CHAPTER II

MATERIAL AND METHODS

Patients

We obtained serum and/or CSF samples from 16 patients with clinically definite MS, diagnosed according to Poser et al.(31) All had clinical exacerbations (See table 1).

Table 1 Multiple Sclerosis Patients Entered into This Study.

Tatal Subjects	16
Age (yrs)	27-54 (median = 30)
Sex	13 Females, 3 males.
Disease Activity	All with clinical exacerbations
Samples	16 serum, 10 CSF samples

The serum and/or CSF samples from another 113 subjects with other neurologic diseases also were analysed. These included 18 with Guillain-Barre'syndrome,4 with post-infectious encephalomyelitis, 2 with post-vaccinal myelitis, 10 with neuropsychiatric SLE, 3 with myasthenia gravis, 32 with central nervous system infection (9 with viral encephalitis, 6 with rabies encephalitis, 6 with tuberculous meningitis, 1 with tuberculoma, 3 with cryptococcal meningitis, 4

with neurocysticercosis, 2 with gnathostomiasis, 1 with eosinophilic meningitis), 8 with cerebrovascular accident, 6 with spinocerebellar degeneration, 12 with muscle dystrophy, 19 with miscellaneous neurologic diseases (2 with acute transverse myelitis, 2 with subacute compined degeneration of cord, 2 with progressive spastic paraparesis, 1 with tetanus, 1 with plasmacytoma and neuropathy, 1 with benign monoclonal IgA gammopathy with neuropathy, 5 with post-vaccinal headache or dizziness, 2 with brain tumor and 2 with spinal cord tumor and 1 with multiple myeloma with neuropathy). Summary see table 2.

All serum and cerebrospinal fluid samples were stored frozen at -20 °C, until the assays eree done.

Table 2 Patients Entered in This Study.

Neurologic Disease		No. of Subject
Multiple Sclerosis		16
Guillain-Barre' Syndrome	Non-MS	18
Post-Infectious Encephalomyelitis	Immune	4
Post-Vaccinal myelitis	mediated	2
Neuropsychiatric SLE	diseases	10
Myasthenia Gravis	(37 cases)	3
Viral Encephalitis		9
Rabies Encephalitis		6
Tuberculous Meningitis	CNS	6
Tuberculoma	infections	1
Cryptococcal Meningitis	(32 cases)	3
Neurocysticercosis		4
Gnathostomiasis		2
Eosinophilic meningitis		1
CVA	Non-immune,	8
Spinocerebellar Degeneration	Non-infectious	6
Muscle dystrophy	neurological	12
Miscellaneous (see above)	diseases(45 cases)	19
Total subjects		130

Determination of anti-MBP antibodies in sera by ELISA

This was performed and modified according to Irie et al (33) and Tabria et al.(34)

Method :

1. Porcine MBP Ø.4 ug/ml (ascertained from the checkerboard) in phoshate buffered saline (PBS 1x), Ph 7.4, 2ØØ ul are added to each well of a 96-wells ELISA flat-bottomed microtiter plate. Cover the plate tightly with plastic wrap and incubated for 3 hours at 37 °C.

2. Empty the plate and wash three times with PBS-tween

3. A 2ØØ ul amount of 2% bovine serum albumin in PBS 1x, pH 7.4 is added to each well. The plate is incubated at 37 °C for 1 hour, then the contents of the wells are shaken out without washing.

4. Immediately add 2ØØ ul of test sera 1 : 1ØØ dilution in PBS 1x pH 7.4 and 5% chick serum. Two positive control and 8 negative control samples are added to every assay. cover the plate and incubate for 1 hour at 37 °C, then wash as step 2.

5. A 2ØØ ul of freshly diluted HRP-conjugated rabbit anti-human IgG, A, M, antibodies in PBS-Tween at 1:5ØØ is added to each well. The plate is incubated for 1 hour at 37 °C and is again washed as before.

6. Immediately add 200 ul of Ø-phenylene diamine substrate at a concentration of Ø.25 mg/ml in 25 ml substrate buffer and 25 ul of 30% H_2O_2 to each well.

7. Allow the substrate to incubate at room temperature for 30 minutes or until color in reactive wells has reahed the desire intensity.

8. Without adding of stooping solution, the plate is examined spectophotometriaclly at 492 nm. in micro ELISA reader.

9. A threshold cutoff point is determined on the basis of sample values (inform of OD = sample OD = mean OD of negative controls) that above two standard deviations of the mean OD of negative controls are regarded as positive.

ELISA for anti-MBP antibodies in CSF

1. Porcine MBP in PBS 1x, Ph 7.4, 100 ul are added to each well of a 96 wells ELISA plate. Cover the plate tightly with plastic wrap and incubate for 3 hours at 37 °C.

2. Decant the contents and wash the wells three times with PBS-Tween.

3. A 100 ul amount of 2% bovine serum albumin in PBS 1x, pH 7.4 is added to each well and incubate at 37 °C for 1 hour. The contents are then shaken out without washing.

4. Immediately add 100 ul of test cerebrospinal fluid 1:2 dilution in PBS 1x, pH 7.4. Two positive control and 8 negative control samples are added to every assay. The plate is covered and incubated for 1 hour at 37 °C and then washed as before.

5. Immediately add 100 ul of 0-phenylene diamine subtrate at a concentration of 0.25 mg/mL in 25 ml substrate buffer and 25 ul of 30 % H_2O_2 to each well.

6. Determine A_{450} of each well after at least 30 minutes incubation at room temperature.

7. Antibody levels are expressed as the numerical difference in optical diensity between the test samples and coresponding control (OD). Values above 5 standard deviations of the mean OD of negative controls are regarded as positive.

To confirm the specificity of the immune reaction for myelin myeline basic protein, which also binds to the Fc region of IgG,(35,38) serum and CSF sample were absorbed with 2.5 ug/ml of purified myelin basic protein for 1 hour at 37 °C and overnight at room temperature, the samples were then centrifuged 800 g for 1/2 hour. The supernatants were then tested for anti-MBP and showed complete absorption.

Quantitation of Immunoglobulin G (IgG) in Patient Sera by Radial Immunodiffusion (RID), Time-Diffusion Method

1. Preparation of buffered agarose-antiserum plates : 2% agarose in K buffer, pH 8, is boiled until completly melt. A 88 ul of goat anti-human IgG (Kallestad Lot 88007176) with a 2.8 ml of 6% polyethylene glycol. and 2.8 ml of 2% buffered agarese are well mixed at 60 °C. A 1 ml of the mixure is applied to the 6-wells plate (Hyland) and 6 of 5 ul wells are made.

2. Bring all agarose plates, reference sera, control sera (in-house-prepardd pooled normal human sera) and patient sera to room temperature before use.

3. Apply 5 uL of reference sera 1, 2, and 3 to each of three wells, using the volumetric pipette. Apply 5 uL of control serum to a fourth well and 5 uL of each patient serum (1:30 dilution in saline) to remaining wells. For additional plates, at least one reference is need per plate.

4. Carefully replace the lid and place the plate in a miost chamber. Incudate at room temperature for 18 hours.

5. Using the viewer to measure the diameter of the precipitin rings.

6. Construct a standard curve by plotting the concentration of each reference serum on the logarithmic scals against the precipitin ring diameter on the linear scale. Connect the adjacent points.

7. Determine patient sample and the control concentrations by locating the ring diameter onthe standard curve and reading the concentration on the logarithmic scale. Samples whose ring diameters are greater than that of the highest reference must be diluted in saline and reassayed.

Quantitation of Albumin in Patient Sera by Biochemical Assay

The assay is according to the method of Price.(37)

Quantitation of IgG and Albumin in Patient Cerebrospinal Fluid

The Kallestad Endoplate Endoplate IgG-Albumin Cerebrospinal Fluid Test Kit (Lot 87007937, 15 wells plate) was used.

Precedure

1. Remove the Endoplate and reference sera from the refrigerator and equilibrate to room temperature (23 °C ± 2 °C).

2. Mix the reference sera and patient CSF samples thoroughly by inverting them several times.

3. Apply 10 uL of reference 1, 2, 3 into each of three wells. A 10 uL of control serum is added into a fouth well and 10 uL of each patient undiluted CSF sample into the remaining wells.

4. In additional plate of the same lot, a reference serum is included.

5. Replace the lid and return the plate to the ziplock bag. Reseal the bag and incubate at room temperature (23 °C ± 2 °C) on a level surfacd.

6. After a mininum of 24 hours, measure the immunoprecipitin ring diameters by using viewer.

7. Construct a reference curve on linear graph paper. Plot the concentration of each reference serum on the ordinate versus the square of the ring diameter on the abcissa. Draw a best-fit straight line as determined by the reference points.

8. Obtain the concentration of the control and test samples by locating the square of each sample's ring diameter on the reference curve and reading the concentration on the ordiante.

Estimation of Intrathecal IgG Synthesis in 12 Patients with Multiple Sclerosis.

1. Matched CSF and serum samples were obtained from 12 multiple sclerosis patients with exacerbations.

2. IgG and albumin levels were measured by biochemical methods. (Methods in detial, see above)

3. Intrathecal IgG Synthesis was estimated by CSF-IgG/CSF-Albumin ratio, the Link-Tabbling IgG index (38) and Tourtellottes empirical formula for daily rate of IgG synthesis(19).

4. Values that indicate intrathecal IgG synthesis :

(a) IgG_c / Alb_c ratio, a value greater than Ø.25 ;

(b) IgG index = $\frac{\text{CSF IgG/CSF albumin}}{\text{(serum IgG/serum albumin)}}$ > Ø.77 (39)

(c) IgG Synthesis (mg/day) =

$$\left[(IgG_c - IgG_s) - (AlB_c - Alb_s) (IgG_s) Ø.43 \right] X 5$$

$$\frac{}{369} \qquad \frac{}{230} \qquad \frac{}{Alb_s}$$

in which subscrip "c" indicates cerebrospinal fluid, subscript "s" indicates serum,"Alb" is albumin, and the Alb and IgG values are concentrations in mg/dL. The rate that reater that 3 mg/day (4Ø) regarded as positive intrathecal synthesis.