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Isoelectric focusing of cerebrospinal fluid proteins for the diagnosis of multiple sclerosis

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ISOELECTRIC FOCUSING OF CEREBROSPINAL FLUID PROTEINS FOR THE DIAGNOSIS OF MULTIPLE SCLEROSIS

by

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THESIS

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INTRODUCTION

I. Background

Multiple sclerosis (MS), one of the most common neurological diseases affecting young adults in developed countries, was first recognized more than a century ago (31). The incidence of the disease increases with increasing latitude, hence its prevalence in temperate climates and among people of European origin (1,31,41). The maximum prevalence is about one per one thousand (41). Near relatives have a greater risk in developing the disease (at least fifteen times that of the general population) and certain HLA types are over represented, but there has been no proof of a definite genetic pattern (31,41). It seems unlikely that either an environmental or a genetic factor is the sole explanation for the disease (41); however, from studies of migrating populations it has been suggested that an environmental factor operates around puberty (1). The disease must be latent for many years, since the average age at the time of diagnosis is thirty years (41). Thus, the shorter the interval is between the onset and initial examination, the greater the likelihood of early diagnosis. The disease process has an average duration of twenty five years (41).

The exact mechanism of the pathology of MS is still obscure. It involves a degeneration of the myelin sheath covering axons in the central nervous system (CNS) resulting in a breakdown in impulse transmission. It has been suggested that this demyelination is a result of the destruction of the oligodendroglial cells which manufacture and maintain the myelin sheath (31). Peripheral myelinated nerve fibers,

however, are unaffected (31,41). It has been observed that one in every twenty patients dies from the disease within five years, one in every seven by the tenth year, and one in every four by the fifteenth year (41). Six to ten percent of the patients have a benign disease and show little or no disability even after twenty five to thirty years (41).

A precise diagnosis of MS is important, firstly, because other more readily treatable diseases should be ruled out (42). Secondly, needless diagnostic procedures can be avoided in early or atypical cases. Thirdly, future personal and family planning can be improved and finally, it provides a population of carefully diagnosed cases which may aid researchers in understanding the etiology and pathogenesis of the disease (42). The diagnosis is still a clinical one (41,42), and is based on a characteristic combination of clinical signs and a history of well-defined attacks and remissions (41). However, the difficulty in symptomatic diagnosis lies in the early or atypical cases, or in disease which is complicated by another condition (41,42). The diagnosis of MS has been confused with meningo-vascular syphilis, nutritional disorders such as Vitamin-B complex deficiency, CNS tumors and some vascular diseases (41). Laboratory tests may be useful in the diagnosis of these conditions; however, such tests may not be diagnostic (41,42).

II. Laboratory Diagnosis

Abnormalities in the cerebrospinal fluid (CSF) are considered to be the most significant changes useful in MS (41,58). This is because identifiable changes are not present in the blood or may reflect associated general disease or complicating pathology rather than the CNS

primary disease process (41,58). CSF is formed from plasma by the filtering and secretory activity of the choroid plexus and is reabsorbed into the blood stream primarily by the arachnoid villi. The mechanism of its production is not fully understood, but due to its low protein concentration, it is considered an ultrafiltrate of plasma (41,56,58). Proteins present in normal CSF have a molecular weight of no more than 160,000 daltons (26,58). Secretory activity by the choroid plexus affects other constituents (26). Circulation of the CSF is slow, allowing long contact with cerebral cells particularly those in certain areas of myelin (55). This may result in the diffusion of solutes into the CSF (38), the analysis of which may reveal an abnormal process. The scope of the clinical pathology of the CSF in MS includes cytology, quantitative and qualitative alterations in proteins, and the immunologic changes involving specific anti-myelin factors (41). Of these, emphasis is placed on the cytology and protein alterations in the routine clinical laboratory.

A. Cytology

CSF cytologic examination has not been a clinically useful test (18) since only 33% of cases of MS showed a pleocytosis greater than 5 cells/cu mm, the upper normal limit (58). When a differential count is done, mainly lymphocytes or plasmacytoid cells are seen (55). B cells and T cells have been distinguished, and it has been observed that there is a higher percentage of T cells in the CSF, relative to the serum, as compared to a relatively lower percentage of B cells (55). Although the CSF cytology is of little prognostic significance, it may be linked

with abnormal immunological reactions. In addition, pleocytosis parallels the incidence of clinical onset and the degree may be a rough index of disease activity and severity (41).

B. Protein Determination

1. Total Protein

Normally, the same classes of serum proteins are present in CSF (26,41,55,58). The ratio of these proteins is similar in both fluids, but the concentration in CSF is 200-250 fold less than in serum (41,48). Thus it has been generally accepted that the bulk of normal CSF proteins originate from the plasma (26,51,58). Some proteins specific to the CSF, namely, the β-trace, γ trace and the tau-transferrin fractions, are probably of cerebral origin (41,58). The normal adult level of total proteins in lumbar CSF is usually 0.2-0.4 g/L (26,58). Therefore, methods for quantitative estimation at this level must be of much greater sensitivity than those for plasma proteins.

If CSF protein is to be used as an indicator of neurological disorders, consideration must be given to the variations in the plasma protein values, the blood-brain barrier permeability, as well as, to the local addition and removal of protein (49,58). These effects vary with different diseases and in different stages of the disease; however, the pattern in MS is unusually constant (41). The total protein is usually normal or only slightly elevated in MS (55). It has been shown that, regardless of the severity and duration of the disease, only about thirty percent of patients show an increased value (41). One author

observed that the average total protein value for MS cases and for normal controls was 0.485 g/L and 0.364 g/L respectively (58). He also suggested that an increase greater than 1.08 g/L argues against MS. The total protein has been considered to be of little value in the diagnosis of MS (18,55). Lamoureux and co-workers (25) discussed a broad CSF protein profile for MS; however, most authors emphasize the importance of an increased gamma globulin fraction.

2. Gamma Globulin Determinations

The characteristic alteration in the CSF proteins of MS patients is a selective increase in the gamma globulin fractions. Kabat and co-workers in 1942 (20) first reported an elevated gamma globulin fraction, in contrast to only a slightly increased total protein in the CSF of MS patients. This finding has been confirmed by many workers who concluded that it may be directly relevant to the pathogenesis of MS, as well as a useful diagnostic sign (41). This led to the development of techniques for the assay of this fraction which became the most widely used laboratory tests for MS (18).

a. Qualitative Tests

Qualitative tests for the estimation of CSF gamma globulins have been supplanted by quantitative and semiquantitative techniques. Some laboratories, however, still employ these tests. In 1912, Lange demonstrated the property of certain pathological CSF to precipitate a gold sol (41,57). Kabat and co-workers (20) showed that gamma globulins were necessary for this reaction, whereas it was inhibited by albumin.

The Lange colloidal gold test was used for many years as an indicator of an increase in CSF gamma globulin. Other colloidal tests were developed such as the gum mastic, benzoin and cephalin flocculation tests (41,57,58). The incidence of positive results in MS cases varied from 29 to 81 percent (41,57). Errors arose from minute traces of contaminants leading to false negative results (41). These tests were finally discontinued because of the instability of the reagents and the variation in results (58).

Precipitation methods were introduced in which CSF was treated with a protein precipitant. A saturated solution of ammonium sulphate was used in the Nonne-Apelt test, whereas the Pandy test incorporated a 10% phenol reagent (41,55,58). These methods require a small volume of unconcentrated CSF and may be useful as indices of globulin elevation. However, they are not sensitive enough to detect protein concentrations at, and just above, the uppper limit of normal, where most of the MS samples lie (41).

b. Quantitative Tests

Quantitative analysis ranges from the differential precipitation of the gamma globulins and albumin with subsequent chemical quantitation of the gamma globulin fraction, to the more accurate immunochemical methods which measure albumin or immunoglobulins of the IgG class. The former was found to be susceptible to errors and gave falsely elevated values, probably as a result of non-specific reactions (41,58). Immunochemical methods were introduced by Kabat and co-workers (20) who observed that 83% of the MS patients studied had an elevated IgG level

when the total protein level was within the normal range. At present, immunochemical analysis include immunoelectrophoresis (65), radial immunodiffusion (6) and electroimmunodiffusion (60). These methods are advantageous in that they are specific for IgG and require very small volumes of unconcentrated CSF. Quantitative chromatography has also been applied to unconcentrated CSF for the estimation of gamma globulins in MS cases (12,58).

The most widely used technique is semi-quantitative and involves a two method operation (18,41,55,58). The total protein is first estimated by a reliable method, and then zonal electrophoresis on cellulose acetate films is carried out on the concentrated CSF. A densitometric evaluation of the ratio of the respective constituents, expressed as a percentage of the total protein, is then made. An increased CSF gamma globulin fraction has been observed in 66% of MS patients by this method (18,55,58). This elevation may result either from local production, or from plasma, secondary to a breakdown in the blood-brain barrier (41,55,58). In addition, an elevated plasma gamma globulin, and other proteins which cross the blood-brain barrier, will be accompanied by an elevated gamma globulin fraction in the CSF. Ackerman and co-workers (2) have demonstrated that relative percent values are more useful than absolute values in differential diagnosis since they reflect locally synthesized IgG. The absolute values for CSF IgG, obtained by immunochemical methods, may be more frequently abnormal than the relative percent values, but they offer no discrimination between the neurological diseases (41,48).

Numerous studies have confirmed that most of the CSF IgG is produced in the CNS in MS patients (58,59). Studies have demonstrated

the synthesis of IgG in vitro by CSF lymphocytes from MS patients (50). As previously stated, IgG may also transfer from the serum to the CSF, where the average concentration is about 300-500 times lower (55,58). Tourtellotte (58) has devised a formula to measure the amount of CNS IgG synthesis per day, and has found that this is constant in most untreated MS cases. The most convenient way of demonstrating this phenomenon is to determine the CSF IgG/albumin ratio (18). Since albumin is synthesized in the liver, an elevated CSF albumin is due to a damaged blood-brain barrier with subsequent infiltration from the plasma (18,58). Although a significantly increased total protein may reflect a damaged blood-brain barrier, the albumin concentration is considered a more sensitive barrier parameter (51). It was observed that only 20% of MS patients have an abnormal barrier (58). This ratio has been evaluated in numerous studies (14,37,38,56) in which quantitation was by immunochemical means.

To correct for variations in plasma IgG and albumin and for greater sensitivity, the IgG/albumin index has been developed (14,45). This index is a quotient of the CSF/serum ratios for IgG and albumin, and increases when local synthesis of IgG occurs. An elevated index was observed in 88% of MS patients in contrast to 18% with other neurological disorders (45). However, a normal index does not exclude MS (35,49).

The highest incidence of abnormal findings in MS CSF has been found on visualization of discrete bands (oligoclonal bands) in the gamma globulin region after agar or agarose gel electrophoresis

(55). This abnormality was first detected by Lowenthal in 1964 on agar electrophoresis and was confirmed by Laterre in 1964 (58). Many other workers have since verified these results using agarose electrophoresis, and have shown that they occur in approximately 90% of MS patients (17,27,36,45,55). These bands may also be seen when the IgG concentration is within the normal range (17). Comparative studies have shown that agarose electrophoresis is the test of choice for MS (4,8,33,35,36,38,44). Recently, the technique has been adapted for rapid clinical analysis using commercially available apparatus and reagents (17).

These oligoclonal IgG bands have also been detected in the CSF, and in some cases, in the serum of patients with neurosyphilis, subacute sclerosing panencephalitis, chronic fungal meningitis and acute bacterial or aseptic meningitis (17,18). They may transiently appear in some patients with Guillain-Barré syndrome or acute Herpes simplex encephalitis (18). It is necessary to analyse a serum sample simultaneously with the CSF, since discrete immunoglobulin bands may migrate from the serum to the CSF in lymphoproliferative disorders (33). These bands have the same mobility in both samples. In most MS cases, however, only the CSF proteins show oligoclonality, and the patterns of bands appear unique for each individual (17,18). More recently, isoelectric focusing, an equilibrium method of high resolving capacity, has been investigated as a technique for greater specificity and sensitivity in the diagnosis of MS.

III. Isoelectric Focusing

A. Theory

The isoelectric point (pI) of an amphoteric molecule, such as a protein, is a physico-chemical constant. It has been defined as the pH at which the molecules do not move relative to the solvent in an electric field (48). The isoionic or isoprotic point, on the other hand, refers to the condition where there is an equal number of protonated basic and deprotonated acidic groups. The accepted definition is the pH which does not change on increasing the concentration of the pure ampholyte (48). When the only complexing ions are protons, as in the case of isoelectric focusing, the difference between the isoelectric and isoionic points is very small and therefore negligible (48).

Isoelectric focusing differs from electrophoresis in that amphoteric molecules such as proteins migrate under an applied voltage to their isoelectric points (pIs) in a pre-established pH gradient (16). This concentration effect gives rise to discrete bands. The concept was introduced in 1912 when Ikeda and Suzuki discovered that a mixture of amino acids assumed an order of increasing pI values form the anode to the cathode during electrolysis (16,48). The practical applicability was hindered by the lack of stable pH gradients (47,48). In 1961, Svensson introduced the "carrier ampholyte" concept which he defined as an ampholyte with good conductance and buffer capacity in the isoionic form (48). These properties are important, for the gradient should remain constant even when substances such as proteins with a certain buffering

capacity are added. For a continuous gradient he found it necessary to have a large number of such ampholytes of small molecular weight. Also, they should be of closely spaced pIs and should migrate with increasing pH in the direction of the current (48). Each ampholyte forms a stationary zone in the neighborhood of its isoionic point. Subsequent removal of the ampholytes is facilitated by their small molecular weights.

Vesterberg introduced the procedure for the synthesis of such ampholytes (48). This involved the condensation of various polyethylene polyamines with α , β-unsaturated carboxylic acids, such as acrylic and maleic acids (47,48,63). This development led to widespread acceptance of the method (48).

The original procedure was carried out in liquid pH gradients, stabilized against gravitational convections by the addition of sucrose (47). Further developments led to the introduction of solid matrix systems, such as gels, to overcome problems of convective mixing and diffusion. For analytical purposes polyacrylamide gels are commonly used, since they provide lower endosmotic effects than gels such as agarose. These effects, if great will result in an unstable pH gradient (47). Also, polyacrylamide gels have good optical clarity. The concentration of the gel must be adjusted so as to minimize molecular sieving effects and to provide a suitable porosity for the mobility of large molecules such as immunoglobulins (47,63). Analytical isoelectric focusing may be carried out in columns or on thin layers of polyacrylamide gel. However, the latter enables the analysis of multiple samples simultaneously.

Preparative isoelectric focusing has also been carried out using granulated gels such as Sephadex (47,63).

Upon isoelectric focusing, proteins condense at their pIs into narrow zones which are stationary in the electric field. Though complexes between proteins and ampholytes have been suggested (48), and since no proof has been produced, it has been postulated that firm protein-carrier ampholyte complexes do not form (48). If loose complexes form due to local electrostatic attraction, they will split up again during focusing since it is very unlikely that they will have the same pIs and they will migrate to different places in the pH gradient (48).

The resolving capacity of isoelectric focusing has been defined as the difference in isoelectric points (ΔpI) with which two proteins can be separated and is evaluated in the formula (48):

$$\Delta \text{ pI} = 3.07 \left(\frac{D \cdot \frac{dph}{dx}}{-E \cdot \frac{du}{dpH}} \right)^{\frac{1}{2}}$$

The parameters $\frac{du}{dpH}$ and D, which represent the mobility and diffusion constants of the protein are fixed; whereas $\frac{dpH}{dx}$ and E, representing the pH gradient and the field strength respectively, are under experimental control (48). Acceptable resolution is obtained for substances with a low diffusion coefficient and a high mobility slope at the isoelectric point, two criteria satisfied by all proteins (48). Such resolution is favored by a high field strength and a low ampholyte concentration (47). However, with high voltage,

Joule heat increases, causing band distortion, convective mixing and denaturation of proteins (47,48). This can be overcome by efficient cooling and the use of a regulated pulse power supply which increases the voltage at predetermined frequencies with low amperage (47). Such equipment is now commercially available.

B. General Applications

The technique has been applied to the separation of complex protein mixtures and isoenzymes, to the determination of the pIs of proteins and for purification purposes (50). It has been predominantly a research tool, but since the advent of commercially available apparatus and reagents more emphasis is being placed on its clinical applications, such as for abnormal variants of hemoglobins, α-1-antitrypsin and other proteins in body fluids (62). It is also used for the study of lipoproteins (47,62), cell membrane proteins including transplantation antigens (62), isoenzymes, metalloproteins, glycoproteins (47) and immunoglobulins (5,62). Body fluids such as plasma, saliva, urine and CSF are used (3). Concentration of the latter two may be necessary depending on the constituent under study and the limits of the method of detection.

Methods of detection include specific staining, zymograms for enzymes (63), immunoprecipitation (19), a two dimensional technique with immunoelectrophoresis as the second technique (3,63), ultraviolet spectrophotometry and radioautography (47). The most commonly used technique has been staining followed by visualization of the bands or densitometric evaluation. One of the limiting factors of the

latter process is the resolving capability of the scanning instrument. For thin layer gels a resolution of at least 50 um is needed (47). With most available scanners the minimum resolving capacity is 1 mm. A soft laser densitometer has been proposed by Righetti and Drysdale (47).

C. Application to Cerebrospinal Fluid Proteins

1. Normal CSF Proteins

Several studies, primarily in Europe, have employed the technique of isoelectric focusing for the analysis of CSF proteins in MS patients. Fossard and co-workers (13) pioneered this application of isoelectric focusing and subsequent studies were primarily concerned with findings in MS (7,10,11,22,36,40,61) and other neurological diseases (21,23,25,28, 29,39). The normal CSF proteins were not extensively studied until recently (43,53).

In one study by Stibler (53), concentrated CSF and serum samples from 32 healthy individuals were analyzed in the pH range of 3.5-11.0. Forty distinct bands in the CSF were observed. The pIs of the main components of 17 normal proteins identified by crossed immunoelectrofocusing were determined at 20°C and are shown in Table 1. Microheterogeneity was observed for all fractions except hemopexin and γ-trace protein of CSF and prealbumin of serum. Upon storage of the concentrated CSF at -20°C the γ-trace protein was displaced from pH 9.3 to pH 8.0. In the latter position, it was reproducible for at least one year when stored at -20°C. The tau-transferrin, which is the asialo form of transferrin,

TABLE 1. THE ISOELECTRIC POINTS OF MAJOR NORMAL PROTEIN COMPONENTS IN CEREBROSPINAL FLUID (53)

PROTEIN	pI
Orosomucoid	2.2
α_1-antichymotrypsin	3.2
haptoglobin	4.1
α_1 -antitrypsin	4.5
ceruloplasmin	4.6
β -trace protein	4.6
prealbumin	4.7
albumin	4.8,4.9
IgA	5.0
α_2-macroglobulin	5.2,5.3
transferrin	5.3,5.4
hemopexin	5.7
C'3-complement	5.8
tau-transferrin	5.9
IgG	6.4-8.9
γ -trace protein	9.3

appeared as a single band at pH 5.9 in only CSF. In two CSF specimens a "double fraction" 1 mm apart was observed at the same pH and was of lower intensity than the single band. It was observed that after desialylation of the sera in these cases the same "double fraction" appeared, whereas only one band was seen with other samples. These bands were identified as double tau-transferrin fractions; however, their origin is still obscure.

Another study on normal CSF samples verified some of these findings (43). Polyclonal IgG was also seen to migrate in the pH region of 4.7-8.6 by immunofixation procedures (28).

2. CSF Proteins in Neurological Diseases

Delmotte in 1971 (10) reported an isoelectric focusing procedure for the analysis of CSF and serum proteins in MS patients. He found that only the CSF samples show a migration of gamma globulin in the "high alkaline region". These findings were confirmed by Kjellin and Vesterberg in 1974 (24). They classified the patterns obtained in various neurological disorders and compared them with those obtained by paper electrophoresis. A fingerprint or "f" pattern rarely seen in MS patients was designated as a pattern corresponding to that observed in the serum sample, and was common in patients with blood-brain barrier damage. Different patterns were observed in the MS patients. It was suggested that they were due to different rates of production or elimination of the CSF immunoglobulins. In subsequent studies, Kjellin (22) noticed that verified MS cases showed oligoclonal bands in the pH region of 8.0-

11.0 and that cathodal bands were more frequent in cases with a progressive course of longer duration. In addition, a double tau-transferrin fraction around pH 5.9 was observed in 30% of 20 verified MS cases.

In 1977 Delmotte (11) observed that 91% of 262 MS patients showed abnormality detected by the presence of oligoclonal bands in the high alkaline region with isoelectric focusing, whereas only 65% were abnormal by agar-gel electrophoresis. In comparison, only 7% of 272 patients with other neurological diseases showed patterns similar to those seen in MS patients by isoelectric focusing. He therefore defined bands in the pH range of 7.8-8.6 as pathological, and bands greater than pH 7.8 as significant for the diagnosis of MS. Numerous studies have verified the occurrence of these fractions in the CSF of MS patients (7,40,61). Other authors have observed the migration of these bands in the pH range greater than pH 8.6 (21,28,40), and noted that these bands belong to the IgG class of immunoglobulins (11,28). Also, no correlation has been found between the age, duration and evolution pattern of the disease and the presence of these bands (7). On comparison with several biochemical parameters, including the total protein, total IgG, kappa and lambda light chain distribution and the lymphocyte count, it was found that only isoelectric focusing gave a uniform distribution of results for all the cases studied (7).

The CSF protein patterns in patients with neurological disorders other than MS have also been studied by isoelectric focusing, though not as extensively (11,21,23,25,29,39,40). In most studies

these patients represented the control population relative to determining a protein pattern specific for the diagnosis of MS. As a result, the CSF protein patterns which differed from those of MS cases (i.e. without "high alkaline bands"), have been ill-defined.

Kjellin and co-workers (21,23,25) observed that a "finger-print" of the corresponding serum gamma globulins ("f" pattern) , indicative of blood-brain barrier damage, occurred frequently in the CSF from patients with various neurological diseases other than MS. These included spinal muscular atrophies, especially amyotrophic lateral sclerosis, muscular dystrophies, such as myotonic dystrophy, and CNS disorders of infectious etiology, such as meningitis, arachnoiditis and post-encephalitic sequelae. These patterns showed a uniform increase of the normal gamma globulins, without the occurrence of "high akaline bands".

The presence of "high alkaline bands" (similar to MS protein patterns) was observed in the CSF from patients with myelopathies of unknown origin (24). This finding was explained by the author's previous observations that more than 60% of these patients were probably suffering from MS, and on follow-up examination the diagnosis in 40% of 45 of these cases was clinically verified as MS. Another study (40) showed that 17% and 35% of the patients with myelopathies and polyneuropathies respectively, displayed "high alkaline bands" in both the serum and CSF samples. These results, however, were inconclusive. Patients with meningoencephalitis also displayed "high alkaline bands" in their CSF samples, however, aberrant protein fractions were also found in the acidic region, two of

which were suspected to be the double tau-transferrin fraction at pI 5.9 (21).

Abnormal protein fraction in the acidic region were also observed in the CSF of patients with limb-girdle dystrophy and facioscapulohumeral dystrophy. These bands have not been identified except for the double tau-transferrin fraction which occurred frequently in the former cases (23). Another study (39), which was carried out on CSF and serum samples from patients with neurological syndromes accompanied by atrophic brain damage, showed that 65% of the 31 cases had abnormal CSF fractions in the acidic pH range between 5.4-5.8, the area where transferrin and other β-proteins migrated. A correlation between these findings and brain damage was not made. It has been suggested that a study of the CSF protein fractions in the acidic pH range may be of diagnostic value in conditions such as CNS tumors, where increased prealbumin and β-trace proteins have been demonstrated by electrophoretic methods (24).

In some instances comparative studies were carried out in which the results obtained by isoelectric focusing were compared to those obtained by electrophoretic methods using paper (22,24) or agar-gels (11). Isoelectric focusing showed a much higher frequency of positive results in the CSF from MS patients. In contrast to this finding, a recent investigation (29), in which agarose electrophoresis was employed as the reference method, showed similar frequencies of positive results in cases with MS as well as other CNS disorders.

The procedures employed in the studies reviewed above are complicated and time-consuming and must be performed only in specialized research laboratories. The lack of easy reproducibility and adequate commercial equipment has resulted in unavailability of CSF isoelectric focusing as a diagnostic laboratory test.

IV. Objective

The purpose of this study is two-fold. First, the technique of isoelectric focusing had to be investigated for its adaptation to routine clinical analysis with the use of commercially available apparatus and reagents. Secondly, it had to be compared with the current diagnostic test for MS, viz. agarose electrophoresis, in terms of specificity, sensitivity, simplicity, cost, reproducibility and the length of time involved.

MATERIALS AND METHODS

I. Instrumentation

The equipment utilized for this assay consisted of a LKB Multiphor 2117 Electrofocusing and Electrophoresis system, a LKB 2103 DC power supply (LKB Western Instrument Inc., Pleasant Hill, CA 94523) and a Lauda/Brinkman refrigerated circulator and bath Model K2/RD (Brinkman Instruments Inc., Westbury, NY 11590). The Multiphor system, which could be adapted for electrophoresis, included a buffer tank, cooling plate and tubing, a cover with electrodes and leads, and a flow meter. The power supply had a "mains input requirement" of 110 V $\pm$ 15%, 50/60 Hz; with a voltage output of 10-2000 V, constant current ranges of 1-10 mA and 20-200 mA, and constant power ranges of 1-11 W and 10-110 W.

II. Reagents

Polyacrylamide gels were part of the LKB Ampholine^{R} PAG plates kit (LKB Western Instruments Inc., Pleasant Hill, CA 94523). The kit consisted of five Ampholine polyacrylamide gel plates, pH 3.5-9.5, 15 electrode strips, 200 sample applicator pieces, five plastic sheets, two templates, two bottles for electrode solution, five experimental result forms and one instruction manual.

The electrode solutions were sodium hydroxide (1 mol/liter) for the cathode and phosphoric acid (1 mol/liter) for the anode.

The fixative consisted of 57.5 gm of trichloracetic acid and 17.25 gm of sulphosalicylic acid dissolved in a mixture of 150 ml of

methanol and 350 ml of distilled water. The stain solution was prepared by dissolving 0.4 gm Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA 94804) in 75 ml methanol, and diluting with 186 ml of trichloracetic acid (1 gm/liter). This was a modification of the preparation outlined by Vesterberg (64).

The destain solution was a mixture of 160 ml glacial acetic acid and 500 ml of ethanol diluted to 2000 ml with distilled water (64). The preserving solution was prepared by diluting 50 ml of glycerol to 500 ml with destain solution.

III. Sample Collection and Storage

A. Patient samples

Various hospitals supplied CSF and, in most cases concurrent serum samples. The CSF was collected by lumbar puncture and was examined by the standard technique of the hospital laboratory for cell count, total protein and percent gamma globulin. In some cases complete CSF analyses were unavailable. The patient population comprised of 13 diagnosed MS cases classified according to MacDonald's criteria (42), 13 cases with probable MS and 49 control patients with other neurological diseases. These controls included 11 patients with chronic infections of the CNS, 15 patients with other chronic inflammatory CNS diseases, three patients with acute infections and 20 patients with chronic non-inflammatory disease of the CNS.

B. Control Samples

Due to the difficulty in procurring CSF samples from healthy individuals a normal control population was not studied. A pooled sample, prepared with CSF from three patients with non-neurological diseases was utilized along with a serum sample from a healthy individual, as a negative control. One CSF sample, obtained at the autopsy of a patient with MS, was used as a positive control. Another control sample used as a check for the reproducibility of the technique, was a diluted serum sample from a patient with an IgG myeloma. This sample, on agarose electrophoresis, showed a monoclonal band in the gamma globulin region. On isoelectric focusing, however, this band resolved into numerous fractions in the IgG region. Variations in the pH gradient were checked by incorporating a mixture of pH markers. These included ferritin, Type I (pI of the main component = 4.5 $\pm$ 0.02 pH units), sperm whale myoglobin (pIs = 8.18 $\pm$ 0.02 and 7.68 $\pm$ 0.02) and cytochrome C (pI = 10.5 $\pm$ 0.02 pH units). They were obtained from Sigma Chem. Co., St. Louis, MO 63178.

C. Storage of samples

CSF and serum specimens were transported at ambient temperatures to the laboratory where they were centrifuged and the supernatant aliquoted and frozen at -20^{o} until the CSF was concentrated. One aliquot of serum and CSF was analysed for oligoclonal bands by agarose electrophoresis using the standard technique in the laboratory (17).

IV. Sample Preparation

A. CSF Samples

Since the CSF protein content is usually low, a very sensitive method is required for the detection of the protein fractions after isoelectric focusing or electrophoresis. However, most of the commonly-used stains and staining methods lack such sensitivity.

It was therefore, necessary to concentrate the CSF proteins prior to analysis by isoelectric focusing or electrophoresis by a suitable concentration technique. However, most available methods introduced various problems especially that associated with the loss of proteins during the concentration process.

1. Comparison of concentration techniques

a. Types of concentrators

A comparative study of three commonly used concentration techniques was carried out to determine the most suitable method. The processes involved ultrafiltration with an Immersible Molecular Separator (Millipore Corporation, Bedford, MA 01730), vacuum ultrafiltration with collodion bags (Sartorius Membranfilter, Beckman Instruments Inc., Anaheim, CA 92806) and concentration by osmotic force with MINICON CS-15 and B-15 concentrators (Amicon Corporation, Lexington, MA 02173). The use of an absorbent polyacrylamide hydrogel ("Lyphogel", Gelman Instrument Co., Ann Arbor, MI 48106) was recommended for a higher recovery of protein (S. Nussenbaum, personal communication); however, retrieval of the concentrated sample from the gel was difficult. Therefore, this process was not included in the investi-

gation.

b. Procedure

One 5 ml and two 2.5 ml aliquots of pooled CSF were concentrated to the "50x" and "20x" levels using the MINICON concentrators and one 2.5 ml and one 5 ml aliquot were concentrated approximately 20-fold in collodion bags. The use of the Immersible Molecular separators required a minimum volume of unconcentrated sample for complete immersion of the concentrators. Two 5 ml aliquots of pooled CSF were concentrated by this technique. The process of concentration was carried out according to the respective manufacturer's directions.

The concentrated samples were measured and the concentration factor was calculated. The total protein level of all the samples, including the unconcentrated pooled CSF sample, was estimated by a dye-binding method, the "Bio-Rad Protein Assay" (Bio-Rad Laboratories, Richmond, CA 94804). The IgG levels were also determined by radial immunodiffusion using the "IgG Ultra Low Level Endoplates" (Kallestad Laboratories Inc., Chaska, MN 55318). The percent recovery for each parameter was calculated and averaged between the duplicate samples. The results are listed in Table 2.

2. Concentration and Storage of CSF samples

As a result of this investigation and the observations discussed on page 57 , the MINICON CS-15 concentrator (Amicon Corporation, Lexington, MA 02173) was used in this study. An aliquot of 2.5 ml of CSF was thawed and concentrated 20-fold. The concentrated CSF was withdrawn by pipetting with a 100 ul capillary tube (Corning

Glass Works, Corning, New York). The tube was then sealed with putty ("Miniseal", Dade Division, American Hospital Supply Corporation, Miami, FL 33152) and labeled. The concentrated CSF was stored at $4^{o}C$ if analysis were to be carried out the next day or at $-20^{o}C$ for future analysis. It was observed that proteins were denatured with a loss in resolution, on repeated thawing and freezing of the concentrated CSF.

B. Serum Samples

Since the protein concentration of serum is approximately 200 times that of CSF, the serum samples were diluted to approximate the protein concentration of the concentrated CSF. It was observed that the application of a greater volume of a dilute solution gave a better pattern than a smaller volume of the concentrated solution (63). This was probably due to the "protein-protein effect" resulting from Van der Waal's forces between the molecules. This effect is greater in concentrated solutions and is due to the close proximity of the molecules. Poor diffusion occurred and a streaking of patterns was indeed noted with concentrated samples. Taking into account the 20-fold concentration of CSF with the concomitant loss of protein, a five-fold dilution of serum samples with saline (0.1 mol/liter).

C. pH Markers

Aqueous solutions (50 ug/ml) of ferritin type I, cytochrome C and sperm whale myoglobin were mixed in the proportion of 1:1:1.

V. Isoelectric Focusing

A. Prefocusing

The polyacrylamide gel plate was prefocused to remove traces of catalysts used during its polymerization. These chemicals, such as ammonium persulfate, give the gel an oxidation capacity which may have adverse effects on the proteins (63). Prefocusing was carried out at 400 V, 30W and a maximum current of 50 mA for 30 minutes. Cooling was provided by circulating water at 4°C through the cooling plate. To ensure good contact between the gel and the cooling plate, and hence, efficient cooling, a few drops of an aqueous solution of Triton X-100 (10 ml/liter) were evenly distributed over the cooling plate. A template, which was used as a guide for sample application, was then carefully placed on the cooling plate, avoiding air bubbles. After spreading a few more drops of the Triton X-100 solution over the template the gel was carefully placed in position. Excess detergent was removed from the perimeter of the gel with absorbent towels. The electrode strips were soaked in the appropriate electrode solution and placed in position on the gel. The cover was then secured, ensuring good contact between the electrodes and the electrode strips. Voltage was then applied.

B. Sample application

The position and mode of sample application were determined by preliminary trials. Various ways for applying the samples were tested. One was to pipette a small volume (e.g. 5 ul) of the highly concentrated samples directly onto the gel. This left a great amount of protein precipitate in the area of application, and the patterns

obtained were smeared. Pieces of cellulose acetate and borosilicate glass fiber filter, as well as the sample applicators supplied by the manufacturer, were soaked in the sample and placed on the gel. Of these, the glass fiber filters gave the best results. This was probably due to its low adsorptive properties, (since it contained no cellulose (65)) and its high absorptive capacity. Filters with cellulose have been found to adsorb basic proteins when placed in the acidic pH range (65). Various volumes of different concentrations of CSF were tested. Also, samples were applied at different positions between the anode and the cathode.

The best patterns were obtained when 100 ul of a 20-fold concentrated CSF sample were applied in the pH range of 6.2-6.5 i.e. midway between positions 3 and 4 on the template. To approximate the protein concentration of normal CSF 25 ul of a five-fold dilution of the corresponding serum sample were applied. Glass fiber filter pieces, 10 mm x 6 mm with thicknesses of 1.5 mm and 0.3 mm, accommodated 100 ul and 25 ul, respectively. The filters were obtained from Millipore Corporation, Bedford, MA 01730. Twenty-five ul of the control serum and the pH markers were also applied.

During the prefocusing period the sample applicators were arranged in the order of application on a glass plate. When prefocusing was completed the samples were pipetted onto the applicators which were then placed on the gel at 6mm intervals. Each run was comprised of twenty-one samples which included the pH markers, the myeloma control sample, the serum and CSF negative controls, the MS control and eight sets of patient samples. Each set consisted of the

CSF and the corresponding serum samples.

C. Focusing

At the end of the prefocusing period the voltage was disconnected, the cover removed and the anode electrode strip replaced with a new one. The samples were placed in position, the cover replaced and the voltage applied. Focusing was carried out for 90 minutes. The initial parameters were 400 V, 50 mA and 30 W whereas the final parameters were set at 1000 V, 15 mA and 30 W.

D. pH measurement

The pH of the gel was measured at 5 mm intervals between the anode and cathode. This was done at the end of each run to check for variations in the pH gradient between runs and also to measure the pIs of the protein fractions. pH measurements were made with LKB Multiphor 2117-111 flat surface pH electrode attached to a Corning pH meter Model 7 (Corning Scientific Instruments, Medfield, MA 02052) at 20°C.

E. Fixation, Staining and Preservation

The gel was immersed in the fixative for one hour to allow for the precipitation of the proteins and the diffusion of the carrier ampholytes, which also stain with protein dyes. The stain solution was prepared and allowed to equilibrate to 60°C in an incubator-oven. Staining was carried out at 60°C for 15 minutes in a covered container. The gel was removed carefully from the stain solution at

one end of the container to avoid the deposition of the stain precipitate on its surface. It was immediately immersed in the destain solution. Destaining was enhanced by magnetic stirring which was facilitated by the use of an LKB staining dish. Stain precipitate on the surface of the gel was removed during destaining by gently wiping with cotton wool soaked in the destain solution. The solution was changed if needed. Destaining for two hours was found to be sufficient. The gel was then placed in the preserving solution for one hour, after which it was allowed to dry overnight on a glass plate at room temperature. The surface of the gel was sticky so that it adhered to a sheet of plastic as it was rolled onto its surface. The protein patterns were then visualized with transmitted light or against a white background.

VI. Evaluation of the protein patterns

A. Immunofixation

The protein fraction of major interest in the CSF of MS patients has been the immunoglobulin IgG. The mobility of this fraction on routine electrophoresis is usually cathodal to the sample application point. On isoelectric focusing, however, it was shown that the IgG fractions migrated both anodally and cathodally to the sample application point at pH 6.3 when a pH gradient of 3.5-9.5 was used (43). The pIs of these IgG fractions in normal CSF and serum samples were in the pH range of 4.7-8.6 (28). Since the anodal region was dominated by other proteins, such as albumin, IgA and the α and β

globulins (28,43), only the region cathodal to the sample application point was studied. In order to locate the focused bands corresponding to the IgG fractions in the patients', as well as the control samples, an immunofixation study was carried out.

1. Sample preparation

The samples included CSF and serum from patients with MS, SSPE and cerebral tumor, negative control serum and CSF, as well as the IgG myeloma serum. The IgG concentration of the CSF samples was estimated by radial immunodiffusion using the "IgG Ultra-Low Level Endoplates". Serum samples were diluted 50-fold with saline (0.1 mol/liter), and the CSF samples concentrated, if required, to adjust the IgG concentration to approximately 0.25 gm/L.

2. Procedure

The technique of immunofixation, as described by Laurenzi and Link (28), was used with some modifications. Isoelectric focusing was carried out as described on page 29. The diluted serum (30 ul) and the corresponding CSF samples (50ul) respectively, were applied on glass fiber filters. Strips of cellulose acetate (Beckman Instruments Inc., Anaheim, CA 92806) were impregnated with anti-human IgG antiserum (Atlantic Antibodies Inc., Westbrook, Maine 04092). When focusing was completed the pH of the gel was measured as described on page . The cellulose acetate strips were then carefully placed on the gel, avoiding air bubbles, along the width of the gel (i.e. extending from the anode to the cathode). The gel was then placed in a

closed container lined with wet paper towels, and incubated at room temperature for two hours. At the end of this period the gel was washed in saline (8.5 gm/L) for 48 hours. Staining, destaining and preservation was carried out as described on page 29. The gel was then visualized for the location of IgG bands, using transmitted light.

RESULTS

I. Analytical variables.

A. Variation of the pH gradient.

Between run variability of the pH gradients has been observed by other workers (43). However, with the use of commercially prepared gels minimal variations of the pH gradient were observed. If the focusing is carried out for very long periods (eg. greater than five hours), or in the absence of cooling, there appears what is termed a "cathodic drift", i.e. the pH gradient is shifted towards the cathode (47). Since the focusing period is relatively short (90 minutes), this phenomenon did not occur. A diagram of the typical pH gradient obtained from two runs is shown in Figure 1.

B. Temperature dependence.

The gel tends to be cooler at the side in contact with the cooling plate resulting in a lower pH. The upper side therefore will have a higher pH, thus forming a temperature gradient from the lower surface to the upper surface (43). This effect results in a loss of resolution, however, the gels which were used in this study were only 1 mm. thick, allowing for an even temperature distribution and only a negligible effect.

C. Amount of protein applied

The detection limit of the staining procedure described is approximately 1 ug. of protein and depends on the carbohydrate

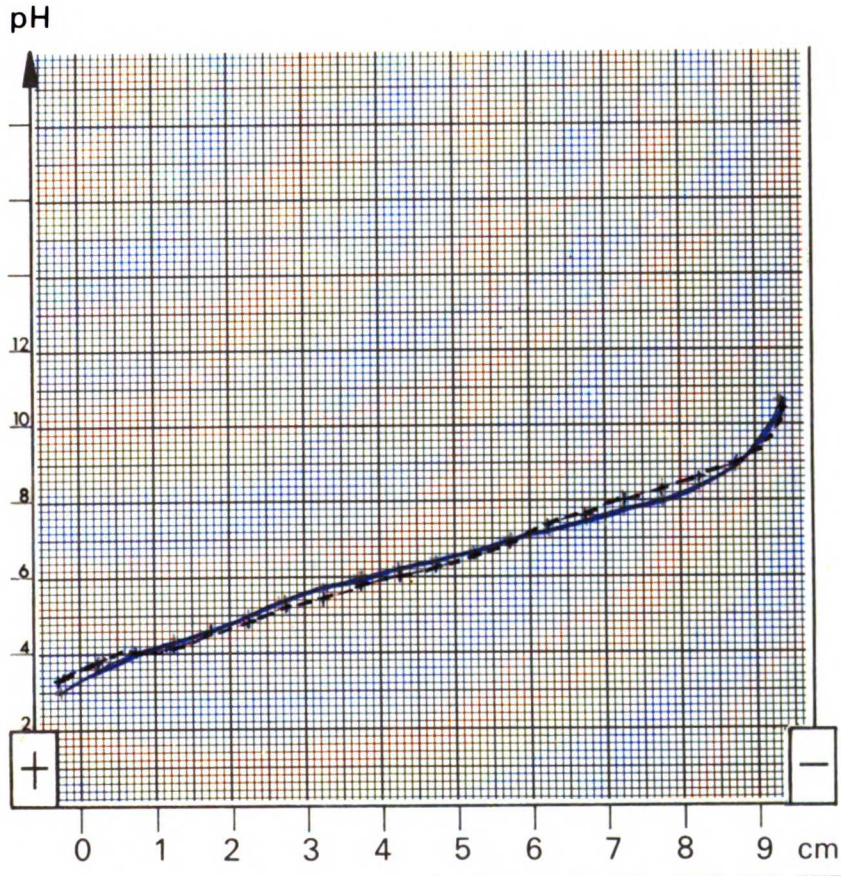

Figure 1. The pH gradients from two Isoelectric Focusing gels.

content of the protein. The greater the carbohydrate content the greater the amount of protein to be applied (63). It is best to apply a constant amount of protein (43,61); however, this will be time-consuming for routine clinical analysis. Figure 2 illustrates the patterns obtained when a constant amount of IgG (48 ug) was applied. The amount actually detected is difficult to estimate since the diffusion rates from the sample applicators through the gels may vary. If the protein concentration is suspected to be high (eg. greater than 0.23 gm/l), as in CSF with gross hemolysis, xanthochromia or of high viscosity, the unconcentrated CSF should be used. Increased protein concentrations were common in patients with acute infections and other neurological diseases with barrier damage. When an excess of protein was applied, smearing of bands in the acidic region was observed. However, this was not the region of interest. CSF samples with IgG concentrations from 0.016 gm/L to 0.20 gm/L gave suitable patterns.

II. Comparison of techniques for the concentration of CSF proteins.

The results of the comparative study on the methods for the concentration of CSF proteins, as described on page 24, are shown in Table 2. The average (n=2) per cent recovery of the total protein and IgG by each method was calculated. These values were 72% for both assays with the collodion bags, 64% and 60% with the "Immersible Molecular Separators" and 59% and 54% with the MINICON concentrators, respectively. The time required for concentration was comparable

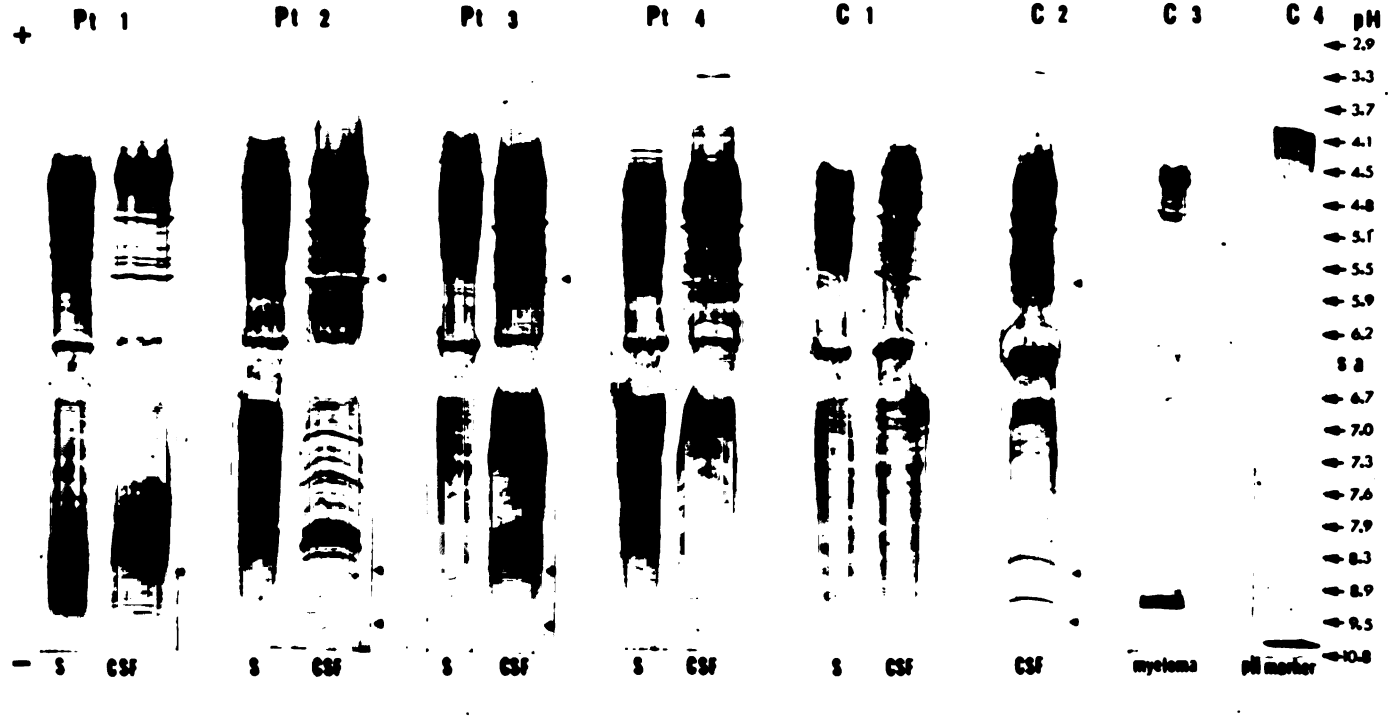

Figure 2. Isoelectric Focusing patterns obtained on application of 48 ug of IgG. The diagnoses of the patients were: Pt.1: SSPE; Pt.2: Clinically definite MS; Pt.3: Probable MS; Pt.4: Stroke. The controls were: C1: negative control; C2:MS control; C3: IgG myeloma control; and C4: pH marker. s a represents the sample application point. Note the double fractions at pH 5.9 (see arrow); the "high alkaline bands" between pH 8.6 and 9.5; and the γ - trace protein at pH 9.5. (S = serum).

TABLE 2. COMPARATIVE STUDY OF CONCENTRATION TECHNIQUES FOR CSF PROTEINS

SAMPLE	Initial Volume (ml)	Final Volume (ul)	Concentration Factor	Total Protein gm/L	% Recovery	IgG gm/L	% Recovery
A. Unconcentrated pooled CSF	———	———	———	.65	———	.064	———
B. Concentrated pooled CSF							
1a. MINICON B-15	5	90	55x	.38	58	.033	52
b. MINICON CS-15 1	2.5	120	21x	.40	62	.038	59
2	2.5	120	21x	.37	57	.033	52
2. Immersible Molecular Separator 1	5	220	23x	.40	62	.036	56
2	5	170	29x	.42	65	.040	63
3. Collodion bag 1	5	320	16x	.48	74	.048	75
2	2.5	100	25x	.45	69	.044	69

for the three methods, and ranged from 30 minutes to one hour, depending on the protein concentration.

III. Identification of the IgG fractions by Immunofixation.

Figure 3 illustrates the CSF and serum IgG patterns obtained by immunofixation for the negative control and for a patient with clinically definite MS. The isoelectric focusing patterns before immunofixation are also illustrated. It can be seen that although the IgG fractions migrated with pIs both anodal and cathodal from the sample application point at pH 6.2-6.5, they dominated the cathodal region, i.e. pH 6.5-8.6 and pH 6.5-9.5 in the negative control and MS CSF samples, respectively. The single band at pH 9.5, seen only in the CSF sample before immunofixation did not react with the IgG antiserum and was probably γ - trace protein (28,43).

IV. Characterization of protein patterns obtained by Isoelectric Focusing.

The presence of oligoclonal bands of IgG in the high alkaline region (pH 8.6-9.5) in CSF and serum samples was verified by immunofixation studies. These studies formed the basis on which the results of the assay were interpreted. Some typical patterns observed in concentrated CSF and dilute serum samples from patients with different neurological diseases are shown in Figure 2. Patterns obtained with the pH markers and control samples are also illustrated. The IgG fractions in the negative control serum and CSF samples, as well as

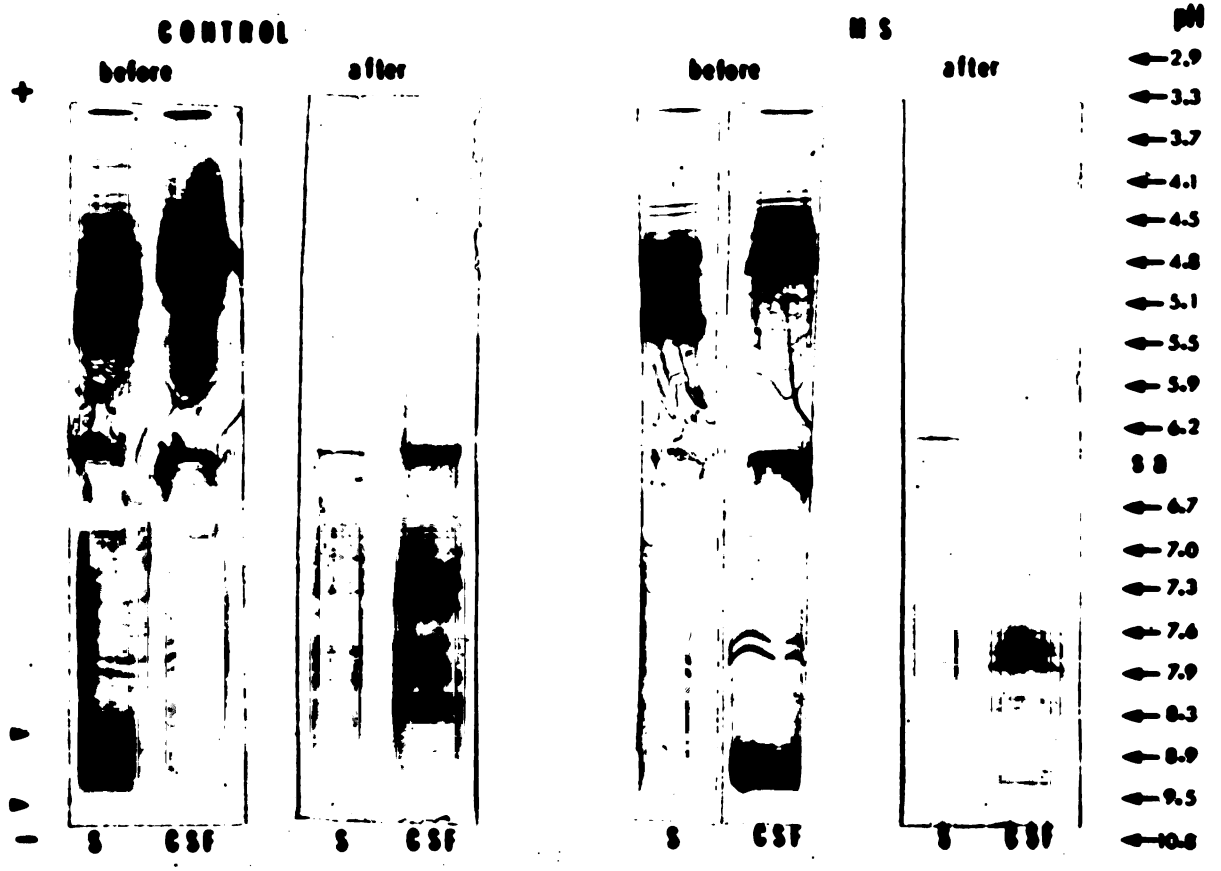

Figure 3. Isoelectric Focusing patterns of the negative control and an MS CSF and serum (S) samples before and after immunofixation with anti - human IgG antiserum. s.a. represents the sample application point.

in samples from patients with neurological disease other than MS, especially of the non-inflammatory type, migrated in the range of pIs of less than pH 8.6. Most of the CSF and, in some cases, the serum samples from patients with MS and chronic inflammatory CNS infections, such as subacute sclerosing panencephalitis (SSPE), had IgG fractions in the pH range of 8.6-9.5. These fractions were called the "high alkaline bands" and their occurrence in the various groups studied is listed in Tables 3 and 4. In some cases a serum sample was not available for assay.

At pH 5.9, the region where the tau-transferrin fraction migrates normally as a single band, a double fraction (as shown in Figure 2) was sometimes seen. This double fraction was only present in CSF, and was noted in the samples from patients listed in Table 5. No correlation between the occurrence of this fraction and the "high alkaline fraction" was observed.

V. Comparison of Isoelectric Focusing and Agarose Electrophoresis.

The comparison of agarose electrophoresis and isoelectric focusing is summarized in Table 3. These assays were not correlated with routine CSF measurements such as total protein, per cent gamma globulin and cell count in all cases. Instead, a summary of the available CSF levels is presented in Table 6. The upper normal limits for these tests were 0.45 gm/L for total protein, a gamma globulin of 12 per cent of the total protein and a cell count of five white blood cells (WBC)/cu.mm (17).

TABLE 3. ELECTROFOCUSING AND AGAROSE ELECTROPHORESIS RESULTS OBTAINED IN THE VARIOUS DIAGNOSTIC GROUPS.

Diagnostic Group	Total Number of Patients	Isoelectric Focusing	Agarose Electrophoresis	
		No. with high alkaline bands	No. with oligoclonal bands	No. with atypical results
Clinically definite MS	13	12	10	1
Probable MS	13	7	6	2
Chronic CNS Infections	11	10	10	
Acute CNS Infections	3	0	1	
Other Chronic Inflammatory Disease of the CNS	15	8	6	3
Chronic noninflammatory disease of the CNS	20	0	1	2

* atypical: presence of only one band in the gamma globulin region.

TABLE 4. THE NUMBER AND DIAGNOSES OF PATIENTS WHICH DISPLAYED "HIGH ALKALINE BANDS" IN THE CSF, OR CSF AND SERUM, IN THE PH RANGE OF 8.6-9.5 ON ISOELECTRIC FOCUSING.

Diagnosis	Total	No. with high alkaline bands in the CSF	No. with high alkaline bands in the corresponding serum
MULTIPLE SCLEROSIS GROUP			
Clinically definite MS	13	12	1
Probable MS	13	7	0
CHRONIC CNS INFECTION			
SSPE	7	7	2
Neurosyphilis	1	1	0
Chronic meningitis of fungal etiology	1	1	0
Chronic meningitis of bacterial etiology	1	1	0
CHRONIC INFLAMMATORY CNS SYNDROME			
Chronic meningeal irritation of unknown etiology	1	1	1
Carcinomatous meningitis	1	1	0
Lupus sclerosis	1	1	1
Granulomatous angitis	1	1	0
Peripneoplastic CNS syndrome	1	1	0
Post encephalitic sequelae	1	1	0
Guillain-Barré syndrome	3	1	0
Unknown diagnosis	1	1	0

TABLE 5. OCCURRENCE OF THE DOUBLE FRACTION AT PH 5.9 IN THE CSF FROM VARIOUS DIAGNOSTIC GROUPS.

Diagnosis	Total No. of Patients	No. with a Double Fraction	No. with high alkaline bands on isoelectric focusing / No. with a double fraction
Clinically definite MS	13	3	3/3
Probable MS	13	2	2/2
SSPE	7	2	2/2
Chronic meningitis of fungal etiology	1	1	1/1
Chronic meningitis of bacterial etiology	1	1	1/1
Epilepsy	1	1	0/1
Myelopathy	2	1	0/1
Perineoplastic syndrome	1	1	1/1

TABLE 6. OCCURRENCE OF ELEVATED TOTAL PROTEIN, GAMMA GLOBULIN AND CELL COUNT IN CSF OF PATIENTS IN VARIOUS DIAGNOSTIC GROUPS.

Diagnostic Group (No. of Patients)	Total Protein* No. elevated/No. tested	Gamma globulin** No. elevated / No. tested	Cell count*** No. elevated / No. tested
Clinically definite MS (13)	3/9	4/6	2/9
Probable MS (13)	4/11	4/8	2/9
Chronic CNS infections (11)	4/5	3/4	2/3
Other chronic inflammatory disease of the CNS (15)	10/12	4/7	3/11
Acute CNS infection (3)	3/3	0/0	3/3
Chronic non-inflammatory disease of the CNS (20)	8/18	3/11	1/15

* upper limit = .45 gm/L

** upper limit = 12 per cent of total protein

*** upper limit = 5 WBC/ cu mm

VI. Comparison of CSF findings in patient groups.

The entire study group consisted of 75 patients with various disorders of the CNS. These patients were classified into various diagnostic groups as listed in Table 3.

A. Clinically definite MS.

The IgG patterns obtained by agarose electrophoresis and isoelectric focusing in the CSF and serum of a patient with clinically definite MS is shown in Figure 4. Agarose electrophoresis revealed discrete oligoclonal bands of IgG, cathodal to the sample application point, only in the CSF. The isoelectric focusing pattern also displayed conspicuous bands cathodal to the sample application point, at pH 6.2-6.5. However, high alkaline bands in the pH range of 8.6-9.5 were seen only in the CSF sample. Of a total of 13 patients 12 showed high alkaline bands with isoelectric focusing. Agarose electrophoresis demonstrated oligoclonal IgG bands in 11 of these patients and an atypical pattern in one. The patterns seen in the latter case are illustrated in Figure 5. Three of the thirteen patients had an elevated CSF total protein level with the highest at 1.32 gm/L. This patient also had an elevated cell count of 12 WBC and a gamma globulin level of 19.1 per cent of the total protein. Both agarose electrophoresis and isoelectric focusing displayed positive results in the CSF and serum samples from this patient. One other patient had an elevated cell count of 13 WBC, whereas three patients had increased gamma globulin values with the highest at 16.1

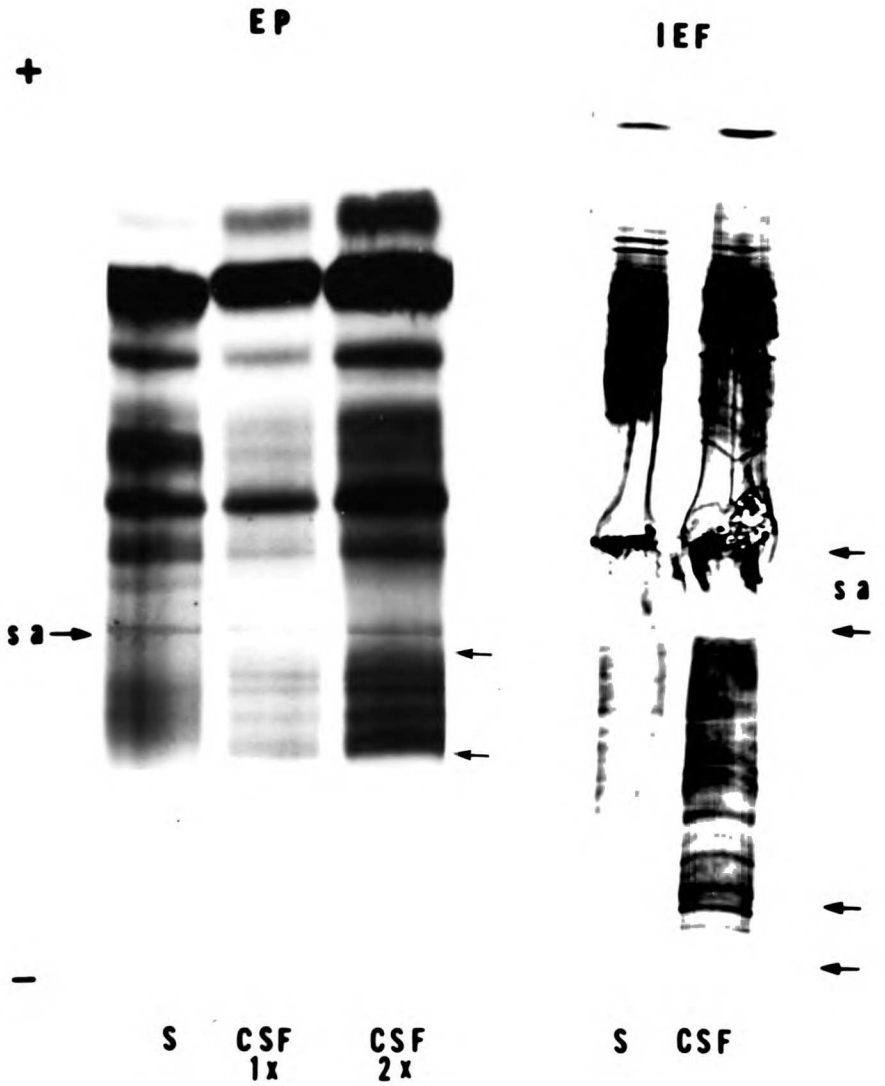

Figure 4. Agarose electrophoresis (EP) and Isoelectric Focusing (IEF) patterns of CSF and serum (S) samples from a patient with clinically definite MS. Arrows show the IgG band patterns. s.a. represents the sample application point.

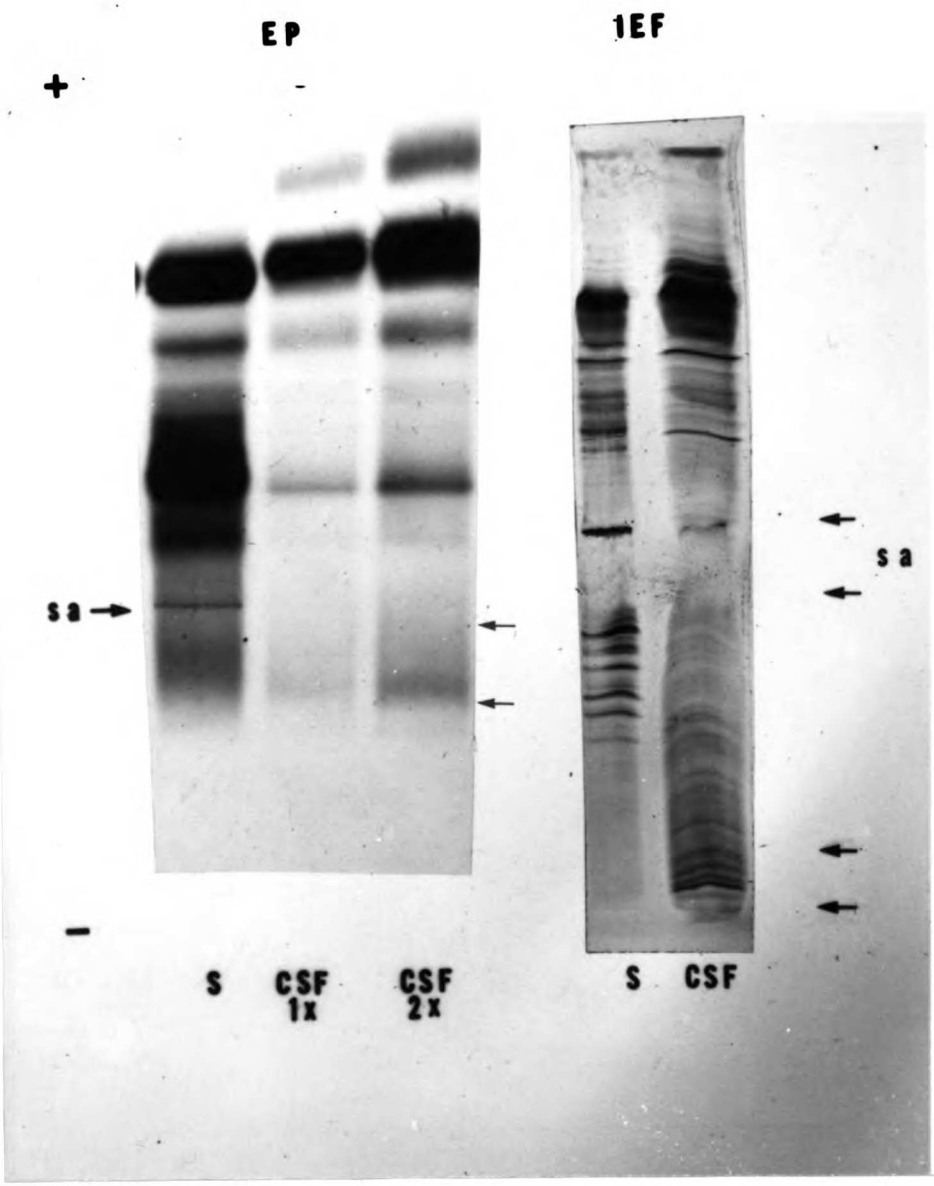

Figure 5: Agarose electrophoresis (EP) and Isoelectric Focusing (IEF) patterns of CSF and serum (S) samples from a patient with clinically definite MS. Note the lack of well-defined bands by EP and the presence of "high alkaline bands" by IEF. (see arrows). s.a. represents the sample application point.

per cent of the total protein.

B. Probable MS.

Seven of 13 patients with probable MS had high alkaline bands by isoelectric focusing. Of these seven cases, five had oligoclonal IgG bands. One was negative and one showed an atypical pattern by agarose electrophoresis. One patient with positive electrophoretic results, and another with an atypical pattern and an increased total protein level (0.54 gm/L), did not display "high alkaline fractions" on isoelectric focusing. Of six of the patients with positive results, two had elevated total protein levels (0.47 and 0.69 gm/L respectively). One of these patients had a normal gamma globulin level and an increased cell count (22 mononuclear cells/cu.mm.), whereas the other had an elevated gamma globulin of 14 per cent and a normal cell count. Two patients with total protein levels within the normal limits had elevated gamma globulin fractions (16 per cent in both cases). These patients showed positive findings by both methods.

C. Chronic CNS Infections

In this group of 11 patients, ten had positive results by the two methods (Table 3). The only patient with negative results by both methods had HSV Type II meningitis. The total protein for this patient was 0.68 gm/L and the cell count was greatly increased (540 WBC, 99 per cent of which were mononuclear cells). The gamma globulin level was not determined.

Seven of the positive patients had SSPE, two of which displayed high alkaline fractions in both the serum and CSF samples. Most of the

SSPE samples were sent from Mexico and unfortunately their CSF profiles were incomplete. Figure 6 illustrates the agarose electrophoresis and isoelectric focusing patterns from one patient with SSPE. One of these patients had a normal total protein level with an increased gamma globulin fraction of 23.1 per cent and a normal cell count. Two patients with meningitis, one fungal, the other bacterial, showed positive findings. Both patients had elevated total protein levels (1.13 and 0.98 gm/L) and gamma globulin fractions (33.6 and 29 per cent), respectively. The cell count was only available for the patient with fungal meningitis and was increased (158 WBC, 80 per cent of which were mononuclear cells). One patient with neurosyphilis had an increased total protein level with a normal gamma globulin fraction and positive electrophoretic and isoelectric focusing results. These patterns are shown in Figure 7.

D. Other Chronic Inflammatory Diseases of the CNS.

The diagnoses and results of patients in this group are shown in Table 7. Positive results by both agarose electrophoresis and isoelectric focusing were obtained in six of 15 patients in this group. One patient with chronic meningeal irritation of unknown etiology displayed high alkaline fractions in both the serum and CSF samples, by isoelectric focusing. However, the agarose electrophoresis results were negative. In this patient, the CSF total protein was highly elevated (2.95 gm/L), with an increased gamma globulin fraction, and the cell count was also increased (35 mononuclear cells/cu.mm). In this patient unconcentrated

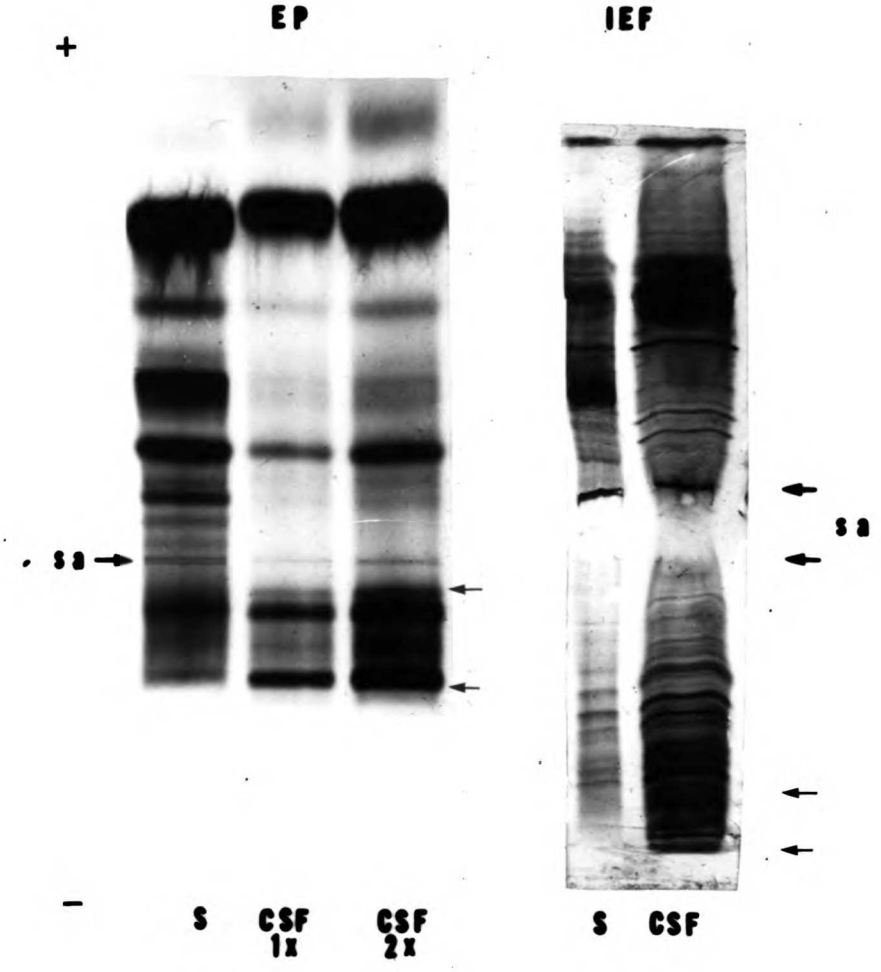

Figure 6. Typical Agarose electrophoresis and Isoelectric Focusing (IEF) patterns of the CSF and serum (S) samples from a patient with SSPE. s.a. represents the sample application point.

Figure 7. Agarose electrophoresis (EP) and Isoelectric Focusing (IEF) patterns of the CSF and serum (S) samples from a patient with neurosyphilis. Note the bands by both methods (see arrows) and similar patterns in the serum and CSF by IEF. s.a. represents the sample application point.

TABLE 7: RESULTS AND DIAGNOSES OF PATIENTS WITH CHRONIC INFLAMMATORY DISEASE OF THE CNS OTHER THAN MS AND CHRONIC INFECTIONS.

Diagnosis	Isoelectric Focusing	Agarose Electrophoresis	
	No. with high alkaline bands	No. with oligoclonal bands	No. with atypical results
Perineoplastic CNS syndrome (1)	1	1	--
Probable poliomyelitis syndrome (1)	0	0	0
Post-encephalitic sequelae (1)	1	1	
Carcinomatous meningitis (1)	1	1	
Chronic inflammatory polyneuropathy (1)	0		1
Cerebral vasculitis (1)	0		1
Granulomatous angiitis (1)	1	1	
Behcet's disease (2)	0	0	1
Lupus sclerosis (1)	1	1	
Chronic meningeal irritation of unknown etiology (1)	1	0	0
Guillain-Barré syndrome (3)	1	0	0
Unknown diagnosis (1)	1	1	

( ) number of patients.

CSF was used for isoelectric focusing, yet the abnormal pattern was readily noted. Two patients with positive results by both methods had increased total protein levels (0.65 and 0.49 gm/L) with elevated gamma globulin fractions (37.7 and 33 per cent) respectively. The cell count was normal in both cases. The diagnosis of the first patient was inconclusive, whereas the second patient presented with lupus sclerosis. Another patient with positive findings by both methods had a normal total protein level, with an increased gamma globulin fraction of 15 per cent, and an increased cell count of 19 WBC. The diagnosis in this patient was a perineoplastic syndrome. In one patient with Behcet's disease, both isoelectric focusing and agarose electrophoresis results were negative, whereas the total protein was increased (0.55 gm/L) with a normal gamma globulin fraction. The cell count was also increased (250 WBC, of which 66 per cent were mononuclear cells). Another patient with Behcet's disease as well as two other patients, one with cerebral vasculitis and one with polyneuropathy, showed atypical electrophoresis patterns. The isoelectric focusing results in both of these patients were negative. Three patients with Guillain Barré syndrome, and one with probable poliomyelitis, had increased total protein levels with increased gamma globulin fractions and normal cell counts. The findings in these patients were negative by both methods with the exception of one patient with Guillain Barré syndrome who displayed positive results only by isoelectric focusing.

E. Chronic Non-Inflammatory Disease of the CNS.

None of the 20 patients in this group displayed high alkaline fractions by isoelectric focusing. Two patients, one with amyotrophic lateral sclerosis (ALS) and one with myelopathy showed atypical agarose electrophoretic patterns whereas oligoclonal bands were seen in one case with peripheral neuropathy. In the patient with ALS only the total protein was increased (0.53 gm/L). The total protein was also increased in one patient with a brainstem glioma (1.04 gm/L), one with cerebellar ataxia (0.53 gm/L), one with idiopathic cerebellar degeneration (0.60 gm/L), one with metachromatic leukodystrophy (1.29 gm/L), and two cases with stroke (0.62 and 0.54 gm/L). The gamma globulin level was normal in all these cases whereas only the patient with metachromatic leukodystrophy showed an elevated cell count of 7 WBC. The agarose electrophoresis findings as well as the isoelectric focusing results were negative. One patient (with depression) had negative results, a normal cell count and a normal total protein yet had an increased gamma globulin fraction of 14.5 per cent.

F. Acute CNS Infections

Three patients, one with a CNS infection with propionbacter and subarachnoid hemorrhage, one with E. coli meningitis and another with tuberculous meningitis, had highly elevated total protein levels (range between 2.02-14.04 gm/L) and cell counts (range between 700-1000 WBCs with 69-97% mononuclear cells). The gamma

globulin per cent were not estimated. None of these patients showed positive results on isoelectric focusing, whereas one patient showed an oligoclonal IgG pattern in both the serum and CSF samples on agarose electrophoresis (Table 3). In these cases unconcentrated CSF was used for isoelectric focusing, since the total protein concentration was highly elevated.

DISCUSSION

I. The technique of isoelectric focusing

Isoelectric focusing, a technique of high resolution capacity for the separation of proteins, has been confined to research laboratories for many years. Electrophoretic methods using cellulose acetate, agar and agarose gels are usually employed for the routine clinical analysis of proteins in biological fluids. The importance of these analyses lies in distinguishing a pathological protein pattern from the normal one. Although isoelectric focusing has a much higher resolution capacity than electrophoretic methods, its application to routine diagnostic work has been hindered both by the technical difficulty of creating pH gradients, and the difficulty in interpreting the various protein patterns. The latter has been due largely to the lack of descriptions of normal isoelectric focusing patterns, especially for CSF.

Some studies (28,43,53) have been done to evaluate these patterns. Numerous studies have shown that CSF gamma globulins, in patients with MS and other chronic inflammatory diseases of the CNS, migrate as discrete oligoclonal bands by agarose electrophoresis (17). The application of isoelectric focusing to the study of CSF proteins in MS and other neurological diseases has not been as widespread, but has continued since Delmotte (10) observed abnormal gamma globulin fractions in the CSF from MS patients. However, the procedures employed by these workers (11,24,43) were too laborious and time consuming for routine clinical analysis. Attempts have been made to

adapt isoelectric focusing for routine analysis with the use of commercially available apparatus and reagents, but these procedures were also complicated and time consuming (40, 61).

One of the inherent difficulties in the analysis of CSF proteins has been the necessity to concentrate the proteins. Trotter and co-workers (61) recommended precipitation of the gamma globulins with ammonium sulfate, for the analysis of this fraction in the CSF of MS patients. This cumbersome concentration procedure was found to be unnecessary. In the present study, a concentration technique was chosen after a comparative study was carried out on three routine methods. With each technique the per cent recovery for IgG and total protein was comparable, indicating that all fractions were lost equally, probably by non-specific adsorption onto the membrane of the concentrator. Although the MINICON CS-15 concentrator (Amicon Corporation, Lexington, MA 02173) gave the lowest recovery for both the total protein and IgG, it was the method of choice for the following reasons. First, it was the only concentrator which was precalibrated for the final concentration required. Secondly, many samples may be concentrated simultaneously, and finally, it was disposable, thus minimizing the risks involved in handling contaminated material.

Another inconvenience in the technique of isoelectric focusing was the long interval between the beginning of the test and the availability of results. In one study (29), this period was approximately three days, however, with the modified staining procedure used in the present study, results may be available within twenty-four hours of sample delivery to the laboratory.

Other improvements which may simplify the operation and enhance resolution are still required such as in the method of sample application. One author (43) observed the best patterns when the samples were applied in basins molded into the gel. However, this method is impractical with the use of commercially available gels. In addition, a more senstive method of detection would allow the use of unconcentrated CSF, thus eliminating the concentration procedure as well as decreasing the sample volume requirement. Finally, differentiation of the IgG patterns between MS and other chronic inflammatory diseases may be feasible if a narrower pH gradient, e.g. pH 7.0-11.0, is used for isoelectric focusing. Such a gradient will greatly increase the resolution of the fractions, enabling better discrimination and densitometric evaluation of the patterns. As a result, greater specificity may be obtained. However, polyacrylamide gels in this pH range are not commercially available at present because of rapid deterioration of these gels on storage (H. Oemke, LKB Instrument Inc., personal communication).

II. Characterization of the protein patterns

Isoelectric focusing yielded several discrete bands of proteins, in the pH range of 3.5-9.5, in CSF and serum samples. The protein of major interest in the CSF of MS patients has been the immunoglobulin, IgG (58). This study, therefore, dealt with the localization and characterization of the patterns of the IgG fractions. An immunofixation experiment with anti-human IgG antiserum was carried out to locate the IgG fractions, and at the same time, verify the presence or

absence of "high alkaline IgG bands".

It was observed that IgG migrated both anodally and cathodally to the sample application point at pH 6.2-6.5. Since it was the dominant protein in the cathodal region, the patterns of the IgG fractions in only this region were studied. The pIs of the IgG fractions in the negative control serum and CSF samples as well as many of the samples from the control patients were between pH 6.5-8.6. IgG fractions in the CSF from MS patients migrated in the high alkaline region with pIs greater than 8.6 and segregated into discrete populations termed "high alkaline bands". These observations were in agreement with those made in other investigations (11,24,28,43); however, the pH range in which these fractions segregated varied among the authors. This discrepancy may have resulted from variations in conditions under which pH measurements were made. Further characterization of these IgG bands is necessary since they may represent subclasses of IgG or fragments of the IgG molecule. It should be noted that one non-immunoglobulin, γ - trace protein, also migrated in the high alkaline region with a pI of 9.5. This finding was also observed by other investigators (28,43). No IgG fractions migrated with pIs greater than that of the γ - trace protein. Hence, the presence of high alkaline IgG bands in the pH range of 8.6-9.5 was defined as positive.

The origin of these IgG fractions is still obscure, and it has been shown that intracellular oligoclonal IgG appeared as a single band on isoelectric focusing, but that secreted IgG (e.g. the IgG in serum or CSF) may resolve into several components as a result of

post synthetic charge alterations (40). Further studies on the mechanism underlying the production and the pathological significance of these IgG fractions are required. Also determination of their antigenic specificity is of great interest.

Characterization of the IgG patterns in the various neurological disorders was not attempted in this study. Previous attempts by some investigators (24,29,40) failed to manifest discrimination between these patterns. In most cases there was an overlap of the patterns, thus the interpretation of the results was difficult. As had been mentioned earlier, the use of a gradient in the pH range of 7.0-11.0 may enable better discrimination of the IgG patterns.

Another aberrant fraction was observed only in CSF from patients with various neurological disorders including MS. This fraction appeared as a double fraction with a pI of 5.9, the region where tau-transferrin, a normal CSF protein usually migrated as a single band. The significance of the occurrence of this fraction is still obscure, although it has been observed in other studies (22,39,53). In one of these studies (53) two healthy individuals displayed these fractions in their CSF. Other workers (22) observed that this fraction was frequently seen in patients with degenerative and demyelinating disorders. They concluded that the occurrence was associated with altered myelin metabolism, and that the bands were decomposition products from destroyed CNS tissue. However, this fraction has been identified by crossed immunoelectrofocusing as a double fraction of tau-transferrin (53). Another study (39) of CSF proteins in patients with neurological syndromes resulting from

various etiologies, but accompanied by severe atrophic brain damage, showed that 14 of 31 patients displayed this fraction. The highest frequency (45%) occurred in patients with cortical atrophy, in contrast to a low frequency (14%) in patients with multiple sclerosis. Further studies on the significance and occurrence of this double tau-transferrin fraction are needed.

III. Interpretation of the IgG patterns in the various patient groups.

Ninety two percent of the MS cases displayed high alkaline IgG bands in the CSF, and in one case, in serum. This finding is in agreement with other studies (7,9,11,22,24,29,40,43,61), some of which showed the occurrence of these bands in all the MS cases (10,40). The populations under study in most instances were small. However, one investigator observed these findings in 92% of 262 MS cases. The number of the high alkaline bands varied among the patients, as has been observed by other authors (11,22,40), but no definite correlation between the number and the pIs of these bands, and the clinical parameters of the disease has been established. Kjellin and Siden (22) graded the number of these bands and compared it with the duration of the disease. They found that discrete bands were more frequently observed in subjects with a 1-10 year duration of disease, and less frequently in patients with either shorter or longer durations. The intensity of the bands also varied among patients. In many of the CSF samples with high gamma globulin levels, from patients with clinically definite MS, dense bands of IgG were observed throughout the pH range of 6.5-9.5. This observation was supported by that of

Kjellin and Siden (22) that the CSF of patients with clinically verified MS displayed conspicuous bands throughout the gamma globulin region but especially in the pH range of 6.4-8.9. They suggested that the different patterns in MS patients may indicate different rates of production or elimination of certain immunoglobulins, probably caused by different viral agents and/or autoimmune reactions, or that there are selective barrier defects for different immunoglobulins.

The CSF and, in some cases, the serum samples from patients with other chronic inflammatory CNS diseases and infection also displayed "high alkaline fractions" of IgG. Other studies support this finding (7,11,24,29). The presence of these bands, like the presence of oligoclonal bands of IgG by agarose electrophoresis, is probably an indication of chronic neural inflammation with a local production of gamma globulins, as suggested by one author (17). Oligoclonal IgG in the high alkaline region was also observed in the serum of one patient with MS and three patients with other chronic inflammatory CNS diseases. Other studies (11,29) showed this finding in higher frequencies, and it has been suggested to be the result of the diffusion of IgG from CSF to serum (24). In this study, all the patients who displayed these fractions in their serum samples had elevated gamma globulin levels in the CSF. The total protein levels in these patients were increased, except for one patient where it was within the normal limits. This latter case may support the suggestion made by these authors (29).

The CSF and serum samples from patients with chronic non-inflammatory diseases of the CNS did not display high alkaline IgG fractions. In some patients conspicuous bands were observed in the pH range of less than 8.6. A low incidence of positive results in such patients has also been reported in the literature (7,11,24,29, 40,61). Kjellin and Vesterberg (24) defined an "f" pattern in these, and other patients with blood-brain barrier defects, in which the IgG fractions showed a uniform increase similar to a "finger-print" of the corresponding serum gamma globulin pattern. The pH region in which this pattern appeared was defined in a later study (22) as pH 6.4-8.9, whereas the high alkaline fractions were seen in the range of pH 8.2-11.0. This "f" pattern corresponded to the patterns seen in cases of cases non-chronic inflammatory CNS diseases, and may indicate blood-brain barrier damage especially in those patients with elevated total protein levels in their CSF.

IV. Comparison of agarose electrophoresis and isoelectric focusing

On comparison of the results obtained by isoelectric focusing with agarose electrophoresis, similar frequencies of positive results were observed in all the diagnostic groups studied. In addition, both methods showed similar frequencies of positive results in cases where other CSF assays were within the normal limits viz. the total protein level, the gamma globulin levels and the cell count. Thus the increased sensitivity of the two methods over the more traditional assays is comparable. In some cases, especially of the chronic inflammatory group, agarose electrophoresis

revealed atypical oligoclonal bands difficult to interpret in the CSF. Isoelectric focusingin two of these cases, one with clinically definite MS and another with probable MS, displayed "high alkaline fractions" in the CSF. Another patient with probable MS had positive results only by isoelectric focusing.

These findings indicate that isoelectric focusing is a more sensitive technique than agarose electrophoresis for the laboratory diagnosis of MS. In addition, a higher specificity is indicated by the lower incidence of positive results in cases with acute CNS infections and chronic non-inflammatory diseases. These conclusions have been supported by other workers (11,29,61).

Negative aspects of isoelectric focusing are that the procedure still involves concentration of CSF samples and visualization of IgG pattern. It is more time consuming and requires a more meticulous technique than agarose electrophoresis. Both techniques involve the use of commercially available reagents and apparatus, and costs and reproducibility are similar. The latter depends to a large extent on the manufacturer's quality control of the reagents and gels which to date have been less than ideal for agarose electrophoresis.

In conclusion, the technique of isoelectric focusing may be used for routine clinical analysis of CSF proteins; however, it offers no major advantages to warrant its replacement of agarose electrophoresis as the routine diagnostic test for MS. In cases where questionable results arise by the latter method, such as those with atypical IgG patterns, isoelectric focusing is advantageous.

SUMMARY

The technique of isoelectric focusing has been adapted for routine clinical analysis of CSF proteins with the use of commercially available apparatus and reagents. Besides analyzing CSF and serum samples from patients with clinically verified MS and probable MS, samples from patients with various other neurological diseases were studied. These included patients with SSPE, Guillain Barré syndrome, neurosyphilis, Behcet's disease, stroke, cerebral tumors, chronic meningitis of fungal, viral and bacterial etiologies, acute CNS infections, and other chronic inflammatory and non-inflammatory diseases. On comparison of these results with those obtained by agarose electrophoresis, the routine diagnostic laboratory tests for MS, a similar frequency of positive results was observed. In some cases a more well-defined pattern was seen by isoelectric focusing. Despite an increased sensitivity and specificity, the complexity of the technique of isoelectric focusing does not warrant its replacement of agarose electrophoresis as the routine diagnostic laboratory test for MS. However, it should be incorporated as a special laboratory test for clinically difficult cases where the results by agarose electrophoresis are questionable.

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