Sodium dodecyl sulfate-agarose gel electrophoresis of urinary proteins: application to multiple myeloma

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We evaluated a new sodium dodecyl sulfate-agarose gel electrophoresis (SDS-AGE) for urinary protein analysis in patients with multiple myeloma (MM; n = 47; ages, 62 ± 2 years, mean ± SE). Abnormal proteinuria (mean = 1872 ± 360 mg/24 h) was present in 95% of the samples; 75% of the patients had some sign of renal dysfunction (glomerular and/or tubular) according to their SDS-AGE pattern. A band suggesting Bence Jones proteinuria (BJP) was detected in 40 vs 33 specimens by routine AGE. Immunofixation identified BJP in 38 patients; the calculated sensitivity of SDS-AGE for BJP was 97%. Excellent correlation (P < 0.0001) was obtained with routine AGE (r = 0.994) and immunonephelometry (r = 0.963) for light chain quantification. SDS-AGE allows easy evaluation of renal dysfunction and shows high sensitivity for BJP detection. In a specialized laboratory, it is useful for following the progress of MM patients through the semiquantification of BJP.

Urinary investigations for patients with multiple myeloma (MM)3 include electrophoretic protein screening (evaluation of renal function, detection of an abnormal protein fraction) followed by immunofixation (IFE) for identification of a monoclonal component [intact immunoglobulin or monoclonal free light chains: Bence Jones proteinuria (BJP)] (1). BJP is detected in the urine of 75% of MM patients and is one of the major laboratory features of this disease (2, 3). Quantitation of BJP by electrophoresis is also used to monitor renal complications of the disease (3) and response to therapy (the amount of Bence Jones proteins excreted in the urine correlates with tumor mass) (4).

Electrophoretic methods (agarose or cellulose acetate) are used routinely for analysis of urinary proteins on the basis of charge and molecular size. These methods require prior concentration of urine, usually by ultrafiltration, which is time-consuming and expensive and may lead to protein loss (5, 6). Some results are particularly difficult to interpret because Bence Jones proteins may comigrate with intact immunoglobulins and other proteins (such as transferrin) (7). Routine agarose electrophoresis (AGE) also shows poor sensitivity for detecting small concentrations of Bence Jones proteins (1).

Recently, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been introduced for qualitative analysis of urinary proteins. SDS-PAGE separates proteins according to their molecular size (8), and free light chain monomers (polyclonal or monoclonal) are visualized as a band of Mr 25 000, easily distinguished from intact immunoglobulins (Mr 150 000). SDS-PAGE also differentiates proteinuria of glomerular (proteins with a Mr >66 000), tubular (Mr <66 000), or mixed origin.

This method is thus a potentially powerful clinical tool for assessing kidney function, an important prognostic factor in MM (9, 10). SDS-PAGE, however, is not widely used in clinical laboratories because it is technically demanding, time-consuming, and expensive (11–14).

In this study, we evaluated, for the first time, a new SDS-agarose gel electrophoresis (SDS-AGE) for analysis of proteins in the urine of patients with MM. Results were compared with those obtained by routine (nondenaturing) AGE and IFE. Free and bound light chains (κ, λ), IgG, and albumin were measured by immunonephelometry.
Materials and Methods

Patients
We studied 47 consecutive patients (mean age, 62 ± 2 years; male, n = 26; female, n = 21) with known MM. In 29 patients (62%), an abnormal fraction was present in the serum (range, 1.0–70.9 g/L, determined by AGE). The serum monoclonal immunoglobulins identified by IFE were IgG, n = 34; IgA, n = 11; and IgD, n = 1; there was one case of light chain disease. We evaluated a control group of 30 consecutive patients (ages, 15–85 years; male, n = 16; female, n = 14) without hematological disorder to assess the specificity of SDS-AGE for BJP. Their urine samples were examined in our laboratory by electrophoresis of urinary proteins; all tested negative for BJP by IFE. All procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Urinary Samples
Samples from 24-h urines were collected in 25-mL Monovette® tubes without additive (Sarstedt) and centrifuged as described above. Stored at −20 °C. Before analysis, urine was resuspended and centrifuged as described above.

Analysis
Routine AGE. Before routine AGE, urine was concentrated 25-fold by ultrafiltration in Minicon® chambers (Amicon). The time required for urine concentration by ultrafiltration varied from 30 min to 4 h. Concentrated urine was analyzed using the Hydragel® kit on an Hydrasys® automated gel electrophoresis apparatus (Sebia). Briefly, 10 μL of concentrated urine was applied in each well (15 wells/gel) and run at 272 V (68 mA) at 20 °C for 7 min. After the gels were dried at 65 °C for 10 min, they were stained with gels were then scanned with a Preference® densitometer (Sebia) using a yellow filter (570 nm) for determination of five different fractions: albumin, α1-globulin, β-globulin, and γ-globulin. A serum protein control, Precinorm® (Boehringer), was run in parallel with urine specimens on each gel for appropriate separation of different fractions. Abnormal fractions present in the γ- or β-globulin zones of the gel were individually integrated and expressed in percentage of total proteinuria and in g/L.

SDS-AGE. Urine was analyzed by SDS-AGE using the Hydragel proteinurie® kit (Sebia), without prior concentration. Urine containing >2 g protein/L was diluted with 9 g/L NaCl to 1 g/L before analysis, according to the manufacturer’s recommendations. Twenty microliters of a 10 g/L SDS solution (with bromphenol blue as a marker) were added to 80 μL of urine and briefly vortex-mixed at room temperature. Five microliters of the treated samples were added to each well (5 wells/gel) and allowed to diffuse for 10 min. A constant voltage of 60 V (10 mA) was applied to the gel for 60 min in an SDS-imidazole (1.0 g/L of SDS and 3.4 g/L imidazole) buffer (150 mL/compartments) by use of a MG 500 power source (Sebia). After the gel was completely dried at 80 °C for 20 min, it was immersed for 30 min in the following aqueous staining solution: 1.33 g/L coomassie blue, 250 mL/L methanol, and 200 mL/L acetic acid. The gel was destained in two successive aqueous baths of 150 mL/L acetic acid before it was immersed in a 150 mL/L glycerol aqueous solution and dried at 80 °C (15 min). Total analysis time was 3.5 h.

On the gel, we detected immunoglobulins (M, 850 000–150 000), haptoglobin (M, 86 000), transferrin (M, 76 000), albumin (M, 66 000), α1-microglobulin (M, 30 000), light chain monomers (M, 25 000), retinol-binding protein (M, 21 000), and β2-microglobulin (M, 12 000). Polyclonal light chains could not be distinguished from monoclonal light chains. SDS-AGE differentiated physiological proteinuria (<150 mg/24 h, mainly albumin) and proteinuria from renal (glomerular, tubular, or mixed) or prerenal origin (Table 1). Five selected SDS-AGE patterns are presented in Fig. 1. Monoclonal light chains (BJP) appear as a broad intense band at M, 25 000 and can be found in the absence (pure prerenal BJP) or in the presence of renal proteinuria.

Urine containing polymeric forms of light chains was

<table>
<thead>
<tr>
<th>Proteinuria</th>
<th>Renal</th>
<th>Tubular</th>
<th>Mixed</th>
<th>Prerenal</th>
<th>Pure BJP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin, 66 000</td>
<td>+ to + + +</td>
<td>+</td>
<td>+ to +</td>
<td>+ + +</td>
<td>Absent or +</td>
</tr>
<tr>
<td>Proteins &gt;66 000</td>
<td>S: absent; NS: +</td>
<td>Absent</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
</tr>
<tr>
<td>Proteins &lt;66 000</td>
<td>Light chains*</td>
<td>Absent or +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Other proteins</td>
<td>Absent</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Absent</td>
</tr>
</tbody>
</table>

* +, positive; + + +, strongly positive; S, selective; and NS, not selective.

Table 1. Type of proteinuria according to the SDS-agarose electrophoresis pattern.
pretreated with β-mercaptoethanol (β-ME) before electrophoresis. Five microliters of 100 mL/L β-ME were added to 100 μL of urine and briefly vortex-mixed at room temperature. A 5-μL sample was loaded onto the gel and allowed to diffuse for 10 min before electrophoresis. After electrophoresis was complete, the gels were scanned, and the results were expressed as a percentage of total proteinuria and in g/L. The stated detection limit for a band was 15 mg/L (manufacturer’s data).

A protein $M_r$ marker LMW® (Pharmacia Biotech) suitable for SDS-PAGE was run on each gel. Band relative mobility ($R_f$) compared with lactalbumin ($M_r$ 14,000) was calculated for each LMW protein (phosphorylase b, $M_r$ 94,000; albumin, $M_r$ 67,000; ovalbumin, $M_r$ 43,000; carbonic anhydrase, $M_r$ 30,000; and trypsin inhibitor, $M_r$ 20,000) in nine different gels on separate days to evaluate the reproducibility of electrophoretic separations. Two urine samples containing high (pure preenal BJP; light chains, 500 mg/L) and low (tubular proteinuria; light chains, 50 mg/L) concentrations of light chains were selected for estimation of the precision (within-run and day-to-day CV, $n = 9$ for each). The high control was treated with β-ME before electrophoresis.

Other biochemical analyses. Total protein was determined on a Hitachi 747® analyzer (Boehringer) by an SDS-pyrogallol red-molybdate technique (Merck-Clevenot) (15, 16). The concentrations of urinary albumin, immunoglobulin G (IgG), and free and bound light chains kappa (Lk) and lambda (La) were measured by laser immunonephelometry on a BNII® analyzer (Behring) (17–19). The urinary Lk/La ratio was calculated (usual values, 1.0 < ratio < 5.2); urinary samples with ratios outside this reference are likely to contain monoclonal light chains (Bence Jones proteins). IFE was performed using the Paragon® IFE kit (Beckman). Samples with total proteinuria <300 mg/L were concentrated up to 100-fold by ultrafiltration in Minicon chambers before IFE.

STATISTICAL ANALYSIS

Results are expressed as mean and SE. Because population distribution was not gaussian, differences between groups were assessed by the Kruskal–Wallis ANOVA, followed by the Dunn’s procedure for multiple comparisons. Correlations between techniques and biological parameters were evaluated by linear regression and ANOVA. Statistical significance was set at $P < 0.05$.

**Results**

TOTAL PROTEINURIA, IMMUNONEPHELOMETRY, AND IFE

Abnormal 24-h proteinuria (>150 mg/24 h; mean, 1872 ± 360 mg/24 h) was found in 95% of the MM patients, with large variations among individuals (136–11475 mg/24 h).

In the control group, total proteinuria ranged from 40 to 18430 mg/24 h (mean, 1840 ± 644 mg/24 h; not significantly different from MM patients); 6 of 30 patients had physiological proteinuria (117 ± 18 mg/24 h). In MM patients, urinary albumin excretion ranged from <20 mg/24 h ($n = 5$) to macroalbuminuria (>300 mg/24 h, $n = 10$). In all but 1 of the 47 MM patients, free and/or bound light chains were detected by immunonephelometry (range, 6–4670 mg/L). Eight had a Lk/La ratio within the normal reference interval; 20 had a ratio > 5.2; and 18 had a ratio < 1. Intact IgG was found in 66% of urine specimens (range, 4–187 mg/L). Urinary BJP was detected by IFE in 38 of 47 urinary samples ($Lk, n = 20; L\alpha, n = 18$).

**Routine AGE**

Albumin values obtained by electrophoresis were significantly higher than those measured by immunonephelometry (187 ± 33 vs 145 ± 26 mg/L; $P < 0.05$); the two techniques correlated significantly for this parameter ($r = 0.958, P < 0.0001$). In ~75% of cases, poor resolution was obtained in the α1-globulin, α2-globulin, and β-globulin zones of the gel. An abnormal fraction, which suggested BJP, was detected in the β- or γ-globulin zones of the gel in 33 of 47 urine samples (70% of cases) and represented 4–95% of the total proteinuria. A significant correlation ($r = 0.950, P < 0.0001$) was noted between this fraction (662 ± 188 mg/L) and light chains measured by immunonephelometry (Lk and Lα, 953 ± 345 mg/L). Samples with an abnormal fraction detected by routine electrophoresis were all positive for BJP by IFE (no false positives). Detailed analysis of 14 negative MM patients by routine electrophoresis is presented in Table 2. In four specimens, protein was <150 mg/L. For the remaining 10 samples, protein concentrations ranged from 180 to 2080 mg/L and consisted of a complex mixture of albumin, α1-globulin, α2-globulin, β-globulin, and γ-globulin. In 10
cases, light chains measured by immunonephelometry were <50 mg/L; one of these had no detectable light chains. BJP κ was identified by IFE in five cases (five false negatives for routine electrophoresis); therefore, the calculated sensitivity for BJP detection was 87% (33 of 38 patients).

**SDS-AGE**

The migration of the molecular weight markers and a urine sample with mixed proteinuria and BJP (identified by IFE) is presented in Fig. 2. The mean CV for the Rq of the reference proteins (LMW control) was 2.7%, ranging from 1.3% (trypsin inhibitor) to 4.0% (albumin). The within-run CV for light chain quantitation was 1% for the high control and 4% for the low control (n = 9). The day-to-day CV was 2% and 5% for high and low controls, respectively (9 days).

Fifty percent of the patients from the control group had a mixed proteinuria, according to their SDS-AGE pattern (Table 1). The mean proteinuria in this group was 2630 ± 1190-1453 mg protein/24 h. Eight samples were identified as glomerular proteinuria, either selective (n = 5) or not selective (n = 3); the total protein was 390 ± 88 and 4500 ± 133 mg/24 h, respectively. There was one case of tubular proteinuria (540 mg protein/24 h) and six cases of physiological proteinuria (<150 mg protein/24 h). Polyclonal free light chain monomers were detected in all urine samples with mixed and tubular proteinuria (n = 16), but there was no evidence of BJP in the control group. This was confirmed by IFE (no false positives).

In MM patients, SDS-AGE patterns were glomerular (n = 13), tubular (n = 9), mixed (n = 13), or prerenal BJP (n = 12), according to criteria summarized in Table 1. Patients with proteinuria of mixed origin excreted significantly (P <0.05) more albumin (453 ± 120 mg/24 h) than those with tubular (76 ± 26 mg/24 h) or prerenal proteinuria (74 ± 19 mg/24 h), as determined by immunonephelometry. A band suggesting BJP was detected in 40 of 47 MM patients (85%), including 8 that were negative in routine electrophoresis (Table 2). IFE detected BJP in 37 of these 40 samples (3 false positives). Among negative SDS-PAGE samples (n = 7), IFE detected BJP λ in one (one false negative). Selected gels from urinary samples that were difficult to interpret by routine electrophoresis are presented in Fig. 3. Specimens with polymeric forms of light chains (47% of cases) were treated with β-ME before quantification (Fig. 4). A significant correlation (r = 0.963, P <0.0001) was found between light chains detected by SDS-AGE (661 ± 204 mg/L) and those measured by immunonephelometry. The two electrophoresis techniques also correlated significantly for light chains (in mg/L): SDS-agarose = 0.9682 × routine agarose − 74.696, r = 0.994, P <0.0001.

**Discussion**

We evaluated, for the first time, a new AGE that uses denaturing conditions (SDS-AGE) for analysis of urinary protein in patients with MM. IFE is the reference method for detection and identification of monoclonal free light chains (Bence Jones proteins), but cannot be used to screen a large number of urinary samples forBJP (1). Despite high sensitivity, immunonephelometry should not be used for BJP quantification with potential under- or overestimation (19–21). Monoclonal proteins may react peculiarly with antisera produced against polyclonal populations of immunoglobulins and light chains (21). Moreover, insufficient information is presently available regarding the usefulness of measuring the urinary κ/λ ratio for BJP identification (17).

Electrophoresis and densitometric scanning of the gel

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**Table 2. Urine specimens from MM patients negative by routine AGE analyzed by immunonephelometry, SDS-AGE, and IFE.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Protein, mg/L</th>
<th>Albumin, mg/L</th>
<th>IgG, mg/L</th>
<th>Light chains</th>
<th>Type of proteinuria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light chains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mg/L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>k/λ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IFE</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>140</td>
<td>46</td>
<td>4</td>
<td>30 (4.0)</td>
<td>&lt;5 BjP κ Glomerular</td>
</tr>
<tr>
<td>2</td>
<td>210</td>
<td>57</td>
<td>13</td>
<td>22 (&gt;5.2)</td>
<td>16 BjP κ Tubular</td>
</tr>
<tr>
<td>3</td>
<td>840</td>
<td>566</td>
<td>9</td>
<td>94 (&gt;5.2)</td>
<td>&lt;5 BjP κ Mixed</td>
</tr>
<tr>
<td>4</td>
<td>510</td>
<td>85</td>
<td>6</td>
<td>48 (4.3)</td>
<td>&lt;5 BjP κ Mixed</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>7</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24 (&gt;5.2)</td>
<td>68 BjP κ Pure BjP</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>14</td>
<td>8</td>
<td>44 (&lt;1)</td>
<td>40 ND Glomerular</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>ND</td>
<td>6</td>
<td>43 (4.4)</td>
<td>&lt;5 ND Tubular</td>
</tr>
<tr>
<td>8</td>
<td>690</td>
<td>95</td>
<td>102</td>
<td>312 (1.9)</td>
<td>15 ND Mixed</td>
</tr>
<tr>
<td>9</td>
<td>220</td>
<td>117</td>
<td>17</td>
<td>24 (&lt;1)</td>
<td>ND ND Glomerular</td>
</tr>
<tr>
<td>10</td>
<td>2080</td>
<td>587</td>
<td>187</td>
<td>64 (4.3)</td>
<td>ND ND Mixed</td>
</tr>
<tr>
<td>11</td>
<td>260</td>
<td>57</td>
<td>35</td>
<td>34 (&lt;1)</td>
<td>ND ND Mixed</td>
</tr>
<tr>
<td>12</td>
<td>180</td>
<td>80</td>
<td>7</td>
<td>0</td>
<td>ND ND Glomerular</td>
</tr>
<tr>
<td>13</td>
<td>280</td>
<td>169</td>
<td>11</td>
<td>44 (2.4)</td>
<td>ND ND Glomerular</td>
</tr>
<tr>
<td>14</td>
<td>1040</td>
<td>453</td>
<td>94</td>
<td>85 (1.4)</td>
<td>ND ND Mixed</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by immunonephelometry.

<sup>b</sup>% of total proteinuria by SDS-AGE. Type of proteinuria was determined according to the SDS-electrophoretic pattern (see Table 1).
is the best available method for both screening and a quantitative approach for measurement of urinary BJP. Routine (nondenaturing) AGE, however, cannot differentiate Bence Jones proteins from intact Ig and shows poor sensitivity for BJP detection. A prior concentration step is required, but there is no agreement on the ideal concentration that should be obtained for BJP screening (22, 23).

In our experience, concentrating urine more than 25-fold leads to excessive background staining. Concentrating urine up to 100-fold or more is time-consuming and not necessarily associated with a gain in sensitivity for BJP detection, with the exception of glomerular proteinuria and pure BJP (Table 2, patients 1 and 5). In addition, prior concentration of urine by ultrafiltration is associated with a loss of low Mᵣ proteins (24), leading to a 30% overestimation of the albumin fraction, compared with immunonephelometry.

SDS-AGE separates proteins according to their molecular mass and does not require prior urine concentration, a substantial improvement over routine electrophoresis. We obtained excellent reproducibility of the Rᵢ (CV <5%, n = 9) of identical proteins (LMW calibrator). These
Renal impairment is observed in chronic MM, which is one of the major complications of MM, and had no signs of renal impairment. Renal failure (acute or chronic) is one of the major complications of MM (26). Renal impairment is observed in ~50% of MM patients during the course of their disease and has been associated with a poor prognosis. Interestingly, most of the severe cases of renal impairment (plasma creatinine >200 μmol/L) were found in patients with mixed proteinuria (43%), rather than in those with glomerular (15%) or tubular (20%) proteinuria or prerenal BJ (7%), identified by SDS-AGE. Light chain excretion has been described as an essential factor contributing to renal failure in MM (26). However, we did not find any relation between light chain proteinuria (determined by SDS-AGE) and renal failure.

A few limitations of the SDS-AGE technique should be emphasized. The most important is that SDS-AGE has no definitive way to distinguish between monoclonal and polyclonal proteins. In the absence of tubular damage (pure BJ or glomerular proteinuria, 50% of our MM samples), we found that a band at $M_r$ 25,000 is always BJ. During tubular or mixed proteinuria, polyclonal light chains are excreted in the urine because of impairment of tubular reabsorption. Because MM can cause tubular damage, a complex mixture of monoclonal and polyclonal light chains may be excreted. We found that a band at $M_r$ 25,000 of greater intensity than other small proteins (α1-microglobulin, retinol-binding protein, and β2-microglobulin) strongly suggests BJ. In patients with tubular or mixed proteinuria, we obtained only two false positives with SDS-AGE, compared with both IFE and immunonephelometry. The presence of substantial quantities of polyclonal light chains in these specimens, however, likely leads to an overestimation of the amount of BJ, as underscored by others (27). In addition, in our IFE negative control group we verified that the SDS-AGE protein pattern produced by polyclonal light chains observed during tubular or mixed proteinuria is unlikely to be confused with monoclonal components (no false positives).

Other difficulties encountered with SDS-AGE are associated with BJ quantitation. Polymeric forms of light chains were present as high $M_r$ bands (≥300,000), smears, or dimers ($M_r$ 50,000) in 50% of our MM urine specimens. Comigration of polymers with albumin on the gel can be suspected by comparing electrophoretic protein patterns with the results of albuminuria (by immunonephelometry) and/or dipstick tests (light chains do not react with tetrabromophenol). Frequencies of 80% for dimers, 30% for tetramers, 20% for higher polymers, and 25% for fragments of Bence Jones proteins have been reported (27). If a large number of MM patients need to be evaluated, we recommend reducing all urinary samples with β-ME before SDS-AGE, with a depolymerization marker run in parallel. We verified that there is no impact of β-ME treatment on the $R_f$ of other proteins. Finally, it should be recalled that there is no reference method for estimation of total urinary protein: the biuret method lacks sufficient sensitivity for urine samples (2).
In conclusion, SDS-AGE appears to be a clinical tool for the laboratory follow-up of MM patients. SDS-AGE has high resolution, sensitivity, and reproducibility. In a specialized laboratory like ours, it is useful for monitoring patient progress through the semiquantitation of BJP. Compared with routine electrophoresis, SDS-AGE combines no need for prior urine concentration with increased sensitivity for BJP detection. Glomerular, tubular, and mixed patterns were easily identified on the gel. Interpreting SDS-AGE protein patterns requires, however, awareness of some pitfalls, especially with regard to light chain polymerization and the presence of mono- and/or polyclonal light chains in urine specimens from MM patients.

We thank all the technical staff in our laboratory for their technical expertise.

References