Development and Comparative Evaluation of Immunoblot Assays for Detecting Autoantibodies to Scl 70 and Jo 1 Antigens in Serum

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We developed rapid 24-h immunoblot assays for detecting autoantibodies to Scl 70 and Jo 1 antigens in serum. In comparative studies, we evaluated the analytical sensitivity of the immunoblot assays and commercial immunodiffusion assays for anti-Scl 70 and anti-Jo 1 autoantibodies with the use of positive control sera, and compared the frequencies of positive and negative results in a group of 116 sera, including specimens from 34 healthy controls and 82 patients with various connective-tissue diseases. The immunoblot assays were >100-fold more sensitive than immunodiffusion for detecting both autoantibodies. Despite greater analytical sensitivity, there were no false-positive results by the immunoblot assay for anti-Scl 70 or anti-Jo 1 autoantibodies in sera from either the controls or the patients. The diagnostic sensitivity of the immunoblot assay for anti-Scl 70 autoantibodies in patients with scleroderma was greater than that of the immunodiffusion assay, 70% vs 20%, and was equivalent in patients with polymyositis, 43%. We conclude that rapid immunoblot assays for anti-Scl 70 and anti-Jo 1 autoantibodies are superior to immunodiffusion assays for clinical use and are suitable for routine use in the clinical laboratory.

Additional Keyphrases: immunodiffusion compared · connective tissue diseases · scleroderma · polymyositis · autoimmune disease

Autoimmune diseases produce various autoantibodies that react with intracellular antigens, including DNA, RNA, proteins, and ribonucleoproteins (1). Autoantibodies to the saline-extractable nuclear antigen Scl 70 (DNA topoisomerase type 1, EC 5.99.1.2) are detectable in sera from 20% to 60% of patients with scleroderma (2-5), but are rarely found in patients with other autoimmune diseases. Anti-Scl 70 autoantibodies are specific for scleroderma. The DNA topoisomerase group comprises a ubiquitous and varied class of enzymes that change the linking of closed-circular DNA molecules by catalyzing the production of transient breaks in the phosphodiester backbones of DNA (6). This class of enzymes is believed to participate in several cellular processes that involve DNA, including control of RNA transcription. The Scl 70 antigen is extractable from chicken erythrocytes (6, 7), calf thymus (7), and rat liver (8).

Autoantibodies to the extractable cytoplasmic antigen Jo 1 (histidyl-tRNA ligase, EC 6.1.1.21) have been reported in 18–36% of patients with polymyositis, an inflammatory autoimmune disease of skeletal muscle (9-12). These antibodies are more common in polymyositis than in dermatomyositis, a cutaneous variant of polymyositis, and are rarely found in other diseases (10-12). Autoantibodies to Jo 1 are strongly associated with interstitial lung disease (13, 14) in polymyositis patients, and nearly all anti-Jo-1-positive patients show clinical or radiographic evidence of lung involvement.

The Jo 1 antigen is one of a family of enzymes, the aminoacyl-tRNA synthetases, that catalyze the attachment of amino acids to their cognate tRNA molecules. The Jo 1 antigen is extractable from HeLa cells (15, 16), calf thymus, and calf liver (17).

Various immunological methods, including double immunodiffusion (Ouchterlony), enzyme-linked immunosorbent assays (ELISA) (7, 9, 17, 18), and immunoblot assays (15–17), have been used to detect and measure serum autoantibodies to the Scl 70 and Jo 1 antigens.2 Immunoblot assays can be performed with crude antigen extracts, but are subject to errors in the interpretation of precipitin lines. ELISAs require purified or recombinant antigens, which are not readily available; the immunoblot assays described thus far are tedious, time consuming, and not suitable for routine use in the clinical laboratory.

We describe the development of rapid immunoblot assays for detecting autoantibodies to the Scl 70 and Jo 1 antigens in serum. The immunoblot assays make use of commercially available, partially purified antigens and commercially available positive control sera. We also compared the diagnostic usefulness of these assays with commercial immunodiffusion assays, using a large group of sera from healthy adults and patients with clinically documented connective-tissue diseases.

Materials and Methods

Patients' sera. We evaluated 116 sera, including specimens from 34 healthy controls and 82 patients with various connective-tissue diseases: systemic lupus erythematosus (n = 10), rheumatoid arthritis (n = 25), antinuclear antibody (ANA)-positive sera at dilutions of 1:160 or greater (n = 30), scleroderma (n = 10), and polymyositis (n = 7). Patients' sera were selected from specimens sent to the Clinical Immunology Laboratory for measurement of ANA. All sera were stored at -20 °C.

Solutions and reagents. Unless otherwise stated, we prepared all solutions in distilled water as follows: solution A, 300 g of acrylamide and 80 g of methylenebisacrylamide per liter; solution B, 1.5 mM Tris buffer (pH 8.8), containing 100 g of sodium dodecyl sulfate (SDS) per liter; solution C, Tris · HCl (0.5 mol/L, pH 8.8), ammonium persulfate (100 g/L), tetramethylthelylenediamine (TEMED, 100 g/L), and bromphenol blue (0.5 mL/L). Transfer buffer (pH 8.2) contained, per liter, 192 mmol of glycine, 25 mmol of Tris, and 200 mL of methanol. The

2 Nonstandard abbreviations: ELISA, enzyme-linked immunosorbent assay; IRMA, immunoradiometric assay; ANA, antinuclear antibody; SDS, sodium dodecyl sulfate; TEMED, tetramethylthelylenediamine; and AMP, 2-amino-2-methyl-1,3-propanediol.
immunoradiometric assay (IRMA) buffer (pH 7.3) contained 50 mMol of Tris, 100 mMol of NaCl, and 0.2 g of NaF per liter. AMP buffer was 2-amino-2-methyl-1,3-propanediol, 5 mM/L. Sample preparation buffer was 1 mMol of solution C, 0.8 mMol of glycerol, 1.6 mMol of the 100 g/L SDS solution, 0.4 mMol of β-mercaptoethanol, 0.2 mMol of bromophenol blue (5 mM/L), and 4 mMol of H2O. Blocking buffer (pH 7.3) was IRMA buffer containing 3 mMol of Tween 20 and 50 g of nonfat dry milk per liter. Stock electrophoresis buffer (pH 8.3) contained 0.96 mol of glycine, 0.25 mol of Tris, and 7.5 g ofs per liter. Wash buffer (pH 7.4) consisted of 10 mMol of Tris, 150 mMol of NaCl, and 0.5 mMol of Tween 20 per liter.

Scl 70 antigen, Jo 1 antigen, and positive controls were obtained from INOVA Diagnostics, Inc., San Diego, CA. Nitroblue tetrazolium (BRL 8NE 301) and 5-bromo-4-chloro-3-indoyl phosphate (BRL 82805A) were obtained from Bethesda Research Labs., Gaithersburg, MD. Alkaline phosphatase conjugated with affinity-isolated (Fab')2 anti-human immunoglobulin antiserum was purchased from Tago, Inc., Burlingame, CA. Nitrocellulose sheets and blot absorbent filter paper were obtained from Hoefer Scientific Instruments, San Francisco, CA.

Equipment. For the immunoblot assays, we used the Bio-Rad Mini Protein® II dual-slab gel and Mini Trans Blot® electrophoretic transfer system (Bio-Rad, Richmond, CA) with gel-pouring accessories and a 25-well incubation tray.

Procedures

Electrophoresis. We prepared 10% resolving mini-polyacrylamide gels (100 × 50 × 0.75 mm), two at a time, by combining 3.35 mL of solution A, 2.5 mL of solution B, 4 mL of H2O, and 100 mL of SDS (100 g/L) in a side-arm flask. This mixture was de-aerated under reduced pressure (~15 mmHg) for 10 min while the gel-casting chambers were being set up. We transferred half of the de-aerated solution to a small beaker, added 10 μL of the ammonium persulfate solution with mixing, then added, with mixing, 5 μL of the TEMED solution. We immediately transferred the solution to the gel-casting chamber with a glass pipette, taking care to avoid air bubbles in the gel. We prepared the second gel and repeated the above procedure with the remaining de-aerated solution. Toothless combs were used to level the tops of the gel mixtures in the casting chambers, and the gels were allowed to polymerize for 15 min. No stacking gels were used. We prepared the buffer for electrophoresis by diluting 60 mL of the stock electrophoresis buffer to 300 mL with H2O. After assembling the polymerized gels in the electrophoresis cell, we added a portion of the diluted electrophoresis buffer to the upper chamber of the cell to a height of 3 mm from the top of the cell, making sure that the gels were covered with buffer. We carefully poured the rest of the buffer into the lower chamber of the cell, making sure that no air bubbles were trapped at the bottom of the gel assembly. A 10-μL aliquot of reconstituted Scl 70 antigen preparation was added to 50 μL of sample preparation buffer, and the mixture was heated at 100 °C for 2 min. A Jo 1 antigen preparation was treated similarly. With a Hamilton syringe, we carefully applied each antigen preparation through the buffer to the top of a separate gel. Electrophoresis was carried out at 200 V for 30 min.

Trans blotting. After electrophoresis, the carefully disassembled gels, along with blotting filter paper and nitrocellulose sheets (cut in the size of the gels), were equilibrated in transfer buffer for 15 min. Following the manufacturer's instructions, we assembled these components into the gel holder and placed it into the Trans Blot cell filled with 1 L of transfer buffer. The cell also contained an ice-filled plastic cup for cooling. Electrophoretic transfer of the fractionated proteins from the gels to the nitrocellulose sheets was carried out at 100 V for 1 h. The nitrocellulose sheets were removed from the gel holder and rinsed thoroughly with wash buffer before being placed in blocking buffer at 40–60 °C for 1 h. The nitrocellulose sheets were again rinsed once with wash buffer and once with IRMA buffer. The sheets were cut into several 5-mm strips, then stored in IRMA buffer until used.

Specimen analysis. The nitrocellulose strips were labeled and placed (one strip/well) in the Bio-Rad incubation tray. All incubations and washes were accomplished by gentle mechanical rocking of the tray containing the nitrocellulose strips in the appropriate solutions for the specified time periods. All specimens were diluted 250-fold in blocking buffer before evaluation. The strips were incubated at room temperature for 1 h with 2 mL aliquots of patients' sera or a negative control, a positive control supplied with the kit, or a positive control (AF/CDC9) from the ANA Reference Laboratory, Centers for Disease Control, Atlanta, GA. After incubation, the tray was carefully emptied of the specimens and the strips washed three times (5 min/wash) with wash buffer. Next, the strips were incubated with 2 mL of alkaline phosphatase-conjugated anti-human immunoglobulin antiserum (diluted 500-fold in blocking buffer) at room temperature for 1 h. After further washing as described earlier, the strips were incubated with 2 mL of alkaline phosphatase substrate reagent (67 μg/L of nitroblue tetrazolium reagent, 100 μL of 5-bromo-4-chloro-3-indoyl phosphate reagent, and 50 μL of AMP buffer) for 5 min at room temperature. The strips were visually inspected and bands, if present, were compared with those on the positive control strips.

Double immunodiffusion (Ouchterlony). We performed immunodiffusion assays with commercial agarose gel plates (INOVA Diagnostics), according to the manufacturer's directions. The NOVA Gel® immunodiffusion kits contain lyophilized antigen preparations (Scl 70 or Jo 1) and positive control sera. We examined the immunodiffusion plates after 24 h and again after 48 h, neting any precipitin lines formed between patients' samples and the antigens. If precipitin lines were observed, their relationships to the positive control precipitin lines were compared to identify sera that contained anti-Scl 70 or anti-Jo 1 autoantibodies.

Results

We compared the analytical sensitivities of the immunoblot and immunodiffusion assay methods with serial dilutions of positive control sera that contained either anti-Scl 70 or anti-Jo 1 autoantibodies. The positive controls were diluted in blocking buffer for the immunoblot assays and in IRMA buffer for the immunodiffusion assays. The highest dilution that yielded a visible band (immunoblot assays) or precipitin line (immunodiffusion assays) defined the endpoint. For detection of anti-Scl 70 and anti-Jo 1 autoantibodies by immunodiffusion, a characteristic precipitin line was not visible at dilutions >16-fold but the immune complex bands of Scl 70 and anti-Scl 70 and of Jo 1 and anti-Jo 1 were detectable by immunoblot assay at dilutions of 4000-fold and 8000-fold, respectively. We determined that serum diluted 250-fold was optimal for analysis by immunoblotting, whereas undiluted specimens were re-
required in the immunodiffusion assay. Typical results of immunoblot assays are presented in Figure 1.

In the comparative clinical study, 34 sera from healthy adults and 82 sera from patients with connective-tissue diseases were evaluated by both methods. Representative nitrocellulose strips from patients' sera found to be positive for anti-Scl 70 and anti-Jo 1 autoantibodies are shown in Figures 2 and 3, respectively. The results of comparative studies with patients' sera show that the immunoblot assay detected anti-Scl 70 autoantibodies in seven of the 10 patients with scleroderma, whereas the immunodiffusion assay detected anti-Scl 70 in only two of those subjects (diagnostic sensitivity, 70% vs 20%). Both methods yielded no false-positive results in healthy controls or in patients with connective-tissue diseases other than scleroderma or polymyositis (e.g., systemic lupus erythematosus, rheumatoid arthritis, and ANA-positive subjects). Both methods detected three of seven patients (43%) with polymyositis or dermatomyositis.

In a separate experiment, the immunoblot assay was performed with a mixture of the Scl 70 and Jo 1 antigen preparations. Three nitrocellulose strips were probed with the positive control sera for the two antigens. Figure 4 shows the bands observed. The Scl 70 and Jo 1 antigens were well separated during the electrophoresis step of the procedure, the Jo 1 antigen migrating ahead of the Scl 70 antigen. Strips prepared in this way might be useful in a screening procedure to test for both autoantibodies simultaneously, although further investigation of this modification is needed to show its reliability for clinical use.
Discussion

The diagnosis of scleroderma or polymyositis is enhanced by the detection in serum of autoantibodies to the Scl 70 and Jo 1 antigens, respectively. In our studies, we showed that, compared with an immunodiffusion assay, the rapid immunoblot assay is much more sensitive for detecting autoantibodies to the Scl 70 antigen. The immunoblot assays we developed yielded reproducible, well-defined bands with each antigen preparation, which greatly simplified the interpretation of results. The mini-polyacrylamide gels in our immunoblot procedures afforded the advantage of using smaller quantities of the reagents and buffers than are required for conventional immunoblot procedures.

Only 70% of the patients diagnosed clinically with scleroderma tested positive for the presence of anti-Scl 70 autoantibodies; only 43% of the patients diagnosed clinically with polymyositis tested positive for anti-Jo 1 autoantibodies. Although these results are consistent with previously published studies, review of the medical records of these patients revealed that all of the patients were on various regimens of immunosuppressive therapy when we were testing their sera. Patients with connective-tissue diseases are frequently treated with immunosuppressive drugs such as prednisone. One study (19) showed that patients on such therapy had lower amounts of autoantibodies in their sera. Therefore, the diagnostic sensitivities of both assay methods for tests performed in untreated patients might be higher than the values we found. Further studies are required to evaluate this possibility.

The use of miniaturized electrophoresis/Trans Blot equipment in the immunoblot assays we developed offers several advantages compared with conventional electrophoresis/immunoblot systems. Much smaller quantities of antigen can be loaded onto the gels, with comparable analytical sensitivity. The electrophoresis time is reduced from 4 h to 1 h, and the quantities of buffers required are much less. In addition, the turnaround time is reduced from 2.5 days to 24 h, and the throughput of specimens is at least doubled. The ready availability of commercial sources of partially purified antigens and commercially available positive controls combined with the use of miniaturized equipment makes the immunoblot assays easily adaptable to the clinical laboratory.

References