Two-Dimensional Gel Electrophoresis of Cerebrospinal Fluid Proteins from Patients with Various Neurological Diseases

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We subjected cerebrospinal fluid (CSF) samples from over 200 patients with various neurological diseases to two-dimensional electrophoresis. The series included non-inflammatory diseases such as epilepsy, amyotrophic lateral sclerosis, and polyneuropathy; and inflammatory diseases such as multiple sclerosis and neuromyelitis. In the resulting electrophoreograms we considered mainly the region of CSF-specific proteins and the area corresponding to the immunoglobulin light chains. The former, at about 35 000–36 000, shows some interesting variations from one CSF to another. Samples from patients with various brain tumors show a specific change. A zone of oligoclonal immunoglobulin light chains appeared in all CSF samples with above-normal gamma-globulin content. These oligoclonal patterns remained constant and characteristic during the course of different diseases for several patients so examined. As expected, differences appeared in the patterns of immunoglobulin light chains from one individual to another, even among a group of patients with the same disease. The extent of the correlation of certain basic patterns with certain diseases cannot yet be determined.

Two-dimensional electrophoresis on polyacrylamide gel (2D-PAGE) according to O'Farrell (1) allows proteins from body fluids to be resolved into several individual species. Proteins in serum (2, 3), urine (4, 5), amniotic fluid (6, 7), and cerebrospinal fluid (CSF) (8–10) have been demonstrated by this high-resolution technique.

Compared with serum, CSF has a very low protein content, usually less than 0.5 g/L. The blood–brain barrier prevents the free diffusion of protein from the blood into CSF. There also is some diffusion of parenchymal proteins of the central nervous system (CNS) into the CSF. Although the concentration is low, most proteins of CSF correspond qualitatively to those of serum. Yet when 2D-PAGE protein patterns for CSF are compared with those for serum and plasma, several protein groups peculiar to CSF can be found (8). Dermer et al. (9, 10) also demonstrated CSF-associated proteins by showing that there were proteins in CSF that were not bound by antibodies to agarose (Sepharose 4B)-coupled whole human serum.

During the last decade neurobiologists have isolated and characterized some of the CSF-associated proteins such as the nervous-tissue-associated S-100 protein (11), the glial fibrillary acidic protein (12) associated with astrocytic glial cells, and the myelin basic protein (13). The composition of proteins in CSF may be significantly altered in certain diseases. Whereas the immunoglobulins in normal CSF originate exclusively from the blood, they can be synthesized intrathecally in inflammatory processes of the CNS. They are mostly characterized by limited heterogeneity and exhibit characteristic band patterns (14) in isoelectric focusing and agarose electrophoresis. These so-called oligoclonal bands can be demonstrated with the help of the isoelectric focusing technique in over 90% of all cases of multiple sclerosis (15).

Very few data are currently available concerning separation of CSF proteins by two-dimensional electrophoresis in inflammatory processes within the central nervous system and other neurological disorders (16, 17). We have used this technique to examine more than 200 CSF samples, chosen from a wide array of neurological conditions. We concentrated our interest on the light-chain region of the patterns. Also, we studied closely the region of non-serum proteins. We report here the results of these analyses.

Materials and Methods

Reagents

Ampholines (pH 3.5–10, pH 5–7) were from LKB, Bromma, Sweden; acrylamide and N,N'-methylene-bisacrylamide were from Bio-Rad, Richmond, CA 94804, and Serva Feinbiochemica, Heidelberg, F.R.G. All other chemicals were commercially available analytical-grade products.

Human Serum, Cerebrospinal Fluid

The CSF was sampled from patients being treated in our Department of Neurology. Five specimens of ventricular fluid were from the Department of Neurosurgery. Lumbar CSF and venous blood, for serum, were obtained concurrently, both by standard procedures. All samples were stored at −20 °C, without preservatives. Before use, CSF samples were concentrated 10- to 20-fold by ultrafiltration (equipment from Sartorius GmbH, Gottingen, F.R.G.). Total protein was measured by the Coomassie Blue Bradford assay (Bio-Rad), as described by Tracy et al. (3). Our normal reference interval for CSF is 200–420 mg per liter of protein. Proteins in serum and CSF were resolved by routine electrophoresis on agarose. We calculated the "IgG index" for CSF for each specimen according to Tibbling et al. (17)—CSF IgG/serum IgG divided by CSF albumin/serum albumin—to demonstrate the presence of any IgG production in CSF. An above-normal value (>0.7 in our laboratory) indicates intrathecal IgG production.

Sample Preparation

Specimens of serum and CSF were prepared for electrophoresis according to the method of Anderson and Anderson (2). We mixed 10 μL of serum with 200 μL of pH 6.5 dissociation buffer (per liter: 30 g of glycerol, 40 g of sodium dodecyl sulfate, 60 g of 2-mercaptoethanol) and heated to 95 °C for 5 min. After cooling, 10 μL of the sample was applied to the isoelectric focusing gel. For analysis of CSF, 20 μL of 10- to 20-fold concentrated CSF was mixed with 10 μL of dissociation buffer and heated to 95 °C for 5 min. After cooling, 20 μL of the sample was applied to the isoelectric focusing gel.

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Electrophoresis

Isolelectric focusing dimension: For first-dimension isoelectric focusing we used the Bio-Rad IEF apparatus (Model 155). The capillary gel columns (14 cm × 0.15 cm i.d.) contained, per liter, 35 g of acrylamide (Bio-Rad), 9 mol of urea, 20 g of NP-40 surfactant, 20 g of 3/4–10 ampholytes, and 5 g of 5–7 ampholytes. The anolyte was 40 mmol/L phosphoric acid, the catholyte was 0.5 mol/L sodium hydroxide. The separation was done at 9000 V h.

SDS-PAGE dimension: For second-dimension (molecular mass) separation we used the Bio-Rad slab gel apparatus (Multiple-Gel Protein Cell, six- or 12-slab gels). The slab gels consisted of a running gel containing 100 g of polyacrylamide per liter. The electrophoretic buffer contained Tris, glycine, and sodium dodecyl sulfate as described by O'Farrell (4). The equilibration buffer used to introduce sodium dodecyl sulfate into and to remove ampholytes from the isoelectric focusing gels was prepared without 2-mercaptoethanol. This buffer eliminated the surface streaks that are commonly seen on silver-stained gels (3) without altering the final spot pattern. After equilibration for exactly 8 min the isoelectric focusing gels were immediately applied to the top surface of the slab gels.

Silver Stain

We compared several silver-staining procedures. That of Oakley et al. (18), which produced the best patterns, was modified in a way that eliminated the nonspecific high-background staining: after the rinse in silver nitrate solution and water, each gel was immersed individually for 30 s in a 200 mg/L solution of sodium thioulate.

For photography we used a Polaroid CU-5 Close-up Land Camera System and Polaroid Land Film Type 665.

Results

A comparison of the patterns of concentrated and unconcentrated CSF revealed no qualitative differences. Therefore, the CSF samples were usually concentrated by ultrafiltration so we could more easily detect important protein groups such as the Ig light chains. We did not use an acrylamide gradient or a stacking gel, but worked with gels of homogeneous pore size (100 g/L polyacrylamide). Addition of a stacking gel did not improve the resolution with our system.

With use of the modified staining method of Oakley et al. (18), several hundred spots of CSF or serum can be demonstrated. Our use of a diluted sodium thioulate solution before the actual development delayed the appearance of the individual spots, but eliminated nonspecific background staining, yielding a clear, homogeneous, yellowish background. This facilitated gel evaluation.

Use of the staining methods described by Wray et al. (19) and Ansorge (20) did not produce patterns as intense as those with the Oakley staining. The techniques of Merrill et al. (21), used mainly for thin-slab gels (30.5 mm), produced only weak staining patterns on thicker gels (1.5 mm). The relatively simple and very sensitive staining method of Adams and Sammons (22) was also compared with that of Oakley (18). As described elsewhere (23), negative staining effects were seen, an effect especially evident in the important region of the immunoglobulin light chains. This made interpretation difficult.

For preliminary evaluation of the protein pattern semiquantitatively, we used visual inspection. Many proteins can be identified by comparison with available maps of two-dimensional patterns for serum (2, 3) and CSF (8). As described by Goldman et al. (8), one notices some CSF-specific protein clusters, i.e., not present in serum (Figure 1). The carbohydrate-deficient "CSF-specific" transferrin (Mr 77 000) exhibited a constant pattern in every CSF. The proteins at Mr 35 000–38 000 comprise the most prominent CSF-specific group. It consists basically of two main parts, a sloping (1–3) and a horizontal group (4–7 in Figure 2). The exact nature of the CSF-specific group, which varies from one CSF to another, is yet to be determined.

Figure 2 shows variations in this CSF-specific group. The patterns of Figure 2a and b are representative of about 90% of the 200 CSF samples we investigated. Of 32 samples from cases of multiple sclerosis 13, or about 41%, showed one or two additional points in the horizontal protein group.

CSF samples from two patients for which beta-globulin values were above normal in the agarose electrophoretogram showed a higher concentration of the "CSF-specific" proteins on the 2D-PAGE patterns (Figure 2a and d). Figure 2c, with a slightly increased beta-globulin fraction, shows the pattern for a patient with a myopathia. Figure 2d, in which the beta-globulin fraction is strongly increased, shows the pattern for a patient with severe unilateral headache.

The investigated ventricular fluids from five different brain tumors show similar patterns in this region. The horizontal protein group (4–7) prevails unequivocally, whereas the sloping group (1–3) is either missing or barely detectable. Figure 2e shows the pattern for a 21-year-old woman with a craniopharyngioma. Similar patterns were found for cases of pinealoma, meningeoma of the wing of the sphenoid, suprasellar tumor, and metastasis to the cerebellum. No agarose electrophoresis was performed because the protein content of ventricular fluid (130–150 mg/L) is too low.

We analyzed not only CSF from brain-tumor patients but also lumbar fluid from five patients with non-Hodgkin's lymphoma who were being treated with methotrexate. In one of these five cases, the CNS was involved. In all cases the horizontal and sloping protein groups were detected on 2D-PAGE patterns (Figure 2f), the horizontal protein group prevailing.
Figure 3 shows a pattern for CSF from a patient with multiple sclerosis. The oligoclonal light-chain pattern seen in the CSF appears to be a result of intrathecal immunoglobulin synthesis, because the corresponding serum shows no oligoclonal pattern. The spots are distributed relatively uniformly across the whole pH range. All of the 32 patients with clinically defined multiple sclerosis showed novel light-chain polypeptides on 2D-PAGE. One such patient, who showed no oligoclonal bands by agarose electrophoresis, did show abnormal immunoglobulin light chains. The number of spots in multiple sclerosis varies from patient to patient, ranging from three to 35. The novel light chains are distributed around the basic fractions of the immunoglobulin light chains. We estimated the protein concentration and calculated the IgG index for every CSF. Of the 32 multiple sclerosis patients, 31 showed an increased gamma globulin and an IgG index >0.7. None of the CSF samples from these multiple sclerosis patients showed a 2D-PAGE pattern that indicated any dysfunction of the blood–brain barrier.

Figure 4 shows the oligoclonal patterns for two additional CNS disorders. The CSF illustrated by the pattern in Figure 4a came from a 22-year-old man with viral myelitis of unknown etiology, which resulted in spastic spinal paralysis. The total protein content was 4.270 g/L, with a calculated IgG concentration of 1.370 g/L. Such an extremely high protein content—which we never found in the CSF of demyelinating diseases—indicates a strongly increased blood–brain barrier permeability. These findings also are consonant with the prealbumin/albumin quotient, which was decreased. We could use unconcentrated CSF in the first dimension because of the high protein concentration.

Dysfunction of the blood–brain barrier caused by various disturbances of the central nervous system changes the pattern characteristically. Transferrin penetrates the barrier even when dysfunction is slight, thereby changing the pattern of CSF-specific transferrin. A more-severe blood–brain barrier dysfunction leads to a decrease in the "CSF-specific" protein group at $M_r$ 35,000–38,000. Alpha- and beta-fibrinogen chains from the blood plasma become easily visible, but the beta-chain of haptoglobin ($M_r$ 43,000) is only sometimes visible (see Discussion). CSF samples from patients with slight dysfunction of the blood–brain barrier show no remarkable change in agarose electrophoreograms. In contrast, samples from patients with a severe breakdown of the barrier show a typically lowered prealbumin/albumin quotient.

A protein group of great importance is the immunoglobulins. They are resolved into heavy and light chains after a two-dimensional run on the slab gel under the dissociating conditions. The light chains are distributed over the entire pH range as six diffuse spots at $M_r$, 22,000–23,000. These six spots were found in each gel for normal CSF, however, with quantitative variations. A different pattern has been found in CSF samples with increased gamma globulin content, from patients with demyelinating and infectious diseases of the central nervous system. Additional immunoglobulin light-chain species appear in this region. The novel immunoglobulins are of restricted heterogeneity and they form an oligoclonal zone (a limited series of well-defined spots). These new polypeptides were identified as immunoglobulin light chains by adsorption to a column of Sepharose with linked Protein A (Protein A from Staphylococcus aureus binds specifically to the Fc part of the heavy chains). We passed CSF from various cases of inflammatory disease (multiple sclerosis, neurolues, myelitis) through a small column and were able to remove the entire oligoclonal pattern in all cases.

![Fig. 2. Six variations of the CSF-specific protein group at molecular weight 35,000–38,000.](image)

A, patient with cerebral insultus; B, patient with multiple sclerosis; C, patient with myopathy and increased $\beta$-globulin in the agarose electrophoreogram; D, patient with severe unilateral headache of unknown etiology and an increased $\beta$-globulin in the agarose electrophoreogram; E, patient with a cranialpneumonia (ventricular fluid); F, patient with non-Hodgkin's lymphoma. The drawing is a composite of forms identified so far. A and B typify about 90% of the 200 CSF samples we studied.

![Fig. 3. The oligoclonal zone of the Ig light-chain region of CSF (A) and the corresponding serum (B) from a 42-year-old man suffering from multiple sclerosis.](image)

Total protein in CSF 610 mg/L; IgG 223 mg/L; Ig index 2.0; prealbumin/albumin quotient normal. Arrow point to clones of Ig light chains (removable with Protein A coupled to Sepharose). Apo A-I lipoprotein is indicated by the parentheses.
The resulting pattern, as expected, shows a high concentration of the haptoglobin chains and a transferrin similar to that of serum. The oligoclonal pattern was not clearly discernible in the corresponding serum in spite of the high blood–brain barrier permeability. The calculated IgG index value was 2. The existing novel light chains on the 2D gel seem to have a higher $M_r$ than those from multiple sclerosis patients. The diffuse basic fractions of the immunoglobulin light chains are not detectable.

Figure 4b shows the oligoclonal zone from a 50-year-old man with brain atrophy that resulted in cerebellar and extrapyramidal disturbance parallel with dementia: difficulties with reading, writing, and speaking. The total protein concentration in CSF was 420 mg/L; the calculated IgG index was 1.9, and the value for the prealbumin/albumin quotient was normal. The gamma-globulin fraction was split into two clear peaks, of equal height, on the agarose-electrophoresis strip. Five discrete spots were distributed across the whole pH range that were not detectable in the corresponding serum. We were unable to find any oligoclonal zones similar to this pattern.

The question arises of how constant the oligoclonal zone of the Ig light chains are during the course of a disease. Figure 5 demonstrates the light-chain proteins in four CSF samples taken during different phases of disease during 10 months from a patient with the diagnosis of viral myelitis. The 2D-PAGE pattern of the first CSF sample in the acute stage shows a definite discrete pattern of spots in the light-chain region. These spots were no longer detectable in the pattern for a CSF sample drawn during clinical remission of the disease. The second sample was collected during remission. The third, and fourth CSF sample, collected during a relapse, exhibit the same spot pattern as depicted with the first CSF sample. The re-emerging spots correspond in charge and size, so these patterns suggest that during a relapse the same oligoclonal immunoglobulins are activated. We also observed a constant pattern for the Ig light chains during the course of a disease from two multiple sclerosis patients and one patient with lymphocytic meningoradiculitis.

**Discussion**

Our results for CSF samples show that in many diseases the agarose electrophoretogram is normal (e.g., no oligoclonal bands in the gamma globulin fraction, no serum-
similar patterns caused by blood–brain barrier permeability. This group of diseases includes Parkinson's disease; epilepsy; discus hernia; cerebrovascular diseases such as insulin and transient ischemic attack; degenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's disease; and also inflammation of the peripheral nervous system (e.g., polyneuropathies) and tumors of the central nervous system. For comparison we used CSF from patients whose complaints were insignificant at the time (e.g., migraine, sciatia).

In a preliminary evaluation of the patterns, we noticed no obvious changes that could be considered typical for a specific disease. The very slight differences that sometimes appeared could not be interpreted as disease-specific, because in such cases only one sample per disease was available to us. In CSF samples from five cases with Parkinson's disease we were unable to detect the Mr 25 000 protein described by Harrington et al. (24).

We believe that the change in the pattern for CSF-specific transferrin is a good indicator of blood–brain barrier dysfunction, in contrast to haptoglobin, which is not. For example, the concentration of haptoglobin can be greatly decreased because of hemolysis. In another example, with slight disturbance of the barrier only Hp 1-1 (Mr 85 000) was able to penetrate, whereas the genetic forms Hp 2-1 (Mr 200 000) and Hp 2-2 (Mr 400 000) are more strongly retained in the blood. The presence in CSF of high-Mr, arginine-rich macroglobulin derived from serum is not well illustrated with our 2D-PAGE system because we used a gel of homogeneous pore size (100 g/L polyacrylamide) instead of a gradient of acrylamide.

An interesting protein group is found at Mr 35 000–38 000 that is characteristic for CSF (Figure 2). Only a single spot, identified as the arginine-rich lipoprotein (a low-density protein from the E class (2)) appears at the same place in patterns for serum. If this CSF-specific group represents a multiple form of lipoprotein E, then it should be possible to bind it completely with immobilized antiserum to human Apo E. In the ventricular fluid of five tumor patients the horizontal fractions were present in high concentrations. It is difficult to ascertain how specific this group is for ventricular fluid or for tumors because normal human ventricular fluid is unavailable. We can only compare our pattern with a recently published pattern for ventricular fluid from a rhesus monkey (26), which lacked this protein group. This group was also detected in all four lumbar fluids of patients with non-Hodgkin's lymphoma. Besides the sloping group, we saw a clear increase in the horizontal protein group in lumbar fluid.

Besides the protein group at Mr 35 000–38 000, we need to determine in the future how many other CSF-associated proteins can be found in a normal CSF with the help of 2D-PAGE, perhaps in combination with other tools. The question also arises of whether there are additional CSF-specific proteins in certain neurological diseases.

Fifty-two CSF samples out of 54 with increased gammaglobulin content showed an oligoclonal zone in the region of the Ig chains. We found a strong correlation between oligoclonal zones and increased IgG index, suggesting that there is an ongoing humoral immune response, particularly in multiple sclerosis, but also in other patients with central nervous system infections. All of 32 patients with definite diagnosis of multiple sclerosis exhibited a pattern similar to that recently described by Tracy et al. (16). Values for total CSF protein in all patients were normal or only slightly above normal. Several authors have reported that the pattern of oligoclonal IgG bands in agarose and isoelectric focusing polyacrylamide gels remain constant during the course of a disease (26, 27). Using the more sensitive method of 2D-PAGE, we could confirm these findings with different diseases producing immunoglobulin clones. We observed, for example, constant patterns for the light chains in CSF from two patients with multiple sclerosis during three months and one year, respectively, with no new spots. An impressive example is a case of a lymphocytic meningoradiculitis with about 45 immunoglobulin light chains newly appearing on the 2D-PAGE pattern. Six months later, we found exactly the same pattern for a sample obtained at the time of a new lumbar puncture. Independent of intensity and number of bands in the gamma-globulin fraction on agarose gels, the number of spots in the light chain region varies from only a few to as many as 50. Link and Koestulas (15) found oligoclonal bands in the CSF of 15% of 906 patients with neurological diseases other than multiple sclerosis (including neurological patients with basically non-inflammatory diseases). We found oligoclonal zones in inflammations or infectious disorders of the CNS such as neurelues, herpes zoster, two cases of Guillain–Barré syndrome, and spinal cord compression.

To what extent do certain specific oligoclonal patterns correlate with a certain disease despite individual differences? In a series of inflammatory diseases with newly appearing immunoglobulin light chains the diffuse basic fraction seemed to be absent, or else only the fractions with more basic isoelectric points were present (e.g., viral myelitis, lymphocytic meningoradiculitis, herpes zoster). Other patterns were found in neurelues or multiple sclerosis, where the diffuse basic fraction appears intense to very intense. At this time we are investigating in detail the light-chains region for a large number of inflammatory diseases.

At present, we doubt that 2D-PAGE and isoelectric focusing are better routine methods for use in detecting oligoclonal immunoglobulins than agarose gel electrophoresis. But, in contrast to electrophoresis on agarose gel and, particularly, isoelectric focusing, 2D-PAGE separates immunoglobulins into heavy and light chains and, in the future, will enable better simultaneous monitoring of all the individual antibody species in CSF samples.

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References
8. Goldman D, Merrill CR, Ebert MH. Two-dimensional gel elec-


