Clinical and Analytical Evaluation of an Enzyme Immunoassay for Myelin Basic Protein in Cerebrospinal Fluid

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Background: RIA of myelin basic protein (MBP) in cerebrospinal fluid (CSF) is commonly used as a biochemical marker of demyelination in patients with multiple sclerosis (MS). Our aim was to develop a sufficiently sensitive ELISA for MBP and evaluate it clinically in patients with MS.

Methods: The ELISA used anti-bovine MBP antibody coated on plates and biotinylated anti-MBP antibody. The bound antibody complex was quantified with streptavidin-horseradish peroxidase. MBP was determined in CSF from 84 MS patients and 55 patients with other neurological diseases.

Results: The respective within- and between-assay CVs were 4.7% and 7.2% at 200 ng/L, and 6.3% and 8.8% at 2000 ng/L. The detection limit was 30 ng/L. Most of the MS patients with acute exacerbations had markedly increased MBP in the CSF. Longitudinal studies of six MS patients with recurrent exacerbation confirmed this observation. MBP concentrations from 78 MS patients, as tested with our ELISA, correlated well with those obtained by RIA (r = 0.9; P < 0.01), but the detection limit of the ELISA was much lower than that of the RIA.

Conclusions: This convenient ELISA with higher sensitivity than the existing assays is a suitable routine assay that provides a diagnostic indicator of myelin breakdown in the central nervous system; moreover, it is an excellent indicator of MS disease activity.

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Materials and Methods

HUMAN SUBJECTS

All CSF samples were obtained by lumbar puncture and were stored at −80 °C until assayed. Samples were taken from 84 Japanese MS patients 14–68 years of age (mean age, 40 years), 55 patients 18–64 years of age (mean age, 45 years) with other neurological diseases (12 with chronic cerebrovascular disease, 14 with amyotrophic lateral sclerosis, 12 with Parkinson disease, 5 with polyneuropathy, 4 with Behcet disease, 5 with meningitis, and 3 with Guillain-Barré syndrome), and 45 patients 19–67 years of age (mean age, 43 years) with nonneurological diseases. In MS patients with exacerbations, samples were obtained within 1 week after the onset of acute exacerbation. CSF samples also were collected at various times.

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Multiple sclerosis (MS)5 causes localized demyelination in the central nervous system. Myelin basic protein (MBP) is the major protein component of myelin (1), and the concentration of MBP or its fragment in cerebrospinal fluid (CSF), as measured by a RIA, correlates closely with the clinical activity of MS and other diseases in which there is myelin breakdown (2–8). The clinical importance of the quantitative determination of MBP in CSF as an index of active demyelination has been reviewed (7, 9, 10). We developed a sensitive, specific, and convenient ELISA for MBP and measured MBP in CSF from patients with MS and other neurological diseases. We also made a serial study of six MS patients for a period of 6 to 30 months to clarify the correlation of the clinical course of the disease and the MBP concentration in the CSF. MBP concentrations obtained by this new ELISA were compared with those obtained by the previously developed RIA.
from six individuals with MS who had recurrent exacerbations for longitudinal studies. The study was approved by the ethics committee of the Utano National Hospital.

**Preparation of MBP and Anti-MBP Antiserum**

MBP was isolated from bovine brain as described by Deibler et al. (11). In brief, an acid extract of brain delipidated with chloroform-methanol was chromatographed on carboxymethylcellulose, pH 10.4, after which the MBP fractions were eluted with a linear salt gradient. In a typical preparation, ~300 mg of MBP was isolated from 500 g of brain tissue. Protein homogeneity for the single band with a molecular mass of 18 kDa was determined by polyacrylamide gel electrophoresis at pH 4.0 (6). Amino acid analysis of the protein showed that its composition corresponded to that of the A1 protein reported by Eylar et al. (12).

Anti-MBP antisera were raised in Japanese White rabbits, using a schedule that favored the induction of specific anti-MBP antiserum without producing experimental allergic encephalomyelitis (13). Briefly, 100 \( \mu \)g of MBP in saline was emulsified with Freund’s incomplete adjuvant (saline:adjuvant, 2:3, by volume), and then injected subcutaneously at six sites on the back of a rabbit; three booster injections were given at 2-week intervals. MBP (100 \( \mu \)g) emulsified with an equal volume of Freund’s complete adjuvant was then injected once a week for 8 weeks. After the final injection, the rabbits were bled, and the antisera were prepared.

The anti-MBP antibody IgG fraction prepared by chromatography on protein A-Sepharose CL-6B (Pharmacia Fine Chemicals) was further purified by affinity chromatography on an MBP-Sepharose 4B column.

**Preparation of Biotinylated Anti-MBP Antibody**

Affinity-purified anti-MBP antibody IgG was biotinylated with 5-(N-succinimidyl-oxycarbonyl)pentyl-d-biotinamide (Dojindo) (14).

**Two-Site ELISA for MBP**

A microtiter plate coated with 100 \( \mu \)L of anti-bovine MBP IgG (3 mg/L) diluted with 10 mmol/L carbonate buffer, pH 9.3, was incubated for 1 h at room temperature. After the plate had been washed twice, the unoccupied protein binding sites in each well were blocked for 1 h at room temperature with 200 \( \mu \)L of 10 mmol/L carbonate buffer, pH 9.3, containing 5 g/L bovine serum albumin. One hundred microliters of a MBP calibrator or CSF sample was added to the wells, and the plate was incubated for 1 h at room temperature. After another washing with washing buffer (50 mmol/L Tris-HCl buffer, pH 7.0, containing 0.2 mol/L NaCl, 10 mmol/L CaCl\(_2\), and 1 mL/L Triton X-100), 100 \( \mu \)L of biotinylated anti-MBP antibody (20 \( \mu \)g/L) was added, and the plate was incubated for 1 h at room temperature. After three more washings, the plate was incubated for 1 h at room temperature with 100 \( \mu \)L of streptavidin-horseradish peroxidase (Amdex) diluted 10 000-fold. After three final washings, the plate was allowed to react for 15 min at room temperature with 100 \( \mu \)L of a substrate solution of 3,3',5,5'-tetramethylbenzidine and H\(_2\)O\(_2\) (Kirkegaard & Perry Laboratories). The reaction was stopped by the addition of 100 \( \mu \)L of 1 mol/L phosphoric acid, after which the absorbance at 450 nm was recorded with an ELISA reader (Labsystems). All washings and incubations were done with gentle shaking.

**Fig. 1. Calibration curve for the MBP ELISA.**

There is a quantitative relationship for human MBP at concentrations of 30–2000 ng/L.

**Fig. 2. CSF MBP in 84 patients with MS (52 patients with acute exacerbation, 32 in remission or the clinically inactive state), 55 patients with other neurological diseases, and 16 with nonneurological diseases.**

There are significant differences between the active MS and any other groups (\( P < 0.001 \)). The dashed line shows the cutoff for MBP.
RIA for MBP
MBPs were measured by a double-antibody RIA described previously (6).

Statistical Analysis
The following methods were used for the statistical analysis: regression analysis for the evaluation of calibration curve; a plot for assessment of the agreement between two methods (15); and the Kruskal–Wallis nonparametric ANOVA. P <0.05 was considered statistically significant.

Results
The calibration curve showed a quantitative relationship for human MBP at concentrations of 30–2000 ng/L (Fig. 1). The within- and between-assay CVs were 4.7% and 7.2% (n = 10) at 200 ng/L and 6.3% and 8.8% (n = 5) at 2000 ng/L.

We assayed CSF samples from 84 patients with MS, 55 patients with other neurological diseases, and 45 patients with nonneurological diseases (Fig. 2). The mean CSF MBP concentration in the patients with nonneurological diseases was 54 ± 24 ng/L (mean ± SD). A MBP concentration >102 ng/L (mean + 2 SD) was considered positive. Of the 55 patients with neurological diseases exclusive of MS, 5 had CSF MBP concentrations above these reference limits: 1 each with chronic vascular disease (110 ng/L), amyotrophic lateral sclerosis (310 ng/L), Parkinson disease (250 ng/L), polyneuropathy (210 ng/L) and meningitis (1000 ng/L).

Patients with MS were divided into two groups composed of 52 who suffered acute exacerbations and 32 in remission or the clinically inactive state. The active-disease group had a markedly increased mean CSF MBP concentration (3076 ng/L) and a high frequency of increased results (81%), whereas most of the inactive MS patients had MBP concentrations that were not increased (mean, 89 ng/L; frequency of increased values, 19%).

To clarify the relationship between changes in CSF MBP and the clinical states of MS, we also measured CSF MBP concentrations in serial samples taken from six individuals with recurrent exacerbation before, during, and after attacks over periods ranging from 6 to 30 months (Fig. 3, case numbers 1–6). CSF MBP values were undetectable or low in most samples obtained before a MS attack, with the value rising rapidly to 500–8000 ng/L at the height of the event. After an acute attack, the CSF MBP decreased rapidly, and within 4 weeks after the exacerbation most patients had CSF MBP values within or below the reference limits. Follow-up studies showed that the maximum MBP concentrations in CSF occurred during the most acute phase of the disease. Thus, good agree-

Days of Illness

Fig. 3. Changes in CSF MBP concentrations in six MS patients with recurrent exacerbation. CSF samples were collected over a period of 6–30 months before, during, and after acute exacerbation. The dashed line shows the cutoff for MBP. Arrows indicate acute relapse.
ment was found between the MBP concentration and clinical symptoms in these six patients.

We compared the results obtained by ELISA and the previously developed RIA for 78 MS samples; MBP was detected in 67% by the ELISA and in 49% by the RIA. There was a good correlation between the CSF MBP concentrations measured by ELISA and RIA ($r = 0.90; P < 0.01$). Fig. 4 shows the mean of the results obtained by the two methods vs the difference between the results obtained by the two methods. The RIA gave higher values than ELISA at low concentrations and lower values than ELISA at high concentrations.

**Discussion**

From an immunochemical point of view, MBP concentrations measured in CSF represent MBP-like materials, not necessarily genuine MBP ($10$). RIA has been used most frequently to measure MBP, largely because of the need for extremely high sensitivity. We previously developed a RIA system for measuring MBP in the CSF as a diagnostic indicator of myelin breakdown in the central nervous system ($6, 16$). The lowest MBP concentration detected by our RIA system in CSF was $\sim 0.5 \mu g/L$, a detection limit similar to that of the existing RIA ($2–9$).

We now have successfully established a sensitive, specific sandwich ELISA for MBP that uses a polyclonal anti-MBP antibody. This sensitive, two-site ELISA system, based on a biotin-streptavidin system capable of measuring a concentration as low as 30 ng/L, gives a genuine, immunologically more highly specific reaction than the RIA. The dilution curves obtained by serially diluting MBP-like material in CSF and purified MBP were parallel.

Our RIA is a competitive assay between $^{125}$I-labeled MBP and CSF MBP. In this assay system, protein concentrations influence the RIA values. Therefore, the RIA gave higher values than the ELISA at low concentrations as shown in Fig. 4. Protein concentrations do not influence the ELISA results. The major advantages of our ELISA are that it does not use radioactive chemicals and that its sensitivity and specificity are much better than the existing RIA. This new assay is simple to perform, requires only a small sample volume, does not require pretreatment of samples before assay, and is highly reproducible. Moreover, all of the required reagents are stable indefinitely.

Increased MBP concentrations have rarely been detected in the CSF of patients with a wide variety of neurological diseases: leukodystrophies, severe anoxia, myelopathies, and encephalopathies caused by irradiation or chemotherapy ($5, 17$); the acute active state in patients with myelopathy, cerebrovascular, and neuro-Behcet diseases ($6, 16$); and brain tumors ($18$). We, however, found increased MBP in the CSF of five patients; one each with chronic cerebrovascular disease, amyotrophic lateral sclerosis, Parkinson disease, polyneuropathy, and meningitis. The RIA and ELISA for MBP in CSF, therefore, are useful for making a clinical assessment of the various neurological diseases in which myelin breaks down acutely. van Engelen et al. ($19$) reported that the concentration of MBP in CSF increases with age. Therefore, we used age-matched references.

The study reported here was performed on MS patients to clarify the relation of clinical status to active myelin destruction by monitoring the MBP in the CSF at sequential times during the course of the disease. Longitudinal studies of individual patients showed a strict temporal relationship between the presence of MBP in the CSF and the peak of the disease. Severe symptoms and dramatic clinical states, however, always were accompanied by the release of MBP into the CSF. Detection of MBP in the CSF should be considered indicative of myelin destruction. Most patients who had increased MBP concentrations in their CSF during the acute phase of disease seemed to be in remission several months later.

Antigenic material that cross-reacts with MBP, or a peptide thereof, may be present in the blood of individuals who have had recent injury to the myelin in the central nervous system. Palfreyman et al. ($20$) detected MBP in sera from patients with head injury. Jacque et al. ($17$) infrequently found MBP in sera from cephalitic patients. Indeed, even when the blood-brain barrier has been broken, the dilution effect caused by the larger blood volume lowers the MBP concentration below the detection limit of most assays. MBP is also a minor component of peripheral nervous systems.

Our new ELISA for MBP in serum samples does not require pretreatment of samples to remove the interfering material usually present in serum. Serum MBP could be determined at concentrations $>30$ ng/L. In unpublished studies, we examined MBP concentrations in the CSF and matched serum samples from 15 MS patients who had acute exacerbation. Increased MBP concentrations were
detected in all CSF samples tested, whereas in serum, MBP was found in only three patients, and at very low concentrations (180, 389, and 200 ng/L). Most serum MBP was not detectable, even in the patients with the highest CSF MBP concentrations. MBP therefore could be detected only rarely in the serum and at low concentrations. The presence of serum MBP does not necessarily correlate with the highest concentration of MBP in the CSF.

Several magnetic resonance imaging methods have been developed recently that may provide an objective measure of MS disease activity (21). More attention needs to be paid to MBP in the CSF and serum as a biochemical marker of MS disease activity. This new ELISA is convenient and simple and can be used routine assays for that purpose.

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References