Capillary-Zone Electrophoretic Analyses of the Proteins and Amino Acid Components in Cerebrospinal Fluid of Central Nervous System Diseases

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Analysis systems for protein and amino acid components in cerebrospinal fluid (CSF) from patients with a variety of central nervous system (CNS) diseases were established employing capillary-zone electrophoresis (CZE). Under the conditions employed, the major proteins in CSF and the concurrent sera were separated into several respective fractions, and in the obtained electropherograms of serum proteins, the % values of areas of each major protein fraction (from γ-globulin to albumin) were in close accord with those in the densitograms of stained protein bands after electrophoresis on cellulose-acetate strips which had been done as a routine laboratory test in the hospital. Some unusual migration patterns of major proteins were observed in various CNS diseases, and an increase of glutamine in hepatic encephalitis was also detected on the electrophorograms. A peak responsible for a minor protein component with the molecular weight of 10000—30000, which was tentatively identified as β-trace protein, was found to be contained in all the CSF samples examined, and its concentration levels were higher in some patients with cerebral infarction and multiple sclerosis. These results suggest that CZE can become a powerful aid in analyses of the protein and amino acid components in CSF for biochemical diagnosis of CNS diseases.

Keywords capillary-zone electrophoresis; cerebrospinal fluid; protein; amino acid; central nervous system disease

Capillary-zone electrophoresis (CZE) has been recognized as a simple and valuable system for analyses of a variety of ionized substances such as proteins and peptides, amino acids, nucleic acid components, biogenic amines, vitamins, sugar derivatives, and drugs etc., but this new technique is not yet fully exploited in the field of clinical biochemistry. Cerebrospinal fluid (CSF) has been chemically studied in connection with neurological diagnosis. Qualitative and quantitative changes in major CSF proteins were reported by earlier researchers who employed electrophoretic techniques on supporting media, like as cellulose acetate strips, agarose-gel and polyacrylamide-gel. According to them, increased penetration of the plasma macromolecular proteins due to disturbance of the blood-CSF barrier function and accelerated immunoglobulin-G (IgG) biosynthesis within the central nervous system (CNS) occur under various pathological conditions in the CNS. It has also been known that glutamine (Gln) which is the most abundant free amino acid in CSF is largely increased in CSF of hepatic encephalitis. Based on the differences in total protein contents between CSF (normally 10—30 mg/dl) and blood plasma (6—8 g/dl), the former contains a relatively larger amount than in serum of low molecular weight (MW) protein components with the MW of less than 30000; some of these CSF low MW proteins with larger mobilities than prealbumin (PA) in continuous density-gradient polyacrylamide-gel electrophoresis (PAGE) and high-voltage agarose-gel electrophoresis were increased in various CNS diseases. The major components of these minor low MW proteins in CSF is a CSF-specific substance with the MW of 28000, which is synthesized in glial cells and called β-trace protein; its CSF levels was often found to be higher in cerebrovascular diseases and multiple sclerosis (MS). In this investigation, the efficiency of CZE as a tool in routine laboratory analyses of proteins and amino acids in CSF of various CNS diseases was evaluated on the basis of results of analyses of three fractions from CSF prepared on differences in the MW of components. Furthermore, in patients from whom both CSF and concurrent sera were taken simultaneously, the quantitative data of serum electrophorograms were compared with those of the densitograms of stained protein bands after electrophoresis on cellulose-acetate strips.

Materials and Methods

Materials Eighty-eight CSF samples were collected by lumbar puncture from 74 patients with various CNS diseases as summarized in Table I, who were admitted to Chiba National Hospital. From 8 of the patients, a blood specimen was collected at the same time as lumbar puncture.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of samples</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral infarction</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Cerebrovascular sclerosis</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Other cerebrovascular disorders</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Meningitis</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Meningoencephalitis</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Other inflammatory disorder in CNS</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Alzheimer's disease</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Parkinson's disease</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Other degenerative disorders in CNS</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Diabetic neuropathy</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Hepatic encephalitis</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Polyneuritis</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Shizophrenia</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Depressive illness</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Neurosis and tension headache</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Other miscellaneous neurological disorders</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>88</td>
<td>74</td>
</tr>
</tbody>
</table>

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from an arm vein for the preparation of concurrent sera. After supplying aliquots required for the routine laboratory tests in the hospital such as measurement of the CSF and serum total protein contents, counting of the CSF cells, electrophoretic fractionation of the serum proteins on cellulose acetate strips, etc., the samples were immediately stored at −20°C for up to one week. CSF samples containing erythrocytes were excluded from the investigation at this stage.

**Fractionation of CSF Samples**

Two milliliters of each of the CSF samples to be examined was first centrifuged in a Centrifrac-30 mini-concentrator (Grace Company) with membranes allowing passage of only substances with the MW of less than 30000; this was done at 740 × g for 30 min at room temperature, then, 1 ml from each of the ultrafiltrates obtained was treated in the same way with Centrifrac-10 (Grace Company) with membranes allowing passage of only substances with the MW of less than 10000. The final ultrafiltrates containing only CSF components with the MW of less than 10000 were taken as the fractions to be analyzed for free amino acids (fraction A), while the concentrates were added with 2 ml of water followed by centrifuging for 60 min in order to obtain fractions of low MW substance free concentrates with the dead stop volume of 50 μl. It was assumed that the fractions thus obtained (fraction B) contained primarily CSF components with the MW of 10000—30000. Intact CSF samples and sera diluted 100 times with 0.8% (v/w) NaCl, as well as fractions A and B from CSF, were subjected to CZE analyses for major proteins (intact CSF and diluted sera), free amino acids (fraction A) and minor low MW proteins (fraction B), respectively.

**CZE Conditions**

CZE was performed employing a Waters Quanta-4000 Capillary Electrophoresis system equipped with a fused silica capillary (75 μm × 60 cm). The electrolyte was used 50 mM sodium tetraborate with or without 1 mM 3-(trimethylammonio)propylsulfonate (Z₃-methyl); Z₃-methyl was needed only to provide good separation in analyses for the major proteins in intact CSF samples and diluted sera. An aliquot of a sample corresponding to a hydrostatic injection of 10 s was injected, and the separation was achieved at 15 kV for 20 min. Detection was carried out at 185 nm. The identifications of PA, albumin (Al), Gln, transferrin (Tf) and IgG in intact CSF and diluted sera were performed by mixed analyses with aqueous solutions of the authentic samples. In analyses of the major proteins in diluted sera, the % values of the area of each peak was obtained by Waters 805 Data Station, while for determinations of the Gln concentrations in fraction A and measurements of the low MW protein component levels in fraction B, prior to the injection, samples to be examined were mixed with an equal volume of aqueous solution of 6-aminocaproate (6ACA) as the internal standard (I.S.) at the concentrations of 500 and 50 μg/ml, respectively.

**Capillary-Isotachophoretic Determination of Gln**

Ten samples (fraction A) were measured to determine Gln concentration using capillary-isotachophoresis (CITP), as reported previously.²²

**Results and Discussion**

Under the conditions employed, neutral peak appeared at the migration time (Mt) of 6.5 min on electropherograms of all the samples examined; in the analyses of intact CSF, peaks identified as IgG (Mt: 7.2 min), Tf (8.1 min), Gln (9.3 min), Al (9.8 min) and PA (11.0 min) were also observed following neutral peak. These electropherograms resembled the densitometric patterns of stained protein bands after electrophoresis on agarose-gel²³ and cellulose acetate strips, except for the presence of a peak for Gln. A typical migration pattern is shown in Fig. 1a, while electropherograms suggesting unusual migration of these major CSF proteins (shown in Fig. 1b—d) were also detected in some patients, as described below.

The peak height ratios of IgG to Al were generally 10—15% (Fig. 1a), but rose to 20% or more (Fig. 1b) in 8 CSF samples from 5 patients with cerebral infarction (1 sample from 1 patient), meningitis (3 from 2), meningoencephalitis (2 from 1) and MS (2 from 2). These had greatly elevated total CSF protein contents of 60—320 mg/dl (the upper normal limit: 30 mg/dl). It was also noteworthy that such relative increases to Al of IgG in CSF were generally (except for 2 samples from an MS patient) accompanied by appearance of a small peak with Mt of 8.6 min on the electropherograms (Fig. 1b). This peak was present on the electropherograms of concurrent sera but not on those of ultrafiltrates with Centrifrac-30, indicating that it was responsible for high MW compounds such as proteins. The corresponding CSF components were assigned from its Mt value to be proteins with the same mobility as Z₃-globulin: Z₃-macroglobulin, haptoglobin polymer and low-density lipoprotein. Indeed, it is known that disturbance in the function of blood-CSF barrier gives the appearance in CSF of these plasmagamic macromolecular proteins which normally can hardly pass through the barrier.¹¹ In our previous study on the CSF proteins employing two dimensional electrophoresis and nitrocellulose blotting techniques, such kinds of protein components with the MW of more than 160000 were detected only in CSF with elevated protein levels in various CNS diseases.²⁵ On the other hand, in 2 CSF samples from 2 MS patients, the elevated CSF total protein contents (80 and 60 mg/dl) with relative increase to Al of IgG were not accompanied by this peak on the electropherograms (Fig. 1c), suggesting that the rises in CSF protein levels in these patients were caused not by accelerated penetration of plasmagamic proteins but by increased production of IgG with in the CNS tissues. Furthermore, on the electropherograms of 4 CSF samples from 3 patients with Parkinson's disease accompanied by
senile dementia and one with Alzheimer's disease, the peak height ratios to Al of PA (generally less than 15%) and TF (generally less than 20%) were increased to ca. 20% and ca. 30%, respectively (Fig. 1d), although the total protein content levels of these CSF samples were normal or only slightly raised (25–40 mg/dl). Clinically interesting was the fact that such increases in CSF of PA and TF expressed by changes in the electropherograms were associated with the occurrence of brain atrophy confirmed by CT (computed tomography) scan tests. This was in agreement with the results of earlier researchers who found increases in the contents of PA and TF in CSF of brain atrophied patients using disk PAGE.26 The above described data on the unusual electropherogram patterns of major CSF proteins under various pathological conditions in the CNS suggested that CZE analyses of intact CSF can offer the same useful information as do conventional electrophoresis systems which require concentration of samples prior to the analyses or complexed procedures for silver staining on supporting media after separation.

The total protein contents of concurrent sera were 6.5 ± 0.4 g/dl (n = 8) and the electropherograms of their 100 times diluted samples resembled those of CSF, except for the absence of PA and Gln peaks, as illustrated in Fig. 2 (the Gln peak present on the CSF electropherograms was not visible due to effects of dilution). They were in close accord with the densitograms of stained protein bands after electrophoresis on cellulose acetate strips which had been done as routine laboratory tests in the hospital. Indeed, as summarized in Table II, the mean % values of area of γ-, β-, α2- and α1-globulin and Al fractions (the main components of γ- and β-globulin fractions were identified as IgG and Tf, respectively) roughly agreed with those of the densitometric data. The concentration ratios in these serum samples of Al and globulin (the A/G values) obtained by CZE and densitometry correlated adequately with each other: r = 0.95 (n = 8), Y = 1.09X − 0.08 (X: CZE, Y: the densitometric data).

The peak for Gln (Mt: 9.3 min), as well as that for neutral substances (Mt: 6.5 min) and NaN3 from the Centricon membranes (8.4 min), was observed on all the electropherograms of fraction A. As described in Materials and Methods, its concentrations were measured by the mixed analyses (1:1, v/v) with 6ACA (500 µg/ml, Mt: 7.0 min) as the I.S. Amino acids other than Gln were not found under these conditions possibly due to their small quantity (Fig. 3a). The mean value of Gln concentrations in CSF examined was 60 ± 17 µg/ml (mean ± S.D., n = 88), ranging between 23 and 270 µg/ml, and a histogram of the distribution exhibited a normal-type pattern within the range of 20–95 µg/ml, giving the only an exception being a greatly increased value of 270 µg/ml in one CSF sample from a hepatic encephalitis patient. The CSF Gln concentrations obtained by CZE were roughly the same as those by the previously developed CITP method22, r = 0.94 (n = 10), Y = 1.04X − 11.7 (X: CZE, Y: CITP). These data clearly
showed that increase of CSF Gli characteristic to hepatic encephalitis\textsuperscript{14–17} can be easily detected by the present CZE system.

The analyses of fraction B gave electropherograms on which only one peak was detected at the M\text{t} of 9.7 min, in addition to the artifact peak for NaN\textsubscript{3} (M\text{t}: 8.4 min) which could not be completely removed even after the preparatory procedures (see Materials and Methods). The CSF component for this peak was tentatively identified as \( \beta \)-trace protein due to its abundance in this fraction of CSF.\textsuperscript{20} As the authentic sample of \( \beta \)-trace protein was not available, its relative content in samples examined was calculated from its relative peak height to 6ACA (50 µg/mL, M\text{t}: 7.0 min) as the I.S. in the mixed analyses (Fig. 3b). The relative values of CSF \( \beta \)-trace protein contents thus calculated ranged from 0.14 to 0.86 with the mean value of 0.38 ± 0.13 (mean ± S.D., \( n = 80 \)), and as illustrated in Fig. 4, a distribution histogram gave two peaks: the major one (\( n = 73 \)) within the range of 0.1—0.6 and the minor one at values beyond 0.6 (\( n = 7 \)). The latter peak was given by samples from 3 cerebral infarction patients and 5 MS patients. The most interesting finding from the viewpoint of clinical biochemistry was that in 2 cerebral infarction patients and 2 MS patients from whom CSF samples were taken twice during the hospital treatments (at acute and recovery phases of cerebral infarction, as well as in malignant and mild phases in MS), the values in the acute phase in cerebral infarction (0.69 and 0.70) and in the malignant phase in MS (0.74 and 0.86) were greater than those in the recovery phase (0.52 and 0.34) and the mild phase (0.44 and 0.62), respectively. These data agreed with the results of earlier researchers who pointed out the parallelism between the elevated CSF \( \beta \)-trace protein levels and the severity of myelin degradation.\textsuperscript{21} The increased \( \beta \)-trace protein in CSF detected by CZE was virtually independent of changes in the results of routine laboratory tests of CSF such as measurement of total protein contents and cell count numbers. Therefore, the CZE analyses of CSF minor low MW proteins in the samples prepared to contain primarily such components seem to be useful as an aid in biochemical diagnosis of CNS diseases, especially in detecting increases in the CSF content of \( \beta \)-trace protein associated with the myelin degradation process in the absence of abnormalities detected in CSF routine laboratory tests.

References and Notes

1) A summary of this study was presented at the 10th Symposium on Analytical Chemistry of Biological Substances, Kyoto, September 1992.
14) S. Brandstäetter, D. Brazlial, \textit{Am. J. Dig. Dis.}, 5, 945 (1960).