Two-Dimensional Electrophoresis and "Ultrasensitive" Silver Staining of Cerebrospinal Fluid Proteins in Neurological Diseases

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Cerebrospinal fluid (CSF) proteins, as resolved by two-dimensional electrophoresis and made visible by silver staining, have been examined in patients with various neurological diseases and normal volunteers. The patterns for 15 of 20 patients with Parkinson's disease showed a protein (M, 25 000) with charge similar to albumin, which was not seen in the patterns for any of 91 normal volunteers. Patterns for 21 of 22 multiple sclerosis patients showed novel immunoglobulin light chain proteins, also not present in the CSF of any normal volunteers. Quantitative analysis by computer-assisted densitometry in Parkinson's disease and multiple sclerosis showed that 20 of 68 and 33 of 85 proteins, respectively, were significantly altered as compared with proteins in the normal population. This ability to characterize both Parkinson's disease and multiple sclerosis molecularly provides a broad baseline for improved clinical diagnosis and may serve as an aid in exploring the underlying pathophysiology. These studies illustrate the potential of applying this methodology in the study of neurological diseases.

Additional Keyphrases: nervous-system disorders · disease "markers" · Parkinson's disease · multiple sclerosis

Examination of cerebrospinal fluid (CSF) is of potential value in searching for changes in neurological diseases because of its clinical availability and its close proximity to the nervous tissue.1

Recently, two-dimensional gel electrophoresis and silver staining of acrylamide gels have enabled over 300 CSF proteins to be examined per individual (1–4). Using this technology, with computer-assisted densitometry (5), we have observed many new protein changes in the CSF from multiple sclerosis (MS) and Parkinson's disease (PD) patients compared with CSF proteins from both normal volunteers and patients with other neurological diseases (6, 7). This ability to observe alterations in protein patterns in neurological diseases is based, in part, on studies involving a large group of normal volunteers. Qualitative CSF protein patterns (including 300 proteins per individual) in these normal individuals are highly reproducible. Quantitatively, five of 67 proteins studied showed an alteration with age (8, 9).

We have used these methodologies to obtain further insight into neurological diseases. PD is of unknown etiology, and drug resistance or disease progression, or both, still take a severe toll on the patients within five to seven years after symptoms develop. A diagnostic test is needed that can distinguish PD from the less-common forms of parkinsonism, such as progressive supranuclear palsy, post-encephalitic parkinsonism, and the parkinsonism resulting from vascular disease. Gamma-globulin abnormality has not been found in PD (10). Oligoclone bands may help to distinguish post-encephalitic parkinsonism from PD (11). Hitherto, protein changes in PD have only been observed with isoelectric focusing (12): up to half of such patients show various alterations in unidentified protein bands, none of which correlate with age, duration of disease, or drug therapy.

MS is a disease of unknown etiology, for which there is currently no specific treatment. Clinical diagnosis and research are both hampered by lack of a diagnostic test, although the typical cases are usually recognized by clinical criteria, which include abnormal visual evoked responses and the presence of CSF oligoclonal bands (13–15).

Specific qualitative and quantitative CSF protein alterations were found in MS and PD in this study. These results demonstrate a more general conclusion: that the improved molecular approach that these methodologies provide will aid in disease classification and may provide clues for understanding the underlying pathophysiology of diseases of the nervous system.

Materials and Methods

Clinical samples: CSF was obtained from the following groups:

• 91 normal volunteers who had no disease symptomatology, were not being treated with or taking any drug, and were clinically examined particularly to exclude any abnormality of the nervous system

• 20 patients with typical idiopathic PD, defined as having the typical clinical features of the disease with no evidence on history, clinical examination, or laboratory testing of other causes of parkinsonism or other disease

• 22 patients with MS who fulfilled McAlpine's criteria (16) for definite disease

The mean age of the PD patients was 55.3 years (range 31–71), of the MS patients 39.3 years (range 23–57), and of the normal volunteers 41 years (range 20–70). The male/female sex ratio was respectively 1.5, 0.63, and 1.125.

Lumbar puncture was performed in the morning in the conventional manner, samples were stored at −70 °C without preservative and thawed before use. Samples between the fifth and eleventh of successive 1-mL aliquots were used in this study.

Reagents: Acrylamide, N,N'-methylenebisacrylamide, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylenediamine, ammonium persulfate, Bio-Lyte carrier ampholytes, and agarose were purchased from Bio-Rad, Richmond, CA 94804. 2-Mercaptoethanol was from Eastman Organic Chemicals, Rochester, NY 14650. Tris base and glycine were from Sigma Chemical Co., St. Louis, MO 63178. Silver nitrate, glycerol, sodium hydroxide, acetic acid, methanol, anhydrous sodium carbonate, phosphoric acid and isobutanol were from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Urea, Nonidet P40 detergent, and

1 Nonstandard abbreviations: CSF, cerebrospinal fluid; MS, multiple sclerosis; PD, Parkinson's disease; 2DE, two-dimensional electrophoresis; IEF, isoelectric focusing; SDS, sodium dodecyl sulfate.

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dithiothreitol were from Bethesda Research Laboratories, Inc., Gaithersburg, MD 20760. Formaldehyde was from Fisher Scientific, Silver Spring, MD 20910. Hydrogen peroxide was from Mallinckrodt Inc., St. Louis, MO 63160. 3,3'-Diaminobenzidine tetrahydrochloride was from Polysciences, Inc., Warrington, PA 18976, and nitrocellulose was from Schleicher and Schuell, Keene, NH 03431. Peroxidase-conjugated monoclonal antibodies to kappa and lambda light chains were from Dakopatts, Accurate Chemical and Scientific Corporation, Westbury, NY 11590. Kits of purified molecular mass protein standards were from Pharmacia Fine Chemicals, Piscataway, NJ 08854 (see below).

Two-dimensional electrophoresis: Protein 2DE was performed as described (17), with minor modifications. Unconcentrated CSF containing 40 μg of total protein per sample, as measured by the method of Lowry et al. (18), was mixed with 5 μL of a solution containing, per liter, 20 g of SDS, 50 mL of mercaptoethanol, 200 mL of glycerol, 20 mL of 3-dimensional ampholyte, and 20 mL of Nonidet P40. Urea was added to give a final concentration of 9.2 mol/L. Samples were then subjected to IEF, with use of a 4:1 ratio of 5/7.3/10 ampholytes. The pH gradient after IEF was measured as described (1), confirming linearity in the range pH 5 through 7, and protein isoelectric points (in urea) were estimated from this gradient. We did not study the most basic CSF proteins, which are not resolved with the usual 2DE method, but do appear on gels run by the non-equilibrium-pH technique (19). We estimate that these comprise less than 10% of the total CSF protein. For the second dimension, where separation is based on molecular mass, we used slab gels. M, markers were phosphorylase b (EC 2.7.7.37), bovine serum albumin, ovalbumin, carbonic anhydrase (EC 4.2.1.1), soybean trypsin inhibitor, and alpha-lactalbumin, all from Pharmacia.

Procedures

Silver staining and photography: Silver staining was performed as described elsewhere (20), except that a second fixation with glutaraldehyde after initial fixation with acetic acid and methanol was added and dithiothreitol was used in place of dichromate (21). Stained gels were transilluminated on a uniform light source (Model T-12; Aristo Grid Products Inc., Port Washington, North Long Island, NY 11050), beside a calibrated density standard (National Bureau of Standards, Gaithersburg, MD 20879). We photographed these gels and the standard with a Mamiya RB 67 pro camera and 140-mm Macro lens, using Kodak 120-mm Tri-X film.

Quantitative analysis: The negatives were digitized with an 1000 HS scanning microdensitometer (Optronics, Chelmsford, MA 01824) and scanned at the 0 to 2 optical-density setting at 100-μm resolution. Digitized images were normalized against the calibrated density standard, and computerized densitometry was performed as described elsewhere (5). Units of protein quantity (O.D. × mm²) were obtained by recording the average optical density for each polypeptide minus a modal background density, and then multiplying by the area occupied by the polypeptide.

Proteins in biological fluids such as CSF are often present in concentrations ranging from relative abundance to as little as 10⁻⁵ of the total. Abundant proteins such as albumin, which are present in saturating quantities on the stained gels, could not be accurately quantified. Conversely, trace proteins that could not be seen in all gels were not quantified in this study. Of the 300 proteins per gel, 109 were consistently present, and of these 68 were quantitatively measured in each PD individual and 85 were measured in MS patients; the same proteins were measured in the normal population. All stained proteins were studied by direct visual comparison for qualitative changes, namely for deletions or additions, but none were found in these samples.

Normalization of quantitative data: The densities of polypeptide spots on respective gels were normalized against "constitutive" proteins, which we defined as those for which the density ratios to each other were constant in all gels studied, had a coefficient of variance <0.5, and had a mean density of <2.5 units. Fifteen polypeptides were thus identified as "constitutive" in this study, and we derived a normalization factor for each gel by dividing the sum of densities of all previously defined constitutive proteins in that gel by the sum of densities of constitutive proteins in an arbitrarily designated "standard" gel. The observed densities of each protein on each gel were then multiplied by that gel's normalization factor to give the normalized densities that are presented in the Results section. This normalization process corrects for variations in image digitization, staining, and initial sample protein content. With use of this method, the quantity of each protein within an individual's CSF varied by less than 5% between independent electrophoretic runs.

Polypeptide identification: Polypeptides were identified by comparison with the published CSF map (1), by comparison with the measured pH (in urea) of an IEF gel, and by co-electrophoresis of the reference proteins of known molecular mass. Immunoglobulin light chains were identified by Western blotting and staining with monoclonal antibodies (6).

Data analysis: Normalized data for normal volunteers were compared separately with data on PD and MS patients, and further statistical treatment was done by using the Statistical Analysis System (SAS Institute Inc., Cary, NC) and an IBM 370/3081 computer. We initially identified significant changes as part of a larger study, using the analysis-of-variance program (ANOVA; then, individual significance was evaluated by use of the two-tailed Student's t-test. Because of the large number of variables quantitated (68 and 85 in PD and MS, respectively), there is an increased possibility that some of the significantly altered proteins appear changed by chance. The best method to determine truly significantly altered proteins will be a prospective study.

Results

The mean concentration of total protein was 0.49, 0.95, and 0.54 g/L for CSF from the PD, MS, and normal groups, respectively.

No qualitative abnormalities in the protein pattern were found in CSF from the normal volunteers (confirming their normality in this respect). However, a newly observed protein, designated protein 129, was observed in 15 of the 20 PD patients (Figure 1). Its M, is 25 000; its pI the same as albumin, and it does not stain with light-chain antibodies after Western blotting to nitrocellulose. The qualitative changes in MS involved the appearance, in 21 of the 22 patients, of many new immunoglobulin light-chain proteins. These proteins were identified as lambda and kappa chains (mainly kappa) by their position on the 2DE gels. This identification was confirmed by detection with monoclonal antibodies after Western blotting to nitrocellulose. These changes, illustrated in Figure 1, were found in CSF from two patients in whom oligoclonal bands were detected by IEF and silver staining (22).

Quantification of the 68 proteins in CSF from PD and normal volunteers showed 20 significant protein changes. The most significant are listed in Table 1 and the relevant proteins are indicated numerically in Figure 1.
The 20 proteins that are significantly altered quantitatively between PD and normal volunteers are numbered on the main gel, as well as the MS proteins referred to in Table 1. The smaller rectangle outlined on the full picture is the location of the new protein (no. 129) in PD, which is illustrated on the right from the same sera of a PD gel. The larger rectangle outlined on the full picture is the location of the new immunoglobulin light chain proteins in MS, which are illustrated below in the same area of an MS gel.

Table 1. The Most Significantly Changed CSF Proteins in PD and MS as Compared with Normal Volunteers

<table>
<thead>
<tr>
<th>Protein Identification no.</th>
<th>Ratio of mean density in normal group to mean density in disease group</th>
<th>p value</th>
</tr>
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<tr>
<td>Parkinson's disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>0.557</td>
<td>.0001</td>
</tr>
<tr>
<td>91</td>
<td>2.88</td>
<td>.0001</td>
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<tr>
<td>2</td>
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<td>.0004</td>
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<tr>
<td>3 apo-A, lipoprotein</td>
<td>1.37</td>
<td>.0006</td>
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<tr>
<td>112</td>
<td>3.679</td>
<td>.0008</td>
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</tr>
<tr>
<td>11</td>
<td>0.697</td>
<td>&lt;.005</td>
</tr>
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<td>37 orosomucoid</td>
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<td>&lt;.005</td>
</tr>
<tr>
<td>48</td>
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<tr>
<td>110</td>
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<td>&lt;.005</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td></td>
<td></td>
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<tr>
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</tr>
<tr>
<td>22</td>
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<tr>
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<td>92</td>
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<td>102 C3-activator</td>
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<tr>
<td>108</td>
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Discussion

The two diseases we studied exemplify the clinical benefits, immediate or potential, of applying high-resolution two-dimensional protein electrophoresis and ultra-sensitive silver staining to the study of neurological diseases. The changes observed here presumably reflect molecular rearrangements in PD and MS that escaped observation with previous methodologies, despite their florid neurological signs and symptoms. In the Lesch–Nyhan syndrome, a disease known to be caused by a single gene defect, blood cells studied by the same methods showed several differences from normal in the gel pattern, reflecting the disordered metabolism secondary to the defective hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) (23). The multiple quantitative alterations in the CSF may also represent
specific secondary metabolic events in these diseases.

We do not know why, in PD, 20 proteins are quantitatively altered. Some of these proteins (numbers 5, 11, 64, 91, 92, 102) are altered in the same direction quantitatively in MS, whereas proteins number 22 and 108 were altered in PD in the opposite direction to that in MS. A common mechanism may be responsible for the quantitative alteration of 10 of the proteins in MS (as shown by the high correlation coefficient of the slope in Figure 2). Nine of these proteins are known to be immunoglobulins, so a common immunological mechanism is likely. This may provide a clue to the functional identification of protein 39, the tenth protein in that group, which may be another immunological component.

The large group of CSF from normal volunteers is well matched with the PD patients, and the protein changes in PD appear to be independent of any mild age factor in the slightly older PD population: 15 of the protein changes observed in PD are found in proteins that are not affected by increased age in normal volunteers, five of the proteins that decrease in the older PD population involve proteins that tend to increase with age, and only protein 11 increases slightly with both this PD group and with normal aging. The age matching was also good between the MS patients and the normal volunteers. The greater number of female MS patients, typical in this disease, does not appear to be responsible for the changes: the only sex-related protein altered in MS is protein no. 111, which is increased in normal women, but decreased in the predominantly female MS population.

The appearance of a new protein, protein 129, in 75% of the PD patients is unlikely to be drug related, because these patients were receiving no drugs at the time of lumbar puncture. It is also unlikely to be an antibody, because electrophoretic of the protein region to nitrocellulose followed by immunoperoxidase staining with anti-kappa or anti-lambda or with antibodies to heavy chain failed to stain protein 129, but did reveal equal amounts of the new immunoglobulin light-chain proteins in MS samples. However, protein 129 does lie in the region of immunoglobulin light chains, and it deserves further investigation. The immunoglobulin light-chain proteins that appear in nearly all MS patients clearly are new immunoglobulin proteins, because they lie in a position on the normal 2DE gels that differs from that of the normal light chains. None of these new immunoglobulin proteins was detected in any of the normal volunteers, even when we electrophoresed 10 times the amount of CSF. The separation on one-dimensional IEF never allowed this observation of new immunoglobulin species in MS to be made as clearly as the 2DE technique does.

Both the qualitative and quantitative changes noted here are of interest in establishing measurable biochemical alterations in PD and MS. Further study of these changes should help both in the diagnosis and in the eventual understanding of the molecular basis for the pathophysiology of PD and MS. Application of this sensitive methodology to other neurological diseases probably will be informative.

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References


