References

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“Microgel Diffusion Blotting” for Sensitive Detection of Antibodies to Extractable Nuclear Antigens
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A fast immunoblotting procedure, termed "microgel diffusion blotting," is used to detect and identify antibodies to extractable nuclear antigens (i.e., to Sm, RNP, and SSB) in patients with rheumatic diseases. The standard method differs from the standard immunoblotting techniques by the use of ultra-thin microgels for polyacrylamide gel electrophoresis: the very thin gel layer allows transfer of proteins to a nitrocellulose membrane by simple diffusion. Principal advantages of this variant technique are its simplicity, rapidity, and reproducibility—characteristics that make the test suitable for routine application. We compared the sensitivity of the test with that of double immunodiffusion in agarose for the evaluation of humoral antinuclear immunity. Microgel diffusion blotting detected antibodies in serum at concentrations <0.001 of those detectable by immunodiffusion.

Additional Keyphrases: rheumatic diseases · double immunodiffusion in agarose compared · screening

Immunoblotting of serum has become a standard technique for detecting serum antibodies to antigenic molecules in a particular protein extract (1,2). It is commonly used for the detection and identification of antibodies to extractable nuclear antigens, such as the protein components of ribonucleoproteins (Sm, RNP, SSA, and SSB), histones, and scleroderma and dermatopolymyositis nuclear antigens (3-5). Detecting these antinuclear antibodies is clinically important in classifying autoimmune rheumatic diseases (4-6). Sm and RNP are closely related nuclear antigens (7, 8), consisting of a similar uracil-rich RNA component and different small protein particles of Mr, 9000-68 000 (8). Antibodies to Sm are serological markers for systemic lupus erythematosus (9); antibodies to RNP, for mixed connective tissue disease (10). SSB, another ribonucleoprotein, is particularly important, because antibodies to it are found in Sjögren's syndrome (7, 11).

The standard design for immunoblotting, vertical polyacrylamide gel electrophoresis of an antigenic protein mixture and electrotransfer of the molecules to an immobulizing matrix, is time-consuming and hardly suitable for antibody screening on a large scale. For the study reported here, we used a horizontal microgel electrophoresis system (12) and ultrathin gels that allow transfer of proteins by simple diffusion. This immunoblotting procedure, which we term "microgel diffusion blotting," is less complicated and easier done than standard immunoblotting (12).

We applied this fast immunoblotting technique for the evaluation of humoral antinuclear immunity (antibodies to extractable nuclear antigens), focusing on the detection of anti-Sm, anti-RNP, and anti-SSB antibodies.

We compared the sensitivity of microgel diffusion blotting with Ouchterlony immunodiffusion in agarose (13) for detecting these antinuclear antibodies.

Materials and Methods
Serum: We evaluated 432 sera obtained from patients with various rheumatic diseases, including rheumatoid arthritis (n = 114), rheumatoid arthritis–scleroderma overlap (n = 9), rheumatoid arthritis–systemic lupus erythematosus overlap (n = 10), scleroderma (n = 27), systemic lupus erythematosus (n = 24), seronegative spondyloarthropathy (n = 57), juvenile chronic arthritis (n = 9), primary Sjög-
ren's syndrome (n = 2), polyarthritis nodosa (n = 9), non-inflammatory joint pain (n = 138), and other rheumatic diseases (n = 33). One of us examined most of these patients and classified them according to the criteria of the American Rheumatism Association, where applicable. If patients fulfilled the criteria for more than one disease entity, we used the term "overlap syndrome."

**Double immunodiffusion (Ouchterlony):** Immunodiffusion in agarose (agarose, 10 g/L, in barbital buffer, 250 mmol/L, pH 8.4) was applied with rabbit thymus nuclear extract (Zeus Scientific, Raritan, NJ) as the antigen source. The lyophilized extract was reconstituted with 2 mL of sterile distilled water. Sera were applied undiluted. If a precipitin line formed, we compared the serum with results for standards of anti-Sm-, anti-RNP-, and anti-SSB antisera (Zeus Scientific), to identify the antibodies.

**Microgel diffusion blotting:** We performed sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) of antigenic extracts, using ultrathin microgels (14), and transferred the proteins to a nitrocellulose membrane by simple diffusion. The two antigenic extracts used were a heterologous thymus extract (the same extract as used for immunoprecipitation) and a homologous nuclear extract from K 562, a human erythroleukemia cell line. The latter cells were grown in suspension, pelleted, transferred to hypotonic buffer (10 mmol/L Tris hydrochloride buffer, pH 7.4, containing 10 mmol of NaCl, 1.5 mmol of MgCl₂, 0.5 mmol of phenylmethylosulfonylchloride, and 3 mL of Triton X-100 surfactant per liter), and homogenized with a Teflon pestle. After lysis of the cytoplasm, the nuclei were pelleted; incubated with DNase and RNase, 500 μg/mL, for 1 h at 20 °C; lyophilized; and dissolved in sample buffer (per liter, 0.1 mol of Tris hydrochloride, pH 6.8, 40 g of SDS, and 100 mL of glycerol containing 10 mL of mercaptoethanol per liter) to contain 10⁸ nuclei per milliliter. This solution was heated at 100 °C and centrifuged (10 min, 400 x g). The supernate was electrophoresed on SDS-PAGE (see below). The second antigenic extract, lyophilized rabbit thymus nuclear extract, was reconstituted with 2 mL of sample buffer, then similarly heated, centrifuged, and electrophoresed.

For SDS-PAGE we used horizontal 43 x 50 x 0.45 mm ultrathin gels (PhastGel Gradient 10-15; Pharmacia, Uppsala, Sweden) on a polyester backing. For electrophoresis, each gel was placed in the chamber of an automated electrophoresis apparatus (PhastSystem, Pharmacia). The separation was performed by applying 60 V·h. After the electrophoresis, we covered the gels with a nitrocellulose membrane (Hybond-C; Amersham International, Amersham, Bucks., U.K.). After wetting the nitrocellulose membrane with pH 8.3 transfer buffer (per liter, 15 mmol of Tris and 192 mmol of glycine), we covered the membrane with three Whatman filter papers and a sponge 10-mm in thickness. The whole assembly was kept between two Perspex plates and placed overnight at room temperature in transfer buffer containing methanol/water (1/5 by vol).

After the transfer, we washed the nitrocellulose papers in pH 7.2 phosphate-buffered saline (per liter, 135 mmol of NaCl, 9 mmol of Na₂HPO₄ , 2.6 mmol of NaH₂PO₄ and 0.5 g of Na₂S) containing 5 mL of Tween 20 surfactant per liter (PBS-Tween) and dried them between two filter papers.

Before incubating the blotted membrane with a patient's serum sample, we diluted the serum 50-fold in PBS-Tween and incubated for 1 h at room temperature. We then added 50 g of non-fat dry milk per liter to the buffer as an additional blocking agent. After incubation, we washed the membrane with PBS-Tween.

To detect immune complexes on the blotting membrane, we incubated the membrane strips with an alkaline-phosphatase-conjugated second antibody (goat gamma-chain-specific antiserum to human IgG, Sigma Chemical Co., St. Louis, MO) diluted 300-fold in PBS-Tween. We measured the alkaline phosphatase activity bound by using a recent method in which 5-bromo-4-chloroindoxyl phosphate is used as substrate (15).

**Results**

The sensitivity of microgel diffusion blotting and double immunodiffusion for detection of antibodies to the ribonucleoproteins Sm, RNP, and SSB was assessed in two ways. First, we determined the greatest dilution of serum at which the immunoblotting pattern or immunoprecipitation line remained detectable; second, we compared the results obtained with both tests in a serological study of patients with rheumatic diseases. We titrated three standard antisera and used them for both microgel diffusion blotting and double immunodiffusion, and the same antigen source (thymus nuclear extract) was used for both tests. The antiserum to Sm was responsible for an immunoblotting line (antibodies to a 17-kDa protein) clearly detectable up to a serum dilution of 1:3200 (Figure 1). The immunoblotting pattern produced by the antiserum to RNP (antibodies to a 68- and 39-kDa protein) remained detectable up to a serum dilution of 1:6400. The anti-SSB immunoblotting pattern (antibodies to a 42- and 39-kDa protein) was present up to a serum dilution of 1:6400. The precipitin line caused by these antisera in a double immunodiffusion assay disappeared at much lower dilutions of serum: for the antiserum to Sm, RNP, and SSB, this was at dilutions of 1:8, 1:8, and 1:4, respectively.

Serum sampled from 432 patients with various rheumatic diseases for the presence of antibodies to Sm, RNP, and SSB was evaluated with both microgel diffusion blotting and double immunodiffusion—a study not designed to evaluate the frequency of these specific antibodies in different rheumatic disease conditions but instead to compare the value of the two described methods for the detection of these antibodies. Table 1 shows the correlation between the results of the two tests. There was a 100% correlation between the results obtained by microgel diffusion blotting with thymus and K 562 nuclear extract for the detection of these three antibodies.

**Discussion**

Immunoblotting is a powerful technique, extremely useful in the evaluation of humoral immunity to auto- or allo-antigens (2, 3). It combines the possibility of high-resolution antigen separation on gel electrophoresis with the sensitivity of a solid-phase immunoassay. It allows the detection and identification of antibodies.

The use of ultrathin microgels facilitates the application of immunoblotting for the routine evaluation of humoral antinuclear immunity. The present technique, microgel diffusion blotting, has obvious practical advantages (12):

- Commercially available prefabricated storable gels with a standardized gel concentration can be used.
The procedure has excellent reproducibility and is highly sensitive for the evaluation of humoral immunity, as is illustrated for the detection of anti-Sm-, anti-RNP-, and anti-SSB-antibodies.

- Microgel diffusion blotting can detect antibodies in serum at concentrations <0.001 of those detectable by immunodiffusion.

In a population survey of 432 patients with various rheumatic diseases, we found no serum with a positive immunodiffusion and a negative microgel diffusion blotting result for one of the studied antibodies. As for the reverse, three of the five sera positive for anti-Sm antibodies on immunoblotting were negative in the immunodiffusion test; one of the four sera positive for anti-RNP antibodies on immunoblotting was negative in immunodiffusion; and two of 14 sera positive for anti-SSB antibodies on immunoblotting were negative by the immunodiffusion test (Table 1).

In addition to positive immunoblotting results, which could be defined as identical to one of the reference sera, many patients' sera gave other banding patterns. The identity of the antigen–antibody complexes responsible for the visualization of these bands remains unknown.

Table 1. Correlation between Microgel Diffusion Blotting (MDB) and Double Immunodiffusion (ID) Results for Detecting Anti-Sm, Anti-RNP, and Anti-SSB Antibodies

<table>
<thead>
<tr>
<th>Anti-Sm antibody</th>
<th>MDB positive</th>
<th>MDB negative</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
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<tr>
<td>ID negative</td>
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<td>427</td>
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<td></td>
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<td>ID negative</td>
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<td>428</td>
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<td>Anti-SSB antibody</td>
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<td>ID negative</td>
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References