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REVIEW

CEREBROSPINAL FLUID PROTEIN ANALYSIS IN DISEASES OF THE NERVOUS SYSTEM

MICHAEL G. HARRINGTON* and CARL R. MERRIL

Biochemical Genetics Section, Clinical Neurogenetics Branch, National Institute of Mental Health, Building 10, Room 4N320, 9000 Rockville Pike, Bethesda, MD 20892 (U.S.A.)

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CONTENTS

LIST OF ABBREVIATIONS

CJD	Creutzfeldt–Jakob disease
CSF	Cerebrospinal fluid
1-DE	One-dimensional electrophoresis
2-DE	Two-dimensional electrophoresis
GBS	Guillain Barré syndrome
IEF	Isoelectric focusing
Ig	Immunoglobulin
LP	Lumbar puncture
MS	Multiple sclerosis

NS	Nervous system
OBs	Oligoclonal bands
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
SSPE	Subacute sclerosing panencephalitis

1. INTRODUCTION

The study of cerebrospinal fluid (CSF) proteins in patients with diseases of the nervous system (NS) has been of clinical value since the introduction of diagnostic lumbar puncture by Quincke in the 19th century [1]. Research and clinical applications of many current CSF protein studies are comprehensively reviewed in three modern texts [2–4]. This review will focus on electrophoretic methods employed in CSF protein analysis.

In clinical CSF studies, measurement of the total protein value (normal range 0.1–0.5 g/l) has proven to be of diagnostic value. Low levels may arise on occasion, but elevation of total protein is the usual occurrence in many NS diseases, especially infections, inflammations, tumors and disease processes which result in the entry of blood into the CSF [2]. However, variations in total protein are quite non-specific and may also occur in systemic disease. Furthermore, there are problems with the measurement of total protein levels in complex mixtures of proteins, as in CSF. Whether the longstanding Lowry method [5], the simpler dye assays [6] or the more recent sensitive silver assays [7] are used, all of these methods are only accurate for individual proteins or very specific mixtures of proteins. These assays rely on the detection of specific reactive groups within the protein molecule and, as the number of such groups varies from one protein to another, the assay reaction kinetics will be different for each protein component in the mixture. Only when a method is based on an identical stoichiometric response for all proteins (such as may arise from a dye binding to the terminal carboxy or amino group), will accurate quantitation of complex protein mixtures be possible.

Many individual proteins have been examined in disease states. For example, total immunoglobulin (Ig), has been found to be frequently elevated in inflammatory neurological diseases. For diagnostic purposes, it is important to distinguish Ig that has been produced within the NS as compared to that derived from plasma. In order to make such an inference, the Ig levels from both CSF and plasma are normalized against their respective albumin levels, on the assumption that a relative increase in Ig in relation to the level of albumin in CSF versus plasma is indicative of a local intra-NS production of Ig. This method has proved of value [8], especially in the diagnosis of multiple sclerosis (MS). More detailed attempts using experimentally derived formulas to estimate the daily NS production of Ig are also of value in monitoring disease [8]. Unfortunately, total Ig is non-specifically altered in many conditions [2] and the formulas are of limited specificity, probably because assumptions of the source of proteins are not always correct in diseases of the NS [9–11].

The study of most individual CSF proteins requires a method with a high re-

solving power. Electrophoresis has provided such a method. Since the initial description by Kabat et al. in 1942 [12], electrophoresis of CSF proteins in NS diseases has involved a wide variety of methods, with different means of protein detection. At least one of the one-dimensional electrophoresis (1-DE) techniques is used in most clinical laboratories to identify the so-called oligoclonal bands (OBs) [13]. These OBs have been most investigated in MS and are now considered useful in supporting the clinical diagnosis of MS [14]. The principle involves the electrophoretic separation of similar amounts of CSF and serum Ig in order to identify discrete bands of abnormal Ig in CSF that are absent in serum. This electrophoretic separation can be conducted in any one of a number of support matrices, such as agarose, cellulose or acrylamide. OBs are present in over 90% of MS patients. The remaining few patients with definite MS that do not have OBs have been shown to have very few or no visible plasma cells in the meninges or adjacent plaques at autopsy [15]. Unfortunately, OBs are not specific for MS. They also occur in a varying percentage of patients with all other inflammatory neurological diseases and, rarely, in patients with non-inflammatory neurological diseases, such as neoplasms, vascular disease, motor neurone disease, Alzheimer's disease, epilepsy, alcoholism and idiopathic vertigo [16].

In subacute sclerosing panencephalitis (SSPE), the majority of OBs are measles-specific antibodies. In MS, the origin of the CSF OBs is unknown: they may arise from a specific, as yet unindentified antigen, multiple antigens or independent from any antigen. One possible explanation is that the OBs represent antibodies directed against viruses that cross-react with myelin basic protein and thus contribute to the pathogenesis of MS. The demonstrated homology between the amino acid sequence of several neurotropic viruses and myelin basic proteins [17, 18] would support this hypothesis.

CSF OBs usually differ between patients with MS, but they remain remarkably constant for most patients, even over ten years [19, 20] and even after immunotherapy [21]. The origin of this persistent and relatively unchanging OB response is unclear, but it is consistent with an immune response to a single antigenic challenge. Furthermore, this phenomenon, in addition to the temporal separation of multifocal lesions, is useful in distinguishing chronic inflammatory diseases, such as MS and SSPE, from acute post-infectious demyelinating diseases and the Guillain Barré syndrome (GBS), in which the presence of OBs is transient. Furthermore, in definite MS, there is no correlation between the number of OBs and the disease activity, severity or prognosis. A report of ten patients that were incorrectly diagnosed as having MS [22] well illustrates an application of CSF analysis: all six mis-diagnosed patients that had CSF examined showed no abnormal immunoglobulin. Therefore, all patients with suspected MS that have normal CSF immunoglobulins should have other diagnoses rigorously excluded.

The OBs are also of value in assessing the prognosis of patients with possible early MS [23–29], those individuals at high risk for MS [30, 31] and those patients with the GBS that are likely to progress to chronic relapsing polyneuropathy [32]. Unfortunately, the occurrence of OBs in numerous disease states severely limits their diagnostic application. Furthermore, of the other disease-

associated protein changes reported in 1-DE studies [33–37], none have become very useful in clinical practice, due to their non-specificity. The goal of clinical CSF protein studies is to obtain a body fluid that closely reflects disordered brain function by revealing an abnormality that will allow accurate diagnosis, prognosis and a rational basis for therapy. Recent advances in protein biochemistry may lead to some improvements in diagnosis and prognosis.

High-resolution two-dimensional electrophoresis (2-DE) with sensitive silver staining methods and computerized analysis [38–41] have been refined and now enable over 1000 proteins to be examined from 20 μl of CSF [42]. One promising example of this approach is the discovery of two 30 000 relative molecular mass proteins in the CSF from all patients with Creutzfeldt–Jacob disease (CJD). These CSF proteins have not been found in patients with other types of dementia [43]. The same proteins have enabled an early diagnosis in two cases of CJD transmitted by human growth hormone [44, 45] and the diagnosis of a sporadic case of CJD when brain biopsy was unhelpful [46].

2-DE of CSF has generally been advocated for research rather than clinical practice [47–53]. While these methods are not yet as automated as many techniques that are presently used in clinical chemistry laboratories, it is our contention that they should immediately supplant the inferior resolution of 1-DE techniques. We consider that they will hold a crucial role in clinical investigations because of their ability to detect diagnostically useful protein changes.

2. SAMPLE COLLECTION, STORAGE AND PREPARATION

Spinal fluid is usually obtained from the lumbar subarachnoid space, in the region of the cauda equina. Changes in ventricular CSF somatostatin were observed in children with dystonia [54], but this source of fluid will only rarely be available, because of the invasive nature of the procedure. Lumbar puncture (LP), unless there is local infection, is relatively safe for most patients: the temporary post-LP symptoms of headache, nausea, vomiting and low back pain occur in less than one third of all patients [2, 3]. Raised intracranial pressure is another relative contraindication for LP, such as when the GBS is associated with intracranial hypertension and papilloedema. In this situation, the LP is preferably performed after a computer tomographic scan (to exclude an unsuspected intracranial pathology) and in liaison with neurosurgical colleagues in case of subsequent clinical deterioration. The reader is referred for full details of the procedure to obtain CSF by diagnostic LP [2].

Prominent protein gradients exist between the ventricular, cisternal and lumbar portions of the CSF [2]. Moreover, there is a gradient of IgG in successive 5-ml aliquots taken at LP from patients [55]. Therefore, studies should be consistent in both the site and aliquot portion of CSF collection.

Age is well established to effect the CSF total proteins [56, 57] and individual proteins [58], but no sex-related differences have been found yet [58]. Thus, age effects must be considered in the interpretation of clinical CSF studies.

Normal ranges for some CSF proteins are established, but there will be slight variation between laboratories because different methods have been used to re-

solve and detect these proteins. Furthermore, the patient's blood must always be examined to differentiate the CSF changes that are independent of blood (for instance, those OBs which occur in MS and GBS) from the systemic changes that occur in systemic infections and gammopathies [59]. Abnormal CSF immunoglobulins are of great value in demyelinating disorders, but only when normal in the patient's blood. An occasional traumatic LP will occur even with the best techniques, and this prohibits reliable diagnostic information. In these circumstances, a repeat examination may be considered after three to ten days, by which time the changes secondary to the blood leakage should have resolved. For immediate patient management, however, it is useful to make approximate allowances for the blood leak prior to the second examination, and estimates of the CSF blood contribution for cells and protein may be calculated [60].

CSF sample storage has to be standardized if there is delay before protein studies are undertaken. There is little published work on the effects of sample storage on CSF proteins. One report indicated that CSF that is lyophilized and stored for longer than four months at $-20°C$ has altered proteins when studied after disc electrophoresis [61]. This report is consistent with artifactural heterogeneity reported after high-resolution 2-DE of *Escherichia coli* proteins that had been lyophilized and stored at $-70°C$ for one month [38]. Based on our experience of 2-DE CSF protein studies, we recommend the general policy of holding the sample at fridge temperature before study, and if this study is not performed within 6 h, the sample should be aliquotted and frozen at $-70°C$. We have not seen protein alterations when samples have been thus stored for over twenty years.

Sample preparation is so varied, and seldom fully described, that a comprehensive critique cannot be described in this review. Generally, the CSF proteins are either denatured, or not, before separation, depending on the aims of the study; for example, study of intact immunoglobulins is restricted to non-denaturing conditions, whereas the highest resolution of large numbers of proteins is achieved with denatured proteins. Interfering substances have been reported to impair certain separation techniques: for instance, sucrose or glycerol impairs resolution in polyacrylamide disc electrophoresis [62]. Methods of concentrating CSF include the use of dialysis, ultrafiltration or temporary adsorption to diatomaceous earth [63]. Sensitive detection methods now enable over 1000 proteins to be observed in small quantities of unconcentrated CSF, as described below. At present for 20 cm $\times$ 16 cm 2-DE gels, we denature 20 μl of unconcentrated CSF with sodium dodecyl sulfate (SDS), dithiothreitol and heat (95°C for 5 min) immediately before electrophoresis.

3. ELECTROPHORESIS

In current clinical practice, CSF protein mixtures are nearly always separated by zonal electrophoresis, where the sample is applied at one point on the matrix and single bands of each protein migrate at their differing speeds from the site of application to either anode or cathode. The rate of migration will depend on the ratio of their charge to mass. In addition to charge and mass, many other influences may participate in the electrophoretic separation of proteins: heat produc-

tion during the procedure will produce convection currents that are minimized by cooling the system; there will always be diffusion due to Brownian motion, the rate depending on temperature, the restrictive properties of the matrix, the protein size and the strength of the electrochemical gradient.

OBs and the other disease-associated protein changes have been reported with greater frequency when methods employ improvements in gel resolution and more sensitive detection. Each matrix for separation of CSF proteins has its own merit/disadvantages. Paper and cellulose acetate are simple and relatively inert; these are still used in some laboratories, but resolution is inferior to more recent techniques [64]. Agar and agarose gels have large pores that allow most proteins to migrate with little sieving effect and are commonly used for 1-DE separations, especially combined with immunological procedures, as in immunoelectrophoresis: an easy, rapid, reliable and sensitive method for simultaneous measurement of IgG and albumin was developed by electrophoresis of CSF in agar/agarose that contained specific antibodies [65]. Further review of agar electrophoresis of CSF is provided in a monograph [66]. Most earlier studies of OBs were performed in agar/agarose: abnormalities have been detected in the majority of patients with definite MS, such as 87% in one of the larger studies [67]. Polyacrylamide and starch gels usually have been used to provide an additional sieving effect on protein migration, but starch gels have largely been abandoned because of impurities and inconsistencies [68]. Polyacrylamide gel electrophoresis (PAGE) can be performed using continuous, but more usually discontinuous buffer systems [69, 70], as the latter improve protein resolution when a large CSF sample volume is applied [71, 72].

Modern electrophoretic techniques (reviewed in ref. 73) used for CSF proteins strive towards greater reproducibility and resolving power, both at the analytical and preparative scales. Three primary modes are utilized: SDS-PAGE, isoelectric focusing (IEF), either in high-quality agarose or polyacrylamide, and a combination of these two modes, high-resolution 2-DE.

SDS-PAGE [74], adding SDS to the PAGE discontinuous buffer system [69, 70], relies on the binding of the highly charged detergent (SDS) to all proteins in a constant ratio to the mass of each protein, with the result that they have uniform charge per unit mass. The electrophoretic separation is then proportional to the differing mass of each protein, irrespective of its original net charge. The polyacrylamide gel provides a sieving effect, the pore size of which can be varied to optimise the separation of specific proteins. This procedure thus allows estimation of the molecular mass of each protein, depending on each protein's relative mobility compared to standards. SDS-PAGE, with silver staining, can detect up to 40 bands of CSF proteins [75]; it allows OB detection in 94% of patients with definite MS, from 0.1 ml of CSF [76].

IEF [77] relies on a separation that is mostly dependent on net charge and is achieved by electrophoresis of proteins within a pH gradient until the p*I* for each protein is reached. This gradient is usually created with commercial carrier ampholytes [78, 79] in high-quality agarose or low-percentage acrylamide gels, or by grafting an immobilized pH gradient [80] onto polyacrylamide gels, as with Immobilines. IEF with carrier ampholytes in polyacrylamide allows detection of

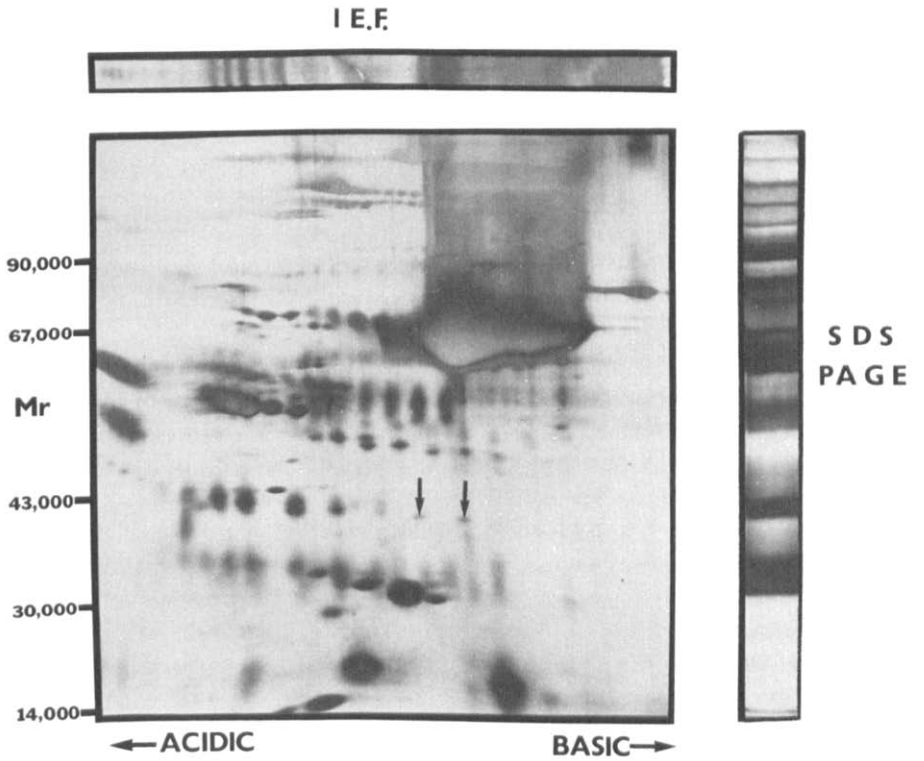

Fig. 1. Aliquots (10 μl) of CSF from a patient with schizophrenia were separated on 1-DE by IEF and SDS-PAGE as indicated, and then, using the same charge and mass separation conditions, 40 μl were resolved by 2-DE [38]. Two 40 000 relative molecular mass proteins (arrowed), that are not present in CSF from normal persons, were identified after 2-DE separation of CSF proteins from a schizophrenic patient. Proteins in all three gels were detected by silver staining [84].

up to 70 bands of CSF proteins [75]. One of the limitations of the method with carrier ampholytes is that gradients are difficult to maintain, especially in the alkaline region, because of an incompletely understood phenomenon called cathodic drift. This drift does not appear to be as relevant with immobilized pH gradients. Unfortunately, Immobiline gels are still not yet ideal, because of chemical instability and the expense of the Immobilines, in addition to interference with the migration of certain proteins (such as many with high molecular mass and/or basic net charge). IEF on high-quality agarose (e.g. ref. 81) allows detection of OBs in 100% of patients with definite MS, but IEF in acrylamide was shown to reveal OBs a little more distinctly than in agarose [82]. There is no doubt about the greater resolution obtained by IEF in either agarose or acrylamide, as compared to simple agarose electrophoresis [83], and this improved resolution may be helpful in early MS. However, all 1-DE methods have equal ability to detect OBs in definite MS.

High-resolution 2-DE [38] uses two successive independent separation systems, IEF and SDS-PAGE, usually in that sequence. The striking increase in resolution of protein mixtures in CSF is illustrated in Fig. 1, where a sample from the same patient is separated by either SDS-PAGE, IEF or 2-DE and stained

Fig. 2. CSF (20 μl) from a patient with CJD was separated using the recently optimized high-resolution 2-DE methods [85]. Arrowed are two 30 000 relative molecular mass proteins that appear to be of value in distinguishing CJD from other types of dementia [43].

with silver. Improvements in 2-DE now enable the separation of more than 1000 spots from 20 μl CSF, as shown in Fig. 2. The trace proteins (arrowed) in both figures could never have been resolved with any of the previous 1-DE methods. These same proteins have also been detected on a miniature 2-DE gel [86] that can be resolved and stained within 6 h [87], as compared to the usual three-day procedure [42].

Study of the known polyacrylamide cross-linkers has revealed that the commonly used bisacrylamide has a background-enhancing effect on the ammoniacal silver stain [88]. In order to reduce this adverse effect, a new cross-linker, diacrylylpiperazine, has been synthesized which lessens the reduction of ionic silver to metallic silver [88]. Further investigation of this new cross-linker in plasma protein 2-DE separations yields better resolution of some high-molecular-mass

and basic proteins [88]. This influence on resolution may be due to the bisacrylamide cross-linker interacting with migration of certain proteins, that is less when diacrylylpiperazine is employed. The resolving and staining advantages of this new cross-linker should be helpful in CSF protein investigations.

2-DE of proteins, reviewed recently (in ref. 89), combined with silver staining, has now been shown to resolve many CSF disease-associated proteins. Those in MS, especially the Ig light chains, are very much better resolved than after 1-DE [51, 90]. The changes identified in CJD, Herpes simplex encephalitis, GBS [43], Parkinson's disease [91], schizophrenia [92] and Alzheimer's disease [93] would never have been detected using 1-DE.

4. PROTEIN DETECTION METHODS

While CSF proteins might be radiolabeled or tagged with fluorescein prior to electrophoresis, this is not usually done in practice, leaving their post-electrophoretic detection to be undertaken on various matrices [94]. Coomassie Brilliant Blue stains are the most commonly used of organic dye stains, with sensitivity limits in the 10–100 ng range. A little less sensitive are Amido Black, Fast Green and Ponceau S. Silver staining, which was long used to stain neurohistological sections, was first utilized to detect phosphorylated proteins separated in polyacrylamide [95]. It was later used to detect CSF proteins on agarose [96] and as a general quantitative protein stain on polyacrylamide [97], with 100-fold more sensitivity than Coomassie Brilliant Blue. Generally, all protein stains are more sensitive on a thin, accessible matrix such as nitrocellulose, as compared to the thicker gels often used in electrophoresis. Thus, after electrophoresis, proteins can be transferred to thin membranes such as nitrocellulose [98] and stained with any of the aforementioned methods, or with immunological reagents [94].

The mechanism of the various silver protein stains depends on certain charged groups in the proteins that allow the reduction of the ionic silver to metallic silver preferentially at the site of each protein [99]. As the content of these electron-donating groups will vary between proteins, the sensitivity and quantitation range will also vary. However, the level of sensitivity appears to be similar for most proteins. Furthermore, quantitative comparison can be made between the same protein in different patient's CSF, unless there is a mutation in the protein that might influence its effect on silver.

5. EVALUATION OF RESULTS

Qualitative analysis of stained electrophoretograms of CSF proteins is usually performed by eye (often by overlay of one stained gel with another) with the intention of identifying changes when compared to both the patient's plasma proteins and a normal CSF pattern. Thus, the goal is to detect any appearance or disappearance of bands on 1-DE gels or spots on 2-DE gels. In the situation where a specific region is expected to contain OBs or the CJD-associated proteins [13, 43], visual scanning is partially satisfactory, but does allow scope for human error. Moreover, with the large number of proteins observed now on 2-DE gels, it

is essential to be able to have automated pattern matching devices. Such computerized matching software has been mainly developed for 2-DE gels [40, 41, 100–104]. 1-DE gels have a serious limit for accurate and sensitive detection of abnormality, namely their limited resolution. This is demonstrated in Figs. 1 and 2, where the small disease-associated proteins that are arrowed on the 2-DE gels could never have been identified on 1-DE gels because of more predominant overlapping proteins. Now that disease-associated protein changes are being identified and found to involve relatively low-abundance proteins [43, 90–93], the critical importance of 2-DE resolution becomes even more apparent.

Quantitative analysis on 1-DE gels with linear densitometry [71, 72] and on 2-DE gels with two-dimensional densitometry of digitized images of the gels [90] allows some measure of the relative amounts of each protein. 2-DE analysis has a considerable advantage over that of 1-DE gels, again because of better protein resolution. Accurate CSF protein quantitation is limited at present, because the staining kinetics for both dyes and silver stains appear to have a complex stoichiometric relationship with each individual protein. While there is a linear range of dye/silver binding on each protein [99], the full binding slopes for each of the CSF proteins have not yet been characterized: until this is done, only approximate values will be obtained, and these values will only represent relative quantities of each protein for intragel comparisons, rather than absolute values.

Complex CSF protein interactions and relations may occur in disease states. Analytical approaches using various forms of artificial intelligence must be applied to address this problem. Multivariate analysis was applied to selected proteins assayed individually by Mancini's technique [105], and suggests this approach might be valuable. Not surprisingly, the poor resolution of 1-DE would limit the accuracy of such analysis, but expert analysis should be more valuable with the 2-DE methods. Heuristic analysis has already provided promising results in the analysis of 2-DE liver proteins [41].

Genetic analysis of CSF proteins has not been employed, again because it is only with 2-DE that large numbers of inherited polymorphic proteins can be assessed. The majority of defined inherited variants have been identified in blood and skin proteins [106] and expression of some inherited variants are limited to brain tissue [107].

Preliminary identifications of polymorphic proteins have been made in CSF [90], but pedigree studies are required to confirm their heritability.

The enormous amount of data available from the 1000 plus proteins on each 2-DE gel from CSF leads to the problem of handling, organizing and interchanging databases. The content of a database for the storage and analysis of this type of data has been addressed in a proposal to develop a human protein index [108]. Critical aspects in the development of a CSF protein database (including methods used and references) include: a reference 2-DE gel image; an index of all of the proteins visualized; identifications/structural studies of proteins; data on specific protein variations (qualitative and quantitative) in physiological and pathological states; CSF/tissue specificity of specific proteins; availability of antibodies to specific proteins. It is important that this database be cross-indexed with the major genomic and protein databases. The bulk of this data should be

frequently updated and available for general circulation. The two modes that might best serve this function would be to have a picture reference accompanied by a standard text file on a floppy disc and/or a regular newsletter. One commercial group (Protein Databases Inc.) [109] is already marketing databases for yeast, *E. coli* and human liver, plasma and lymphocyte proteins. Another company, Large Scale Biology Corp., are building a general database of human plasma proteins [110], based on published work [111, 112].

As there are a great number of proteins that are common to both plasma and CSF, there should be a great deal of interchange between the plasma and CSF databases. Our CSF protein maps have been/will be published [42, 113] and we intend to make our own database of CSF protein information available in the future. We would hope that other laboratories will also contribute to this normal and disease-associated CSF protein database.

6. ADDITIONAL STUDIES

CSF proteins of specific interest in human diseases that are identified after electrophoresis, can be further studied in various ways. Electrotransfer of the separated proteins to nitrocellulose [98] can enable binding studies with lectins, receptors, other proteins, DNA, RNA or whole cells [94].

Moderate sized protein spots/bands on a gel can be excised and used as immunogen to raise antibodies. Trace spots resolved on high-resolution 2-DE gels, however, are present in quantity insufficient for raising antibodies with conventional techniques.

An additional approach to characterizing the CSF proteins of interest from the highly purified protein on a 2-DE gel is to obtain their amino acid composition. Better still is to define their partial amino acid sequence. Using the refined methods of microsequencing [114], we have achieved this with three disease-associated trace CSF proteins [115]. This sequence data is being used to synthesize oligopeptides and oligonucleotides to study the genetic and cellular origin and function of these proteins. This approach, like the aforementioned antibody production, cannot be applied to trace proteins on 2-DE gels without additional purification.

7. SUMMARY

Electrophoretic studies of human CSF proteins from patients with diseases of the NS are reviewed. Various 1-DE methods are of similar value in identifying the non-specific OBs, which are helpful in the diagnosis of MS and recurrent GBS. In early and subclinical MS, OBs are of prognostic value, with IEF gels having the greater resolution. Silver-stained 2-DE gels provide the equivalent information to the OBs on 1-DE gels, with even greater sensitivity, and yield additional disease-associated protein data. Two proteins have proven to have diagnostic value in CJD and other changes that are still being evaluated have been identified in Parkinson's disease, GBS, Alzheimer's disease, schizophrenia and Herpes simplex encephalitis. The vastly improved CSF protein information

obtained with silver-stained 2-DE gels heralds both a change from the relatively limited applications with 1-DE methods and also the need to adopt this approach in the routine clinical laboratory.

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