Two-Dimensional Gel Electrophoresis of Cerebrospinal Fluid Proteins

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Two-dimensional electrophoresis, with isoelectric focusing in the first dimension and sodium dodecyl sulfate/polyacrylamide gel electrophoresis in the second, has been adapted for the high-resolution analysis of cerebrospinal fluid proteins. Proteins were detected with a new, highly sensitive silver stain that made visible more than 300 polypeptides from 60 μL of spinal fluid, in highly reproducible patterns. We have mapped these patterns, noting differences between the proteins observed in spinal fluid and plasma, and have prepared a partial map of cerebrospinal fluid proteins.

Additional Keyphrases: peptides - "mapping" of CSF proteins - comparison with plasma

A technique allowing simultaneous observation of many different proteins would be of obvious utility in searching for diagnostic markers in disease and physiologic correlates of behavior. Already, significant advances have been made by using isoelectric focusing (IEF),⁵ either alone or in conjunction with crossed immunoelectrophoresis. Forty distinct bands were seen after IEF and crossed immunoelectrophoresis of cerebrospinal fluid (CSF) (1), and 22 CSF protein bands were demonstrated after polyacrylamide gel electrophoresis (PAGE) (2). These protein patterns have been found to be of diagnostic interest. Delmotte and Gousette (3) detected an abnormal alkaline gamma-globulin region in 91% of 262 patients with multiple sclerosis. Kjellin and Stibler (4) found CSF protein abnormalities in all patients with spinal muscular atrophy and in 11 of 13 patients with muscular dystrophy; they reported abnormalities mainly in the alkaline and highly alkaline pH fractions in amyotrophic lateral sclerosis and in myotonic dystrophy and abnormal acid pH fractions in limb girdle and fascioscapulohumeral muscular dystrophy (4). Vitamin B₁₂ deficiency and infection were both accompanied by increases in alkaline pH fractions (5).

In 1975 O'Farrell (6) detailed the use of two-dimensional gel electrophoresis, a technique with the capacity to resolve complex mixtures of thousands of proteins. Samples are subjected to IEF, then PAGE, to produce a gel pattern of proteins. The position of the proteins is determined by their isoelectric point (pI) and relative molecular mass (Mr). The stained density of each polypeptide on the gel is a function of its concentration.

A highly sensitive stain is required to realize the potential improvement this electrophoretic method offers in the study of CSF because: (a) radioisotope labeling of CSF proteins in animals is too expensive and in humans too hazardous to be practicable; (b) CSF must otherwise be obtained in prohibitively large amounts; and (c) gel patterns become locally distorted when more than 0.1 μg of any protein is present in a single spot. Therefore, a stain sensitive to proteins that are present in only trace amounts is necessary to demonstrate the minor spots (0.1% or less of total gel protein). Trace proteins are often the major species present on a gel; in Escherichia coli, 80% of the proteins seen in cell lysate gels with autoradiography are trace proteins (6).

Elsewhere, a silver stain for proteins that is more than 100 times as sensitive as Coomassie Brilliant Blue and is especially useful in the analysis of body fluids has been described (7). We now more completely describe the application of this stain in the electrophoretic analysis of CSF, and demonstrate some differences and homologies between CSF protein patterns and plasma protein patterns. We have made preliminary identification of 26 CSF proteins.

Materials and Methods

Protein Standards

Orosomucoid, fibrinogen, immunoglobulin G, transferrin, and ceruloplasmin were purchased from Sigma Chemical Co., St. Louis, MO 63178. High- and low-density lipoproteins were generous gifts of Dr. Brian Brewer. Actin was prepared by Dr. Sidney Shifrin from human macrophages and lymphocytes according to the method of Lazarides and Lindberg (8).

Purified α₁-antitrypsin, transferrin, ceruloplasmin, prealbumin, and antithrombin III were prepared by immunoprecipitation with specific antibodies. We added 50 μL of serum or concentrated CSF to 5 μL of commercially prepared antiserum purchased from Miles Biochemicals, Elkhart, IN 46515, or Calbiochem-Behring Corp., La Jolla, CA 92037, and incubated for 12 h at 4 °C. After samples were centrifuged at 12 600 × g for 2 min, we added 5 μL of rabbit anti-goat IgG serum (Miles) and incubated for an additional 12 h at 4 °C. After being centrifuged (2 min, 12 600 × g) the immunoprecipitates were washed twice with ice-cold phosphate-buffered physiological saline (20 mmol/L, pH 7.0) before the standard sample preparation described below.

Sample Preparation

Of the first 10 mL of spinal fluid withdrawn (with informed consent) from normal controls or from subjects with neurologic disease, we used 1 to 2 mL of these experiments. Phenylmethylsulfonylfluoride (Sigma) was added to a final concentration of 1 mmol/L to inactivate serine proteases. Samples were dialyzed overnight at 4 °C against 1 to 2 L of a 100 g/L solution of polyethylene glycol (Fisher Scientific, Pittsburgh, PA 15219), to decrease the salt concentration and increase the protein concentration fourfold. These samples were then stored at −70 °C. Plasma samples were obtained by centrifuging heparinized venous blood 5 min at 12 000 × g at room temperature in Eppendorf tubes. The plasma was then transferred to fresh tubes, phenylmethylsulfonylfluoride

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⁵ Nonstandard abbreviations used: IEF, isoelectric focusing; CSF, cerebrospinal fluid; and PAGE, polyacrylamide gel electrophoresis.

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Two-Dimensional Electrophoresis

IEF and PAGE were performed as described by Merril et al. (7), with minor modifications. Fifteen milliliters of a solution containing, per liter, 20 g of sodium lauryl sulfate (specially pure; Gallard-Schlesinger, Carle Place, NY 11514), 50 mL of mercaptoethanol (Bio-Rad, Richmond, CA 94804), 200 mL of glycerol (Baker and Anderson, Morristown, NJ 07960), 20 mL of 3/10 Biolyte (Bethesda Research, Lab., Rockville, MD 20850) was added to 15 \( \mu \)L of fourfold concentrated CSF or 0.5 \( \mu \)L of plasma, and the mixture was heated for 5 min at 95 °C and cooled to room temperature. After cooling, urea was added to make a final concentration of 9.2 mol/L.

The usual ampholine mixture used was 16 mL of 5/7 and 4 mL of 3/10 ampholines (Bio-Rad) per liter. Two milliliters of 3/10 ampholines per liter was used when effective resolution of some basic proteins, including the IgG alkaline chains, was required.

Staining

The silver stain was performed as described by Merril et al. (7) and Switzer et al. (9). This stain is based on the specific reduction of silver to its metallic form in the presence of polypeptides. Gels are incubated in a solution containing methanol and acetic acid and pretreated with paraformaldehyde before adding silver nitrate, copper nitrate, and silver diammine. The silver is then selectively reduced in the presence of ethanol, formaldehyde, and citric acid.

Periodic acid–Schiff staining for glycoproteins was done according to the method of Zacharius et al. (10) except that gels were fixed in a solution containing 500 mL of methanol and 120 mL of acetic acid per liter. During the step involving 10 mL/L periodic acid, gels that had previously been silver stained were destained by this oxidant. Destained gels were then stained for glycoproteins.

pH Gradient Measurement

After isoelectric focusing, gels were removed from their glass tubes, blotted dry, and sliced with a hand-built slicer made of razor blades spaced at 1.1-mm intervals. These gel slices were then placed in 16-mm diameter microwells (Linbro

was added (1 mmol/L final concentration) and samples were stored at −70 °C.
Fig. 4. Composite normal human CSF pattern, qualitatively representing the proteins that are generally visible in spinal fluid after silver staining. Because protein concentrations vary over a wide range, many of these proteins can be seen only faintly. Gels must be intensely stained to make some of these proteins visible, whereas others are obscured by such staining.

Fig. 5. A difference pattern between CSF proteins and plasma proteins: groups A, B, C, D, G, and J are found primarily in CSF; groups E, F, H, and I are found primarily in plasma.

Scientific, Hamden, CT 06517) in groups of three (15 mg total weight). After adding 100 μL of degassed 20 mmol/L KCl, we read the pH at room temperature with a surface electrode (Corning Glass Works, Corning, NY 14830) and a Century SS pH meter (Beckman Instruments, Fullerton, CA 08854), after several minutes of equilibration.
Gel Analysis

Gels were sealed in water-filled transparent plastic sleeves (20th Century Plastics, Los Angeles, CA 90016). A stereoscope (type ST-4 Wild; Buntion Instrument Co., Rockville, MD 20850) equipped with 3X and 8X oculars was used to superimpose regions of different gels. Intergel differences were enhanced by alternately illuminating each gel. The light source was a T12 Aristo grid lamp (Port Washington North, Long Island, NY 11050). Photographs were made on type 665 Positive/Negative Polaroid film with a Mamiya RB67 camera equipped with a 1:45, 140 macro lens and a Polaroid back. Data analysis was also performed with a computer program that is under development in collaboration with Drs. Lewis Lipkin and Peter Lemkin, who are utilizing a DEC Systems 20/20 computer (11).

Polypeptides were tentatively identified by: (a) running standard proteins that co-electrophoresed with unknown proteins; (b) comparing polypeptide positions with positions ascertained for serum by Anderson et al. (12); and (c) corroborating some identifications with the glycoprotein stain. Color produced by the silver stain is nonuniform, which aided some assignments, as will be described later.

Results

The pH gradient of the first-dimension isoelectric focusing gels (Figure 1) was linear in the range 5.3 to 7.0. The effective M1, range in the second dimension 100 mL/L PAGE gels (Figure 2) was approximately 13 000 to 200 000. These gradients are highly reproducible.

A composite map of CSF proteins (Figure 3), constructed by analyzing electrophoretic patterns of the same CSF sample, electrophoresed on multiple occasions with variations in the

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**Fig. 6.** Enlargements of specific areas of CSF and plasma gels

Ac, actin; Alb, albumin; α1 AT, α1-antitrypsin; AT III, antithrombin III; C III A, C III activator; α F, α-fibrinogen; β F, β-fibrinogen; γ F, γ-fibrinogen; GcG, Gc globulin; Hapt, haptoglobin; IgG, IgG heavy chain; Tr, transferrin. All gels are positioned with the acid side to the left and higher M1 upwards. I. Protein clusters found primarily in CSF (A, B, C, D, and E); γ-fibrinogen is present in CSF, but is fainter than in plasma. II and III are CSF and plasma patterns, respectively, compared for the same subregion. γ-Fibrinogen is present in much lower concentration in the CSF gel than in the plasma gel, but two clusters of polypeptides (D and G) are present in much higher concentration in CSF. IV and V are the CSF and plasma patterns, respectively, of a more basic gel region (for orientation, note the position of albumin); three CSF polypeptides (J) are present in only trace amounts in plasma.
Fig. 7. CSF proteins identified by three different methods: (a) co-electrophoresis of purified proteins; (b) glycoprotein stain; and (c) position analysis, with the Anderson map of serum (12) as a reference.

The proteins identified, listed with the superscript letters corresponding to the method(s) of identification, are: prealbumin, a-c globulin, albumin, C3 activator, transferrin, and C3. The proteins identified with a chain, demonstrate more than 300 individual polypeptides in a single CSF sample. Treatment of the sample with protease before electrophoresis eliminates all components detectable under the conditions described. These polypeptides were consistently present in CSF samples from 20 unrelated individuals. Many of these proteins are members of clusters whose components have minor charge differences secondary to posttranslational modifications, such as sialization, deamidation, methylation, or phosphorylation, or minor differences in Mr. The two-dimensional system clearly separates many proteins that would have overlapped in a one-dimensional system, whether the technique used in that dimension was PAGE or IEF.

To compare CSF and plasma (Figure 4), we used an initial volume of plasma that was 1/120 that of CSF. The patterns are generally similar, except that six clusters of polypeptides were more prominent in CSF. These are shown diagrammatically in Figure 5, with photographic enlargements in Figure 6. The most acidic of these, Group A, is composed of ~15 acidic polypeptides (Mr ~30 000) that stain yellow-brown.

Group B CSF proteins consists of at least five polypeptides that stain blue and have an Mr of ~36 000. Group C CSF proteins include at least 15 polypeptides (Mr ~38 000) that may stain reddish or yellow. A number of brown-staining proteins (Mr ~47 000) make up group D CSF proteins, which are also present in lesser relative amounts in plasma and in serum. Groups E, F, H, and I have been tentatively identified as fibrinogen and fibrinogen γ, β, and α-chains, respectively, and are present in much lower amounts in CSF than in plasma; they are greatly diminished after clotting. The polypeptides in group G CSF proteins are brown-staining and streaked, probably because of microheterogeneity. Group J, present in greater concentration in CSF than in plasma, have an Mr slightly less and a pI somewhat more basic than that of transferrin.

Twenty-six CSF proteins have been tentatively identified (Figure 7). Of these proteins only two, high- and low-density lipoproteins (not shown), stained blue, suggesting that this may be a characteristic of lipoproteins. Other appeared yellow, brown, or red. Glycoproteins were identified with the periodic acid–Schiff base stain.

Discussion

Utilizing two-dimensional electrophoresis and the silver stain, we have shown that more than 300 polypeptides can be made visible in CSF. In examining the CSF polypeptide patterns of 20 individuals, we prepared two to three electropherograms for each individual, but observed no qualitative differences in protein patterns among the individuals in this study. Some of the polypeptides present in trace amounts required more intensive staining to become visible; however, once the electropherograms were adequately stained, no protein deletions or aberrations in Mr or charge were observed in these individuals. By analyzing electropherograms of purified proteins and gels containing identified proteins, such as are found in human serum [in which Anderson et al. (12)] have identified many of the proteins], we have been able to tentatively identify 26 of the proteins in the CSF electrophorograms patterns. These "identified" CSF polypeptides can now be used as internal markers for isoelectric and Mr gradients in clinical CSF gels. No variations or deletions were observed for any of the polypeptides in the CSF samples in these studies. However, even with electrophoretic techniques with less resolution and sensitivity, some protein changes have been
reported in certain disease states (3, 4, 13). With the order of magnitude increase in sensitivity and resolution offered by the current technology, a survey of diseases of the central nervous system should reveal new protein variations.

Previous findings of CSF protein abnormalities in disease can now be re-analyzed with a more accurate and discriminating tool. Appropriate applications would include: (a) diseases in which a viral etiology is suspected but unproven; (b) diseases in which the presence of a "marker" protein in CSF could help the clinician make a diagnosis or follow a response to therapy; (c) diseases that are genetically transmitted or otherwise well defined but for which the cause is unknown; and (d) diseases that are well defined and believed to cause changes in CSF proteins.

Additional studies with this CSF technology potentially include the identification of proteins by co-electrophoresis with known purified proteins, by specific antibody precipitation, by utilization of known protein variants in disease, or by physiologic and pharmacologic manipulations that alter proteins in known ways. Specific tissues and, occasionally, cell types may be electrophoresed to identify possible sources of CSF proteins. It may be possible to monitor certain aspects of nervous system development through the appearance of trace proteins in CSF, to explore the efficacy or mode of activity of a drug by observing effects on selected enzymes in CSF, and more profitably to search for unknown mediators of behavior that may circulate in CSF. Proteins derived mainly from brain and spinal cord are already known to appear in CSF. One such protein, acetylcholinesterase, has been demonstrated to be present in CSF, and evidence indicates that it has a central origin (14–20). CSF acetylcholinesterase has been shown to increase after treatment with chlorpomazine (21, 22), and has been detected in CSF with PAGE (23). A second enzyme, dopamine-β-hydroxylase (24), has also been measured in CSF; its activity has been found to vary with central noradrenergic activity.

A major difficulty in the clinical application of this technique is the analysis of the large amount of information contained in each electropherogram. Two-dimensional CSF electropherograms contain both positional and quantitative polypeptide information. Detection and processing of this information is difficult and time consuming. Maps of the usual CSF patterns, together with an optical device for gel comparison can, in our experience, suffice for the detection of many variations in protein pattern; however, the problems of accurate positional and quantitative analysis must eventually be addressed with computerized image processing and data reduction. The tactics of this approach are being worked out by several groups (11, 25, 26).

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References