Analysis for Cerebrospinal Fluid Proteins by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

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Cerebrospinal fluid (CSF) proteins with molecular masses of <150 000 Da were identified by immunoblotting after two kinds of nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). With PAGE 1 (17–27% gradient gel), CSF proteins were clearly separated into seven to nine bands with molecular masses of 3000–67 000 Da; seven bands were identified as β2-microglobulin, lysozyme, prealbumin, free k and λ chain, apolipoprotein A-I, glycoproteins, and albumin by immunoblotting. With PAGE 2 (10–20% gradient gel), proteins were clearly separated into 11–16 bands with molecular masses of 15 000–150 000 Da; 11 were identified as prealbumin, free k and λ chain, apolipoprotein A-I, glycoproteins, albumin, α1-antitrypsin, transferrin (separated into two bands), immunoglobulin fragments, haptoglobin, and IgG. We analyzed CSF samples collected from 81 patients with cerebrospinal signs by these SDS-PAGE methods and observed prominent bands in some cases.

Additional Keyphrases: immunoblot technique · Alzheimer disease · leukemias · brain disorders · prealbumin · albumin

Analysis for proteins in cerebrospinal fluid (CSF) can be useful for diagnosing various neurological diseases.4 Total protein (TP), albumin (Alb), and immunoglobulins in CSF are usually measured by spectrophotometric or immunometric methods in clinical laboratories. In addition, analysis for various proteins (1, 2) and detection of oligoclonal bands (3–5), monoclonal immunoglobulin (6), and transferrin variants (7, 8) are carried out by agarose gel electrophoresis (1, 2), cellulose-acetate membrane electrophoresis (2), polyacrylamide gel electrophoresis (PAGE) (8), PAGE-isoelectric focusing (5), sodium dodecyl sulfate (SDS)-PAGE (4, 9), and two-dimensional PAGE (7). Of these methods, SDS-PAGE is very useful because many kinds of proteins in CSF can be easily fractionated and detected without concentration of the original sample (4, 9). In these studies, however, we focused the analyses on proteins with relative molecular masses (Mr) of >60 000. Here, we report nonreducing SDS–gradient slab polyacrylamide gels (SDS-PAGs) specific for analysis for proteins with Mr <40 000 in CSF and demonstrate the clinical usefulness for the determination of small-Mr proteins in CSF.

Materials and Methods

Specimens

CSF specimens were collected from patients by lumbar puncture for routine clinical-diagnosis tests. The untested remainder of the specimens was used in this study. Each sample was centrifuged and the supernate was stored at −80°C until use. We excluded CSF samples showing visual evidence of erythrocytes. The remaining samples were derived from 81 patients (37 males, ages 5–78 years, mean 46.9 years; 44 females, 3–85 years, mean 45.3 years). The diagnoses of the patients were reported by the attending doctors: 5 cerebrovascular diseases (2 cerebral infarction, 2 cerebral aneurysm, and 1 hydrocephalus), 12 malignancies with cerebral signs [2 acute myelocytic leukemia, 3 acute myelomonocytic leukemia (AMMoL), 2 acute lymphocytic leukemia, 2 malignant lymphoma, 2 brain tumor, and 1 spinal cord tumor], 5 infectious diseases (2 meningitis, 1 cytomegalovirus disease, 1 lymph node tuberculosis, and 1 Creutzfeldt–Jakob disease), 19 neurodegenerative diseases (5 Alzheimer disease, 2 Parkinson disease, 3 cerebral spongylisis, 2 spinocerebellar degeneration, 2 progressive supranuclear palsy, 2 amyotrophic lateral sclerosis, 2 spinal canal stenosis, and 1 ossification of posterior longitudinal ligament of cervical spine), 9 neuroimmunological diseases (5 multiple sclerosis, 2 Paget disease, 1 Sjögren syndrome, and 1 Guillain–Barré syndrome), and 31 other diseases with cerebrospinal signs.

Reagents

SDS-PAG (10–20%, 84 mm wide × 70 mm high × 1.0 mm thick, with 12 wells, each 4 mm wide), acrylamide, N,N'-methylene-bis-acrylamide (Bia), Tris-glycine buffer for electrophoresis, Sepasol 1 (denaturing solution; 20 g of SDS, 5 g of bromphenol blue, and 300 mL of glyceral per liter of 0.25 mol/L Tris·HCl, pH 6.8), and reagents for immunoblotting were obtained from Daiichi Pure Chemical Co. (Tokyo, Japan). 2D-Silver stain-II, also from Daiichi, was composed of (a) fixing reagent, including thiouracil; (b) pretreatment reagent, including dithiothreitol, guanaraldehyde, and thioures; (c) staining reagent A, including silver nitrate; (d) staining reagent B, including ammonium hydroxide; (e) concentrated developer, including citric acid, formicaldehyde, and sodium thiosulfate; and (f) stopper solution, including ammonium hydroxide. The pre-
Table 1. Composition of Solutions I and II

<table>
<thead>
<tr>
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<th>I</th>
<th>II</th>
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<tr>
<td>39% Acrylamide-1% Bis solution, mL</td>
<td>17</td>
<td>27</td>
</tr>
<tr>
<td>1.5 mmol/L Tris-HCl buffer (pH 8.8), mL</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Distilled water, mL</td>
<td>to 40</td>
<td>to 40</td>
</tr>
<tr>
<td>Ammonium persulfate, mg</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>TEMED, μL</td>
<td>25</td>
<td>25</td>
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cise compositions of these reagents are confidential. \(N,N,N',N'-\)tetramethylethylenediamine (TEMED) was obtained from BDH Ltd. (Poole, UK). Electrophoresis calibration kits for \(M_r\) determination were obtained from Pharmacia AB (Uppsala, Sweden). Alb, IgG, prealbumin (thyroxine-binding prealbumin, TBPA), \(\alpha_1\)-acid glycoprotein (\(\alpha_1\)AG), and transferrin (TF), all of human origin, were obtained from Sigma Co. (St. Louis, MO). SDS-PAGs (17–27%) were prepared in our laboratory.

Methods

Preparation of SDS-PAGs (17–27%). SDS-PAGs (17–27%) were prepared by pouring 40 mL of each of solution I and II (Table 1) into a two-vessel gradient-former (84 mm wide \(\times\) 70 mm high \(\times\) 1.0 mm thick; Daiichi Pure Chemical Co.). We used combs that are 1.0 mm thick with 12 teeth, each 4 mm wide.

Protein determination. The concentration of TP in CSF was determined by the method of Watanabe et al. (10). Alb and TBPA in CSF were determined by immunonephelometry (11).

Electrophoresis. Electrophoresis on two kinds of SDS-PAG, 17–27% (PAGE 1) and 10–20% (PAGE 2), was performed with a cassette electrophoretic apparatus (Daiichi Pure Chemical Co.) according to Laemmli (12). Equal volumes of CSF and the denaturing solution were mixed and heated to 95°C for 5 min. To preserve S-S bonding of protein, no reducing agent was added to CSF. A portion of the mixture (10 μL when TP in CSF was <0.4 g/L, 5 μL when TP was 0.4–0.8 g/L, 2 μL when TP was 0.8–1.6 g/L, and 1 μL when TP was 1.6–2.4 g/L) was loaded onto each well of the gel so that <500 ng/mm² of protein was applied. Electrophoresis was carried out at 50 mA per gel at room temperature for 40 min. After the run, gels were stained by 2D silver stain-II by the method of Oakley et al. (13). The \(M_r\) of each protein was estimated with electrophoresis calibration kits (Pharmacia). Pooled CSF was used for quality control and measured in every run.

Silver staining. The gel was soaked in 200 mL of the first fixing solution (methanol:acetic acid:distilled water, 10:2:8 by vol) and shaken for 10 min, then soaked in 200 mL of the second fixing solution (methanol:acetic acid:fixing reagent:distilled water, 6:2:1:11 by vol) and shaken for 15 min, soaked in 200 mL of the pretreatment solution (methanol:pretreatment reagent:distilled water, 10:1:9 by vol) and shaken for 10 min, and soaked in 200 mL of distilled water for 5 min. The gel was then stained, while shaking, in 200 mL of the silver staining solution (staining reagent A:staining reagent B:distilled water, 1:1:18 by vol) for 15 min; rinsed three times in 200 mL of distilled water for 2 min; and developed, while shaking, in 200 mL of the developer solution (10 mL of concentrated developer and 190 mL of distilled water) for 3 min. Developing was stopped by adding 10 mL of stopper solution, and the gel was washed with de-ionized water and then scanned at 465 nm with a Cliniscan 2 electrophoresis densitometer (Helena Laboratories, Beaumont, TX).

Immunoblotting. Proteins in gels were transferred electrophoretically to a polyvinylidene difluoride microporous membrane and identified by an enzymatic immunoassay involving sequential reactions of primary antibody, secondary antibody conjugated to peroxidase, and 3,3’-diaminobenzidine (14).

Results

Electrophoretic Patterns

Proteins with \(M_r\) of 3000–67 000 in human CSF were fractionated into 7–9 bands on PAGE 1, and those with \(M_r\) of 15 000–150 000 gave 11–16 bands on PAGE 2 (Figure 1). The number of bands for patients varied. As shown in Figure 1, proteins with \(M_r\) <20 000 were most clearly separated on PAGE 1. The protein in each band was characterized by immunoblotting. Seven bands on PAGE 1 and 11 bands on PAGE 2 were identified (Figure 2). The properties of the bands B1–B15 are shown in Figure 3.

Detection of \(\beta_2\)-Microglobulin

B2 was clearly separated on PAGE 1 and immunoreacted with anti-\(\beta_2\)-microglobulin antibody (lane 1, Figure 2A). B2 was prominent in some samples collected from patients with malignancy and infectious diseases.

Detection of Hemoglobin (Hb)

Blood can appear in CSF by traumatic tap or pathological processes such as cerebral bleeding. We excluded
traumatic-tap CSF from this study. We could detect the presence of 20 ng of Hb on PAGE 1 (lane 3 in Figure 1 and lane 21 in Figure 2A). In cases of cerebral bleeding, Hb always appeared in two bands, one with an Mr of 14 000 (B3) and another with an Mr 15 000, as shown in lane 3 of Figure 1. In the presence of large amounts of Hb, additional bands appeared in slower and faster migrating regions, as shown in lane 4 of Figure 1.

Detection of Lysozyme

B3 on PAGE 1 was reactive to anti-lysozyme antibody in five of seven patients who had prominent B3. The most remarkable reactivity was observed in two patients with AMMOL (lane 2, Figure 1). In two of seven patients, B3 was immunonegative to both anti-Hb and anti-lysozyme antibodies. These results showed that the B3 comprised Hb, lysozyme, and other proteins.

Detection of Proteins on PAGE 2

The clear bands of B8, B9, B11, and B15 and faint bands of B7, B10, and B12–B14 were detected in all samples examined on PAGE 2 (81 samples). B7 was a faint and broad band, suggesting that this band comprised various proteins. B9 was prominent in all samples examined and this band comprises a1-antitrypsin, hemopexin, and a1-antichymotrypsin, on the basis of immunoblotting (Figure 2B). Transferrin was identified in two bands (B10 and B11). B12 immunoreacted with anti-IgG and anti-Fc antibodies in all samples examined. Anti-haptoglobin (Hp) antibody blotted differently in different patients, mostly on B13 and rarely on B12. In the samples as shown in lane 5 of Figure 1, anti-Hp antibody reacted on B12, whereas the immunoreactivity was observed in a prominent band of B13 as shown in lane 6 of Figure 1 and lane 26 of Figure 2B.

Semiquantitative Evaluation of Protein Content

We examined the linear range of our method by using pure protein (Figure 4). The results showed that a protein content <100 ng/mm² may be quantified. We took the TBPA band (B4) as an internal reference band, and we measured the concentrations of TBPA in CSF by
immunonephelometry. A relative concentration ($C_i$) of the band $i$ may be calculated as follows:

$$C_i \text{ (mg/L)} = (B_i/B4) \times \text{TBPA (mg/L)}$$

where $B_i$ and $B4$ are integral values of the band, by densitometry.

Concentrations of B2–B6 were determined by PAGE 1 and those of B7–B11 were determined by PAGE 2. Effect of sample volume on $C_i$ values (Table 2) and reproducibility (Table 3) suggest that variation of relative concentration of each band except C8 could be presumed by determining $C_i$ values if the total amount of protein loaded was <500 ng/mm².

We measured $C_i$ values of CSF specimens from 81 patients and calculated the C/Alb values in which Alb was determined by immunonephelometry. The mean, SD, and CV of each C/Alb and diseases in which C/Alb values were higher than the mean + 2SDs are shown in Table 4. The CVs of C2/Alb and C3/Alb were large, whereas those of C6/Alb, C9/Alb, and C11/Alb were not. For C7/Alb, three of five patients with Alzheimer disease had values higher than the mean + 2SDs.

Discussion

CSF proteins with $M_r <150 000$ were separated by nonreducing SDS-PAGE and identified by immunoblotting. This SDS-PAGE system was very effective in identifying the proteins with $M_r <20 000$, such as B2 and B3. Combination of this SDS-PAGE with a silver staining method is very useful for detecting a small amount of protein.

HB always appeared in double bands, one with an $M_r$ of 14 000 at B3 and the other with an $M_r$ of 15 000, which seemed to correspond to the $\alpha$ and $\beta$ chain of Hb (lane 3, Figure 1). B3 was prominent in the CSF of two of three AMMol patients with accompanying cerebral signs. In these cases, a main component of B3 was lysozyme, because it was reactive against anti-lysozyme antibody but not against anti-Hb antibody. However, there were some cases in which B3 was prominent but scarcely reactive against either anti-lysozyme or anti-Hb antibodies. In these cases, proteins other than Hb and lysozyme were increased. B7 was a small component in CSF proteins. Considering width of the band and immunological heterogeneity, B7 seems to contain several proteins, such as $\alpha_1$AG and Gc-globulin. B10 and B11 were immunoreactive against anti-Tf antibody. B10 may be tau transferrin. B12 immunoreacted to anti-IgG and anti-Fc antibody in all CSF samples and anti-Hp antibody in one sample, suggesting that B12 contained IgG heavy chain and Hp type 1-1.

The intensity of color developed by silver staining varies for different kinds of proteins, and the linear
range is narrow and also affected by the staining method (15, 16). However, with this silver staining kit, we could semiquantitatively determine the concentrations of the bands except Alb and immunoglobulins in the range of <100 ng/mm², by using an internal reference. We took the TBPA band (B4) as an internal reference band and determined the C_i for each band, because TBPA appeared to be constant in various diseases. Then we calculated C/Alb values and compared the C/Alb concentrations of the various bands found in CSF from patients. C2/Alb and C3/Alb varied widely in various diseases. C7/Alb was increased higher than the mean + 2SDs in three of five patients with Alzheimer disease. The reason why C7/Alb was increased in Alzheimer disease is unknown.

In conclusion, this nonreducing SDS-PAGE system was effective in clearly separating CSF proteins, particularly small-Mᵦ proteins (Mᵦ < 20 000) and also in detecting specific abnormalities with small amounts of protein in CSF.

We thank Daiichi Pure Chemical Co. for offering us the reagents used in SDS-PAGE.

References