (1,050 and 980 CTR units), and high (1,900 and 2,280 CTR units) antibody concentrations were assayed with the A-format cups. The antibody levels in each sample were measured six times in five independent runs on 5 different days, and the overall precision was calculated.

Statistical analysis. The antibody concentrations for the different groups were expressed as the means and standard deviations. The Mann-Whitney U rank-sum test was used for comparison of means, and significance was set at a P value of <0.05. Specificity and sensitivity for anti-Ro/SSA and anti-La/SSB were calculated at different cutoff values, and the results were graphically presented by using receiver operating characteristic (ROC) curves (38) (see Fig. 1). Coefficients of variation (CVs) for precision studies were determined by standard methods.

RESULTS

Antibody concentration. The mean antibody concentration in anti-Ro/SSA-positive sera was 3,644 ± 2,158 CTR units (range, 245 to 9,236 CTR units), and that in sera from the control group was 105 ± 11 CTR units (range, 95 to 160 CTR units) (P = 0.00001). The mean antibody concentration in anti-La/SSB-positive sera was 3,212 ± 2,433 CTR units (range, 285 to 7,413 CTR units), and that in sera from the control group was 105.9 ± 17 CTR units (range, 95 to 185 CTR units) (P = 0.00001).

Sensitivity, specificity, and precision studies. As expected, whether Ro52 and Ro60 antigens were coated on two different particles or together on a single particle, there were no substantial differences in the performances of the two reagent formats. With the cutoff set at 200 CTR units, as determined by using ROC curves (Fig. 1), the sensitivities were 88.2% for anti-Ro/SSA and 95.2% for anti-La/SSB, and the specificities were 97.6 and 98.1%, respectively. The intra-assay CVs ranged from 4.3 to 10.9% for anti-Ro/SSA and from 2.8 to 12.5% for anti-La/SSB; interassay CVs were 6.5 to 13.2% and 8.2 to 14.5%, respectively.
Anti-Ro52 and anti-Ro60 prevalences in SLE and SS patients. Among the anti-Ro/SSA-positive samples, the Ro60 antigen was recognized by 66% of the test sera and the Ro52 antigen was recognized by 95% of the test sera; 34% of the Ro/SSA-positive sera were reactive only with the Ro52 antigen, indicating that anti-Ro52 is the most common antibody specificity recognized by anti-Ro/SSA autoantibodies. No differences were found between the prevalence of anti-Ro60 and anti-Ro52 in relation to SLE or SS disease (Table 1).

DISCUSSION

In recent years, molecular biochemical and recombinant DNA technologies have contributed to more precise antigen identification as well as the availability of purified antigen preparations, thus allowing the development of a wide range of immunochemical methods (26). The refinement of the analytical techniques has made autoantibody testing available in an increasing number of clinical laboratories, and in turn, the competent diagnosis of autoimmune disease has become a major responsibility of clinical laboratories worldwide. The study described here was designed to evaluate the analytical performance of a new immunoassay, the Copalis system, based on recombinant polypeptides derived from cDNAs coding for Ro60, Ro52, and La/SSB, for determination of the immune status of autoimmune patients.

The Copalis system was previously shown to be effective in detecting human viral antibodies (H. A. B. Multhaupt, E. Coruzzi, P. Ortz, L. Vesperini, T. Juan, and M. J. Warhol, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. V5, 1999), and it was proven to be very practical and easy to handle (6). Our results demonstrate that the Copalis technology can also be applied to the detection of anti-Ro/SSA and anti-La/SSB antibodies in patients with CTDs.

This new assay showed a high degree of specificity and good sensitivity for both antibodies, comparable to reports of studies that have used traditional ELISA and immunoblot methods (4, 5, 14, 23, 25). In addition, the system was very practical and particularly suitable for use for everyday routine analysis because it enabled detection of antigen-coated microparticles with multiple specific antibodies in a short time and in the same assay. Furthermore, the system might have a broader application since it may also be used for other assays (i.e., viral and hormonal serology), thus making its use more cost-effective.

Since Ro52 and Ro60 antigens were coated on two different particles in one of the two reagent formats used in the present study, it was also possible to separate the two antibody specificities in order to assess the prevalence of each antibody. Ninety-five percent of the anti-Ro/SSA-positive serum samples recognized the 52-kDa antigen, and in 34% of the serum samples, it was the only antibody present, whereas only 5% of the serum samples were reactive with only the 60-kDa protein. These data are consistent with those obtained by Ben-Chetrit et al. (3), who showed that most of the anti-Ro/SSA antibodies are directed against the Ro52 antigen in CTD patients.

We also evaluated the prevalence of the two antibody specificities in relation to the clinical diagnosis. Indeed, some studies have suggested that the separate analysis of the two Ro antibodies may be important in the differentiation of SLE from SS, as anti-Ro60 would be prevalent in patients with SLE and anti-Ro52 would be prevalent in patients with SS (3, 29, 32). Other studies, however, were less positive on this point, having found overlapping features between antibody specificity and the kind of disease (1). We tested 70 anti-Ro/SSA-positive serum samples (36 from patients with SS and 34 from patients with SLE) and found no significant differences in antibody prevalence in the two groups. Although we cannot draw any
definite conclusions due to the limited number of serum samples tested, we cannot confirm that differentiation of anti-Ro60 from anti-Ro52 can be of practical clinical value for the differential diagnosis of SLE and SS.

The results of our study indicate that because of its good analytical performance and practicability, this new immunoassay based on coupled-particle light-scattering technology is an efficient diagnostic tool and may be used for the detection of anti-Ro/SSA and anti-La/SSB antibodies in patients with autoimmune disorders.

REFERENCES


