Isoelectric Focusing of Gamma Globulins in Cerebrospinal Fluid from Patients with Multiple Sclerosis

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The technique of isoelectric focusing has been adapted for rapid clinical analysis for globulins in cerebrospinal fluid with use of commercially prepared horizontal-slab acrylamide gels. The globulin fraction is concentrated by ammonium sulfate precipitation, which allows more of the relevant protein to be applied, use of a wider range of total protein concentrations, and higher resolution than is true for previously described methods. Critical variables include a constant concentration and volume of IgG, a constant low temperature of the acrylamide gel, and sensitive staining with Coomassie Brilliant Blue G-250. The apparatus used is adaptable for other electrophoretic procedures in the clinical laboratory, and the use of commercially prepared gel slabs is more convenient, more reproducible, and requires less time than other methods.

Additional Keyphrases: diagnostic aid • electrophoresis • proteins

Isoelectric focusing (IF) represents a major advance in the high-resolution separation of proteins. In principle, proteins are separated according to their isoelectric point in pH gradients that are formed by electrolysis of amphoteric buffer substances known as "carrier ampholytes." This technique can demonstrate heterogeneity of protein preparations which appear to be homogeneous by other gel-electrophoretic methods.

The cerebrospinal fluid (CSF) of patients with multiple sclerosis was recently reported by Delmotte to show an abnormal homogeneity of the gamma-globulin region by isoelectric focusing in 91% of 262 patients (1). Only 7% of 272 CSF specimens from patients with other neurological diseases showed an abnormal pattern. (The traditional method of electrophoresis on agar-gel showed pathological results in only 65% of multiple sclerosis patients.) Similar but less explicit data on a smaller number of patients were reported independently by Kjellin and Siden (2).

When we attempted to reproduce these findings, we found that in the methodology described by Delmotte (3) or Kjellin and Vesterberg (4) a constant amount of IgG was not used for each sample. The importance of applying a constant amount of IgG when attempting to compare qualitative differences in protein pattern is almost self-evident, and has also been emphasized by Link (5), when agar-gel electrophoresis is used. Furthermore, we were unable to obtain a high percentage of abnormal alkaline bands in the cerebrospinal fluid of multiple sclerosis patients by published methods (3, 4), because the pattern smeared when CSF samples with high total protein were focused. We therefore attempted to purify the globulin fraction to eliminate other proteins (e.g., albumin). This method proved successful and is described here. In order to make this technique simple and reproducible for use on a day-to-day basis in a clinical laboratory, we adapted our technique so that a commercially prepared acrylamide gel with ampholytes in the pH 3.5–9.5 range can be used.

Materials and Methods

Apparatus

An LKB Multiphor electrophoresis unit with polyacrylamide gel electrofocusing kit and electrophoresis kit (LKB Instruments, Inc., Rockville, Md. 20852), and a No. 3-1155 Power Supply (Buchler Instruments, Fort Lee, N.J. 07024) were used for both the electroimmunodiffusion and isoelectric focusing procedures. The protein-concentrating apparatus and collodion bags were purchased from Schleicher and Schuell, Inc., Keene, N.H. 03431.

Reagents

We used ammonium sulfate, 3.9 mol/liter; ammonium sulfate, 2.0 mol/liter; Coomassie Blue G-250, 400 mg/liter of perchloric acid (35 g/liter) solution; aqueous hemoglobin (Sigma Chemical Co., St. Louis, Mo. 63178, 7 g/liter); AEID electroimmunodiffusion kits (Antibodies, Inc., Davis, Calif. 95616); "PAGplate" kits (LKB Instruments); perchloric acid, 35 g/liter; acetic acid, (50 ml/liter); "Preserving Solution" [200 ml of glycerol, 250

1 We used several lots of this dye with no problems.
ml of methanol, 70 ml of glacial acetic acid, all diluted to 1 liter with water; sodium chloride, 50 mmol/liter; sodium hydroxide, 1 mol/liter; and phosphoric acid 1 mol/liter.

Procedure

Freshly obtained cerebrospinal fluid is centrifuged and stored at 4 °C (if the assay is to be performed within 48 h) or frozen at −20 °C (for up to 4 months). The IgG concentration is first determined by electroimmunodiffusion of 2 μl of unconcentrated CSF as described in the AEID instructions. The volume of CSF containing 100 μg of IgG (or 5.0 ml, whichever is smaller) is determined and concentrated to 0.1 ml in the collodion bag apparatus. One hundred microliters (0.1 ml) of cold (4 °C) 3.9 mol/liter ammonium sulfate is added and mixed immediately and thoroughly. The mixture is then incubated for 30 min at 4 °C, and centrifuged. The supernatant fluid is discarded and the precipitate is washed once with 2.0 mol/liter ammonium sulfate and centrifuged. This precipitate is dissolved in 2 ml of 50 mmol/liter sodium chloride and the solution is then concentrated to 10 μl vs. de-ionized water in the collodion bag apparatus.

The LKB PAGplate gel may be cut to the appropriate size according to the number of samples. A thin layer of mineral oil is applied to the plastic backing of the gel and the template provided with the kit, to ensure good contact with the cooling plate. The template is placed on the cooling plate and the gel is placed on the template. The electrode strips provided with the PAGplate kit are moistened with 1 mol/liter sodium hydroxide (for the cathode) or 1 mol/liter phosphoric acid (for the anode). Excessive dampness of the electrode strips can cause distortion of the protein pattern. The sample may be applied by one of several methods, but we have found that small pieces of Whitman 3MM chromatography paper are quite satisfactory as sample applicators; they should be cut into 5 × 10 mm pieces and laid on the cathode side of the gel, guided by the template provided in the PAGplate kit. Finally, 10 μl of CSF sample is pipetted onto the applicator. A 5-μl sample of the hemoglobin solution is included with each run, as a marker.

The Multiphor cooling plate is maintained at 10 °C throughout the procedure by circulating cold tap water or a circulating water bath. The gel is focused using a step-wise increase in voltage (in 200-V increments every 10 min) to 1000 V. The voltage is then increased to 1100 V for an additional 40 min. The sample-applicator papers are removed 30 min after the start of the focusing procedure. Some time may be saved if the gel is prefocused for 30 min (while preparing the sample) before sample application. The prefocusing is performed in 200-V increments every 10 min. After the gel is applied, the increments are continued to 1000 V as described above and the focusing continued for an additional 40 min at 1100 V. The focusing procedure then takes only 60 min after sample application. The focused gel is placed directly in the Coomassie Blue G-250 staining solution for 4 h at 37 °C. It is then rinsed by placing it in the perchloric acid solution for 15 min and intensified by placing it in the 50 ml/liter acetic acid solution for 45 min. It is then placed in “Preserving Solution” for 30 min and then subsequently dried in the open room-temperature air. The results may be read by inspection at this stage and the stained gel may be kept as a permanent record if it is covered with a plastic sheet.

Results and Discussion

The horizontal polyacrylamide gel offers several advantages over the gel rod procedure for isoelectric focusing (6): (a) handling of many gel rods is avoided; (b) all samples are focused under identical conditions, allowing side-by-side comparison; (c) the flat gel bed is easier to handle for pH measurements, staining, and destaining; (d) the flat gel bed is more easily cooled, allowing higher voltages and consequently shorter runs; and finally, (e) the flat gels may be preserved and stored in a notebook for a permanent record.
The main difficulty with using unfractionated CSF was that the pattern smeared when more than 200 μg of albumin was applied.

Because the globulins were the fraction of interest, it is desirable to partly purify this group of proteins. The CSF had to be concentrated to an IgG concentration of 1 g/liter before 3.9 mol/liter ammonium sulfate would precipitate the gamma-globulins. Although the pH gradient of the gel may be measured with surface electrodes, we have found the hemoglobin reference is sufficient for routine qualitative evaluation. Two or more bands cathodal to the most anodal prominent minor hemoglobin band (Figure 1) are considered to be pathological “alkaline” bands.

The Coomassie Blue G-250 was used for staining, and the bands were intensified with acetic acid treatment as has been previously described (7, 8). This staining method is more sensitive than is the case for Coomassie Blue R-250 or Amido Black, and there is less background staining of amphoniles (7, 8).

The use of commercially prepared acrylamide gels allows for a more reproducible and economical (fewer wasted gels and CSF samples) procedure. Furthermore, the use of thin gels allows better cooling and consequently a shortened focusing time because higher voltages can be used. The equipment used in our procedure is adaptable to other electrophoretic or immunoelectrophoretic needs of the clinical laboratory. The use of partly purified IgG resulted in a more distinct pattern and a more sensitive method because there was no smearing by other proteins.

Using this method, we have examined the CSF of 25 patients with clinically defined multiple sclerosis and 20 patients with other neurological diseases (amyotrophic lateral sclerosis, pseudo tumor cerebri, cerebrovascular accident, and senile dementia). Twenty-three of the multiple sclerosis patients (92%) and none of the controls had abnormal “alkaline” bands. This confirms the work of Delmotte and Gonsette (1). Our method gave reproducible and dependable results and the material cost was no greater than for agarose-gel electrophoresis, which is not as discriminative. The slight increase in technician time was easily compensated by the increased diagnostic accuracy.

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