THE DEVELOPMENT OF AN IMMUNOASSAY TO MEASURE MYELIN BASIC PROTEIN AUTOANTIBODIES IN HUMAN BLOOD AND THE DETERMINATION OF ITS DIAGNOSTIC VALUE IN MULTIPLE SCLEROSIS

By

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A thesis submitted in conformity with the requirements For the degree of Master of Science Department of Laboratory Medicine and Pathobiology Faculty of Medicine, University of Toronto

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Finally, my eternal thanks to Konstanty Lorenc, who taught me courage and perseverance, by example.

I wish to dedicate this thesis to the many generous adults and children with MS who gave of themselves for this study.
Multiple Sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) with possible autoimmune aetiology. Although the autoantigen responsible for MS has not been conclusively identified, myelin basic protein (MBP) has been proposed as a candidate autoantigen.

Antibodies against MBP were detected in the cerebral spinal fluid (CSF) of MS patients and the antibody levels correlated with active MS disease status. To date, both the presence of MBP autoantibodies and their clinical utility in MS is controversial.

In order to address this issue, we developed a serum/plasma assay, hence avoiding the invasive technique of lumbar puncture required for CSF collection. Using plasma from 94 patients with clinically definite MS, we have achieved a clinical sensitivity of 77% and a specificity of 95% through the measurement of circulating MBP autoantibodies, alone. This simple, rapid and non-invasive assay offers a tool for the diagnosis and monitoring of the progression of MS.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xi</td>
</tr>
</tbody>
</table>

## CHAPTER ONE

### INTRODUCTION

A. Multiple Sclerosis

<table>
<thead>
<tr>
<th>I</th>
<th>Epidemiology, Genetics and Immunology</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Epidemiology</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>(ii) Genetics</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>(iii) Immunology</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II</th>
<th>Diagnosis of Multiple Sclerosis</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Diagnostic Criteria</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>(ii) Clinical Features</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>(iii) Treatment</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

B. Pathogenesis of Multiple Sclerosis

<table>
<thead>
<tr>
<th>I</th>
<th>Myelin</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>The Blood-Brain Barrier</td>
<td>15</td>
</tr>
<tr>
<td>III</td>
<td>The Glia and Microglia</td>
<td>16</td>
</tr>
<tr>
<td>IV</td>
<td>Autoimmune Pathogenesis</td>
<td>16</td>
</tr>
</tbody>
</table>

| (i) T cell Autoimmunity in MS | 17 |
| (ii) B cell Autoimmunity in MS | 19 |
C. Candidate Autoantigens and Biochemical Markers of MS

I. Autoantigens of myelin and oligodendrocyte origin

(i) Myelin Basic Protein
(ii) Proteolipid Protein
(iii) Myelin associated glycoprotein and Myelin/oligodendrocyte glycoprotein

II. Non-myelin Antigens

(i) S-100B
(ii) αB Crystallin
(iii) Neuron Specific Enolase
(iv) Thrombomodulin

D. MBP Autoantibodies and their Diagnostic Value in MS

E. Experimental Objectives

CHAPTER TWO

MATERIALS AND METHODS

A. Materials

I. Blood Collection

II. MBP Autoantibody ELISA

B. Methods

I. Sample Size Justification

II. Patient Selection and Inclusion Criteria

(i) MS Patients
(ii) Other Autoimmune Disease Controls
(iii) Normal Healthy Controls

III. Blood Collection and Analysis

IV. MBP Autoantibody ELISA

(i) Microtiter Plate Preparation
(ii) ELISA Protocol
CHAPTER THREE

MBP AUTOANTIBODY ELISA DEVELOPMENT AND ASSAY PERFORMANCE

A. Assay Optimization

I Coating Optimization

(i) Microtitre Plate Selection
(ii) Coating Buffer Selection
(iii) Coating Concentration Optimization

II Blocking Buffer Selection

III Plasma/Serum Dilution Optimization

(i) Dilution Factor Determination
(ii) Non-Specific Binding Reduction

IV Conjugate Dilution Optimization

B. Assay Performance

I Precision

(ii) Intra-Assay Precision
(ii) Inter-Assay Precision
(iii) Precision Results

II Analytic Recovery

III Interference

IV Matrix Analysis
CHAPTER FOUR

CLINICAL VALIDATION OF CIRCULATING MBP AUTOANTIBODIES IN MS PATIENTS

A. MBP Autoantibody ELISA
   I  Diagnostic Parameters: MBP IgG 72
   II MBP Autoantibody Levels and Disease Status 78
   III MBP Autoantibody Levels and Disability 80
   IV MBP IgM Autoantibody Levels and Disease Status 80
   V Analytic Specificity of the MBP Autoantibody (IgG) Response 83
   VI Clinical Specificity 88

B. Western Blot Analysis for the Detection of MBP Autoantibodies (IgG) 91

C. Summary 95

CHAPTER FIVE

CLINICAL UTILITY OF OTHER BIOCHEMICAL MARKERS IN MULTIPLE SCLEROSIS

A. Myelin Basic Protein 96
B. Neuron Specific Enolase 99
C. S-100B 101
D. Thrombomodulin 103
E. Summary 106
CHAPTER FIVE

DISCUSSION

A. Assay Performance 110
B. Clinical Utility of MBP Autoantibodies in MS 113
   I Specificity of the MBP Autoantibody Response 114
C. Diagnostic Utility of a Biochemical Panel in MS 116
D. Pathological Significance of Circulating MBP Autoantibodies 117

FUTURE STUDIES 120
REFERENCES 122
APPENDIX 135
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Disease course in Multiple Sclerosis</td>
<td>9</td>
</tr>
<tr>
<td>2.</td>
<td>Potential Mechanisms for Demyelination</td>
<td>14</td>
</tr>
<tr>
<td>3.</td>
<td>The human MBP family consisting of isoforms and charge isomers</td>
<td>23</td>
</tr>
<tr>
<td>4.</td>
<td>ELISA Configuration</td>
<td>44</td>
</tr>
<tr>
<td>5.</td>
<td>Selection of Microtiter Plate</td>
<td>50</td>
</tr>
<tr>
<td>6.</td>
<td>Optimization of MBP Coating Concentration</td>
<td>54</td>
</tr>
<tr>
<td>7.</td>
<td>Selection of Optimal Plasma Dilution Factor</td>
<td>56</td>
</tr>
<tr>
<td>8.</td>
<td>Plasma Dilution Buffer Optimization</td>
<td>57</td>
</tr>
<tr>
<td>9.</td>
<td>Drug Interference by Solumedrol in the MBP Autoantibody ELISA</td>
<td>67</td>
</tr>
<tr>
<td>10.</td>
<td>Matrix Analysis of Matched Serum and Plasma Samples</td>
<td>69</td>
</tr>
<tr>
<td>11.</td>
<td>Standard Curve for MBP Autoantibody ELISA</td>
<td>71</td>
</tr>
<tr>
<td>12.</td>
<td>MBP Autoantibody (IgG) Levels in Clinically Definite MS patients</td>
<td>74</td>
</tr>
<tr>
<td>13.</td>
<td>Receiver Operating Curve for MBP Autoantibody (IgG) ELISA</td>
<td>75</td>
</tr>
<tr>
<td>14.</td>
<td>MBP Autoantibody (IgG) Levels in Clinically Stratified MS patients</td>
<td>79</td>
</tr>
<tr>
<td>15.</td>
<td>Correlation of IgG Autoantibodies and EDSS score</td>
<td>81</td>
</tr>
<tr>
<td>16.</td>
<td>MBP Autoantibody (IgM) Levels in Clinically Definite MS Patients</td>
<td>82</td>
</tr>
<tr>
<td>17.</td>
<td>MBP Autoantibody (IgM) Levels in Clinically Stratified MS patients</td>
<td>84</td>
</tr>
<tr>
<td>18.</td>
<td>Evaluation of PLP Autoantibodies (IgG) in Clinically Definite MS Patients</td>
<td>85</td>
</tr>
<tr>
<td>19.</td>
<td>Evaluation of NSE Autoantibodies (IgG) in Clinically Definite MS Patients</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>20</td>
<td>Evaluation of S-100B Autoantibodies (IgG) in Clinically Definite MS Patients</td>
<td>87</td>
</tr>
<tr>
<td>21</td>
<td>MBP IgG in Diabetic Patients</td>
<td>89</td>
</tr>
<tr>
<td>22</td>
<td>MBP IgG in Rheumatoid Arthritis Patients</td>
<td>90</td>
</tr>
<tr>
<td>23</td>
<td>MBP Autoantibody Western Blot: Evaluation of MS patients</td>
<td>92</td>
</tr>
<tr>
<td>24</td>
<td>MBP Autoantibody Western Blot: Evaluation of Sensitivity</td>
<td>93</td>
</tr>
<tr>
<td>25</td>
<td>Evaluation of Assay Sensitivity</td>
<td>94</td>
</tr>
<tr>
<td>26</td>
<td>Serum Concentration of MBP in Clinically Definite MS Patients</td>
<td>97</td>
</tr>
<tr>
<td>27</td>
<td>Serum Concentration of NSE in Clinically Definite MS Patients</td>
<td>100</td>
</tr>
<tr>
<td>28</td>
<td>Serum Concentration of S-100B in Clinically Definite MS Patients</td>
<td>102</td>
</tr>
<tr>
<td>29</td>
<td>Serum Concentration of Thrombomodulin in Clinically Definite MS Patients</td>
<td>104</td>
</tr>
<tr>
<td>30</td>
<td>Serum Thrombomodulin Concentration in Stratified MS Patients</td>
<td>105</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Familial Risks for MS</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>Coating Buffer Selection</td>
<td>52</td>
</tr>
<tr>
<td>3.</td>
<td>Intra-Assay Precision</td>
<td>61</td>
</tr>
<tr>
<td>4.</td>
<td>Inter-Assay Precision</td>
<td>62</td>
</tr>
<tr>
<td>5.</td>
<td>Analytic Recovery of MBP Autoantibody (IgG) in three normal human plasma samples</td>
<td>64</td>
</tr>
<tr>
<td>6.</td>
<td>MBP Autoantibody ELISA: Interference by Plasma Constituents</td>
<td>66</td>
</tr>
<tr>
<td>7.</td>
<td>Diagnostic Value of MBP Autoantibody ELISA</td>
<td>76</td>
</tr>
<tr>
<td>8.</td>
<td>Determination of Diagnostic Parameters</td>
<td>77</td>
</tr>
<tr>
<td>9.</td>
<td>Summary of Diagnostic Utility of all Biochemical Markers Tested</td>
<td>107</td>
</tr>
<tr>
<td>10.</td>
<td>Diagnostic Utility of a Biochemical Panel in MS</td>
<td>108</td>
</tr>
<tr>
<td>11.</td>
<td>MBP Autoantibody Evaluation in Query MS Patients</td>
<td>136</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

AD  Alzheimer's Disease
ADEM  Acute Disseminated Encephalomyelitis
Ag  Antigen
AIDS  Acquired Immunodeficiency Syndrome
BBB  Blood Brain Barrier
Blotto  Solution of Carnation Skim Milk
BSA  Bovine Serum Albumin
CDMS  Clinically Definite Multiple Sclerosis
CNPase  2’3’-Cyclic Nucleotide-3’-Phosphodiesterase
CNS  Central Nervous System
CSF  Cerebral Spinal Fluid
CT scan  Computerized Tomography scan
CV  Coefficient of Variation
DOB  Date of Birth
EAE  Experimental Allergic Encephalomyelitis
EAP  Experimental Allergic Panencephalitis
EDSS  Expanded Disability Scaled Score
ELISA  Enzyme Linked ImmunoSorbant Assay
FCA  Freund's Complete Adjuvant
g  gram
GαMBP  goat anti-myelin basic protein immunoglobulin G
GC  Galactocerebroside
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd</td>
<td>Gadolinium</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
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<tr>
<td>HAM</td>
<td>Human Lymphotrophic virus type-1-Associate Myelopathy</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<tr>
<td>H$_2$SO$_4$</td>
<td>Sulfuric Acid</td>
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<tr>
<td>IDDM</td>
<td>Insulin Dependent Diabetes Mellitus</td>
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<td>IFNβ</td>
<td>Interferon beta</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IgM</td>
<td>Immunoglobulin M</td>
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<td>IL2-R</td>
<td>Interleukin 2 Receptor</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>LP</td>
<td>Lumbar Puncture</td>
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<tr>
<td>LR</td>
<td>Likelihood Ratio</td>
</tr>
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<td>LSDMS</td>
<td>Laboratory-Supported Definite Multiple Sclerosis</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin Associated Glycoprotein</td>
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<td>MBP</td>
<td>Myelin Basic Protein</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<td>µl</td>
<td>microliter</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>ml</td>
<td>milliliter</td>
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<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
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<td>μmol</td>
<td>micromole</td>
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<tr>
<td>MOG</td>
<td>Myelin/Oligodendrocyte Glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NKC</td>
<td>Natural Killer Cell</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative Predictive Value</td>
</tr>
<tr>
<td>NSE</td>
<td>Neuron Specific Enolase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>ON</td>
<td>Optic Neuritis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid Protein</td>
</tr>
<tr>
<td>PPMS</td>
<td>Primary Progressive Multiple Sclerosis</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive Predictive Value</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RαMBP</td>
<td>Rabbit anti-myelin basic protein immunoglobulin G</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operating Curve</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RRMS</td>
<td>Relapsing-Remitting Multiple Sclerosis</td>
</tr>
<tr>
<td>S-value</td>
<td>Subtracted value</td>
</tr>
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<td>SC</td>
<td>Spinal Cord</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
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<td>SPMS</td>
<td>Secondary Progressive Multiple Sclerosis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
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<tr>
<td>Tm</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetra-methyl Benzidine</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tris Buffered Saline + Tween-20</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopeia units</td>
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<tr>
<td>V</td>
<td>volt</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

A. MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the human central nervous system (CNS). Pathologically, the disease presents focal areas of myelin destruction, known as plaques or lesions (Vollmer, 1999). With the focal deterioration of the insulating myelin sheath, the lesions result in decreased conduction velocity through naked regions of the axons affected. Such primary demyelination manifests as the symptoms of MS which include motor weakness in one or more limbs, optic neuritis, diplopia, paraesthesia, fatigue and eventually paralysis and early morbidity (Pender, 1995).

Although the aetiology is unknown, geographic, genetic, and immune factors acting co-ordinately determine disease onset and severity. Given the early onset of the disease (with diagnosis in children increasing in frequency) but usually the third or fourth decade of life, MS is one of the leading causes of neurological impairment in young adults.
Epidemiology, Genetics and Immunology

Epidemiology

Over 400,000 North Americans are currently diagnosed with the disease (Vollmer, 1999). Approximately 1 in 1,000 individuals of Northern European origin will be diagnosed with MS in their lifetime, with less prevalence in African and Asian populations (Matthews, 1991). The disease is twice as prevalent in females than in males (Matthews, 1991). Population based studies have shown that prevalence of MS increases with increasing latitude from the equator (Acheson, 1963); migration studies reinforce that environment or climate is important in the acquisition of the disease. Migration from high to low risk areas during childhood has been found to afford some protection from MS, while migration from high to low risk areas after the age of 15 results in the same chances of developing MS as the area of origin (Alter et al., 1966). The importance of age in the determination of risk has lead some researchers to search for a causative agent that may be acquired during childhood, related to climate or geography, such as a virus or bacterial infection. Numerous viral agents, including canine distemper virus (Chan, 1977), measles (Brody et al., 1970), rubella, herpes zoster and Epstein-Barr viruses (Symington et al. 1978; Johnson et al., 1980) have been implicated in the aetiology of MS, however viral associations in MS are weak. Moreover, the high incidence of MS in populations living in hot climates, such as Sardinia and Sicily (Granieri and Rosatti, 1982; Rosatti et al., 1986), reinforce the observation that epidemiological trends are difficult to interpret in all cases of MS.
Table 1: Familial Risks for MS (Genetic Factors)

- Familial MS
  
  Up to 20% of MS subjects have at least one relative with MS

- Empiric recurrence risks (age-adjusted)

  MS parent-risk for child: 4%
  MS subject-risk for sibling: 4-5%
  MS twin with MS-risk for co-twin (fraternal): 3-5%
  MS twin with MS-risk for co-twin (identical): 26-36%

Vollmer, 1999
(ii) Genetics

Despite its appearance as a relatively sporadic disease showing variable familial trends, population-based studies in twins have revealed a genetic component that does not follow classic Mendelian genetics. Ebers and colleagues (1986) found a much higher concordance of the disease in monozygotic (25.9%) than dizygotic twins (2.3%) and non-twin siblings (1.9%) (Table 1). The basis of this association of risk with genetics is not yet well defined and the only confirmed genetic factor predisposing to MS is the HLA-DR-DQ haplotype DRw15, DQw6, Dw2 (Pender, 1995).

Human leukocyte antigens (HLA) class II molecules regulate the immune response against peptide antigens. They determine whether the individual will react immunologically to a given antigen and they shape the T cell repertoire (Hillbert, 1994). Class II molecules are cell membrane bound heterodimers (α and β chain) which are encoded by highly polymorphic genes in the HLA or major histocompatibility locus (MHC) on the short arm of chromosome 6 (Hogancamp et al., 1997). The association of HLA locus appears to be less important than that observed in other autoimmune diseases like rheumatoid arthritis and diabetes (Bell and Lathrop, 1996) and since most MS patients do not share this haplotype, other loci may be essential to the definition of genetic susceptibility in MS. Recent evidence using large sets of polymorphic markers to screen the whole genome has revealed multiple genetic loci associated with multiple sclerosis (Ebers et al., 1996; Haines et al., 1996; Sawcer et al., 1996; Kuokkanen et al., 1996), however each of small effect. Taken together, MS may not be a single gene disease.
(iii) Immunology

The precise mechanism by which demyelinating lesions are formed in MS is not known, however the most promising theory is that demyelination occurs as a result of aberrant immunology resulting in immune attack of myelin. The association between histocompatibility phenotype and susceptibility in MS has immunological implications. Furthermore, clinical features in MS, such as the presumed long latent period, the chronic nature of the disease and the pattern of acute attacks followed by remission are suggestive of an immunologically mediated disease. Pathologically, MS lesions are marked by an infiltration of numerous immune cells, especially T lymphocytes and macrophages (Pender, 1995). Histological and immunological similarities between MS and the classic animal model of the disease, experimental allergic encephalomyelitis (EAE), have suggested that the disease may be T cell mediated (Esiri, 1991). However, evidence of humoral immunity in MS is also abundant as reflected by the presence of B cells and plasma cells within MS lesions and the elevated levels of CSF IgG in MS patients.

Despite evidence of recovery of myelin/myelin protein- specific T cells from both the MS lesion and peripheral blood of MS patients, as well as reports of specific B cell responses to myelin components, the specific antigen towards which a damaging immune response might be directed has not been identified. Moreover, the triggering mechanism which would initiate an autoimmune attack on myelin is not known. Despite advances in technology since Charcot’s (Charcot, 1877) first description of MS in the 1870’s, the heterogeneous nature of MS has hindered the definite aetiology of the disease.
(II) Diagnosis of Multiple Sclerosis

(i) Diagnostic Criteria

At the present time, there is no simple diagnostic test for MS. The diagnosis of MS currently remains primarily one of clinical evaluation. Since the time of Charcot’s first observations, the definitive aspect of the diagnosis of MS has centered around the demonstration of lesions disseminated in time and space. In order to justify this diagnosis, a patient must either present with the occurrence of more than one attack (exacerbation/relapse) or progression of symptoms over many months, as well as, evidence of multiple discrete anatomical loci of disease in the white matter of the central nervous system (CNS) (Miller, 1998). Perhaps the most widely accepted diagnostic scheme was put forth by Schumacher and his colleagues in 1965. Patients are classified as having “clinically definite, probable or possible” MS depending on the number of the following criteria described below which are applicable (Miller, 1998):

1. Age at onset between 10-50 years;
2. Objective neurological deficits present on examination referable to CNS dysfunction;
3. Neurological symptoms and signs indicative of CNS white matter disease;
4. Dissemination in time: two or more attacks (lasting at least 24 hours) and separated by at least 1 month (an attack is defined as the appearance of new symptoms or signs or worsening of previous ones) or the progression of symptoms and signs for at least 6 months;
5. Dissemination in space: two or more noncontiguous anatomical areas of brain involved;
6. No alternative clinical explanation.

The diagnosis of “clinically definite MS” requires that patients’ meet five or six of the criteria, always including the last one. Patients who fulfill fewer than 5 of the criteria, but
always including the last, are diagnosed with either "clinically probable or clinically possible MS".

The Schumacher criteria for the diagnosis of MS is based solely on clinical examination and history. With the advent of neuroimaging techniques in the 1970's and advances in electrophysiology in the 1980's, demyelinating lesions suggestive of MS became visually apparent in some cases even prior to their clinical manifestations. In addition, abnormalities in CSF immunoreactivity, such as the presence of oligoclonal bands, increased IgG synthesis and elevated IgG, became useful diagnostic criteria, but none were definitive. The existing clinical criteria did not accommodate the new laboratory techniques for diagnosing MS. In 1983, Poser and his colleagues published a new set of diagnostic guidelines which added the demonstration of para-clinical lesions (i.e. lesions visualized by MRI or CT scan or evoked-potential testing) as part of the clinically definite MS (CDMS) diagnostic criteria, as well as a new diagnostic category of laboratory-supported definite MS (LSDMS) which required the detection of oligoclonal bands in the CSF or increased CNS synthesis of IgG, as measured by the CSF IgG index* (Link, 1991).

These novel techniques were incorporated into the diagnostic criteria of MS, however positive MRI results or the presence of oligoclonal bands alone were not sufficient. Although lesions visualized through MRI correlated well with relapsing disease activity in the majority of MS patients (Comie et al., 1998), neither the number nor the size of the lesions reflected disability in the individual patient. Frequently, patients presenting with severe neurological deficit had little evidence of active lesions by MRI. Likewise, some patients with high lesion load have been reported to be asymptomatic (Fazekas et al., 1998). Furthermore, although oligoclonal bands are present in the CSF of approximately 90% of

*CSF IgG Index is determined by the ratio CSF/serum IgG: CSF/serum albumin.
clinically definite MS patients, their presence is not specific to MS as they can be detected in other inflammatory neurological diseases, such as paraneoplastic syndromes, post-infection encephalitis and meningitis (McLean et al., 1990). The non-specific nature of these diagnostic tools reflects the heterogeneity of this disease.

Unambiguous diagnostic criteria for MS is of extreme importance for the patient, for prognostic purposes. At the present time the definitive diagnosis of MS is often times a lengthy process, which renders the patient anxiously uncertain for days or months regarding the diagnosis and prognosis. In addition, defining a population of patients with "probable or possible" MS aids in the prospective evaluation of novel diagnostic tests for the disease (Poser et al., 1983). The clinical trials for testing novel MS therapies are now multi-centered and require a minimization of the subjectivity involved in the clinically-based diagnostic work-up performed by the examining neurologist. Resolution of these issues could be achieved if a simple, rapid and clinically sensitive diagnostic test was available to both family physicians and neurologists alike.

(ii) Clinical Features

Specific clinical signs and the temporal pattern of their presentation in the individual MS patient indicate the clinical course of the disease. Each disease course is remarkably different from the other, although the precise definition of the forms of the disease remains a constant source of debate and undergoes continuous revision. To summarize, the majority of cases of MS initially run a relapsing and remitting course (Figure 1: A) (Matthews, 1991). This form of the disease is characterized by alternating phases of exacerbation/relapse (acute
Figure 1: Disease course in Multiple Sclerosis

A. Relapsing-remitting MS (RRMS) with characteristic alternating phases of acute attack of neurological deficit (relapse) and recovery (stable)
B. Secondary-progressive MS (SPMS) with characteristic increases in neurological deficit with or without relapses
C. Primary-progressive MS (PPMS) with characteristic steady progression of neurological impairment from onset without distinct relapses
attack of neurological deficit) of symptoms and subsequent recovery with little or no residual
deficits (Stinissen et al, 1997; Thompson et al, 1997). The duration of relapse ranges from
24 hours to months before any remission of the symptoms is observed. In many cases,
patients first experience some degree of optic neuritis. Improvement/recovery after the first
attack is sufficient to identify remitting disease (Vollmer, 1999).

On average, the proportion of patients with relapsing-remitting MS is 85% (Vollmer, 1999). Patients older than 40 usually have the progressive form (Vollmer, 1999) of the
disease known as secondary-progressive (SPMS) characterized by significant deficits that
increase over time with or without relapses (Figure 1: B) (Thompson et al, 1997). Many
patients with relapsing-remitting MS (RRMS) progress to SPMS on average ten to fifteen
years after the initial symptoms present, although some older patients (over 40 years at
onset) experience the secondary progressive disease course from the outset.

Primary progressive MS (PPMS) is less common and is characterized by patients
with a slow and steady progression of impairments from onset of presentation without
distinct attacks (Figure 1: C) (Al-Omaishi et al, 1999). Rarely, a more malignant form of
MS occurs and is called the Marburg’s variant. Marburg’s disease is characterized by an
acute fulminant monophasic course resulting in death 3 weeks to 6 months after the onset of
initial symptoms (Lassman et al., 1981). Conversely, some MS patients can remain
asymptomatic for 15 years and are considered to follow a “benign” disease course. Disease
course is variable between individual MS patients resulting in a continuum of severity which
reflects an equally variable pathology and possibly aetiology.
(iii) Treatment

(a) Interferon β₁(1a and 1b)

IFNβ-1b (BETASERON), and IFNβ-1a (Rebif/Avonex) are both considered effective “first line” treatments in RRMS, in that they reduce relapses and MRI lesions, both indicators of disease activity in RRMS. Although the mechanism of action has not been clearly defined for the β-interferons, their effects are likely that of immunomodulation because β-interferons are anti-inflammatory cytokines. They may also have significant capability of slowing down lymphocytic trafficking across the blood-brain-barrier, a theory suggested because they tend to suppress the development of gadolinium (Gd)-enhancing MRI lesions.

(b) Glatiramer Acetate (Copaxone)

Copaxone consists of a mixture of L-amino acids glutamate, tyrosine, alanine and lysine, that make up an immunodominant region of myelin basic protein. Copaxone’s efficacy lies in its ability to reduce the number of relapses in the individual patient. Copaxone is thought to downregulate immune responses directed against myelin components by stimulating the generation of suppressor cells that are capable of reducing immune response by release of anti-inflammatory cytokines. Although this drug has been shown to reduce relapse rate and MRI lesion activity, Copaxone has no effect on short-term disease progression as measured through the EDSS.

(c) Methylprednisolone (Solu-Medrol)

Methylprednisolone is a corticosteroid which is used to treat inflammation. Methylprednisolone is commonly administered to MS patients during acute relapse because it is deemed to have the effect of closing the damaged blood-brain barrier and reducing inflammation in the CNS (van den Noort and Holland, 1999).
(d) Paclitaxel

Paclitaxel (Taxol) is a well known anti-cancer agent. Recent evidence (Cao et al., 2000) has shown that paclitaxel is an effective agent in attenuating the clinical manifestations of experimental allergic encephalomyelitis (EAE), the classic T cell-mediated animal model of MS. Paclitaxel may prove to be a valuable early treatment for MS and a Phase II clinical trial for paclitaxel is currently underway at St. Michael’s Hospital MS Clinic in Toronto.
B. PATHOGENESIS OF MULTIPLE SCLEROSIS

Studies on the pathology of the demyelinating lesions in MS have led to the identification of numerous key players in the multifactorial pathogenesis of the disease (Figure 2). The release of components of the myelin sheath, evidence of blood-brain barrier (BBB) damage, astrocytic proliferation early in the formation of the plaque, gliosis, as well as infiltration of macrophages and B cells within the lesion, and CD8+ T cells surrounding the lesions are consistent features in MS pathology. These pathological characteristics facilitated both the identification of contributory factors in MS and provided clues as to which biochemical markers might offer the most utility in the diagnosis and prognostic monitoring of MS.

(I) Myelin

Central to the pathogenesis of the disease is the degeneration of the myelin sheath. This multilamellar sheath is formed by membranous extensions of oligodendrocytes within the CNS (Peters, 1960a; b). Its ability to insulate axons and facilitate rapid nerve conduction is attested to by its high lipid content (70% of the total weight). The protein components of myelin, representing the remaining 30%, have been the focus of the majority of research on the mechanism of MS because not only did their administration to naïve animals induce demyelinating disease, but activated lymphocytes against these myelin proteins have been recovered from MS patients.

The proteolipid protein (PLP) family (PLP and DM20) constitutes the majority of the myelin protein, accounting for approximately 50% of the total protein content. The myelin basic protein (MBP) family constitutes approximately 35% of the total protein content.
Figure 2: Potential Mechanisms for Demyelination (modified from Mak, 1999)

1) Disruption of the bilayer. 2) Deimination of MBP by PAD. 3) Further disorganization of the bilayer. 4) Shedding of MBP. 5) Activation of astrocytes and the induction of astrogliosis and homing to myelin. 6) Digestion of MBP by cathepsin D and MMP generating MBP peptides. 7) Sensitization of lymphocytes. 8) MMP digests BBB allowing sensitized lymphocytes into the brain. 9) Autoimmune phase begins.
Other myelin proteins, representing the remaining 15%, include 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase), myelin associated glycoprotein (MAG), myelin/oligodendrocyte glycoprotein (MOG) and others.

Whether immune and inflammatory cells initiate demyelination by binding to any one specific myelin protein or the immune response is generated secondary to demyelination by the leakage of myelin components from the CNS into the periphery has not been determined. Nonetheless, T cells reactive to MBP, MBP-specific antibody-secreting B cells and macrophages have been localized to the areas of demyelination in MS patients. The measurement of the protein constituents of myelin, or likewise immunoreactivity against them, may not conclusively define the role of these proteins in the pathogenesis of the disease, however they certainly offer possible diagnostic and prognostic utility.

(II) The Blood-Brain Barrier (BBB)

Damage to the blood-brain barrier (BBB) appears to be a necessary component of pathology leading to demyelination, not only in EAE, through the use of pertussis toxin or the like prior to adoptive transfer of autoreactive T cells, but also in MS. Under normal physiological conditions, access of immunomodulatory cells to the CNS is limited due to the extensive tight junctions of the endothelium comprising the BBB. Deterioration of the BBB should be an early event in the onset of the disease, occurring prior to demyelination if MS is autoimmune in nature. Presumably, damage to the endothelial barrier in the CNS results in leakage of endothelial proteins into the CSF and eventually the peripheral blood. Measurement of endothelial proteins in the CSF or blood of MS patients has been performed (Washington et al., 1994). One such protein, thrombomodulin, has been detected in the CSF of MS patients and patients with other neurological diseases (Tsukada et al., 1995; Frigerio
et al., 1998). Although non-specific, utilization of a marker denoting endothelial cell damage, prior to clinical manifestations of the disease, would offer a means of determining high risk patients and may aid in the confirmation of MS in clinically probable or possible MS patients.

(III) The Glia and Microglia

Activated macrophages and microglial cells may be involved in the demyelination process through the production of inflammatory cytokines, activation of components of the complement cascade, by functioning as antigen-presenting cells or enhancement of the opsonization of myelin components (Noseworthy et al., 2000). The formation of astrocytic scars is a prominent feature in MS pathology. The use of astrocyte markers such as GFAP and S-100B for the diagnosis of MS has not been assessed.

(IV) Autoimmune Pathogenesis

The aetiology of MS remains unknown, however, by far the most compelling theory of MS pathogenesis is that the demyelination process occurs as a result of autoimmunity, but the target antigen has not been identified. Pathologically, active lesions are characterized by the infiltration of numerous immune and inflammatory cells, including lymphocytes and activated macrophages, especially in the periventricular white matter of the brain, the optic nerve and tract, corpus callosum and brain stem (Brosnan and Raine, 1996). Although the CNS is usually an immune privileged site, the BBB is damaged during acute inflammation in the CNS, as serum proteins have been found throughout the lesions (Brosnan and Raine, 1996), resulting in novel access of lymphocytes to myelin and myelin components.
In efforts to delineate the autoimmune pathogenesis of MS, in vitro and in vivo model systems for MS have been useful. The first and classic animal model for MS is experimental autoimmune encephalomyelitis (EAE) which is induced in mice injected with an antigen such as MBP or PLP in conjunction with Freund's complete adjuvant. Adoptive transfer of T cells isolated from EAE mice to normal mice, and the ensuing development of EAE in the normal mice, reinforced the hypothesis that MS was a T cell-mediated disease (Archelos et al. 2000). Bornstein and Appel (1961), however demonstrated that sera from EAE mice could induce demyelination of myelinated rat cerebellum in vitro cultures. The serum factor responsible for demyelination was later shown to be myelin-specific immunoglobulin (IgG). Therefore, despite past and current belief that MS is a T cell-mediated autoimmune disease, the humoral response in MS is indicative of a possible B cell driven pathology, as well.

(i) T cell Autoimmunity in MS

At the center of MS research is the examination of the cellular autoimmune attack by cytotoxic T lymphocytes (CD8\(^+\)). Both CD8\(^+\) and CD4\(^+\) T cells (helper T cells), of which the former is generally more prominent in MS tissue, have been identified surrounding MS lesions (Pender, 1995). Expression of the interleukin-2 receptor (IL2-R) on some of the infiltrating cells has suggested that they are activated T cells (Pender, 1995). Although it is unknown whether T cells become activated in the periphery or if naïve T cells become activated at the site of the lesion, these immune cells gain access to the CNS tissue through a compromised BBB. In fact, although the ratio between CD4\(^+\) and CD8\(^+\) cells were found to be higher in blood than in the CNS tissue, these cells have also been recovered from the peripheral blood of secondary progressive MS patients.
The association of HLA in familial studies of MS underscores a protein/peptide driven immune response. Since demyelination is the focus of the pathology in MS, protein constituents of myelin were first suspected as the antigens driving activation of T cells. Of the myelin proteins, MBP was especially implicated, given the ability of MBP and the adoptive transfer of MBP reactive T cells to induce EAE. Moreover, analysis through T cell proliferation assays revealed that T cells were significantly more reactive against MBP in MS patients than normal controls or patients with other neurological diseases. Moreover, MBP-specific T cells occurred at a significantly higher frequency in patients with clinically active MS than in those in stable courses of the disease (Johnson et al., 1986). Specific epitope studies have identified amino acid residues 87-106 as the immunodominant epitope for MBP reactive T cells (Martin et al., 1990) and further support the importance of MBP-specific cell-mediated autoimmunity in the disease. Despite the extensive focus of autoreactivity to MBP and although at a reduced frequency of detection, reactive T cells against other myelin and non-myelin antigens have been recovered from MS patients. PLP and PLP-peptide-specific T cells have been evaluated for their contribution to the autoimmune pathogenesis in MS, in light of the immunogenicity of PLP in the induction of EAE. Moreover, MOG, MAG, S-100B and several heat shock proteins, such as αB crystallin, from the peripheral blood of MS patients have been demonstrated to generate increased proliferation of T cells, albeit not to the same extent as that observed using MBP (Pender, 1995). Taken together, the variable T cell responses to different autoantigens in MS patients strengthens the supposition that differences in the pathology and immunological activity of lesions between individual patients may be under separate autoimmune pathogenic mechanisms.
(ii) B cell Autoimmunity in MS

Serum antibodies to numerous ‘self’ antigens have been reported in healthy individuals without intentional immunization (Coutinho et al., 1995) and have been termed “natural autoantibodies”. Natural autoantibodies consist of IgM, IgA, and IgG isotypes, of which the later constitute the majority (Avrameas, 1991), are non-pathogenic, tend to be polyreactive and are of low titer and low affinity (Lacroix-Desmazes et al., 1998). Their physiologic significance is not known, however during antigen-driven B-cell differentiation, somatic hypermutations may occur, resulting in unwanted autoreactive B-cell clones. Normally, auto-reactive B cells are regulated by germinal centers of secondary lymphoid tissues and undergo apoptosis (Archelos et al., 2000). This surveillance may be diverted in MS and the production of autoantibodies which cause tissue damage could ensue.

Although the precise mechanism by which self-tolerance is diverted in MS is not known, several mechanisms have been proposed. The first of these is the dysregulation of T cell-dependent B cell activation, which is dependent on two signals by the antigen and T cells. Altered expression of CD19 or CD22 co-receptor molecules on B cells which are involved in antigen induced signalling, excessive B cell differentiation, or an imbalance of the Fas/Fas ligand mediated control of apoptosis for B cells may all contribute to an increase in B cell differentiation (Archelos et al, 2000). Alternatively, autoantibodies may be generated by B cell superantigens, even with an intact immune system (Silvermans, 1997) or via molecular mimicry. The theory of molecular mimicry in MS is a long standing concept which involves a susceptible individual acquiring a bacterial or viral antigen that contains molecular/structural similarity to self antigens (Fujinami and Oldstone, 1985). It was proposed as a mechanism to explain the sensitization of T cells in the periphery which then home to the CNS, since the autoimmune theory depended heavily on sensitization prior to
demyelination. The pathogen-specific immune responses were postulated to cross-react with self epitopes to cause tissue damage and disease (Albert et al., 1999; Gran et al., 1999). Whatever the mechanism by which self-reactive B cells are generated, their presence has been detected in MS lesions, to variable degrees (Guseo et al., 1975; Esiri, 1977) and are elevated during lesional activity (Esiri, 1977).

B cells have effector functions traditionally through a number of mechanisms including the secretion of antibodies, the activation-dependent release of cytokines and the mutual activation of T cells. Since BBB breakdown is an early event in MS (Katz et al., 1993) coinciding with the development of a lesion, B cells, their autoantibodies and complement have new access to the CNS and can initiate tissue damage. Because many of the B cells/plasma cells detected at the CNS lesion are IgG secreting cells, and furthermore secrete antibodies to MBP, MAG, MOG, and PLP (Van der Goes et al., 1999), autoantibodies against such myelin components may be released. These antibodies can initiate demyelination via several possible mechanisms including complement activation with subsequent assembly of membrane attack complex, release of inflammatory mediators through stimulation of Fc receptors on natural killer cells (NKC), macrophage or mast cells (Brosnan and Raine, 1996) or opsonization of myelin (Van der Goes et al., 1999).

The pathogenic contribution of autoantibodies has been examined using flow cytometry by Van der Goes and colleagues (1999). They found that when monoclonal antibodies against several myelin components including MBP, PLP, Galactocerebroside, and MOG were incubated with myelin, phagocytosis of the myelin resulted and the degree of opsonization of myelin was dependent on the isotype of the antibody, the epitope recognized and the antibodies' ability to fix complement. The evidence supporting humoral autoimmunity in MS pathogenesis suggests that autoantibodies are associated with disease
activity. Therefore, they show promise as diagnostic indicators of MS activity and disease progression.
C.  CANDIDATE AUTOANTIGENS AND BIOCHEMICAL MARKERS OF MS

I. Autoantigens of myelin and oligodendrocyte origin

(i) Myelin Basic Protein (MBP)

Myelin basic protein is a cationic membrane-associated protein found in the myelin membrane (Moscarello, 1997) and represents 30% of all CNS myelin proteins (Lees and Brostoff, 1984). In humans, there are four main isoforms generated by alternative splicing of a single transcript (Figure 3): 21.5 kDa, 18.5 kDa and the 17.2 kDa isoforms (Kamholz et al., 1986; 1988). The 18.5 kDa isoform, in which exon 2 is spliced out, is 170 amino acids in length (Carnegie, 1971) and is the most prominent isoform in mature human myelin (Moscarello, 1997; Schmidt, 1999).

Each isoform exists as several components or “charge isomers” termed C1-C8, based on the net positive charge, the result of post-translational modifications such as phosphorylation, methylation, glycosylation and deimination of arginine (Moscarello, 1997). The C8 isomer, being the least cationic and found to be the most post-translationally modified isomer in the spectrum, is generated by the deimination of 6 arginyl residues citrulline, at positions 25, 31, 122, 130, 159, and 170 of the amino acid sequence (Wood and Moscarello, 1989). Peptidylarginine deiminase (PAD) is a calcium dependent enzyme which converts arginyl residues within peptide bonds to citrulline (Pritzker and Moscarello, 1998). PAD is the only known enzyme which generates citrulline with proteins and thus is presumed to be the enzyme responsible for the generation of citrulline in the C8 isomer. T cell reactivity to C8 was greater in MS patients than in controls and more pronounced as compared with C1, the most positively charged isomer in the continuum (Tranquill et al.,

* isomer is not used in the mass spectrometric sense but in a biological sense
Figure 3: The human MBP family consisting of isoforms and charge isomers (Moscarello, 1997)
The role of C8 in the pathogenesis of MS was initially proposed because the C8 isomer was found to be elevated 2-3 fold in the MBP isolated from the white matter of 13 MS patients (Moscarello et al., 1994). In studies of MBP isolated from infants less than 2 years of age, the C8 charge isomer accounted for 80-90% of the total MBP. The predominance of C8 in the brains of MS patients suggested developmental immaturity of myelin which may result in easier degradation (Moscarello et al., 1994). Citrullinated MBP has been reported to be more susceptible to proteolytic digestion by cathepsin D (Pritzker et al., 2000). Moreover, the observations that C8 exhibited reduced interactions with the lipid bi-layer (Wood and Moscarello, 1989), as compared with other charge isomers in the spectrum, and that loose myelin was enriched with citrullinated MBP, whereas compact myelin contained the more cationic MBP charge isomers (Cruz and Moscarello, 1985), suggested that the loss of electrostatic interactions may contribute to the de-stabilization of myelin in MS patients. Therefore, the complete role of charge microheterogeneity of MBP in the pathogenesis of MS remains a focus of much research (Moscarello et al., 1994).

Although the precise mechanism by which MBP is released from the myelin membrane has not been entirely worked out, measurements by several groups has revealed that MBP in the CSF, when measured by RIA, can be a reliable indicator of disease activity (Whitaker, 1977; Whitaker, and Herman, 1988). Patients exhibited the highest levels of CSF MBP if they were experiencing acute exacerbation/relapse (Whitaker, 1977; Thompson et al, 1985; Warren et al, 1985; Freqin et al, 1992 and Ohta et al. 2000), whereas the majority of clinically stable/remitting patients had levels of CSF MBP comparable to that observed in other neurological disease controls. Furthermore, in patients in clinical remission who had significantly elevated MBP levels, sub-clinical demyelination was also detected by MRI (Thompson et al. 1985) which supports the role of MBP in CSF as a marker for myelin
destruction. CSF MBP levels were reduced to normal levels when previously relapsing patients were treated with high doses of methylprednisolone (Frequin et al., 1992). This reduction in CSF MBP correlated well with a decrease in intrathecal IgM synthesis, which is also known to increase during relapse. Taken together, MBP, having also significantly correlated with EDSS score during relapse and the number of gadolinium enhanced lesions on MRI, has shown promise as an indicator of MS pathology (Thompson et al., 1985; Frequin et al., 1992). Unfortunately, the clinical utility of MBP in the serum of MS patients has not been pursued because the extent of MBP peptide generation by circulating proteases has not been determined, resulting in the lack of a sensitive assay which specifically measures circulating MBP/MBP-peptides. Measurement of MBP in serum would perhaps provide a simple means of monitoring the progression of the disease in the individual patient.

(ii) Proteolipid Protein (PLP)

Proteolipid protein (PLP) is a 30 kDa hydrophobic protein which constitutes approximately 50% of the total myelin protein. PLP is expressed in CNS myelin and is found in the myelin membrane (Lees and Brostoff, 1984). During the formation of myelin, an alternative spliced product of the PLP gene, called DM20, is expressed at high levels. However, PLP is the dominant form of the protein in mature myelin (Schmidt, 1999).

PLP was first proposed as an autoantigen in MS due to its ability to induce EAE when injected with Freund's complete adjuvant (FCA) into naïve animals (Hashim et al., 1980). In addition, adoptive transfer of PLP-specific activated T cells results in inflammation of the spinal cord (SC) but less so in the medulla, optic nerve and periventricular white matter. Despite evidence of PLP-peptide-specific T cell recovery from the blood of MS patients (Correale et al., 1995), a pathogenic role for PLP autoantibodies
has not been demonstrated. Nevertheless, B cells secreting anti-PLP antibody have been reported in MS patients (Sun, et al., 1991; Sellebjerg, et al., 2000). Sellebjerg and colleagues (2000) found not only that anti-PLP antibody secreting cells could be recovered in the CSF of 50% of patients with possible onset symptoms of MS (POSMS), but also that their levels weakly correlated with disease activity, as measured by the EDSS and CSF levels of MBP. Likewise, Warren and Catz, 1994 measured anti-PLP antibodies in the CSF of MS and optic neuritis patients and found that, although at a markedly lower frequency, anti-PLP antibodies could be detected in the CSF of patients without demonstrable anti-MBP antibody. Moreover, this group hypothesized that the mutually exclusive antibody levels for PLP and MBP in MS are a reflection of two immunologically distinct forms of the disease. Patients with elevated levels of anti-PLP were proposed to have a more rare form of the disease, which although demyelinating, was less inflammatory than the anti-MBP common form of MS. Warren’s results may reflect the heterogeneity of MS.

(iii) **Myelin associated glycoprotein (MAG) and Myelin/oligodendrocyte glycoprotein (MOG)**

Myelin associated glycoprotein (MAG) and myelin/oligodendrocyte glycoprotein (MOG) both represent minor protein components of myelin, accounting for 1% and 0.01-0.05% of the total myelin protein (Quarles et al., 1992; Amiguet et al., 1992).

MAG exists as two isoforms generated by alternative splicing of the mRNA transcript from a single gene. Mature myelin expresses the smaller isoform, while the larger isoform is expressed during myelination (Quarles et al., 1992). With respect to its contribution to the pathogenesis of MS, MAG-specific T cells have been demonstrated to induce inflammatory lesions in specific regions of the brains of Lewis rats (Berger et al.,
Furthermore, CD4+ T cells from the peripheral blood and CSF of MS patients have been shown to be reactive against MAG (Zhang et al., 1993). To date, humoral responses to MAG have not been reported.

Existing as several isoforms derived by alternative splicing, MOG's role in the generation of cell-mediated and humoral immune responses in MS has been extensively studied in recent years. Anti-MOG antibodies have been demonstrated to induce demyelination when co-transferred with MBP-specific T cells. Although MOG-specific T cells have been recovered from the CSF and peripheral blood of 50% of MS patients, research on MOG has been centered on B cell responses. MOG-specific autoantibodies have been detected in both the CSF and serum of MS patients by several groups (Xiao et al., 1991; Reindl et al., 1999).

However, provided that disease in traditional animal models of MS cannot be induced by the immunization with MOG and given their low expression in myelin, the measurement of MAG and MOG and their immune responses would likely pale in comparison to the diagnostic utility of more immunogenic and strongly expressed myelin proteins, such as MBP.
II Non-myelin Antigens

(i) S-100B

S-100B (ββ homodimer) is a 21 kDa acidic calcium binding protein expressed primarily in astrocytes, oligodendrocytes (Kimura et al., 1986; Richter-Landsberg and Heinrich, 1995) and Schwann cells, as well as other cells and tissues including adipose tissue, skeletal muscle, the retina, salivary glands and immune cells (Zimmer et al. 1995). It has been used as a biochemical marker for melanoma and successfully used in the diagnosis of stroke (Takahashi et al. 1999). In addition, autoantibodies against S-100B have been detected at high frequency in the serum of Alzheimer’s disease (AD) patients (Jankovic and Djordjijevic, 1991).

S-100B was first suspected in the pathogenesis in MS with the recent observation that experimental autoimmune panencephalitis (EAP), an extensive inflammatory reaction with CNS involvement in the Lewis rat, was induced by the adoptive transfer of S-100B specific T cells (Kojima et al., 1997). The distribution of lesions in this model of MS was remarkably similar to several subsets of patients with MS (Schmidt et al., 1997). T cells isolated from the serum of clinically definite MS patients have been found to be reactive to S-100B, however this response was half of that observed for MBP (Schmidt, S. et al., 1997). Although perhaps more tightly regulated than MBP T cells, S-100B specific T cells have been suggested in the pathogenesis of MS based on the EAP model. To date, the B cell response to S-100B in MS has not been investigated, and should prove to be interesting in light of the cell-mediated responses to this protein in animal models. S-100B has been measured in the CSF of MS patients by microcomplement fixation assay (Michetti et al., 1979). Seventy percent of MS patients had CSF S-100 concentrations significantly elevated as compared with controls and 82% of the MS patients in acute phases of disease course
exhibited significantly elevated concentrations of this glial protein (Michetti et al., 1979). Since S-100B is present in oligodendrocytes (Kimura et al., 1986; Richter-Landsberg and Heinrich, 1995), death of oligodendrocytes may be suggested by high levels of this protein in the CSF. Serum S-100B concentrations in MS patients have yet to be evaluated.

(ii) \( \alpha B \) crystallin

\( \alpha B \) crystallin is a 23 kDa heat shock or “stress protein” which, although expressed in many tissues, is possibly over-expressed in CNS myelin, oligodendrocytes and astrocytes in MS patients (Van Noort et al., 1995). Since \( \alpha B \) crystallin has been shown to induce EAE in Biozzi ABH mice (Thorea et al., 1997) and T cell responses against \( \alpha B \) crystallin have been observed in MS patients, \( \alpha B \) crystallin has been suggested as an autoantigen in MS.

Immunohistochemical studies demonstrated that \( \alpha B \) crystallin was highly elevated in oligodendrocytes within early developing MS lesions but not within inactive lesions or healthy white matter (Van Noort and Amor, 1999). Although a minor component in myelin, the observation that \( \alpha B \) crystallin triggers a much stronger T cell response than any other myelin protein when it is present at stress induced levels (Van Noort and Amor, 1999) suggests that \( \alpha B \) crystallin may contribute to the demyelination process and may represent a useful diagnostic marker in MS.
(iii) Neuron Specific Enolase (NSE)

This glycolytic enzyme, responsible for the dehydration reaction of 2-phosphoglycerate to phosphoenolpyruvate (Lehninger et al., 1993), is active as a dimer. In cells of neuronal lineage, isoform switching occurs between αα, αγ, and γγ, however each subunit is encoded by a different gene (Sensenbrenner et al., 1997). The expression of NSE (αγ and γγ) has been correlated with structural and functional differentiation processes of the CNS such as the differentiation of oligodendrocytes and the formation of large amounts of membrane structures (Sensenbrenner et al., 1997). In addition, γ enolase is related to the differentiation and synaptic activity of neuronal cells (Schmechel et al., 1980; Sensenbrenner et al., 1997). NSEγγ has been thought to be expressed in high levels in neurons and neuroendocrine cells exclusively, but recent work has shown that it may be expressed also in glial cells which normally contain the αα isoform of the enzyme (Schmechel et al., 1980). Sensenbrenner and her colleagues (1997) have shown that NSE is selectively expressed in developing oligodendrocytes that stain for GC(galactocerebroside) and MBP. The expression of NSE in oligodendroglial cells is associated with the period during which myelination takes place.

NSE has been proposed as a biological marker for both neuronal tumors (glioblastoma, astrocytoma, and Schwannoma) as well as small cell lung carcinoma (Jorgensen, 1999) and NSE can be reliably detected in reactive astrocytes after brain injury (Lin et al., 1994). Since NSE is developmentally regulated, the fact that transformed cells, which contain the αα dimer in normal adult cells, express NSEγγ implies that an increase in glycolytic activity is required. NSE must be upregulated in the altered glial cell so as to adapt to new metabolic demands (Sensenbrenner et al., 1997). Injured glial cells may express fetal characteristics of progenitors. This putative conversion to early developmental
stages may be favourable for the repair and regeneration process through the neurotropic
effect of NSE\textsubscript{\gamma}. Although NSE has not been suggested as a marker in MS, it deserves
serious consideration. Moreover, NSE\textsubscript{\gamma} but not the \textalpha\textalpha dimer has a proposed
neuroprotective and neurotrophic effect on a broad spectrum of neurons (Hattori et al.,
1995). NSE\textsubscript{\gamma} levels are elevated in the CSF of patients with not only brain tumors but also
Creuzfeld-Jakob disease and cerebrovascular accident, possibly in a
neuroprotective/neurotrophic response to injured neurons. NSE autoantibodies have also
been detected as elevated in the serum of patients with Alzheimer’s disease (AD) as well as
various psychiatric disorders (Jankovic and Djordjijevic, 1991). Therefore, based on its
role during CNS development, its potential for repair of neuronal injury, its presence in adult
oligodendrocytes, and the detection of NSE autoantibodies in other neurological diseases,
NSE should be examined as a potential marker for MS.

(iv) **Thrombomodulin**

Thrombomodulin (Tm) is a 75 kDa endothelial cell surface transmembrane
glycoprotein which functions to indirectly activate protein C, by binding to thrombin and
altering its procoagulant activity (Dittman and Majerus, 1990). Thrombomodulin has been
isolated from both placenta and lung, has a half-life of 19 hours in the circulation (Dittman et
al., 1988) and is thought to be released into the circulation when the endothelial cells are
damaged (Tsukada et al. 1995). Therefore, damage to endothelial cells precedes
thrombomodulin release into the circulation.

Several investigators have examined the CSF and sera of MS patients for levels of
thrombomodulin as an indicator of damage to the blood-brain barrier. In one study, Tsukada
and colleagues (1995) used the Fuji Tm ELISA kit to evaluate circulating Tm concentrations
in 36 MS patients and 13 human lymphotropic virus type-1-associated myelopathy (HAM) patients. They found not only that relapsing MS and chronic progressive MS patients have Tm levels significantly elevated when compared with controls but also HAM patients exhibited elevated levels above the controls. They concluded that thrombomodulin could be useful as a biochemical indicator of endothelial cell damage in inflammatory diseases, including MS and HAM. These results, however, could not be repeated by Frigerio and her colleagues (1998), using the Diagnostica Stago Tm ELISA kit, but not the Fuji kit, to measure circulating Tm. They reported that patients with non-MS inflammatory neurological disease had Tm serum levels higher than patients with progressive MS. A possible explanation may be that Tsukada and colleagues, using the Fuji assay reported mean Tm levels at approximately 2.5 ng/mL in healthy controls, whereas Frigerio and colleagues reported a mean concentration of 22 ng/mL in their control samples, a 10 fold higher concentration. Therefore, the discrepancy between the two groups may be due to differences in analytic sensitivity between the two different assays. Further work is required to establish the utility of thrombomodulin as a biochemical indicator of blood-brain barrier damage.
D. MBP AUTOANTIBODIES AND THEIR DIAGNOSTIC VALUE IN MS

Although the predominant autoimmune theory in MS is centered around T cells, the role of B cells and autoimmunity in MS remains an active area of research. Towards this end, B cells were suspected to be involved in MS following the identification of elevated CSF IgG compared with other proteins in patients with MS (Kabat et al., 1948). Elevated IgG index in the CSF of MS patients was examined as a diagnostic tool. In addition, by using agar gel electrophoresis, the CSF proteins could be better separated and “extra bands” were visualized in inflammatory neurological diseases including MS, encephalitis and meningitis. These bands were thought to represent IgG, later proven to be IgG and were referred to as oligoclonal banding (Link, 1967). CSF oligoclonal banding suggested that the IgG was of intrathecal origin, i.e. synthesized in the CNS. With the evidence of elevated IgG in MS, as oligoclonal bands and IgG index, much work focused on determining the antigen to which IgG was responding.

Following the identification of MBP as the antigen associated with EAE, much research has focused on the examination of MBP’s contribution to the elevated level of IgG present in the CSF. Panitch and colleagues (1980) first identified MBP autoantibodies in the CSF of MS patients by solid phase radioimmunoassay (RIA). MBP autoantibody levels were reported highest in MS patients experiencing acute exacerbations and lower in remitting MS patients. However, the detection of MBP autoantibodies in the CSF has remained controversial. This is reflected in numerous reports, which document inconclusive or unsuccessful attempts by a variety of assays, including immunoblotting, RIA and ELISA (Colombo et al. 1997; Agius et al. 1999; Reindl et al. 1999; Brokstad et al. 1994; Lennon and MacKay, 1972; Schmid et al. 1974; and Gutstein et al. 1978).
The most consistent examination of MBP autoantibodies in the CSF has been performed by Warren and Catz (1986-present). Their extensive work rests on the supposition that MBP autoantibodies are frequently complexed with MBP and require acid dissociation with 1N acetic acid in order to release them for their detection. It should be noted that the diagnostic utility of MBP autoantibodies rests in this domain and that their diagnostic success lies in studying patients defined as having active disease (Warren and Catz, 1986), that is patients in relapse. Throughout their work, Warren and Catz have utilized acid dissociation of CSF samples, to break MBP autoantibody complexes, and their results are reported in sub-classified groups of CSF autoantibody responses. These responses are free (non-dissociated, % bound radioactivity), total (dissociated), bound (total-free) and a final free/bound ratio. All patients experiencing acute exacerbations have demonstrated elevated levels of free MBP autoantibodies. Patients in the progressive form of the disease had elevated bound levels of anti-MBP. Elevated levels of either free or bound anti-MBP were not detected in patients with clinically inactive disease. This group reasoned that long-term and/or repeated MBP release into the systemic circulation is likely to enhance the immune memory for anti-MBP synthesis. In further support of the increased frequency of anti-MBP responses, patients with optic neuritis (ON) were examined for their autoantibody response to MBP. Warren and Catz found that ON patients demonstrated elevated levels of MBP free antibodies by RIA (Warren and Catz, 1994). It appears that anti-MBP is associated with active phases of disease in MS especially since MS patients frequently present initially with attacks of ON.
Since IgG synthesis within the BBB has been documented as a consistent feature of MS (Tourtelotte, 1970; Warren and Catz, 1985), MBP autoantibodies have been presumed to be intrathecally synthesized. Antibodies raised against MBP have been isolated from the CSF using a two-step affinity purification process (Warren and Catz, 1991). This purified source of anti-MBP was used to determine the specific epitope to which the autoantibodies were directed. Interestingly, the autoantibodies reacted with synthetic peptides including residues 61-106 and later further refined to amino acids 85-96 of MBP as a minimal epitope (Warren et al., 1995). This minimal epitope correlated well with the epitope defined by the T cell work (Martin et al., 1990). The definition of a specific epitope has been extremely important in reducing the skepticism surrounding whether the autoantibody response in MS is a non-specific/non-sense event.

Despite the work conducted using the CSF, the presence of MBP autoantibodies in the systemic circulation has not been conclusively determined. Previous work has favoured measurement in the CSF and numerous investigators have reported limited success with their detection in serum. These experiments, using serum samples are plagued by problems with high background using a variety of assay methods. This is likely the result of insufficient optimization of their assays for the enhanced sensitivity required for MBP autoantibody detection at low titers in a matrix which contains so many other proteins. Furthermore, the lack of consistent methodology between investigators may account for the apparent discrepancy between their collective results. To illustrate this point, Reindl and colleagues (1999) investigated the detection of anti-MOG and anti-MBP IgG antibodies in the sera and CSF of MS patients. Patient samples were first screened by Western blot, however subsequent ELISA evaluation was limited only to that of anti-MOG. The sensitivities reported by this group represented seropositivity as assessed by immunoblotting alone.
Taken together, they achieved positive detection of anti-MBP IgG in 28% of the total MS patients studied. Western blotting was likely a poor choice as a means of MBP autoantibody detection. The Western blot technique is typically less sensitive than ELISA: the nature of these autoantibodies in serum may be of relatively low titer, thus the detection of 28% represents the failure of immunoblotting to sensitively measure low levels of autoantibodies.

Likewise, Cruz and colleagues (1987), although successful in detecting 32% of MS patients as positive for anti-MBP antibodies in the CSF by immunoblot, they failed to detect any bands when matched serum samples were examined. ELISA results also showed a higher detection in the CSF (44%) of their 25 MS patients compared with 8% detected in serum. Cruz, however, reported high background in the ELISA. MBP is a highly cationic protein; its charge may be the contributing factor for the high background in the ELISA due to non-specific charge interactions which typically plague the sensitivities of ELISAs using positively charged proteins (Pesce. et al., 1986). The sensitivity of the ELISA largely depends on the reduction of non-specific binding by other serum factors.

By the same token, Brokstad and colleagues (1994) using both Western blot and ELISA failed to detect MBP autoantibodies in either the CSF or the serum of MS patients. According to their ELISA protocol, serum samples were diluted only 1:100. Furthermore, they utilized an extraordinarily low dilution of 1:350 of the conjugated detector antibody. This likely gave rise to extremely high background and diminished the sensitivity of the assay.

Although a clinical sensitivity of ~ 90% can be achieved using MBP autoantibody levels in the CSF, this method is unsuitable as a diagnostic tool for several practical reasons. Lumbar puncture (LP) is an invasive technique. Besides being uncomfortable to the patient, complications associated with LPs include prolonged headaches post-procedure, a risk of
infection and lengthy time duration for both patient and physician, since a typical lumbar puncture may take several hours. The performance of LPs are reserved for experienced neurologists. Moreover, the RIA described by Warren and Catz (1986-present) is fairly cumbersome, given the requirement of acid dissociation before testing. In all likelihood, widespread utility of the para-clinical test would likely be inhibited by the obstacle of commercializing such an assay. A simpler measurement is one in which MBP autoantibodies are measured in the blood of MS patients. Venipuncture has much less associated complications, is usually performed as a standard procedure in any initial diagnostic work-up, and can be performed by individuals without any neurological background. Moreover, should MBP autoantibodies be predictive of disease progression and have prognostic utility, blood sampling can be performed frequently. In addition, acid dissociation may not be required as these circulating autoantibodies may have alternative methods of release and may not be present in complexed form as readily. A simple ELISA can be easily commercialized, could be integrated into the diagnostic work-up in MS and may facilitate the monitoring of patients involved in novel MS therapeutic trials.
E. EXPERIMENTAL OBJECTIVES

Traditionally, a good biochemical marker for any disease is characterized by a high clinical sensitivity and specificity for only the disease of interest. In the case of MS, numerous biochemical markers, including MBP, MBP and PLP autoantibodies, S-100B and thrombomodulin have been examined. Although these possible diagnostic measures have shown some extent of non-specificity for MS and variable clinical sensitivities, ranging from 0-50%, were observed, these biochemical markers have shown promise as diagnostic and prognostic tools attested by the fact that clinical sensitivities generally increased in patients experiencing active disease. This observation is especially characteristic of MBP autoantibody measurement in the CSF of MS patients. Given the invasive nature of lumbar puncture, its unsuitability as a widely accepted diagnostic measure and since the majority of these other markers have been measured in the CSF, clinical sensitivity and specificity of MBP autoantibodies and other markers must be re-confirmed in the blood of MS patients.

The diverse clinical manifestations of MS, variations in immunological evidence of pathology between patients and the presence of immune responses to different autoantigens suggests not only that MS is a heterogeneous disease but also emphasizes that accurate diagnosis with high sensitivity and specificity may mandate that multiple markers be incorporated into one test for the definitive diagnosis and progressive monitoring of the disease.

The purpose of this study was to develop an immunoassay to measure MBP autoantibodies in the blood of MS patients, thereby avoiding the invasive technique of lumbar puncture required for CSF collection. We propose that by examining matrix effects within the developed assay, both serum and plasma samples may be used. In addition,
different autoantibody classes, that is IgG and IgM, may better indicate the disease status of the individual patient.

In the determination of the assay's clinical utility, clinically definite MS patients' sera/plasma should exhibit elevated titers of MBP autoantibodies when compared with titers in healthy controls, therefore offering a high clinical sensitivity and specificity. If MBP autoantibodies are involved in the pathogenesis of demyelinating lesions, we expect that their levels should increase during periods of active demyelination resulting in relapse or exacerbation of clinical signs in MS patients. The diagnostic utility of the developed MBP autoantibody assay will be compared with the clinical utility of assays measuring other biochemical markers for MS.
CHAPTER 2.

MATERIALS AND METHODS

A. MATERIALS

(I) Blood Collection

Butterfly needles (23 gauge) were obtained from Veinsystems (Abbott Laboratories). Heparin and serum vacutainer tubes and 10 cc tuberculin syringes were obtained from Becton-Dickenson. Plasma and serum fractions were aliquoted into 0.5 ml and 1.5 ml screw top polypropylene tubes (Sarstedt).

(II) MBP Autoantibody ELISA

Unfractionated myelin basic protein was obtained from Dr. Moscarello’s laboratory. MaxiSorp 96 well microtiter plates were obtained from Nunc. Protease-free bovine serum albumin (BSA) was purchased from Equitech-Bio and all buffer components were obtained from BDH. Heparin was obtained from Sigma. Goat anti-human IgG-HRP, goat anti-human IgM-HRP, and donkey anti-goat IgG-HRP were all purchased from Jackson. Tetramethylbenzidine (TMB) substrate was obtained from Moss.
B. METHODS

(I) Sample size justification

MS patient sample size was determined by currently available patients. Under the assumption of a conservative estimate of 2 standard deviation difference between MS patients and controls, the minimum sample size of 10 patients provides 80% power to detect a type I error at p=0.01.

(II) Patient Selection and Inclusion Criteria

(i) MS Patients

Consecutive MS patients, from 26 April, 2000 to 4 October, 2000, meeting the diagnosis of clinically definite MS, as described by Poser et al (1983), seen in consult at the St. Michael's Hospital, MS Clinic, Toronto Ontario, were offered study inclusion. All patients were provided the Letter of Information and the content of the letter was discussed with them. Consent was obtained prior to blood collection using patient consent forms and baseline assessments were made in the clinic including DOB, sex, date of blood collection, when available date of symptom onset, date of diagnosis, as well as a detailed neurologic examination, determination of disease severity score (EDSS: Expanded Disability Scaled Score) and categorization of current disease status (active relapse, remission, primary/secondary progressive).
(ii) **Other Autoimmune Disease Controls**

Blood samples from ten diabetes type I (IDDM) patients were previously collected for other studies (courtesy of Dr. H-M. Dosch, Hospital for Sick Children, Toronto, Ontario). Blood samples from fifteen rheumatoid arthritis patients were obtained (Dr. E. Keystone, Mt. Sinai Hospital, Toronto, Ontario). Patients were provided the Letter of Information, the content of the letter was discussed with them and control consent forms were provided by the examining physician.

(iii) **Normal Healthy Controls**

Blood samples from ninety eight apparently healthy individuals, with no previous symptoms of MS, were drawn at SynX Pharma, Inc. DOB and gender were recorded at the time of blood collection and a general health questionnaire was used to record any other diseases in the individual donors.

(III) **Blood Collection and Analysis**

Following patient consent, phlebotomy was performed on ninety-six MS patients. Each patient was assigned an SMH number at the time of blood collection to ensure the confidentiality of the patients and provide anonymous analysis of the blood until clinical utility of the test was assessed. The blood was collected into matched heparinized and serum Vacutainer tubes (Beckon-Dickenson) and centrifuged at 3,000 rpm for 30 minutes. Each patients’ plasma and serum fractions were aliquoted and frozen at −20°C until use. The patient information was obtained from the clinic charts after analysis by ELISA.
(IV) MBP Autoantibody ELISA

(i) Microtiter Plate Preparation

All odd column wells of 96 well microtiter plates (MaxiSorp, Nunc) were coated with 125 μl of 8 mg/L unfractionated myelin basic protein (bovine) in 100 mM carbonate/bicarbonate buffer, pH 9.6. All even column wells were coated with 125 μl of 100 mM carbonate/bicarbonate buffer (modified Crimando and Hoffman, 1992). Plates were sealed with ELISA plate sealer (Costar) and incubated at 4°C overnight. Plates were then washed three times with 10 mM PBS + 0.05% Tween-20, 300 μl/well. All wells were subsequently blocked with 250 μl/well of 2% w/v BSA (Equitech-Bio) in carbonate/bicarbonate buffer, to reduce non-specific binding, and were incubated at 4°C overnight.

(ii) ELISA Protocol (Figure 4)

Plates were washed three times with 10 mM PBS + 0.05% Tween-20. Plasma test samples were diluted in the dilution buffer which consisted of 10 mM PBS, 0.05% Tween-20 and 5 USP/ml heparin (Sigma) (Pesce et al, 1986) at a working dilution of 1:320. 100 μl of each diluted test sample was added to the matched MBP and adjacent MBP-free wells. Affinity purified goat polyclonal antibody against bovine unfractionated MBP was diluted in a calibrant of normal human plasma, diluted at 1:320 in the dilution buffer, and tested at 0, 25, 50, 100 and 200 ng/ml to form a standard curve. Negative controls were run in quadruplicate and consisted of neat dilution buffer. The wells were incubated for 1 hour at room temperature. Plates were then washed three times with 10 mM PBS + 0.05% Tween-20. Wells containing test samples and the negative control were incubated with 100 μl/well
Purified MBP (8 mg/L) is passively absorbed to microtitre plate

Diluted patient plasma is added to the wells

60 minutes, RT Incubation

Purified anti-human IgG/IgM-HRP is added to the wells

60 minute, RT Incubation

TMB substrate is added to the wells

Incubate 2.5 minutes, RT dark

Reaction is stopped with 1N H₂SO₄

Optical Density is read at 450nm

Figure 4: ELISA Configuration
of goat anti-human IgG (Fc) conjugated to horseradish peroxidase (Jackson) or goat anti-
human IgM conjugated to horseradish peroxidase (Jackson), diluted 1:15,000 or 1:20,000
respectively, in dilution buffer for 1 hour at room temperature. The wells containing the goat
polyclonal antibody were incubated with 100 µl of donkey anti-goat IgG conjugated to
horseradish peroxidase (Jackson), diluted 1:7,000 in dilution buffer for 1 hour at room
temperature. After three washes with 10 mM PBS + 0.05% Tween-20, 100 µl of tetra-
methyl benzidine (Moss) was added to each well and incubated in the dark for 2.5 minutes.
The reaction was stopped with 1 N H₂SO₄, 100 µl/well. Optical density was read on a
SoftMax microtiter plate reader (Molecular Devices) at 450nm. Absorbance values from the
non-coated well were subtracted from the OD 450nm value in the MBP coated well from the
same sample. This subtracted value (S-value) reflects the specific antibodies present in the
sample. The subtracted values obtained from the standard curve has been used to quantitate
the amount of IgG in each test sample.

(V) Western Blot Protocol for the Detection of MBP Autoantibodies (IgG)

The equivalent of 2 µg unfractionated myelin basic protein (bovine) per lane was
separated by 12.5% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) at
180V for 60 minutes at room temperature (Laemmli, 1970) as well as, 6 µl of precision
molecular weight markers (Biorad). The gels were then transferred to Immobilon P PVDF
membranes (Millipore) at 100V for 180 minutes at 4°C using 10% methanol in transfer
buffer. Following electroblotting, the membranes were blocked with 5%Blotto/TBS and
allowed to incubate for 60 minutes, shaking at room temperature and then incubated at 4°C
overnight. The membranes were washed for 30 minutes at room temperature, shaking with
TTBS. The membranes were secured in the MiniCell Protean III multi-sample apparatus.
Thirty-eight normal serum samples and forty-two clinically definite MS samples were randomly chosen and diluted 1:10 in 1%Blotto/TBS. Diluted serum samples (240 μl of each sample) were loaded into separate lanes on the apparatus. The diluted serum samples were incubated for 60 minutes at room temperature, rocking and all sera was subsequently aspirated off of the membrane using a vacuum pump. The membranes were washed three times, each for 5 minutes with TTBS. Goat anti-human IgG (Fc) conjugated to horseradish peroxidase (Jackson) was diluted 1:2,000 in 1% Blotto/TTBS. Membranes were incubated with the conjugated antibody for 120 minutes, shaking at room temperature. The membranes were subsequently washed three times, each for 5 minutes, shaking at room temperature with TTBS. The immunoblots were developed using a TMB substrate kit as per the manufacturer's instructions for 3 minutes. The reaction was stopped with UF water.

(VI) **Solid phase ELISA for the detection of PLP, S-100B and NSE Autoantibodies**

(i) **Microtiter Plate Preparation**

All odd column wells of 96 well microtiter plates (MaxiSorp, Nunc) were coated with 125 μl of 8 mg/L of either purified water soluble PLP, bovine S-100B (Sigma) or NSE (a gift from SynX Pharma, Inc.) in 100 mM carbonate/bicarbonate buffer, pH 9.6. All even column wells were coated with 125 μl of 100 mM carbonate/bicarbonate buffer (modified Crimando and Hoffman, 1992). Plates were sealed with ELISA plate sealer (Costar) and incubated at 4°C overnight. Plates were then washed three times with 10 mM PBS + 0.05% Tween-20. 300 μl/well. All wells were subsequently blocked with 250 μl/well of 2% w/v BSA (Equitech-Bio) in carbonate/bicarbonate buffer, to reduce non-specific binding, and were incubated at 4°C overnight.
(ii) ELISA Protocol

Plates were washed three times with 10 mM PBS + 0.05% Tween-20. Plasma test samples were diluted in the dilution buffer which consists of 10 mM PBS, 0.05% Tween-20 at a working dilution of 1:320. 100 µl of each diluted test sample was added to the matched antigen (Ag) and adjacent Ag-free wells. A mouse monoclonal antibody raised against PLP, a monoclonal antibody raised against NSE (SynX Pharma, Inc.), and a rabbit polyclonal antibody raised against S-100B (Dako) were diluted 1:1000 in the sample dilution buffer and served as positive controls for each of the respective assays. Negative controls were run in quadruplicate and consisted of neat dilution buffer. The wells were incubated for 1 hour at room temperature. Plates were then washed three times with 10 mM PBS + 0.05% Tween-20. Wells containing test samples and the negative control were incubated with 100 µl/well of goat anti-human IgG (Fc) conjugated to horseradish peroxidase (Jackson), diluted 1:15,000 in dilution buffer for 1 hour at room temperature. The wells containing the positive controls were incubated with 100 µl of either goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson), for the S-100B autoantibody assay or goat anti-mouse IgG conjugated to horseradish peroxidase (Biorad), diluted 1:7,000 in dilution buffer for 1 hour at room temperature. After three washes with 10 mM PBS + 0.05% Tween-20, 100 µl of tetramethyl benzidine (Moss) was added to each well and incubated in the dark for 2.5 minutes. The reaction was stopped with 1 N H₂SO₄, 100 µl/well. Optical density was read on a SoftMax microtiter plate reader (Molecular Devices) at 450nm. Absorbance values from the non-coated well are subtracted from the OD 450nm value in the MBP coated well from the same sample. This subtracted value (S-value) reflects the specific antibodies present in the sample. The subtracted values obtained from the standard curve has been used to quantitate the amount of IgG in each test sample.
Sera Concentrations of MBP, S-100B, NSE\gamma, and Tm

MBP, S-100B, NSE and Tm concentrations were measured in 90 matched MS patient serum samples, by two-site, in-direct ELISA as per the manufacturer’s instructions for the Smart MBP kit, Smart S-100B kit, Smart NSE kit and Smart Tm kit (SynX Pharma, Inc.) respectively. The clinical decision limit was determined by the evaluation of 103 normal healthy donors by the manufacturer and was defined as the mean + two standard deviations of the normal population tested. It should be noted that not all MS patient sample analysis could be included in this work, as some of the serum samples were hemolyzed and pose interference problems in the NSE assay.
Numerous optimization experiments must be conducted during the development of an immunoassay in order to maximize analytic and clinical sensitivity, as well as minimize non-specific interference within the assay. Since most assays for specific antibodies are qualitative, these experiments generate an assay which minimizes false positive results and allow for a low detection limit required to detect low titer antibody levels (Micallef and Ahsan, 1994), while maintaining low levels of imprecision. Upon the completion of the fully optimized assay, experiments to determine performance characteristics are conducted to show that the assay is precise and repeatable.

A. ASSAY OPTIMIZATION

Some of the more critical optimization experiments and their results are described in the following sections. For each experiment the parameter tested which offered the highest signal: noise ratio using a rabbit polyclonal control or MS patient sample, while minimizing the S-value in normal control sera was chosen as optimal.
Microtiter Plate Type

Figure 5: Selection of Microtiter Plate

Efficiency of MBP adsorption to four brands of microtiter plates was assessed by calculating the coefficient of variation (%CV) between 16 determinations of a polyclonal antibody control (RoMBP) and dilution buffer alone for each of the four plates. The ELISA protocol outlined in the Methods section was modified, as described in the Results section.
(I) Coating Optimization

(i) Microtitre plate Selection

In order to determine which plastic surface was most suitable for the passive absorption of unfraccionated MBP, MBP was added to the wells of Immunolon 4 HBV flat bottom plates (Dynatech), PolySorp flat bottom plates (Nunc) and Corning #25880-96 flat bottom plates (Fisher) and MaxiSorp C bottom plates (Nunc) at 1 µg/ml overnight. Affinity purified rabbit antibody specific for MBP was diluted 1:1000 and was added to the wells. 16 determinations (OD450nm) for the rabbit antibody and wash buffer alone demonstrated that the MaxiSorp plates offer the highest signal to noise ratio (Figure 5). The coefficient of variation for both the polyclonal control and the buffer alone are both less than 10% as compared with the high CVs of the Corning and Immunolon plates. This indicated that MaxiSorp plates offered a more reliable measurement of antibody while maintaining an excellent signal to noise ratio. Therefore, MaxiSorp microtitre plates were the optimal solid phase for this assay.

(ii) Coating Buffer Selection

Since MBP is a cationic protein, its solubility and therefore its ability to bind to the solid phase may be affected by the pH of the coating buffer. In order to determine whether pH affected coating efficiency, MBP was coated to MaxiSorp plates (1 µg/ml) in 100 mM carbonate/bicarbonate buffer, pH 9.6, 10 mM PBS, pH 7.4 and 150 mM citrate/phosphate buffer, pH 5.5. The rabbit anti-MBP antibody was added to wells and the resulting 24 determinations illustrated that no difference between the coating buffers was observed with respect to CV and signal to noise ratio (Table 2). We chose 100 mM carbonate buffer because we were familiar with the stability of this buffer.
Table 2: Coating Buffer Selection

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Mean</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM carbonate/bicarbonate buffer, pH 9.6</td>
<td>2.67</td>
<td>3.46</td>
</tr>
<tr>
<td>10 mM PBS, pH 7.4</td>
<td>2.46</td>
<td>3.29</td>
</tr>
<tr>
<td>150 mM citrate/phosphate buffer, pH 5.5</td>
<td>2.51</td>
<td>5.98</td>
</tr>
</tbody>
</table>

MBP was passively absorbed to the microtiter plate wells at 1 mg/L in 3 different coating buffers: 100 mM carbonate/bicarbonate buffer, pH 9.6; 10 mM PBS, pH 7.4; 150 mM citrate/phosphate buffer, pH 5.5. Affinity purified RαMBP IgG was added to each of 24 wells containing MBP. The ELISA protocol, outlined in the Methods section, was followed. The mean absorbance values of the 24 determinations, for each coating buffer examined, and standard deviations were calculated in order to determine the co-efficient of variation (%CV).
(iii) Coating Concentration Optimization

By testing 5 MS patient and 11 normal control plasma samples on microtitre plates coated with 8 μg/ml, 4 μg/ml, 2 μg/ml and 1 μg/ml of unfractionated MBP, we observed that the best distinction between S-values for MS and normal plasma occurred when the plate was coated with 8 μg/ml of MBP (Figure 6).

(II) Blocking Buffer Selection

The selection of a blocking agent in any assay is critical to reduce non-specific adherence of antibody to any exposed binding sites on the microtitre plate well while not masking any exposed epitopes on the coating antigen.

Several blocking proteins and their concentrations in different buffers were tested by examining the signal to noise ratio using S-values for a rabbit polyclonal antibody and the S-values of normal plasma. The following blocking agents were examined at various concentrations in both carbonate buffer, pH 9.6 and 10 mM PBS, pH 7.4: BSA, casein, hydrolyzed casein, poly-lysine, ovalbumin, poly-vinyl alcohol and carnation skim milk. BSA (2%, w/v) in carbonate/bicarbonate buffer, pH 9.6 because it offered the highest signal to noise ratio using the rabbit anti-MBP IgG antibody and most effectively minimized background in the normal plasma samples tested.
Figure 6: Optimization of MBP Coating Concentration
Microtiter plates were coated with 8 μg/ml, 4 μg/ml, 2 μg/ml, and 1 μg/ml of unfractionated MBP. MBP autoantibody titers were measured in plasma from normal healthy controls (n=11) and MS patients (n=5). The best distinction between the two groups was achieved using 8 μg/ml of MBP as the coating concentration.
(III)  Plasma/Serum Dilution Optimization

(i)  Dilution Factor Determination

The optimal serum/plasma dilution factor was chosen by running 11 normal samples and 5 clinical samples in the anit-MBP IgG ELISA. The choice of 1:320 as the plasma dilution was based on the fact that the point of inflection for the majority of the normal samples in their serial dilution was observed at this dilution factor (Figure 7). Furthermore, the greatest distinction between OD values (OD450nm) obtained using MS sera, compared with the normals was seen at this dilution.

(ii)  Non-Specific Binding Reduction

Traditional measures of reducing non-specific binding include the addition of BSA, high salt concentrations, and high detergent concentrations. These experiments were performed however any addition of these materials resulted in a total decrease in the signal to noise ratio when MS patient sera was tested.

Since MBP is a cationic protein, non-specific charge interactions were hypothesized to contribute to the lack of distinction between normal and MS patient MBP autoantibody titers. We tested the effect of the addition of two polyanions, heparin and dextran sulfate at several concentrations in the plasma dilution buffer. Heparin (5USP) was chosen because it maintained excellent signal to noise ratio in the polyclonal control (rabbit) while reducing the detectable levels of MBP autoantibodies in normal plasma. Figure 8A demonstrates that at the optimal dilution factor of 1:320, only 2 of the initial 24 MS patient samples has antibody titers elevated above the mean+2SD of the normal range (n=11). Clinical detection, via sensitivity dramatically improved when the antibody dilution buffer was modified to include heparin (Figure 8B). The difference in sensitivity between the two assays illustrates that
Figure 7: Selection of Optimal Plasma Dilution Factor
Serial two-fold dilutions, starting at 1:20, of plasma from normal donors (n=11) were assayed for MBP autoantibodies (IgG) by ELISA, as outlined in the Methods section. The plasma dilution of 1:320 represents the point of inflection for the titration of the majority of plasma samples examined.
Figure 8: Plasma Dilution Buffer Optimization
A. ELISA results without addition of heparin
B. ELISA results with the addition of heparin
assay protocols which are not well tailored to the protein of interest lack adequate sensitivity. This affords a possible reason as to why some investigators achieved successful detection of MBP autoantibodies where others had previously failed. Upon examination of those reports denying the presence of anti-MBP, the assay protocols employed simple buffers, which did not appear to be specific for such a cationic protein.

(IV) Conjugate Dilution Optimization

Upon the selection of affinity purified anti-human IgG and IgM HRP conjugated antibody, optimization of the dilution factor for each antibody is performed by serially adding concentrations of the conjugated antibody to wells incubated with plasma from a normal control and an MS patient. We chose 1:15,000 as the conjugated antibody dilution for the goat anti-human IgG antibody and 1:20,000 for the goat-anti-human IgM antibody.

The optimization experiments outlined above were some of the more critical experiments in defining our sensitive assay. In addition to these experiments, we also tested the effect of duration of each step and examined two substrates for HRP, TMB and OPD, as well as the duration of plate development. The final fully developed assay was constructed by optimization of each step of the ELISA protocol.
B. ASSAY PERFORMANCE

In order to validate a newly developed assay, and determine whether the values obtained in the assay are accurate and correct, many parameters must be examined and are usually set forth by guidelines published by the International Federation of Clinical Chemistry (Micallef and Ahsan, 1994; Grotjan and Keel, 1996). Some of the most common assay performance characteristics include assay sensitivity, specificity, intra-assay and inter-assay precision, analytic recovery and interference. In addition, when other tests are commercially available, patient results are compared with those obtained by these other independent methods of assay (Micallef and Ahsan, 1994).

(I) Precision

In the determination of assay reproducibility, analysis of control samples, with known amounts of analyte (e.g. addition of specific autoantigen to sample), is performed. These control samples ideally mimic concentrations observed in patient samples, especially at or near the clinical decision limit of the assay. Reproducibility is expressed as the coefficient of variation (standard deviation divided by the mean x 100 % = CV) of the patient sample, in duplicate results, for the determination of intra-assay (within-assay) and inter-assay (between-assay) precision (Micallef and Ahsan, 1994). An acceptable level of imprecision for assay results is dependent on the clinical application and on intra-individual biologic variation (Micallef and Ahsan, 1994), however CV less than 10 % are usually acceptable.
(i) **Intra-assay precision**

Also referred to as within-sample, within-batch precision, intra-assay precision is simply determined by assaying, in one run, 10-20 replicates of samples containing a range of analyte concentrations distributed along the standard curve (Gosling and Basso, 1994). After calculating the mean, SD and CV for each set of replicates, a graph relating precision to analyte concentration is plotted, which is referred to as the within-sample, within-batch precision profile.

(ii) **Inter-assay precision**

Also referred to as within-sample, between-batch precision, inter-assay precision is assessed by using samples of low, medium and high concentrations which usually represent diagnostically relevant concentrations. The mean, SD and CV for each concentration is calculated from separate assay runs/batches. That is each result is the average of duplicate determinations if the patient samples are assayed in duplicate (Gosling and Basso, 1994).

(iii) **Precision Results**

Plasma from three MS patients, of high, medium and low titres for MBP autoantibodies were assayed in replicates each of 20 in one run to determine intra-assay precision (Table 3). Likewise, these plasma samples were assayed in replicates of five in five different runs to determine inter-assay precision (Table 4). The results illustrate that coefficient of variation values (%CV) at all levels for both intra-assay and inter-assay experiments are in acceptable ranges, that is less than 10%. Therefore, samples evaluated in duplicate are reliable indicators of the true levels of MBP autoantibodies and the evaluation
Table 3: INTRA-ASSAY PRECISION

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean OD_{450nm} (S-value)</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>0.029</td>
<td>6.75</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.091</td>
<td>7.81</td>
</tr>
<tr>
<td>Sample 3</td>
<td>0.217</td>
<td>8.42</td>
</tr>
</tbody>
</table>

MBP autoantibody (IgG) titers were measured in the plasma of 3 MS patients. The mean subtracted absorbance value and standard deviation of 20 determinations of each patient sample was used to assess precision by calculating the coefficient of variation (%CV).
Table 4: INTER-ASSAY PRECISION

<table>
<thead>
<tr>
<th>n=5</th>
<th>Mean OD$_{450nm}$ (S-value)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>0.027</td>
<td>5.85</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.093</td>
<td>5.27</td>
</tr>
<tr>
<td>Sample 3</td>
<td>0.225</td>
<td>9.46</td>
</tr>
</tbody>
</table>

MBP autoantibody (IgG) titers were measured in the plasma of 3 MS patients. The mean subtracted absorbance values and standard deviation of 5 determinations for each of 5 separate assay runs were used to assess precision between assays by calculating the coefficient of variation (%CV).
of samples can be reliably accomplished using separate preparations of the ELISA components. The assay is precise and repeatable.

(II) Analytic Recovery

In order to assess analytic recovery, a known amount of pure analyte (the protein of interest, in this case anti-MBP IgG) or a small volume of a sample with a very high analyte concentration is added into samples (Miller and Levinson, 1996). When the samples are assayed, the observed results should equal the endogenous concentrations plus the concentration within the calibrant (a matrix mimicking the matrix of the samples to be tested which contains many proteins, in this case 1:320 dilution of plasma/serum in the previously described dilution buffer) (Micallef and Ahsan, 1994). When analytic recovery is consistently greater or less than 100%, there may exist a disparity between the calibrant and patient sample matrices or the assay may be susceptible to interfering substances in the patient samples.

Plasma from two separate MS patient samples was added to plasma from each of three normal donors and were assayed for anti-MBP IgG. Table 5 illustrates that subtracted OD values* for both MS patient samples was within a 5% margin of recovery. Traditionally, analytic recovery experiments are performed by adding a known concentration of purified analyte (in this case purified anti-MBP IgG) into normal samples. However, this can not be performed for this assay because purified MBP IgG is not commercially available. Nevertheless, this recovery experiment illustrates that MBP autoantibody titres are not subject to interference by normal plasma constituents.

*subtracted OD450nm is obtained by subtracting the absorbance value obtained in the no-MBP coated well from the absorbance value obtained in the matched MBP coated well for each sample tested.
Table 5. Analytical Recovery of MBP Autoantibody (IgG) in three normal human plasma samples

<table>
<thead>
<tr>
<th>Plasma Sample</th>
<th>Endogenous MBP Autoantibody (Mean OD₄₅₀nm S-value)</th>
<th>Expected MBP Autoantibody (Mean OD₄₅₀nm S-value)</th>
<th>Observed MBP Autoantibody (Mean OD₄₅₀nm S-value)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.008 0.008</td>
<td>0.361 0.228</td>
<td>0.380 0.238</td>
<td>105 104</td>
</tr>
<tr>
<td>2</td>
<td>0.014 0.014</td>
<td>0.361 0.228</td>
<td>0.348 0.229</td>
<td>96 101</td>
</tr>
<tr>
<td>3</td>
<td>0.006 0.006</td>
<td>0.361 0.228</td>
<td>0.342 0.227</td>
<td>95 100</td>
</tr>
</tbody>
</table>

Plasma from two MS patient samples was added to plasma from each of three normal donors and was assayed.
Endogenous MBP Autoantibody is the absorbance value obtained in the normal samples without the addition of additional antibody.
Expected MBP Autoantibody is the absorbance value obtained in the clinical samples used prior to its addition to normal samples.
Analytic recovery was determined by calculating the %Recovery (Observed/Expected MBP Autoantibody x 100%) for each sample.
(III) **Interference**

The effect of interfering substances such as hemoglobin, lipids or bile acids are generally appreciated by the inclusion of hemolyzed, lipemic or icteric blood in the normal cohort of samples examined (Miller and Levinson, 1996). However it is important to test these possible interfering substances in the developed assay by adding known quantities of hemoglobin, lipid and bilirubin into patient samples. Moreover, the extent to which specific pharmacotherapeutic drugs for the disease of interest interfere with the testing of the analyte is also critical. By evaluating patient samples containing added increasing quantities of common therapeutics, the extent of possible drug interference can be determined.

In order to assess whether standard interference compounds, such as hemoglobin, bilirubin and lipid prevent the accurate measurement of MBP autoantibodies, 3 concentrations each of hemoglobin, bilirubin and triglyceride was added to the plasma from one MS patient. Each sample was assayed for MBP IgG in duplicate. When compared with the subtracted OD values achieved by the control samples (MS patient plasma without any additional compounds), samples with elevated hemoglobin, bilirubin and triglyceride yielded similar OD values (Table 6). The three concentrations of each compound tested represent values outside the normal range, as well as within the normal range. Therefore, MS patients who present with hemolyzed, icteric or lipemic blood samples can still be reliably tested for MBP autoantibodies by this assay.

In the assessment of possible interference of common MS pharmaceutical agents, increasing doses of methylprednisolone (SoluMedrol), IFNβ (Avonex/Rebif), Glatiramer Acetate (Copaxone) and Paclitaxel were added to the diluted plasma of one MS patient. With the exception of SoluMedrol (Figure 9), the remaining three drugs did not significantly decrease the endogenously detected titer of MBP autoantibodies. SoluMedrol concentrations
Table 6. MBP Autoantibody ELISA: Interference by Plasma Constituents

<table>
<thead>
<tr>
<th>Interfering Substance</th>
<th>Level Tested</th>
<th>Mean OD$_{450nm}$ (S-value) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/L)</td>
<td>167.00</td>
<td>0.370 ± 0.014</td>
</tr>
<tr>
<td></td>
<td>83.50</td>
<td>0.363 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>41.80</td>
<td>0.364 ± 0.006</td>
</tr>
<tr>
<td>Bilirubin (μmol/L)</td>
<td>132.00</td>
<td>0.368 ± 0.024</td>
</tr>
<tr>
<td></td>
<td>66.00</td>
<td>0.350 ± 0.021</td>
</tr>
<tr>
<td></td>
<td>33.00</td>
<td>0.368 ± 0.019</td>
</tr>
<tr>
<td>Triglyceride (g/L)</td>
<td>5.30</td>
<td>0.400 ± 0.024</td>
</tr>
<tr>
<td></td>
<td>2.65</td>
<td>0.382 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>1.33</td>
<td>0.367 ± 0.010</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.366 ± 0.002</td>
</tr>
</tbody>
</table>

Three concentrations of interfering compounds were added to the plasma from one MS patient and were run in the assay to determine whether MBP autoantibody titers can be reliably detected in hemolyzed, icteric or lipemic samples.
Figure 9: Drug Interference by SoluMedrol in the MBP Autoantibody Enzyme Linked Immunosorbant Assay (ELISA)

Increasing concentrations of Solumedrol were added to the plasma of one MS patient to determine interference effects. Examination of the subtracted absorbance values (OD450nm, S-value) obtained for each concentration of Solumedrol indicates that concentrations of 375 mg/ml or greater in the plasma of an MS patient, interferes with the accurate detection of MBP autoantibodies by ELISA.
commencing with 375 mg/ml significantly inhibited the accurate detection of MBP autoantibodies in the patient's sample. The recommended dose for this drug ranges between 10-6,000 mg. Since 375 mg/ml translates to over 50g in the average individual, the detection of MBP IgG in a patient receiving this treatment would remain unaffected.

(IV) Matrix Analysis

A matrix is defined as constituents of a sample, exclusive of the analyte itself (Grotjan and Keel, 1996). Matrix effects in an immunoassay are recognized by inaccurate values or dissimilar values between matrices within the same test sample and result through a variety of mechanisms, including interfering with the binding of the analyte to a primary antibody, thus preventing accurate detection.

Numerous papers report inconclusive or unsuccessful attempts of MBP autoantibody detection by several assay techniques (Colombo et al., 1997; Agius et al. 1999; Reindl et al., 1999; Brokstad et al., 1994; Lennon and MacKay, 1972; Schmid et al., 1974; and Gutstein et al., 1978). One possible reason for failure of MBP IgG detection in serum, proposed at the outset of this project, was that autoantibody levels in serum are low or undetectable because they are removed with the formation of the fibrin clot. In order to address this possibility, comparative matrix analysis was performed for serum and heparinized plasma. Figure 10 illustrates that subtracted OD values for MBP IgG in matched serum and plasma samples from MS patients (n=83) provide excellent correlation ($R^2 = 0.81$). Therefore, both serum and plasma samples can be used in the assay to measure MBP IgG. Limited MBP IgG detection by others (Brokstad et al., 1994; Cruz et al, 1987) is likely a result of high background in the assays used to date.
Figure 10: Matrix Analysis of Match Serum and Plasma Samples

MBP Autoantibody (IgG) titers were measured in matched heparinized plasma and serum samples from 83 MS patients to determine the effect of matrix in the assay. The resulting subtracted absorbance values for both plasma and serum samples exhibit a correlation coefficient of 0.81.
(V) Standard Curve

It is important to have a standard curve that will provide a means of quantitating the concentration of autoantibody in a sample by using the linear portion of the curve. Ideally, purifying the autoantibodies from the blood of a high titer MS patient and using them as the calibrators in the assay would facilitate the most representative quantitation of autoantibodies in each assay run. The majority of commercially available assays for specific human antibodies are qualitative. In order to quantitate the results achieved in this assay, a standard curve using affinity purified anti-MBP IgG, raised in goat, was obtained. Figure 11 illustrates that using three separate assay runs, the standard curve exhibits linearity from 0 to 100 U/mL. This standard curve has been used to quantitate the amount of IgG in each patient sample.

(VI) Summary

Through a battery of optimization experiments, an ELISA to measure MBP autoantibodies in the blood of MS patients has been developed. Precision studies have shown that the assay is precise and repeatable at both high and low antibody titers. Analytic recovery experiments indicated that normal plasma constituents do not interfere with the accurate detection of MBP autoantibodies using this ELISA and both serum and plasma are suitable matrices for their measurement. Hemolyzed, icteric and lipemic blood samples do not pose any problem in the assay and circulating concentrations of several common MS drugs do not inhibit the detection of MBP autoantibodies. The developed assay is simple, inexpensive and rapid (2 hours).
Figure II: Standard Curve for the MBP autoantibody ELISA

Standard curve is achieved using affinity purified polyclonal antibody diluted in normal human plasma at the working dilution. Linearity of the standard curve is achieved from 0-100 U/ml.
CHAPTER 4

CLINICAL VALIDATION OF CIRCULATING MBP AUTOANTIBODIES IN MS PATIENTS

A. MBP AUTOANTIBODY ELISA

The overall efficiency of a fully developed immunoassay is determined by the proportion of all patients for whom the test correctly predicts the presence or absence of disease (Micallef and Ahsan, 1994). The clinical utility of MBP autoantibody measurement was examined by using the developed ELISA to measure anti-MBP IgG and IgM in clinically definite MS patients. We hypothesized that MBP IgG should correlate with disease activity, as was observed in the CSF (Warren and Catz, 1986). Detection of anti-MBP IgG in MS patient sera by Western blot was examined to determine whether this technique, which has been employed by other investigators, is sensitive enough to measure low titers of MBP autoantibodies in the circulation. Moreover, if the autoimmune response in MS is specific to MBP, autoantibodies to other myelin and non-myelin proteins should be negligible in comparison.

(I) Diagnostic Parameters: MBP IgG

Ninety-eight normal healthy subjects (age range 20-66, mean age = 36, approximately equal number of males and females) were run on the ELISA, in duplicate. Plasma samples from ninety-four clinically definite MS patients (age range 18-63, mean age = 38, male and female) were also run on the ELISA. Using the mean + 2SD of the normal
subjects studied as the clinical cut-off (dashed line), 77% of the MS patients tested exhibited elevated levels of MBP autoantibodies (IgG) in plasma (p<0.001), whereas only five normal samples exhibited IgG levels marginally above clinical cut-off (Figure 12). Receiver Operating Curves (ROC) are constructed by plotting the sensitivity vs. the specificity of the test using multiple measurements as possible clinical decision limits. Determination of the optimal clinical cut-off by ROC plot (Figure 13) illustrates that using the mean + 2SD of the normal samples as the clinical decision limit offers high sensitivity (77%) and specificity (95%).

The diagnostic parameters are summarized in Table 7 and their determination is shown in Table 8. Sensitivity and specificity describe attributes of the test when the actual clinical diagnosis is known (i.e. the proportion of those with MS who actually have a positive test result or the proportion of those who are healthy who will have a negative test result). Since, in clinical practice one does not usually know who has the disease, positive predictive value (PPV) and negative predictive value (NPV) are useful in determining how likely a patient is to have the disease given a positive test result. PPV is the proportion of patients with a positive test result who have the disease. NPV is the proportion of patients with a negative test result who do not have the disease. Although useful, PPV and NPV vary according to the prevalence of the disease (i.e. the proportion of people undergoing the test who actually have the disease). Sensitivity and specificity are usually stable regardless of the prevalence of disease in the population in which the test is conducted while predictive values vary considerably in different populations of patients. Likelihood ratios (LR) permit a calculation of the probability of disease for a specific test result and specific disease prevalence. Likelihood ratios with a value greater than 10 are usually judged to be of high
Figure 12: MBP Autoantibody (IgG) Levels in Clinically Definite MS Patients

Plasma from MS patients (n=94), age range 18-63 (mean age = 38), male and female, was assayed for MBP autoantibodies (IgG) by ELISA, as outlined in the Methods section. Using the mean + 2SD of the normal subjects (dashed line), clinically definite MS patients tested had elevated levels of MBP autoantibodies (IgG) in plasma (p<0.001).
Figure 13: Receiver Operating Curve for MBP Autoantibody (IgG) ELISA

Determination of the optimal clinical cut-off by ROC plot illustrates that using the mean + 2SD of the normal samples as the clinical decision limit offers high sensitivity and specificity.
**Table 7: Diagnostic Value of MBP Autoantibody ELISA**

<table>
<thead>
<tr>
<th>Clinical Decision Limit</th>
<th>mean + 2SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>77 %</td>
</tr>
<tr>
<td>Specificity</td>
<td>95 %</td>
</tr>
<tr>
<td>PPV</td>
<td>94 %</td>
</tr>
<tr>
<td>NPV</td>
<td>82 %</td>
</tr>
<tr>
<td>LR</td>
<td>14.8</td>
</tr>
</tbody>
</table>

Using the mean + 2SD of the normal subjects tested as the clinical decision limit, the above diagnostic parameters were calculated (refer to Table 8 for the definition of each diagnostic parameter).
Table 8: Determination of Diagnostic Parameters

<table>
<thead>
<tr>
<th>DISEASE</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>-</td>
<td>c</td>
<td>d</td>
</tr>
</tbody>
</table>

Sensitivity = \[ \frac{a}{a + c} \]

Specificity = \[ \frac{d}{b + d} \]

PPV = \[ \frac{a}{a + b} \]

NPV = \[ \frac{d}{c + d} \]

LR = \[ \frac{\text{sensitivity}}{1 - \text{specificity}} \]

PPV = Positive Predictive Value
NPV = Negative Predictive Value
LR = Likelihood Ratio
diagnostic value. This assay offers a likelihood ratio of 14.8 and thus is a reliable diagnostic tool for MS.

(II) MBP Autoantibody Levels and Disease Status

Seventy-four MS patients were stratified on the basis of disease status at the time of blood collection, irrespective of disease course. Patients in relapse (n=26) had MBP IgG autoantibody levels significantly elevated (p<0.001, Student’s t-test) compared with those patients in stable courses of the disease (n=48) (Figure 14). Therefore, at first glance MBP autoantibodies (IgG) in plasma appear to be reflective of disease activity in this patient population. If this is the case, MBP IgG may be a useful biochemical marker for monitoring MS progression in patients undergoing novel therapies. It should be noted however that this experiment does not demonstrate that MBP IgG levels increase within individual patients experiencing relapses. In order to validate whether IgG levels are indicative of disease status, a long-term prospective case-control study would need to be performed in which individual patients MBP IgG would be matched at both stable and relapsing periods in disease course. Unfortunately, given the nature of the disease, that is patients may be stable for months to years without exhibiting acute attacks, this type of long-term study is beyond the scope of my project.

In the majority of clinical trials examining therapeutic efficacy of MS drugs, patient disease course determination is critical in the screening of patients for study inclusion. Traditionally this is accomplished by neurological history and MRI results. Sixty-five MS patients with clearly defined disease course, that is primary progressive (n=14), relapsing-remitting (n=33) and secondary progressive (n=18), were examined for differences in MBP IgG levels. Results from the Kruskal-Wallis test (a non-parametric alternative to one-way
Figure 14: MBP Autoantibody (IgG) Levels in Clinically Stratified MS Patients
When clinically separated into MS disease states, patients in relapse (n=26) had MBP IgG autoantibody levels significantly elevated (p<0.001) compared with those patients in stable courses of the disease (n=48).
ANOVA) indicated that there was no significant difference (p>0.05) in MBP autoantibody (IgG) levels between the patients exhibiting these disease courses. Therefore, MBP autoantibody levels are not useful in aiding the stratification of patient disease course in MS.

(III) MBP Autoantibody Levels and Disability

The Expanded Disability Scaled Score (EDSS) (Kurtzke, 1983) provides a clinical method of assessing neurological impairment in MS. The scale ranges from 0 (no impairment) to 10 (death) with intervals of 0.5. Scores from 0-5.5 usually reflect increasing levels of functional sensory and motor impairment and scores greater than 6 reflect increasing deficits in ambulation. Figure 15 demonstrates that in patients with recorded EDSS scores at the time of blood collection, MBP IgG levels do not correlate with disability as measured by the EDSS. These results were to be expected since most diagnostic measures in MS fail to correlate well with EDSS scores. These results may not so much reflect a deficit in the testing of MBP autoantibodies but more so in the failure of the EDSS to accurately reflect disease activity in MS patients. EDSS is a cumulative assessment of deficit and reflects inactive disease primarily. Autoantibody measurements reflect active disease.

(IV) MBP IgM Autoantibody Levels and Disease Status

Plasma samples from the previously described normal and patient cohort were assayed for MBP IgM autoantibodies by ELISA. Using the mean + 2SD of the normal samples (dashed line), elevated MBP IgM levels were observed in only 30% of the clinically definite MS patients tested (Figure 16) but none of the controls showed elevated levels. When the patients exhibiting elevated levels of MBP IgM were stratified into stable or
Figure 15: Correlation of IgG Autoantibodies and EDSS score
MBP autoantibody titers were measured by ELISA (refer to Methods section) in 56 MS patients for whom EDSS score was recorded at the time of blood collection. The resulting subtracted absorbance values (y-axis) were correlated to EDSS score for each patient. A correlation coefficient of 0.0014 was obtained.
Figure 16: MBP Autoantibodies (IgM) in Clinically Definite MS Patients

MBP autoantibodies (IgM) were measured by ELISA, as described in the Methods section. Elevated MBP autoantibody (IgM) levels (greater than the mean + 2SD = 40 U/ml, dashed line) were observed in 30% of the clinically definite MS patients tested (n=94).
relapse disease status (Figure 17), no significant difference was observed between the two patient groups (p>0.05, Student’s t-test). MBP IgM levels also did not correlate with EDSS scores (data not shown). It is interesting to note that only one patient was experiencing her first relapse. This patient had the highest level of MBP IgM. The IgM response is short lived and usually precedes that of IgG when humoral immunity is initiated. It would be interesting to collect samples from more patients experiencing their first attack and determine whether MBP IgM is also dramatically elevated in these patients.

(V) Analytic Specificity of the MBP Autoantibody (IgG) Response

In order to further evaluate the specificity of the autoantibody response to MBP in MS patients, other autoantibody responses to both myelin proteins, as well as, non-myelin CNS-specific proteins were examined. Plasma samples from the same normal and patient cohort were assayed by direct ELISA for proteolipid protein (PLP), which accounts for 50% of the total myelin protein (Figure 18), neuron specific enolase (NSE), a neuronal marker (Figure 19) and S-100b, an astrocyte marker (Figure 20). Analysis of autoantibodies for all three proteins by Student’s t-test revealed that there was no significant difference in the levels between normal and MS patients. The patients who exhibited elevated PLP autoantibody levels also had elevated MBP IgG titers. This was unexpected because Warren and colleagues (1994) claimed that CSF autoantibodies to both MBP and PLP were never detected simultaneously in the same patient. On the other hand, demyelination should result in an antibody response to any myelin antigen. In the case of PLP only 9 patients showed an antibody response suggesting that PLP was not as good an immunogen as MBP.
Figure 17: MBP Autoantibody (IgM) Levels in Clinically Stratified MS Patients
When clinically separated into MS disease states, patients with elevated anti-MBP IgM that were in relapse (n=15) had MBP IgM autoantibody levels that were not significantly elevated (p>0.05) compared with those patients in stable courses of the disease (n=7).
Figure 18: Evaluation of PLP Autoantibodies (IgG) in Clinically Definite MS Patients

As measured by direct ELISA (refer to Methods section), PLP IgG autoantibody levels were not significantly different between normal (n=98) and MS patients (n=94). Dashed line represents the mean +2SD (0.041) of the normals tested.
Figure 19: Evaluation of NSE Autoantibodies (IgG) in Clinically Definite MS Patients
As measured by direct ELISA (refer to Methods section), NSE IgG autoantibody levels were not significant different between normal (n=98) and MS patients (n=94). Dashed line represents the mean +2SD (0.140) of the normals tested.
Figure 20: Evaluation of S-100B Autoantibodies (IgG) in Clinically Definite MS Patients
As measured by direct ELISA (refer to Methods section), S-100B IgG autoantibody levels were not significant different between normal (n=98) and MS patients (n=94). Dashed line represents the mean +2SD (0.175) of the normals tested.
(VI) Clinical Specificity

In order to accurately validate the diagnostic potential of the MBP autoantibody assay, it is critical to demonstrate that the antibody response to MBP was specific for MS patients. To date, MBP autoantibody (IgG) levels have been tested in nine patients with insulin-dependent diabetes mellitus (IDDM) (Figure 21) and fifteen patients with rheumatoid arthritis (RA) (Figure 22). While only one diabetic patient exhibited elevated MBP IgG titers, 5 of the 15 rheumatoid arthritis patients had elevated MBP autoantibody levels. In general, patients with autoimmune diseases tend to be reactive to multiple antigens. The results obtained using the MBP autoantibody assay may be a reflection of this phenomenon in these clinical cases.

It will be more prudent to test patients with clinical symptoms similar to MS, to determine the true utility of the assay in differentially diagnosing MS from diseases such as sarcoidosis involving the CNS, Behcet’s disease and systemic lupus erythematosus (SLE). Plasma samples are currently being collected from patients with these and other neurological conditions at St. Michael’s hospital, for this purpose.
Figure 21: MBP IgG in Diabetic Patients
Using the mean + 2SD of the normal subjects tested (n=98) at the clinical cut-off (dashed line), one insulin dependent diabetes patient (IDDM) of the nine tested had elevated levels of MBP autoantibodies (IgG), as measured by ELISA (refer to Methods section).
Figure 22: MBP IgG in Rheumatoid Arthritis Patients
Using the mean + 2SD of the normal subjects tested (n=98) at the clinical cut-off (dashed line), five rheumatoid arthritis patients (RA) of the tested had elevated levels of MBP autoantibodies (IgG), as measured by ELISA (refer to Methods section).
B. WESTERN BLOT ANALYSIS FOR THE DETECTION OF MBP AUTOANTIBODIES (IgG)

In order to evaluate Western blot analysis as a secondary method of MBP autoantibody detection, normal (n=38) and clinically definite MS patient samples (n=42) were analysed by Western blot for MBP specific IgG (Figure 23). None of the patient samples demonstrated a definitive band at 18.5 kDa, corresponding to MBP. This is likely the result of poor sensitivity of the Western blot technique on a whole. Figure 24 illustrates the results of serial two-fold dilutions of a polyclonal antibody to MBP (raised in rabbit). Bands corresponding to MBP were clearly visible until dilutions greater than 1:16,000 were used. When the same series of dilutions was run in the ELISA (Figure 25), the subtracted OD values which are inclusive of those achieved in the MS patients correspond to dilutions greater than 1:32,000. This accounts for the absence of bands when patient samples were examined. Moreover, the variation in background for the MS patients tested by Western blot (Figure 23) can not be adjusted for as is done in the ELISA format, by the addition of heparin (Alcantara et al., 1999) and the subtraction of individual samples’ background, and merely compounds the difficulty in achieving reasonable sensitivity by this method.
Molecular weight Markers

250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
15 kDa

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 23: MBP Autoantibody Western Blot-Evaluation of MS Patients

Example of Western blot for the detection of MBP Autoantibodies. Lanes 1-18 represent separate MS patient samples. The equivalent of 2 μg/lane of unfractionated MBP was separated by 12.5% SDS-PAGE. Subsequent to transferring to PVDF membranes, 240 μl of each patient’s sera (1:10 dilution) was added to the PVDF membranes. Goat anti-human IgG-HRP was allowed to react with the membrane. The blots were developed using TMB as a substrate (refer to Methods section). No bands were detected at 18.5 kDa, where MBP was expected.
Figure 24: MBP Autoantibody Western Blot-Evaluation of Sensitivity

Each lane represents a dilution of affinity purified rabbit polyclonal antibody added to PVDF membranes containing MBP, as described in the Methods section.
Figure 25: Evaluation of Assay Sensitivity
Serial dilutions of affinity purified rabbit anti MBP IgG were assayed by ELISA (refer to Methods section). The subtracted absorbance values achieved at dilutions greater than 1:32,000 represent the absorbance values achieved with the majority of MS patient samples tested.
C. SUMMARY

Measurement of plasma MBP autoantibodies (IgG) by ELISA is effective in achieving the clinical objectives of high sensitivity (77%) and specificity (95%). Circulating anti-MBP IgG in MS appears to be an indicator of disease activity as levels were increased in patients who were experiencing relapse. The measurement of MBP autoantibodies by ELISA was also shown to be a more sensitive technique than Western blotting and appears to have the highest opportunity for clinical relevance compared with the measurement of autoantibodies to PLP, NSE and S-100B.
CHAPTER 5

CLINICAL UTILITY OF OTHER BIOCHEMICAL MARKERS IN MS

The measurement of MBP autoantibodies offers diagnostic potential for MS. Given that a single autoantigen responsible for the aberrant immune pathogenesis has not been conclusively determined, numerous autoantigens may be involved. Likewise, the humoral response in MS does not act in isolation of other immune or pathogenic factors. Since demyelination is the central aspect of MS pathology, the measurement of other major myelin proteins such as MBP, in the circulation may offer clinical utility. Therefore, MBP was measured in the blood of our MS patient cohort. Serum concentrations of other candidate autoantigens, S-100B and NSE, were also measured. Since damage to the BBB is considered an early event in MS pathogenesis, the measurement of thrombomodulin, an endothelial cell protein, was performed to assess clinical utility in MS.

A. MYELIN BASIC PROTEIN

Ninety clinically definite MS patient serum samples were assayed for myelin basic protein concentrations by quantitative indirect ELISA (Smart MBP ELISA kit; SynX Pharma Inc., Mississauga). The components of this ELISA cannot be disclosed. The clinical decision limit or clinical cut-off (0.13 ng/mL) was established previously using 60 normal donor samples. Samples containing concentrations of MBP greater than this value were considered positive. Figure 26 demonstrates that using this clinical cut-off (dashed line), 43% of the patients tested had elevated concentrations of circulating MBP. Moreover, MBP
Figure 26: Serum Concentration of MBP in Clinically Definite MS Patient
Analysis by indirect ELISA, as described in the Methods section, revealed that serum concentrations of MBP were elevated in 43% of MS patients tested (n=90), using the mean +2SD as the clinical cut-off (0.130 ng/ml, dashed line). Inset includes the two patients who had extraordinarily high serum MBP.
concentration in patients experiencing relapse was not significantly different from those patients in stable courses of the disease and no definitive pattern with respect to disease course could be ascertained. The half-life of MBP in serum is approximately two hours whereas IgG can persist in the circulation for 23 days. Therefore, the lack of correlation between MBP and IgG levels may be the result of an immune response generated by a transient release of MBP into the circulation and subsequent clearance. While MBP autoantibody testing may be more useful in diagnosing MS, the measurement of MBP itself may indicate activity of lesions which would possibly precede clinical symptoms of relapse and may suggest immunodominant epitopes if the MBP material can be identified.
B. NEURON SPECIFIC ENOLASE

Ninety clinically definite MS patient serum samples were assayed by quantitative indirect ELISA (Smart NSE ELISA kit; SynX Pharma Inc., Mississauga). The clinical decision limit (8.34 ng/mL) was established previously using 77 normal donor samples. Figure 27 demonstrates that using this clinical cut-off (dashed line), 40% of the patients tested had elevated concentrations of circulating NSE. These results are interesting because as discussed earlier NSE autoantibody levels were not elevated in the same MS patient cohort. Elevated circulating NSE concentrations in these patients, while not contributing directly to the autoimmune pathogenesis of MS, may be a reflection of the proportion of patients with axonal degeneration secondary to demyelination. This possibility can not be confirmed at present due to the lack of histological examination of brain biopsies in this patient cohort.
Figure 27: Serum Concentration of NSE in Clinically Definite MS Patients

Analysis by indirect ELISA, as described in the Methods section, revealed that serum concentrations of NSE were elevated in 40% of MS patients tested (n=90), using the mean +2SD as the clinical cut-off (8.34 ng/ml, dashed line). Inset includes the patient who had extraordinarily high serum NSE.
C. S-100B

Ninety clinically definite MS patient serum samples were assayed by quantitative indirect ELISA (Smart S-100 ELISA kit; SynX Pharma Inc., Mississauga). The clinical decision limit (0.02 ng/mL) was established previously using 103 normal donor samples. Figure 28 demonstrates that only 7.8% of the MS patients had elevated concentrations of S-100. These results are what would be expected given the lack of autoantibody response for this glial marker in the same patient cohort. However, the observed increased concentration in select patients may correlate with the extent of gliosis in these patients or astrocyte/oligodendrocyte breakdown.
Figure 28: Serum Concentration of S-100B in Clinically Definite MS Patients
Analysis by indirect ELISA, as described in the Methods section, revealed that serum concentrations of S-100B were elevated in 7.8% of MS patients tested (n=90), using the mean +2SD as the clinical cut-off (0.02 ng/ml, dashed line).
D. THROMBOMODULIN

Since thrombomodulin concentrations have been measured previously in the CSF and serum of MS patients and appeared to correlate with disease activity (Tsukada et al., 1995; Frigerio et al., 1998) ninety clinically definite MS patient serum samples were assayed by quantitative indirect ELISA (Smart Tm ELISA kit; SynX Pharma Inc., Mississauga). The clinical decision limit (46 ng/mL) was established previously by the manufacturer. Figure 29 illustrates that 66% of the MS patients had elevated concentrations of thrombomodulin (Tm). Since thrombomodulin is an endothelial cell marker, it is possible that the especially elevated Tm levels correlated with lesions of the blood brain barrier (BBB). However, when patients were stratified on the basis of disease status at the time of blood collection, no significant difference was observed between patients in stable versus relapsing courses of their disease (p>0.05, Student’s t-test) (Figure 30). The extent to which the elevated Tm levels actually correlate to the severity of damage to the BBB can not be evaluated without evidence from MRI. Thrombomodulin has been implicated in a host of various diseases ranging from coagulation disorders to pancreatic insufficiency and thus this marker may not carry enough specificity for the diagnosis of MS on its own.
Figure 29: Serum Concentration of Tm in Clinically Definite MS Patients

Analysis by indirect ELISA, as described in the Methods section, revealed that serum concentrations of Tm were elevated in 66% of MS patients tested (n=90), using the mean +2SD as the clinical cut-off (46 ng/ml, dashed line). Inset includes the patients who had extraordinarily high serum Tm.
**Figure 30: Thrombomodulin Concentrations in Clinically Stratified MS Patients**

When clinically separated into MS disease states, patients in relapse (n=26) had Tm concentrations, as determined by ELISA (refer to Methods section) that were not significantly different from the stable patients (n=48). Dashed line represents the clinical cut-off (46 ng/ml).
E. SUMMARY

Serum concentrations of thrombomodulin, MBP and NSE were shown to have clinical utility in MS and the diagnostic parameters have been summarized in Table 9. When MBP, Tm and MBP autoantibody measurements are all considered in the diagnostic work-up of the MS patients, clinical sensitivity increases to 95% which can not be achieved by the measurement of any of these markers alone (Table 10). The adoption of a panel of markers may optimize the usefulness of each of these markers in the diagnosis of MS.
Table 9: Summary of Diagnostic Utility of All Biochemical Markers tested

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP IgG</td>
<td>77 %</td>
<td>95 %</td>
</tr>
<tr>
<td>MBP</td>
<td>43 %</td>
<td>95 %</td>
</tr>
<tr>
<td>NSE</td>
<td>40 %</td>
<td>99 %</td>
</tr>
<tr>
<td>S-100B</td>
<td>8 %</td>
<td>95 %</td>
</tr>
<tr>
<td>Tm</td>
<td>66 %</td>
<td>96 %</td>
</tr>
</tbody>
</table>

All markers were assayed by ELISA as described in the Methods section.
Table 10: Diagnostic Utility of a Biochemical Panel in MS

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP IgG + Tm</td>
<td>93 %</td>
</tr>
<tr>
<td>MBP IgG + MBP</td>
<td>90 %</td>
</tr>
<tr>
<td>Tm + MBP</td>
<td>77 %</td>
</tr>
<tr>
<td>All three markers</td>
<td>95 %</td>
</tr>
</tbody>
</table>

All markers were assayed by ELISA, as described in the Methods section. Sensitivity was determined by calculating the number of MS patients with elevated concentrations of any one marker in the above combinations.
CHAPTER 5

DISCUSSION

The diagnosis of MS relies heavily on clinical examination and neuroimaging techniques. Due to the heterogenous nature of the disease, accurate and rapid diagnosis is made difficult, especially when the differential diagnosis includes diseases such as Behcet’s disease, sarcoidosis with CNS involvement and monophasic demyelinating disease including optic neuritis and acute disseminated encephalomyelitis (ADEM). ADEM is especially important in the differential diagnosis of MS in children. With the recommendation for earlier treatment in MS, earlier and reliable diagnostic measures are of paramount importance. Moreover, recent trends are showing that treatment may be more effective if tailored to individual sub-classification of disease course, i.e., secondary progressive, primary progressive, relapsing-remitting). Drugs such as interferon β1 are more effective in relapsing-remitting MS than secondary progressive MS. To date, clinical stratification of patients is achieved through the patients’ history and only in primary progressive using MRI (there is more spinal cord lesion involvement in PPMS). Inclusion criteria include specified disease course for almost all drug trials. A reliable biochemical marker which is indicative of disease activity, as well as disease course, could be employed not only for the diagnosis of MS and the prognostic monitoring of the disease for clinical trial but also may represent a means of determining disease sub-type for patient study inclusion. The work presented in this thesis is suggestive that MBP autoantibodies, as well as, the measurement of other biological markers can be used in the para-clinical work-up and monitoring of MS progression.
A. ASSAY PERFORMANCE

Several investigators have established that autoantibodies against several myelin and non-myelin proteins can be detected at various frequencies in the CSF of MS patients. At the forefront of this work is the detection of MBP autoantibodies in the CSF of MS patients. While Panitch (1980) reports a clinical sensitivity of 81% in his MS patients, Warren and Catz (1986) observed a sensitivity of 55% when free (no acid dissociation) levels were assayed and a clinical sensitivity of 56% when bound fractions were measured. Warren and Catz (1999) subsequently reported that 90-95% of MS patients with active disease had elevated levels of MBP autoantibodies in the CSF. It is important to recognize that the high sensitivity they reported was a reflection of a restricted population of patients, that is only patients in active relapse. Therefore, for comparative purposes, it may be more useful to consider their reported total sensitivity of 77.7% as a CSF reference value when compared to values measured in the peripheral circulation. Nevertheless, the method of acid dissociation appears to have utility since it offers the possibility of measuring total antibody in distinguishing patients’ disease activity and shows promise as a prognostic measure, should a proper long-term prospective study be performed in the future.

Numerous investigators have reported various levels of success in the detection of MBP autoantibodies in the circulation, which has contributed to the controversy surrounding the utility of measuring these antibodies in serum. The lack of consistent methodology between investigators may account for the apparent discrepancy between their collective results and the data which has been presented in this thesis.

To illustrate this point, Reindl and colleagues (1999) investigated the detection of anti-MOG and anti-MBP IgG antibodies in the sera and CSF of MS patients. Patient
samples were first screened by Western blot for antibodies to MBP and MOG, however subsequent ELISA evaluation was limited only to that of anti-MOG. The sensitivities reported by this group represented seropositivity (i.e. the sera used in the Western blot demonstrated a band corresponding to MBP) as assessed by immunoblotting alone. Taken together, they achieved positive detection of anti-MBP IgG in 28% of the total MS population studied. Furthermore, the frequency of seropositivity by Western blot increased in patients with clinically definite MS as compared with patients with laboratory-supported definite MS. They hypothesized that anti-MBP responses accumulated over time. The detection of 28% of MS patients as having MBP autoantibodies by western blot by Reindl and colleagues was not observed in our study, as we failed to identify any seropositive patients. The Western blot technique is much less sensitive than ELISA as was illustrated by the titration of a polyclonal control antibody: the nature of these autoantibodies in serum is of relatively low titer, at an equivalent polyclonal dilution, especially compounded with issue of high background as seen through the Westerns performed, it is understandable that patients could not be detected as positive.

Likewise, Cruz and colleagues (1987), although successful in detecting 32% of MS patients as CSF positive for anti-MBP antibodies by immunoblot, they failed to detect any bands when matched serum samples were examined. ELISA results also showed a higher detection in the CSF (44%) of their 25 MS patients compared with 8% detected in serum. Cruz, however, reports high background in their ELISA results. MBP is a highly cationic protein; its charge may be the contributing factor for high background in the ELISA due to non-specific charge interactions which typically plague the sensitivities of ELISAs using positively charged proteins (Pesce et al., 1986). This obstacle was recognized early during the development of our current ELISA protocol. It became apparent that the addition of a
polyanion was required to aid in the charge neutralization of the MBP. After optimization experiments were performed, heparin was chosen because it significantly improved the distinction between control and MS patients while maintaining an excellent signal to noise ratio with the positive control. The sensitivity of the ELISA largely depends on the reduction of non-specific binding by other serum factors. However, the addition of heparin to serum samples has been found to produce non-specific reactivity on Western blots, while other tests, such as the ELISA remained unaffected (Alcantara et al. 1999). Thus, an increase in sensitivity is not likely possible using this method of non-specific binding reduction, for Western blotting.

By the same token, Brokstad and colleagues (1994) using both Western blot and ELISA failed to detect MBP autoantibodies in either the CSF or the serum of MS patients. In their ELISA protocol serum samples were diluted only 1:100. From our optimization experiments such a high dilution diminished the differentiation of MBP autoantibody levels between control and MS patients. Furthermore, they utilized an extraordinarily low dilution of 1:350 of the conjugated detector antibody. This likely gave rise to extremely high background and diminished the sensitivity of the assay. In this thesis, a dilution of 1:15,000 of the conjugated antibody is required to achieve acceptable background.

Finally, to illustrate the necessity of subtracting individual background levels for each sample tested, Garcia-Merino and colleagues (1986) achieved the highest detection of MBP autoantibodies in the serum (41%), as compared with the previous published reports. Our success in using the detection of circulating MBP autoantibodies by ELISA (77%), compared with this group, was due to the reduction of non-specific binding, not only through the use of heparin, but also by the elimination of individual sample variation in non-specific
adherance to the microtiter plate by using subtracted OD values (Crimando and Hoffman, 1992).

B. CLINICAL UTILITY OF MBP AUTOANTIBODIES IN MS

Through the development of a highly sensitive ELISA, we have achieved a clinical sensitivity of 77% by the measurement of anti-MBP IgG. Moreover, because levels of IgG in the stratified patient cohort were significantly elevated in the relapsing patients versus patients in a stable/remitting phase of their disease, it appears that circulating MBP IgG is indicative of disease activity. This observation was also reported by numerous investigators who measured anti-MBP IgG in the CSF. Since the nature of the disease dictates that relapses have various frequencies of occurrence, ranging from 2 months to years, a long-term prospective follow-up of a patient cohort, though beyond the scope of this project, is required to substantiate the hypothesis that MBP IgG levels increase in the individual patient experiencing relapse.

Despite reports by other groups (Reindl et al, 1999), there did not appear to be any significant difference in the titers of MBP autoantibodies (IgG) between relapsing-remitting and progressive (secondary/primary) patients. Therefore, it seems that the proposed hypothesis that autoantibody levels accumulate over time is incorrect (Reindl et al., 1999). Any difference between patients is likely a reflection of continued demyelination and release of MBP, prompting the proliferation of secretory memory B cells and may correlate with the presence of new lesions by MRI. Nevertheless, the absence of any distinction between disease course sub-groups through the measurement of these autoantibodies is disappointing; circulating MBP autoantibodies (IgG) alone can not be used as a biochemical marker for the stratification of MS patients during clinical drug trials.
With respect to anti-MBP IgM levels, only 30% of the MS patients exhibited circulating elevated titers, as measured by ELISA. Although one patient with an extremely elevated anti-MBP IgM level, was experiencing her first relapse in MS, no significant difference was found between relapsing and stable patients on the whole. In fact, no definitive feature of the patients with elevated anti-MBP IgM could be ascertained. Elevated IgM in the CSF during episodes of relapse has been previously established in the literature, however, little focus has been devoted to determining the antigenic characterization of this IgM response. In one group of RRMS patients, MBP-specific IgM responses correlated well with CSF IgM indices (Annuzziata et al., 1997). Furthermore, patients with high IgM anti-MBP suffered from fewer attacks, with a less rapid frequency and exhibited a decrease in EDSS score more readily as compared with low IgM anti-MBP patients. It appears that anti-MBP IgM primary response is the product of specific antigenic stimulation. Patients with high IgM followed a more favourable/benign course during follow-up. Therefore, a long-term study examining prognosis, as well as duration and time between attacks, may be useful in the determination of the significance of elevated anti-MBP IgM in the circulation.

Therefore, in summary, MBP autoantibodies may be reliably detected in the circulation of MS patients. Moreover, the IgG class of antibodies may be a better indicator of disease activity than IgM.

(I) Specificity of the MBP Autoantibody Response

In a detailed and extensive study for determining the specificity of MBP autoantibody response in the CSF, Warren and Catz (1999) determined that 98% of other non-MS controls did not exhibit elevated anti-MBP antibodies. Contrary to their findings numerous reports documented anti-MBP responses in a variety of other patients including, catatonic
schizophrenia (Rimon et al. 1986), subacute sclerosing panencephalitis (Panitch et al. 1980),
cerebrovascular accidents (Schmid et al., 1974), Alzheimer’s disease (Singh et al., 1992) and
AIDS (Maimone et al. 1994).

Little research has been reported for the clinical specificity of the MBP autoantibody
response in serum, presumably because many investigators have had such limited success in
their detection in MS patients. In one diabetes (IDDM) patient and 5 of 15 rheumatoid
arthritis patients, elevated levels of anti-MBP autoantibodies were detected by our method.
These results might best be explained by considering that in general, patients with
autoimmune diseases tend to be reactive to multiple antigen. The results obtained by testing
IDDM and RA patient samples in the MBP autoantibody assay may be a reflection of this
phenomenon. In fact, recent evidence has shown that T cells reactive to MBP can be
recovered from NOD mice, an animal model for insulin-dependent diabetes mellitus (Winer
et al..). Furthermore, research on rheumatoid arthritis, a systemic autoimmune disease, has
shown that RA-specific autoantibodies recognize citrulline containing epitopes preferentially
(Schellekens et al, 1998; Girbal-Neuhausser et al, 1999). Therefore, alternatively it is
possible that the seropositive RA samples (Figure 22) are recognizing citrulline within the
MBP (for a discussion of citrullinated MBP refer to page 23). The substantiation of this
hypothesis can be examined by citrullinating NSE, which is non-reactive with RA patients,
and determining whether the samples become seropositive for citrullinated NSE
autoantibodies. Preliminary attempts to deiminate NSE in vitro have not been successful due
to precipitation of the protein and low yields of final product.

Since MBP autoantibodies were the most elevated in the MS patients tested, as
compared with PLP, NSE or S-100B autoantibodies, the autoantibody response in MS
appears to be specific for myelin basic protein. The 6% of patients who presented elevated
anti-PLP IgG all exhibited elevated titers of MBP autoantibodies as well, in disagreement with Warren and Catz (1994) who found mutual exclusion. Based on this mutual exclusion of anti-PLP and anti-MBP levels in the CSF, they postulated two immunologically distinct forms of the disease that reflect differences in the extent of inflammation. Although perhaps valid in the CSF, no such distinction can be made in the serum.

C. DIAGNOSTIC UTILITY OF A BIOCHEMICAL PANEL IN MS

The evaluation of autoantigenic markers. MBP, S-100, NSE and Tm showed promise for the diagnosis of MS. Of the 43% of patients who had elevated concentrations of MBP in serum, no difference in levels were observed between patients in relapse and those who were stable. Previous work performed in the CSF demonstrated that MBP was a reliable indicator of relapse, correlated well with the number of gadolinium-enhanced lesions on MRI, EDSS scores, intrathecal IgM synthesis (Lamers et al, 1998) and levels of CSF MBP correlated well with CSF free anti-MBP levels (Warren and Catz, 1987). Correlation between EDSS score, MBP and MBP autoantibodies was not observed in our study. However, the Smart MBP ELISA kit used in this study to measure MBP does not disclose whether MBP and MBP peptides can be detected in the serum. Since the nature of MBP in the serum is not known, it is not certain if the absolute levels of MBP including MBP peptides can be measured by this method. Determination of MBP peptide detection by ELISA could be determined by digesting full length MBP into peptide fragments and measuring their concentration in the ELISA. Nevertheless, the utility of measuring circulating MBP in conjunction with MBP autoantibodies offers and excellent clinical sensitivity of 90% and should be incorporated into the diagnostic work-up in MS.
Likewise thrombomodulin, while failing to correlate with traditional measures of disease activity, was detected at elevated concentrations in 66% of the MS patients studied. Presumably, damage to the endothelial cells forming the BBB releases Tm into the CSF and subsequently is transported into the peripheral blood. Future studies using MRI may reveal that such increased Tm concentrations correlate with not only BBB damage but also with the presentation of new lesions. Therefore, Tm may be released prior to the onset of clinical symptoms of relapse. When either serum Tm or anti-MBP IgG are measured, 93% of MS patients can be detected as positive. While the addition of Tm to the differential diagnosis of MS may exacerbate problems with clinical specificity, it offers excellent clinical sensitivity in the diagnosis of MS.

D. PATHOLOGICAL SIGNIFICANCE OF CIRCULATING MBP AUTOANTIBODIES

CSF anti-MBP antibodies are not disease specific, however they are associated with disease activity. Considering the presence of natural autoantibodies in normal healthy individuals (Coutinho et al. 1995; Lacroix-Desmazes et al. 1998), as well as their presence in other autoimmune (RA) and psychiatric patients, circulating MBP autoantibodies also may not be disease specific. It is popular belief that the polyspecific nature of autoantibodies implies that any generated antibody response to myelin basic protein is non-specific and is an epiphenomenon. Despite this, there is no question that the immune response to MBP is indeed specific. Fab\(^2\) fragmentation, sub-class determination of the MBP autoantibodies in serum as restricted to IgG1 and IgG3 (Garcia-Merino et al, 1986) and specific epitope mapping (Warren and Catz, 1992; 1993) suggest that autoantibodies against MBP may play a
central role in the pathogenesis of the disease. Antibodies specific to protein antigens typically belong to the IgG1 and IgG4 sub-class (Garcia-Merino et al, 1986) and usually belong to both subclasses simultaneously. The identification of IgG1 or IgG3 sub-class restriction by Garcia-Merino’s group (1986) suggests a monoclonal or oligoclonal origin, perhaps a reflection of the oligoclonal nature of the IgG response in the CSF.

Much controversy surrounds the site of production of MBP antibodies in MS patients. Circumstantial evidence dominates the field with the proposal that increased IgG synthesis in the CSF implies that MBP autoantibodies are of intrathecal origin. However, much evidence exists for the in vitro demonstration that MBP autoantibodies in serum induce demyelination. Whether B cells recruited to the CNS generate the anti-MBP responses and are leaked to the circulation or the response is initiated peripherally through the surveillance of the peripheral circulation has not been elucidated. Until the true origin of this MBP specific humoral response can be identified, the pathogenic role of MBP antibodies remains enigmatic.

MS is a heterogeneous disease: clinical presentation and neuropathology are diverse between patients (Laman et al, 1998). Recent recommendations for the development of novel diagnostic measures in MS incorporate the fact that MS is a disease mediated immunologically: markers may be identified more easily in accessible peripheral fluids, i.e. blood, urine and CSF (Laman et al., 1998). Such a marker should meet acceptable assay performance requirements and correlate with disease stage.

Based on the evidence presented in this thesis, should MBP autoantibodies in the blood of MS patients mimic the diagnostic benefit of MRI and identify possible MS patients (POSMS) as having the disease, whether used alone or in conjunction with MBP or Tm
concentrations. MBP antibody measurement would offer clinicians a rapid, sensitive, non-invasive and inexpensive tool for the diagnosis and monitoring of the progression of MS.
FUTURE STUDIES

1. In this study we demonstrated that circulating MBP autoantibodies are elevated in relapsing patients as compared with patients in stable courses of MS and this observation has also been reported when anti-MBP IgG was measured in the CSF (Warren and Catz, 1986). In order to determine whether circulating MBP autoantibodies are measures of disease activity in the individual patient, a long term prospective study in which MBP autoantibody levels could be measured at relapse and during subsequent remission would facilitate our understanding of their predictive value in determining the occurrence of relapse and would help indicate whether these antibodies could be used in the progressive monitoring of patients receiving novel MS therapies. Moreover, by incorporating this ELISA into trials in which patients undergo frequent MRI scans, the correlation of MBP autoantibodies and lesion load or the development of new lesions might also help to substantiate the hypothesis that MBP autoantibody levels are indicative of disease activity and active demyelination.

2. Since IgM antibody production is usually a first response to an antigen, we hypothesized that circulating anti-MBP IgM may be an indicator of relapse. Although no statistically significant difference was observed in clinically stratified MS patients, the IgM class of MBP autoantibodies may be present at elevated levels during the first attack of MS. Therefore, the collection of plasma from MS patients at the time of first relapse and during clinical remission should be performed to determine if anti-MBP IgM has diagnostic utility in clinically possible/probable MS patients.
3. The differential diagnosis in MS is especially important in children. To date, blood from approximately 30 paediatric patients with either MS, optic neuritis, ADEM or epilepsy have been collected in a blinded manner. Upon the completion of sample collection, evaluation of MBP autoantibodies in these children should be performed to determine whether MBP autoantibodies are clinically useful in the differential diagnosis of paediatric MS.

4. The ELISA developed in this study uses unfractionated MBP as a coating antigen. Much research in our laboratory has examined the role of the C8 charge isomer in the pathogenesis of MS. The examination of autoantibody responses to C8 compared with C1 (the most positively charged fraction of MBP) may reveal that a stronger autoantibody response is generated against the C8 charge isomer of MBP. Moreover, since C8 contains citrulline, preferential autoantibody response to this charge isomer of MBP may help explain why some of the rheumatoid arthritis patients studied in our work demonstrate "MBP" autoantibodies.
REFERENCES


APPENDIX

We evaluated MBP autoantibodies (IgG) by ELISA in matched CSF and serum samples of seven additional query MS patients. CSF samples were diluted 1:10 in the plasma dilution buffer, as described in the Materials and Methods section. The clinical diagnoses, results from laboratory tests, and results from the MBP autoantibody ELISA for each patient are summarized in Table 11. All seven patients studied had no detectable levels of MBP autoantibodies (IgG) in the CSF, as measured using our ELISA. Four of the seven patients had a definitive diagnosis of MS. However, in those patients with clinically definite MS, all four patients had elevated titers of MBP autoantibodies in their serum.

According to reports by other investigators (Warren and Catz, 1986), elevated CSF MBP IgG is associated with active disease. Since only one patient was experiencing a relapse at the time of lumbar puncture, we cannot validate whether MBP IgG is detectable in the CSF of relapsing MS patients. MBP autoantibodies (IgG) in the CSF of MS patients should be evaluated in the future, upon the inclusion of a larger patient cohort.
Table 11: MBP Autoantibody Evaluation in Query Multiple Sclerosis Patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serum MBP IgG</th>
<th>CSF MBP IgG</th>
<th>MRI</th>
<th>Oligoclonal Bands</th>
<th>Diagnosis</th>
<th>Disease Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMH 01</td>
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